European Commission

Draft (Renewal) Assessment Report prepared according to the Commission Regulation (EU) N° 1107/2009

Lenacil

Volume 3 – B.6 (AS)

Rapporteur Member State : Belgium

Co-Rapporteur Member State : Austria

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Version History

When	What
November 2007	Initial DAR Draft Assessment Report (DAR) – prepared in the context of the application for the first inclusion of the a.s. in Annex I to Council Directive 91/414/EEC.
June 2016	Updated Draft Assessment Report (DAR) – prepared in the context of specific provisions included in Regulation (EU) No 540/2011: 'If a decision on the classification of lenacil under Regulation (EC) No 1272/2008 of the European Parliament and of the Council identifies the need for further information on the relevance of the metabolites IN-KE 121, IN-KF 313, M1; M2, M3, Polar B and Polars, the Member States concerned shall request the submission of such information. They shall ensure that the notifier provides that information to the Commission within six months from the notification of such a classification decision'.
December 2018	Draft Renewal Assessment Report (DRAR) – prepared in the context of the application for renewal of approval of the a.s. according to Reg (EU) No EU 844/2012. *Note: The RAR is a stand-alone document containing the evaluations already displayed in the original DAR, as well as the new assessments. The revision of the initial DAR has been done in accordance with SANCO/10180/2013 rev.1 (March 2013), with changes to the original text – resulting from assessment of new studies (or reconsideration of old studies or studies that were not yet previously peer-reviewed) – being highlighted by means of yellow shading.*
February 2019 May 2019	Draft Renewal Assessment Report (DRAR) – prepared in the context of the application for renewal of approval of the a.s. according to Reg (EU) No EU 844/2012.Update taking into account remarks of the APDESK of EFSA: correction of the table of contents. Further update after submission of the Appendix E of the GD pertaining endocrine effects. *Note: The RAR is a stand-alone document containing the evaluations already displayed in the original DAR, as well as the new assessments. The revision of the initial DAR has been done in accordance with SANCO/10180/2013 rev.1 (March 2013), with changes to the original text – resulting from assessment of new studies (or reconsideration of old studies or studies that were not yet previously peer-reviewed) – being highlighted by means of yellow shading.*

In the following table, an overview is given of the studies performed in vol.3 B.6

Table B.6.0. Reference of studies conducted with the test substance Lenacil

Study	Reference	Report n°	a.s.	Batch n°	Purity %
B.6.1 ADME studies					
ADME, main study	[redacted] (1996)	HLR 62-94	[^{14}C] Lenacil, Lenacil	No 391, B634-88 No19615,20300	>98% 98%
Interspecies *in vitro* metabolism	Pineiro Costas (2016)	WIL Research Project 512721	[^{14}C]Lenacil	CFQ42525	97.6%
B.6.2 Acute toxicity studies					
Oral	[redacted] (2001a)	ACD 004/013224/AC	Lenacil	141712003	98.6
Percutaneous	[redacted] (2001b)	ACD 005/013220/AC	Lenacil	141712003	98.6
Inhalation	[redacted] (2001)	ACD 021/013229	Lenacil	141712003	98.6
Skin irritation	[redacted] (2001c)	ACD 006/013201/SE	Lenacil	141712003	98.6
Eye irritation	[redacted] (2001d)	ACD 007/013273/SE	Lenacil	141712003	98.6
Skin sensitisation	[redacted] (1992)	HLO 34-92	Lenacil	B634-91	98.2
Phototoxicity	Westerink (2016)	WIL Research Project 511052	Lenacil	B0634-142	98.6
B.6.3 Short term studies – 6.8.2 supplementary studies(°)					
28-d rat, oral	[redacted] (2002a)	ACD 001/010098	Lenacil	141712003	98.6
28-d dog, oral	[redacted] (2001)	ACD 003/013230	Lenacil	141712003	98.6
90-d (4-wk rec.) rat, oral	[redacted] (2002b)	ACD 002/013903	Lenacil	141712003	98.6
Additional 90-d (4-wk rec.) rat, oral (°)	[redacted] (2004)	ACD 055/024499	Lenacil	141712003	98.6
90-d mouse, oral	[redacted] (1991)	HLR 293-91	Lenacil	B634-91	98.2
90-d dog, oral	[redacted] (2002)	ACD 022/014297	Lenacil	141712003	98.6
B.6.4 Genotoxicity studies					
Salmonella/microsome assay (*in vitro*)	Russell (1977)	HLR 601-77	Lenacil	B634-50	n.s.
Salmonella/microsome assay (*in vitro*)	D'Amico (1994)	HLR 413-94	Lenacil	B634-107	n.s.
Salmonella/microsome assay (*in vitro*)	May (2001)	ACD 016/013217	Lenacil	141712003	98.6
UDS study (*in vitro*)	Riach (1989)	IRI 6135	Lenacil	8906	n.s.
Chromosomal aberrations (*in vitro*)	Allais (2001)	ACD 017/013707	Lenacil	14171003	98.6
Chromosomal aberrations (*in vitro*)	Kellum (2017)	047303003	Lenacil	047303003	98.6

Study	Reference	Report n°	a.s.	Batch n°	Purity %
Mouse lymphoma (*in vitro*)	Clare (2003)	ACD 053/023530	Lenacil	141712003	98.6
Mouse micronucleus assay (*in vivo*)	[redacted] (2001)	ACD 018/013472	Lenacil	141712003	98.6
B.6.5 Long term studies					
Long-term toxicity and carcinogenicity, rat, oral	[redacted], 2003	ACD 045/024288	Lenacil	141712003	98.6
Long-term toxicity and carcinogenicity, rat, oral	[redacted] (2004)	ACD 045/042214	Lenacil	141712003	98.6
Oncogenicity, mice, oral	[redacted] (1994)	HLR 336-93	Lenacil	B634-91	98.2
B.6.6 Reproduction studies					
2-generation, rat, oral	[redacted] (2002)	ACD 019/010186	Lenacil	141712003	98.6
2-generation, rat, oral	[redacted] (2003)	ACD 020/023865	Lenacil	141712003	98.6
Developmental, rat, oral	[redacted] (1978)	HLR 405-78	Lenacil	B634-61	*Ca.*100
Developmental, rat, oral	[redacted] (1996)	HLR 996-96	Lenacil	DP B 634091 [redacted] 18759	98.5
Embryo-foetal development, rat, oral (preliminary)	[redacted] (2003)	ACD 057/030001	Lenacil	141712003	98.6
Embryo-foetal development, rat, oral	[redacted] (2003)	ACD 058/032316	Lenacil	141712003	98.6
Developmental toxicity, rabbit, oral	[redacted] (1991)	HLR 626-91	Lenacil	B634-91	98.5
B.6.8.2 Supplementary studies					
Thyroid study, 20 weeks, rat, oral	[redacted] (2004)	ACD 060/033946	Lenacil	141712003	98.6
B.6.8.3 Studies assessing endocrine effects					
ER binding assay, *in-vitro*	Nabb (2018a)	DuPont R. No.: 49349	Lenacil	047303003	99.33
AR binding assay, *in-vitro*	Nabb (2018b)	DuPont R. No.: 49367	Lenacil	047303003	99.33
ER transactivation assay, *in-vitro*	Rijk (2018a)	DuPont R. No.: 49351	Lenacil	047303003	99.33
AR transactivation assay, *in-vitro*	Rijk (2018b)	DuPont R. No.: 50113	Lenacil	047303003	99.33
6-d uterotrophic assay, rat, oral	[redacted] (2018)	DuPont R. No.: 49350	Lenacil	036402003	99.33
Aromatase inhibition assy, in vitro	Rijk (2019)	FMC-51364	Lenacil	047303003	99.33
Steroidogenesis assay, in vitro	Verkaart (2019)	FMC-51365	Lenacil	047303003	99.33

Study	Reference	Report n°	a.s.	Batch n°	Purity %
Thyroid mechanistic assay	[redacted] (2019)	49352	Lenacil	[redacted] 32157 (lot -151), [redacted] 321 (lot 152)	98.8%

n.s.: not specified (despite GLP study)

B.6.1 (CA 5.1) Absorption, distribution, metabolism and excretion.

B.6.1.1. Absorption, distribution, metabolism and excretion by oral route

B.6.1.1.1. Absorption, distribution, metabolism and excretion of [2-^{14}C]-lenacil ([2-^{14}C]-DPX-B634) in the rat ([redacted] 1996)

RMS reported more details for the [redacted] study :

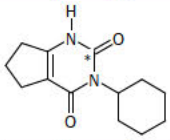
Study report: HLR 62-94

Guideline: study is in compliance with Dir EEC 87/302/EEC, equivalent to OECD test guideline n° 417 (1984).

GLP status: yes (no attest of competent authority)

Material and methods:

Male and nulliparous, nonpregnant ♀ Crl:CD®BR rats, Lenacil, ([redacted] sample No.: 19615, 20300; purity >98%) and [2-^{14}C]-lenacil ([redacted] sample No.: 19614, 19614-02, also referred to as ([2-^{14}C]-DPX-B634); purity 98%, specific activity: 17.78 µCi/mg) were used. Lenacil was labeled with ^{14}-C on the pyrimidine ring, as clarified in following scheme:



Lenacil was administered to animals by gavage. Eight dosing groups were constituted, according to **table B.6.1.1.1-1**.

Table B.6.1.1.1-1
Absorption, distribution, metabolism and excretion of [2-^{14}C]-lenacil in the rat ([redacted], 1996): dosing groups.

Group	Aim	Dose	Specific activity of [2-^{14}C]-lenacil dose solution (dpm/µg)		Number of animals	
Group A	To examine metabolism, tissue distribution, and elimination of [2-^{14}C]lenacil after:	Single oral dose of 10 mg/kg [2-^{14}C]-lenacil	20.1 x 10^3	18.3 x 10^3	5	5
Group B		Seven consecutive doses of "cold" lenacil (10 mg/kg) followed by a single dose of [2-^{14}C]-lenacil (10 mg/kg)	18.2 x 10^3	19.5 x 10^3	5	5
Group C		Single oral dose of 1000 mg/kg [2-^{14}C]-lenacil	172	166	5	5
Group D	To determine plasma pharmacokinetics of [2-^{14}C]-lenacil after:	Single oral dose of 10 mg/kg [2-^{14}C]-lenacil	17.1 x 10^3	23.6 x 10^3	6 (cannulated in the jugular vein)	6 (cannulated in the jugular vein)
Group E		Single oral dose of 1000 mg/kg [2-^{14}C]-lenacil	166	195	6 (cannulated in the jugular vein)	6 (cannulated in the jugular vein)
Group F	To measure tissue residues of total radioactivity at T_{max} and $T_{max/2}$ of [2-^{14}C]-lenacil after:	Single oral dose of 10 mg/kg [2-^{14}C]-lenacil	29.7 x 10^3	17.8 x 10^3	6	6
Group G		Single oral dose of 1000 mg/kg [2-^{14}C]-lenacil	136	232	6	6
Group H	To determine biliary elimination of total radioactivity of [2-14C]-lenacil after:	Single oral dose of 10 mg/kg [2-^{14}C]-lenacil	19.5 x 10^3	17.8 x 10^3	6 (cannulated in the bile duct)	6 (cannulated in the bile duct)

Group A: Urine and faeces were collected 12, 24, 36, 48, 72, 96,120, and 144 hours following dosing. Upon collection, these samples were stored at -20°C. At approximately 144 hours, rats were euthanised with either chloroform or CO_2. Blood was withdrawn by heart puncture with heparinised syringes, transferred to heparinised tubes, and refrigerated until analysed. The following organs and tissue samples were obtained and weighed: heart, lungs, liver, kidneys, spleen, testes (or ovaries and uterus), brain, thyroid, adrenals, G.I. tract tissue and content. And samples of skin, muscle and fat. The contents of ther G.I. were removed and treated as a separate sample. The femurs were excised for bone marrow isolation. The residual carcass was weighed and maintained with all organs and tissues at -20°C until analysed.

Groups B & C: excreta and tissues were taken and stored as described for Group A.

Group D: blood was collected at 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, and 72 hours after dosing. Each blood sample was centrifuged to separate plasma from whole cell fraction. Excreta were taken and stored as described for Group A.

Group E: blood and excreta were collected and stored as described for Group D.

Group F: at the time of maximum blood concentration, as determined in Group D, 6 rats (3/sex) were sacrificed and the same tissues than in Group A were collected. At the time when plasma concentration of ^{14}C fell to half the plasma concentration at T_{max} ($T_{max}/2$) as determined in Group D, 6 rats (3/sex) were sacrificed and the same tissues were collected. Excreta were collected and stored, but not analysed.

Group G: at the time of maximum blood concentration, as determined in Group E, 6 rats (3/sex) were sacrificed and the same tissues than in Group A were collected. At the time when plasma concentration of ^{14}C fell to half the plasma concentration at T_{max} ($T_{max}/2$) as determined in Group E, 6 rats (3/sex) were sacrificed and the same tissues were collected. Excreta were collected and stored, but not analysed.

Group H (biliary excretion): bile was collected at 12h and 24h and stored at -20 C until analysis. No other samples were obtained from this group.

Analysis of excreta and tissues:
Tissue concentrations were determined by combustion followed by radioassay by LSC. Whole blood concentrations were determined by direct combustion and radioassay. Urine, plasma, cage washes and bile concentrations were determined by direct radioassay of aliquots. Faeces were mixed with water to give a paste and aliquots of the pastes combusted and radioassayed. Freeze-dried faeces were extracted with organic solvents and the extracts analysed by HPLC. Urine was analysed directly by HPLC. Some urine and faecal extracts were enzyme treated (□-glucuronidase/sulphatase) to assess the nature of any conjugated species. The major metabolites were identified by chromatographic comparison with authentic reference standards by LC-MS.

The study is accepted.

Findings:

No adverse effects were observed in the treated animals under the different lenacil dosing regimens.

***Absorption*:**

Oral absorption was rapid after low dose administration (10 mg/kg), as suggested by the T_{max} which was seen after 1 h in ♂ and after 3.5 h in ♀ (**table B.6.1.1.1-2**).

When the dose increased 100×, oral absorption was delayed (T_{max} was observed at 24 h for high dose in ♂).

The AUC was increased by a factor of 14.5 for ♂ and 12.7 for ♀. AUC for ♀was 2× higher than for ♂, and this could be related to a slower excretion rate in ♀, as suggested by the T½ of elimination. In both sexes, $T_{1/2}$ elimination was longer than 24 h. (**table B.6.1.1.1-2**).

Radioactivity was detected in red blood cells (RBCs) as well as in plasma up to 72 h post dosing. There was no apparent binding to RBCs. There was a significant sex and dose differences in plasma radioactivity profiles. The time to reach T_{max} was dose dependent and AUCs were dose-proportional.

Table B.6.1.1.1-2 Absorption, distribution, metabolism and excretion of [2-^{14}C]-lenacil in the rat ([redacted],1996): radioactivity in plasma.

Time post dosing (h)	Radioactivity (µg lenacil equiv./ mL) RBC/plasma			
	1 x 10 mg/kg bw		1x1000 mg/kg bw	
Sex	♂	♀	♂	♀
0.50	0.38/1.45	0.58/1.95	1.98/0.68	NS/NS
1	0.52/1.51	0.52/1.87	1.23/0.92	5.65/7.37
2	0.56/1.45	0.50/1.51	0.21/0.90	13.6/6.56
4	0.18/0.75	0.49/1.54	0.47/1.33	11.0/6.89
6	0.24/0.63	0.57/1.96	0.99/1.89	10.1/10.4
8	0.21/0.49	0.44/1.40	0.69/1.83	9.44/14.3
12	0.16/0.29	0.36/1.02	0.88/2.71	9.41/13.5
24	0.19/0.13	0.32/0.40	1.83/3.09	10.6/10.0
36	0.11/0.08	0.22/0.24	1.12/2.03	8.01/7.25
48	0.12/0.05	0.20/0.19	1.07/1.75	7.76/4.02
72	0.15/0.03	0.16/0.13	1.89/1.19	3.79/3.13
AUC (µg × h/mL)	15.7 ± 3.1	44.8 ± 7.3	227 ± 39.1	569 ± 163
β (1/h) elimination rate constant	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
$T_{1/2}$ (h)	30.7 ± 9.1	44.0 ± 12.8	47.3 ± 12.2	32.2 ± 8.3
C_{max} (µg/mL)	1.11 ± 0.64	2.05 ± 0.34	3.29 ± 0.86	17.2 ± 2.3
T_{max} (h)	1.0 ± 0	3.5 ± 2.78	24 ± 12.0	7.33 ± 1.16

NS: no sample taken.

After a single oral low dose of ^{14}C-labeled lenacil, radioactivity was mainly excreted into urine within 12-24h. Urine represents the main excretion route after low dose, single or repeated. There were no important quantitative differences between ♂ and ♀ rats. After repeated dosing, urinary excretion was increased and a slight delay in excretion occurred which could suggest an increase in oral absorption or an induction of biotransformation of lenacil

During the 0-144h period, total urine excretion represented a mean of **59%** (♂: 59.17%; ♀: 58.83%) of the administered oral low dose, increasing to a mean of **78.9%** (♂: 71.99%; ♀: 85.79%) after repeated dosing but decreasing to **6.8%** (♂: 8.28%; ♀: 5.29%) after high dose On the same period, total faecal excretion represented a mean of **32%** (♂: 30.99%; ♀: 33.91%) of the administered oral low dose, decreasing to a mean of **15.4%** (♂: 18.99%; ♀: 11.77%) after repeated dosing but increasing to **84%** (♂: 81.41%; ♀: 86.51%) after high dose suggesting that the compound is not absorbed when the dose is increased (saturation of intestinal absorption, **table B.6.1.1.1-3**).

No $^{14}CO_2$ or other volatile radioactivity was detected in Group C (<0.1% of the dose); therefore, none was collected from the other groups.

Recovery of radioactivity ranged between 92 and 100%.

Table B.6.1.1.1-3 Absorption, distribution, metabolism and excretion of [2-^{14}C]-lenacil in the rat ([redacted],1996): % of radioactivity in excreta, cage wash and carcass.

Tissues sampling time (h) /administered dose	Radioactivity recovered (mean cumulative % of administered dose)					
	10 mg/kg bw		1000 mg/kg bw		Repeated 10 mg/kg bw	
Urine	♂	♀	♂	♀	♂	♀
0-12	47	47.2	1.04	0.58	50.76	67.49
0-24	54.7	54.71	3.78	2.35	59.8	78.69
0-36	56.4	56.37	5.54	3.71	62.53	81.63
0-48	57.43	57.33	6.71	4.54	64.55	83.11
0-72	58.2	58.11	7.36	4.92	67.46	84.01
0-96	NC	58.51	7.72	5.06	70.04	85
0-120	58.97	58.71	8.04	5.08	71.4	85.46
0-144= total	**59.17**	**58.83**	**8.28**	**5.29**	**71.99**	**85.79**
Faeces						
0-12	14.39	13.77	11.02	1.18	2.34	3.08
0-24	26.2	29.53	66.21	46.21	8.86	5.85
0-36	28.48	32.23	73.4	67.46	13.4	7.65
0-48	29.25	33.25	78.34	83.21	15.63	9.37
0-72	29.87	33.56	80.4	85.02	17.2	11.14
0-96	NC	33.71	80.81	85.37	17.88	11.51
0-120	30.65	33.84	81.11	86.17	18.59	11.67
0-144= total	**30.99**	**33.91**	**81.41**	**86.51**	**18.99**	**11.77**
Cage washes	1.26	0.47	3.32	2.15	3.94	1.99
Tissues	0.16	0.11	0.06	0.02	0.18	0.17
Residual carcass	0.78	0.49	0.17	0.14	0.75	0.76
Total recovered dose	**92.30**	**93.79**	**93.26**	**92.93**	**95.85**	**100.49**
Absorbed [$]	**61.37**	**59.9**	**11.83**	**7.6**	**76.86**	**88.71**

*NC: not collected, N=5/sex; **italics bold**: fraction 0-48h considered in part for the estimation of the oral absorption*
[$]: taking into account urine excretion, tissues, residual carcass

Conclusion oral absorption value:

Based upon the urinary excreted radioactivity after a single dose (10 mg/kg b.w.), oral absorption represents 59% of the administered low dose. Taking into account the metabolite identification in urine ~~and faeces~~, it appears clearly that oral absorption is more important as also suggested by the results of repeated dosing (7 doses) where urine radio-activity amounts to 72(♂)-86(♀)% at 144h. In contrast, at high dose (1000 mg/kg b.w.), urinary excretion is limited to about 8%, indicating a saturation effect.

In the repeated dose toxicity studies in rats and dogs and in the carcinogenicity study in rodents, the liver was identified to be at target. For this reason, the excretion via the bile (6% in ♂ and 17% in ♀ which was determined at 12 and 24 hours after oral administration could potentially be taken into account in the determination of the total absorption - see table B.6.1.1.1-6). It is of note that the estimation of urinary radioactivity pertains to a repeated oral administration, while the bile collection to a single oral administration, but since a single administration would conceivably provide an underestimation as compared to a multiple administration scheme (in terms of absorption), a summation of the two figures appears appropriate.

Therefore, the total gastrointestinal absorption, after repeated low-dose administration (the most relevant), taking into account urine excretion, tissues and residual carcass levels, would amount to 77+6 (bile) =**83%** in the ♂, and 89 (17% bile) ~**100%** in the ♀.

RMS: during the former evaluation, it was considered that the oral absorption value could be derived from both urine and faecal metabolites. (see table B.6.1.1.1-5). However, it is uncertain whether faecal metabolites could be regarded absorbed as an enteral metabolism (by intestinal bacteria) is generally not considered eligible to be inserted in the absorbed fraction (it remains not evident to discern metabolism from intestinal flora from that derived from the biliary source). However, this revisal does not compromise the overall conclusion, *i.e.* a quasi complete absorbed fraction of lenacil after repeated oral dosage.

***Distribution*:**

At T_{max}, maximal tissue concentration was observed in the gastrointestinal tracts- as well as in its content. High concentrations were identified in excretory organs such as liver, and kidneys ~~and heart~~ (see **table B.6.1.1.1-4**)

At 144 h after oral administration of lenacil, radioactivity was still detected in a large part of tissues at a very low concentration, with a higher concentration in excretory organs. At that time, at 10 mg/kg bw, ♂rat carcass retained the highest concentration representing 0.78% of the administered dose.

The increase of total residue concentration between the low and high dose groups was less than the 100-fold increase in the dose. There was no evidence of tissue accumulation after single dose administration.

Table B.6.1.1.1-4 Absorption, distribution, metabolism and excretion of [2-^{14}C]-lenacil ([2-^{14}C]-DPX-B634) in the rat ([redacted] 1996): Tissue distribution of lenacil

Tissues	Radioactivity in tissues in µg equivalents/gr tissue at T_{max} (144 h)			
Dose	10 mg/kg bw		1000 mg/kg bw	
	♂	♀	♂	♀
Adrenals	1.49/0.02	0.98/0.02	3.13/0.64	2.50/1.18
Blood	1.42/0.10	1.35/0.1	5.93/1.19	3.11/1.05
Bone	0.58/0.02	0.57/0.02	2.02/-	1.38/0.19
Brain	0.21/0.0	0.13/0.01	0.5/0.09	0.74/0.16
Fat	0.78/0.01	0.39/0.0	1.65/0.23	3.73/0.44
GIT contents	12.1/0.02	52.8/0.01	1215/3.73	10629/1.11
GIT tissue	48.5/0.03	16.8/0.02	85.2/2.26	906/0.54
Heart	1.89/0.03	0.97/0.03	3.15/0.47	2.57/0.43
Kidney	3.68/0.06	4.38/0.09	11/1.03	17/1.16
Liver	3.23/0.07	3.07/0.06	11.6/1.22	5.23/0.80
Lung	1.29/0.08	1.24/0.07	5.34/1.0	2.98/0.78
Marrow	0.89/0.01	0.73/0.01	2.38/-	2.53/1.10
Skeletal muscle	0.94/0.03	0.81/0.03	2.82/0.44	2.07/0.33
Skin	0.64/0.06	0.88/0.08	4.22/1.80	2.54/1.09
Spleen	1.35/0.03	0.73/0.03	2.81/0.32	2.88/0.67
Testes/ovary	1.27/0.02	0.86/0.02	1.92/0.27	4.09/0.42
Thyroid	1.46/0.08	0.80/0.06	2.95/1.04	2.25/0.55
Uterus		0.75/0.02		3.12/0.36
Carcass	0.46/0.08	0.34/0.06	7.06/2.07	12.5/1.88

***Metabolism*:**

The results presented in **table B.6.1.1.1-5**, show that rats excreted lenacil into 6 fractions/metabolites. Repeated dosing affected quantitatively but not qualitatively the metabolic profile. Parent compound (peak 6) was excreted in faeces at a high concentration after a single high dose administration confirming saturation of oral absorption at high dose. Faecal extracts showed profiles similar to those seen in urine. Parent compound was excreted in faeces of ♀at a much higher concentration than in ♂after a single oral low dose.

A total of 58.16% and 56.51 % of the administered dose was characterised in urine of ♂ and ♀, respectively, after a single oral low dose, reaching 69.79% and 80.85% after repeated dosing.

These results indicate that lenacil is extensively metabolised by the rat. The major biotransformation pathway was hydroxylation of either the cyclohexyl or cyclopentenyl ring, or both rings.

The major component in urine (**peak 5**, 3-31% of administered dose) was identified as a **hydroxylated metabolite** of lenacil with the OH group on C5 or C6. **Peak 4** (0.4-20% of administered dose) was identified as **IN-KD304, a hydroxylated lenacil metabolite**. **Peak 3** (0.65-15%) and **peak 2** (0.5-12% of the administered dose) were identified as **two di-hydroxylated lenacil metabolites**; the most abundant metabolite was di-hydroxylated on the cyclopentenyl ring. Although components peak 2 and peak 3 are both dihydroxylated on the cyclopentenyl ring, the actual sites of the di-hydroxylation are different, giving the different retention characteristics. **Peak 1** (0.5-5%)

was identified as a **di-hydroxylated metabolite of lenacil** with a hydroxyl group on both the cyclopentenyl and cyclohexanyl rings.

No glucuronide or sulfate conjugates were released by glucuronidase or sulfatase.

The metabolic pathway of lenacil in rodents is proposed in figure B.6.1-1.

Table B.6.1.1.1-5 ADME of [2-^{14}C]-lenacil in the rat ([redacted],1996): urine and faecal analysis by HPLC equipped with radioactive flow monitor.

Radioactive component peak*	Retention time (min)	Mean % of dose excreted in pooled urine					
		10 mg		1000 mg		Repeated 10 mg/kg bw	
		♂	♀	♂	♀	♂	♀
1	11-12	4.53	3.21	0.90	0.49	5.21	3.77
2	27-28	6.89	6.68	0.60	0.52	10.5	11.58
3	31-32	12.29	10.82	1.73	0.65	14.3	14.6
4	34-35	6.83	12.78	0.41	1.08	8	19.81
5	**36-37**	**27.62**	**23.02**	**3.24**	**3.01**	**27.76**	**31.09**
6 = parent	50-53	ND	ND	0.92	0.47	ND	ND
Total		**58.16**	**56.51**	**7.8**	**6.22**	**69.79**	**80.85**
		Mean % of dose excreted in pooled faeces					
1	11-12	3.37	1.11	ND	ND	6.3	3.26
2	27-28	3.82	ND	ND	ND	1.51	0.93
3	31-32	8.56	ND	ND	ND	4.85	2.76
4	34-35	2.25	2.59	ND	ND	ND	0.36
5	36-37	5.69	2.98	ND	ND	2.91	0.61
6= parent	50-53	2.50	27.62	81.43	83.56	6.34	1.40
Total		**26.19**	**34.3**	**81.43**	**83.56**	**21.93**	**9.3**
Sum U+F		**84.35**	**90.81**	**89.23**	**89.78**	**91.72**	**90.15**
Sum U+F - parent= ~~oral absorbed dose~~		**82.35**	**63.2**	**6.8**	**0**	**85.38**	**88.75**

ND: not detected; *See metabolic scheme for the identification of metabolites.

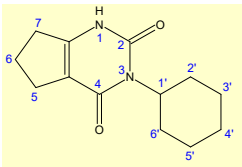
Table B.6.1.1.1-6 ADME of [2-^{14}C]-lenacil in the rat ([redacted],1996): radioactive components excreted in bile from rats dosed with 10 mg/kg bw lenacil

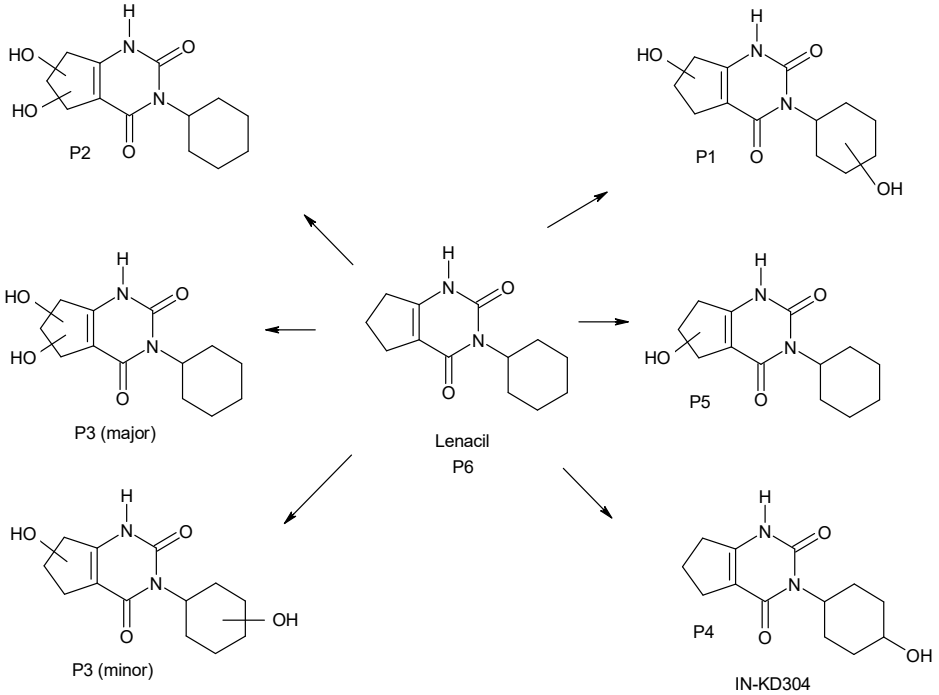
Radioactive component peak*	Retention time (min)	Mean % of dose recovered as radioactive components excreted in pooled urine	
		10 mg/kg bw	
		♂	♀
1	11-12	1.47	1.90
2	23-24	No peak	6.70
3	27-28	1.18	1.12
4	31-32	1.98	2.52
5	34-35	1.39	2.48
6	36-37	No peak	2.12
Total		**6.02**	**16.84**

***Excretion*:**
After a single low dose exposure, the majority of the dose was excreted in urine (59%); remaining radioactivity was excreted in faeces (31-34%). After repeated dosing, radioactivity excreted in the urine increased (72-86%) and less was detected in faeces (12-19%) and this could indicate an increase in oral absorption or an induction of biotransformation of lenacil. When the oral dose was high, reaching 1000 mg/kg bw, there was a saturation of intestinal absorption. Approximately 6-17% of the low dose was recovered in the bile at 24h (**table B.6.1.1.1-6**). Similar profile of metabolites to those seen in faeces and urine were identified in bile. These results could suggest that enterohepatic circulation occurs.

Figure B.6.1-1 Proposed metabolic pathway of [2-^{14}C]-Lenacil in animals

RMS: note that the actual hydroxylation of lenacil in order to obtain main metabolite P5=M35=INKQ961 is on the C5 or C6 (IUPAC nomenclature), and not "C3 or C4" (please also refer to B.6.8 and Vol.1 point 3.4.2).





B.6.1.2. Absorption, distribution, metabolism and excretion by other routes

The dermal absorption on the representative formulation is reported in the product DRAR. No other *in-vivo* ADME study was present and deemed necessary. However, an *in-vitro* metabolism study on isolated hepatocytes from rodent (rat, mouse), dog and human hepatic tissue was conducted and reported under B.6.1.3 below.

B.6.1.3. *In vitro* comparative metabolism

B.6.1.3.1 Interspecies comparison of in vitro metabolism of [Pyrimidine-2-^{14}C]Lenacil in mouse, rat, dog and human hepatocytes (Piñeiro Costas N, 2016).

Study report: Project 512721
Guideline(s): the study procedures were in compliance with the following guidelines:
The United States Food and Drug Administration: Guidance for Industry: Safety Testing of Drug Metabolites (February 2008) and followed international recommendations (1-11): Listing of international recommandations, see below:

1. *The Organisation for Economic Cooperation and Development (OECD) Good Laboratory Practice Guidelines (1997).*
2. *Bjornsson T.D., Callaghan J.T., Einolf H.J., Fischer V., Gan L., Grimm S., Kao J., King S.P., Miwa G., Ni L., Kumar G., McLead J., Obach R.S., Roberts S., Roe A., Shah A., Snikeris F., Sullivan J.T., Tweedie D., Vega J.M., Wrighton S.A., The conduct of in vitro and in vivo drug-drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective, Drug Metab. Dispos. 31(7) (2003), p. 815-32.*
3. *Huang S-M., Temple R., Throckmorton D.C. and Lekso L.J., Drug interaction studies: Study design, data analysis, and implications for dosing and labeling. Clin Pharmacol Ther 81 (2007), p. 298-304.*
4. *Huang S-M., Strong J.M., Zhang L., Reynolds K.S., Nallani S. et al., New era in drug interaction evaluation: US Food and Drug Administration update on CYP enzymes, transporters, and the guidance process. J. Clin. Pharmacol. 48 (2008), p. 662-670.*
5. *The United States Food and Drug Administration Good Laboratory Practice Regulations.*
6. *The United States Environmental Protection Agency Good Laboratory Practice Regulations.*
7. *Li A.P. (1997). Primary hepatocyte cultures as an in vitro experimental model for the evaluation of pharmacokinetic drug-drug interactions. Adv. Pharmacol. Series 43, 103-130.*
8. *Loretz L.J., Li A.P., Flye M.W. Wilson A.G. (1989). Optimization of cryopreserved procedures for rat and human hepatocytes. Xenobiotica 19(5), 489-498.*
9. *Ruegg C.E., Silber P.M., Mughal R.A., Ismail J., Lu C., Bode D.C., Li A.P. (1997). Cytochrome-P450 induction and conjugated metabolism in human hepatocytes after cryopreservation. In vitro Toxicology 10(2), 217-222.*
10. *Li A.P., Lu C., Brent J.A., Pham C., Fackett A., Ruegg C.E., Silber P.M. (1999). Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. Chem. Biol. Interact. 121, 17-35.*
11. *Gebhardt et al. (2003). New hepatocyte in vitro systems for drug metabolism: metabolic capacity and recommendations for application in basic research and drug development, standard operating procedures. Drug Metabolism Reviews 35(2&3), p 145-213.*

GLP status: yes (directive 2004/10/EC on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances) with deviation.

Deviations:
The method development part and the pilot incubation experiment were performed non-GLP for determination of the optimal experimental conditions for the analysis of the test item and possible metabolites.
Analysis of test item in vehicle for concentration, homogeneity was not performed, however, to limit the impact, the test item preparation was performed with approved procedures and documented in detail. Preparations were visually inspected for homogeneity prior to use.
These deviations did however not affect the integrity of the study.

The study is accepted.

Materials and Methods

[Pyrimidine-2-^{14}C]lenacil (batch CFQ42525, chemical purity not provided, radiochemical purity 97.6%, specific activity 4.74 bMBq/mg) was incubated at a single concentration (20 µM) for 1 ± 1, 30 ± 1, 60 ± 1, 90 ± 1 and 120 ± 1 minutes in duplicates with ♂CD-1 mouse (batch HEP136023, Biopredic international, Rennes, France), Sprague Dawley rat (batch HEP134033, Biopredic international, Rennes, France), Beagle dog (batch HEP185038, Biopredic international, Rennes, France) and human (pool of 10 donors, batch JKK, IVT Bioreclamation, Baltimore, Maryland, USA) hepatocytes in suspension. The incubation medium was a phosphate-buffered saline; its composition was as follows:

Components	Concentration (g/L)
$CaCl_2$ (anhydrous)	0.10
$MgCl_2.6H_2O$	0.10
KCl	0.20
KH_2PO_4	0.20
NaCl	8.00
$Na_2HPO_4.7H_2O$	2.16
D-Glucose	1.0
Sodium pyruvate	0.036

The cryopreserved hepatocytes were previously stored in liquid nitrogen until use; their viability was measured by Trypan blue exclusion. The metabolic activity of the hepatocytes was confirmed for each species by measuring one CYP dependent enzymatic activity (phenacetin metabolism) and phase II enzymatic activity (7-hydroxycoumarin metabolism).

After incubation, samples were analysed for metabolic stability and metabolic profile by LC-PDA-(RAD)MS, followed by radioactivity measurements by scintillation. Only peaks with >5% of total radioactivity in the chromatogram were profiled by MS. Reference compounds used were Lenacil TGAI (batch FEB12HE004), metabolites: IN-KE121 (AS1630, batch EXP-15-DA9895) and IN-KF313-002 (AS1631, batch 70409).

Findings

A linear relationship was observed with the developed analytical method between the [pyrimidine-2-^{14}C]Lenacil concentration and the response when using LC-MS.
[Pyrimidine-2-^{14}C]lenacil was chemically stable under the incubations conditions used.
For all four species, the hepatocytes viability after incubation was above 50% of initial value (which was 82% immediately after thawing from liquid nitrogen) and similar viability percentages were obtained in hepatocytes treated with the solvent control or [pyrimidine-2-^{14}C]lenacil, indicating that it was not cytotoxic in the conditions used.
The metabolic activity of all batches used met the criteria indicating that the quality was sufficient for use in the study.

Radioactivity chromatograms allowed to identify peaks for the mouse, rat, dog and human hepatocyte incubations with [pyrimidine-2-^{14}C]lenacil at different incubation time points.

A total of 42 radioactive peaks (including [pyrimidine-2-^{14}C]lenacil) was found in the different incubations (15, 39, 7 and 6 metabolites for mouse, rat, dog and human, respectively), of which 7 metabolites (M24, M25, M26, M32, M33, M35, M39) represented more than 5% of total radioactivity of the chromatogram in at least one species (5/1, 5/1, 0/1 and 0/1 metabolite(s)/parent for mouse, rat, dog and human, respectively).

No human-specific metabolite was detected. **Tables B.6.1.3.1-1** to **B.6.1.3.1-4** summarise data on the percentage of the total radioactivity detected in each individual peak for all incubation time points of mouse, rat, dog and human hepatocyte incubation samples, respectively.

A comparison of significant peaks (containing at least one value ≥5% of total radioactivity in at least one species) is given in **tables B.6.1.3.1-5**.

It is notably shown that, after 120 minutes of incubation with human (and dog) hepatocytes, most (≥90%) radioactivity was still associated with the parent compound, contrasting with a value <5% in the rat, in which extensive metabolisation was seen (also found in the mouse but to a more limited extent).

Table B.6.1.3.1-1 : Percentage of total radioactivity resulting from the analysis of hepatocytes incubation samples - Mouse

Time point (min)	Incubation	% of total radioactivity of the chromatogram																				
		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21
1	1																					
	2																					
30	1																					
	2																					
60	1																				0.55	
	2																				0.67	
90	1								0.19							0.39					1.07	
	2								0.39							0.34					1.50	
120	1																			1.21		
	2						0.39													1.28		

M: metabolite peak; TI: test item; blank cells = ND: radioactivity not detected; bold values are ≥ 5% of total radioactivity.

Table B.6.1.3.1-1 : Percentage of total radioactivity resulting from the analysis of hepatocytes incubation samples - Mouse (continued)

Time point (min)	Incubation	% of total radioactivity of the chromatogram																				
		M22	M23	M24	**M25**	M26	M27	M28	M29	M30	M31	**M32**	**M33**	M34	**M35**	M36	M37	M38	**M39**	**TI**	M40	M41
1	1																			**100**		
	2																			**100**		
30	1				2.19							1.23	1.84		**8.06**				2.45	**84.23**		
	2				1.59							1.88	1.40		**6.02**				2.84	**86.27**		
60	1				3.36					1.05		2.95	3.86		**11.64**				4.36	**72.23**		
	2				4.67					1.01		2.17	3.47		**10.98**				3.42	**73.60**		
90	1		0.34		**5.68**	0.58				1.70	0.49	4.17	**5.39**		**18.29**				**5.58**	**56.14**		
	2				**5.14**					1.70		3.64	4.27		**18.47**				**5.48**	**59.09**		
120	1				**6.46**	0.40				3.28	0.56	**5.35**	**6.21**		**19.19**				**5.40**	**51.92**		
	2				**6.85**	0.49				1.67	0.64	4.98	**5.17**		**18.28**	0.79			**5.37**	**54.09**		

M: metabolite peak; TI: test item; blank cells = ND: radioactivity not detected; bold values are ≥ 5% of total radioactivity.

Color code: percentage of total radioactivity

< 5%
≥ 5% and < 10%
≥ 10% and < 50%
≥50%

Table B.6.1.3.1-2 : Percentage of total radioactivity resulting from the analysis of hepatocytes incubation samples - Rat

Time point (min)	Incubation	% of total radioactivity of the chromatogram																				
		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21
1	1																					
	2																					
30	1											0.63	0.38	0.46	0.50					0.55		
	2											0.41	0.19	0.30						0.52		
60	1					0.47	0.26		0.34			1.07		0.77	1.28				0.30	0.86		
	2			0.62		0.35	0.93				0.42	1.00	0.77	1.16	1.12				0.27	0.62		
90	1			0.42		1.58	1.21		0.75		0.79	0.96	1.58	1.88	2.21	0.58	0.54	0.42		0.88		
	2	0.34	0.37	1.27		1.20		0.34	0.37		1.20		2.73	1.95	2.21	0.19				0.82	0.79	
120	1			1.63		3.08	1.76		0.97		1.14		4.44	2.38	3.08	0.92	0.53			0.79		
	2		0.78	0.60	0.78	1.16	2.35		0.45	0.52	0.97		4.18	1.45	2.83	0.30	0.71		0.48	1.12	0.63	0.30

M: metabolite peak; TI: test item; blank cells = ND: radioactivity not detected; bold values are ≥ 5% of total radioactivity.

Table B.6.1.3.1-2 : Percentage of total radioactivity resulting from the analysis of hepatocytes incubation samples – Rat (continued)

Time point (min)	Incubation	% of total radioactivity of the chromatogram																				
		M22	M23	**M24**	**M25**	**M26**	M27	M28	M29	M30	M31	**M32**	M33	M34	**M35**	M36	M37	M38	M39	**TI**	M40	M41
1	1																			**99.71**		
	2																			**100**		
30	1		2.10	**5.89**	**10.14**	**6.35**				0.50		**11.11**			**11.15**			0.80	2.57	**46.87**		
	2		2.97	4.45	**9.71**	**5.63**				0.70		**9.67**			**10.56**		0.56	0.89	2.48	**50.96**		
60	1	0.64	3.08	**8.47**	**17.28**	**7.96**				1.45	0.77	**15.40**	1.45		**13.73**			1.07	3.46	**19.89**		
	2	0.27	3.16	**7.75**	**16.01**	**7.72**			0.39	1.50	0.54	**16.44**			**13.39**		0.54	1.20	2.51	**21.84**		
90	1		4.00	**10.25**	**23.88**	**7.21**	0.42			1.25		**15.79**			**12.58**			1.13	1.96	**7.75**		
	2	0.64	4.46	**8.09**	**21.16**	**6.70**	0.82		0.49	1.95		**17.04**	0.82		**12.25**		0.56	0.94	2.02	**8.28**		
120	1		2.77	**9.11**	**24.24**	**7.00**			0.84	1.54	0.84	**16.28**		0.31	**10.82**	0.40		1.19	0.40	3.56		
	2	0.82	4.40	**7.49**	**25.13**	4.85		0.82	0.71	2.09	1.12	**15.73**		0.26	**10.25**		0.67	1.38	0.75	3.91		

M: metabolite peak; TI: test item; blank cells = ND: radioactivity not detected; bold values are ≥ 5% of total radioactivity.

Color code: percentage of total radioactivity

< 5%
≥ 5% and < 10%
≥ 10% and < 50%
≥50%

Table B.6.1.3.1-3 : Percentage of total radioactivity resulting from the analysis of hepatocytes incubation samples – Dog

Time point (min)	Incubation	% of total radioactivity of the chromatogram																				
		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21
1	1																					
	2																					
30	1																					
	2																					
60	1																					
	2																					
90	1																					
	2																					
120	1																					
	2																					

M: metabolite peak; TI: test item; blank cells = ND: radioactivity not detected; bold values are ≥ 5% of total radioactivity.

Table B.6.1.3.1-3 : Percentage of total radioactivity resulting from the analysis of hepatocytes incubation samples – Dog (continued)

Time point (min)	Incubation	% of total radioactivity of the chromatogram																				
		M22	M23	M24	M25	M26	M27	M28	M29	M30	M31	M32	M33	M34	M35	M36	M37	M38	M39	TI	M40	M41
1	1																	0.29	0.49	**99.22**		
	2																		0.19	**99.81**		
30	1											0.80			0.95				0.85	**96.94**	0.45	
	2											0.44			1.43				0.93	**97.20**		
60	1											1.20			2.79				0.63	**95.38**		
	2											0.67			2.32				1.13	**95.88**		
90	1				0.55							1.75			3.75				1.65	**92.30**		
	2					0.39						1.22			4.14				1.12	**99.70**	0.44	
120	1											2.47			3.85				1.05	**92.34**		0.29
	2				0.29							1.62			3.34				1.62	**93.12**		

M: metabolite peak; TI: test item; blank cells = ND: radioactivity not detected; bold values are ≥ 5% of total radioactivity.

Color code: percentage of total radioactivity

- < 5%
- ≥ 5% and < 10%
- ≥ 10% and < 50%
- ≥50%

Table B.6.1.3.1-4 : Percentage of total radioactivity resulting from the analysis of hepatocytes incubation samples – Human

Time point (min)	Incubation	% of total radioactivity of the chromatogram																				
		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21
1	1																					
	2																					
30	1																					
	2																					
60	1																					
	2																					
90	1																					
	2																					
120	1																					
	2																					

M: metabolite peak; TI: test item; blank cells = ND: radioactivity not detected; bold values are ≥ 5% of total radioactivity.

Table B.6.1.3.1-4 : Percentage of total radioactivity resulting from the analysis of hepatocytes incubation samples – Human (continued)

Time point (min)	Incubation	% of total radioactivity of the chromatogram																				
		M22	M23	M24	M25	M26	M27	M28	M29	M30	M31	M32	M33	M34	M35	M36	M37	M38	M39	**TI**	M40	M41
1	1																		0.5	**99.5**		
	2																			**99.76**		0.24
30	1											0.86			0.82				0.90	**97.43**		
	2											0.92			1.28				0.48	**97.00**	0.32	
60	1											1.82			1.49				0.69	**96.00**		
	2											2.20			1.75				0.57	**95.48**		
90	1											2.30			1.48				0.99	**94.90**	0.33	
	2											1.91			2.37				0.89	**94.48**	0.35	
120	1											2.12			2.45				0.83	**94.35**	0.25	
	2											2.40			1.73	0.28			1.10	**94.13**	0.35	

M: metabolite peak; TI: test item; blank cells = ND: radioactivity not detected; bold values are ≥ 5% of total radioactivity.

Color code: percentage of total radioactivity

- < 5%
- ≥ 5% and < 10%
- ≥ 10% and < 50%
- ≥50%

Table B.6.1.3.1-5: Comparison of significant peaks (containing at least one value ≥ 5% of total radioactivity in at least one species) across species.

Time point (min)	Incubation	% of total radioactivity of the chromatogram															
		Metabolite 24				Metabolite 25				Metabolite 26				Metabolite 32			
		M	R	D	H	M	R	D	H	M	R	D	H	M	R	D	H
1	1																
	2																
30	1		**5.89**			2.19	**10.14**				**6.35**			1.23	**11.11**	0.80	0.86
	2		4.45			1.59	**9.71**				**5.63**			1.88	**9.67**	0.44	0.92
60	1		**8.47**			3.36	**17.28**				**7.96**			2.95	**15.40**	1.20	1.82
	2		**7.75**			4.67	**16.01**				**7.72**			2.17	**16.44**	0.67	2.20
90	1		**10.25**			**5.68**	**23.88**	0.55		0.58	**7.21**			4.17	**15.79**	1.75	2.30
	2		**8.09**			**5.14**	**21.16**				**6.70**	0.39		3.64	**17.04**	1.22	1.91
120	1		**9.11**			**6.46**	**24.24**			0.40	**7.00**			**5.35**	**16.28**	2.47	2.12
	2		**7.49**			**6.85**	**25.13**	0.29		0.49	4.85			4.98	**15.73**	1.62	2.40

TI: test item; blank cells = ND: radioactivity not detected; bold values are ≥ 5% of total radioactivity.

Table B.6.1.3.1-5: Comparison of significant peaks (containing at least one value ≥ 5% of total radioactivity in at least one species) across species (continued)

Time point (min)	Incubation	% of total radioactivity of the chromatogram															
		Metabolite 33				Metabolite 35				Metabolite 39				TI (lenacil, parent)			
		M	R	D	H	M	R	D	H	M	R	D	H	M	R	D	H
1	1											0.49	0.5	**100**	**99.71**	**99.22**	**99.5**
	2											0.19		**100**	**100**	**99.81**	**99.76**
30	1	1.84				**8.06**	**11.15**	0.95	0.82	2.45	2.57	0.85	0.90	**84.23**	**46.87**	**96.94**	**97.43**
	2	1.40				**6.02**	**10.56**	1.43	1.28	2.84	2.48	0.93	0.48	**86.27**	**50.96**	**97.20**	**97.00**
60	1	3.86	1.45			**11.64**	**13.73**	2.79	1.49	4.36	3.46	0.63	0.69	**72.23**	**19.89**	**95.38**	**96.00**
	2	3.47				**10.98**	**13.39**	2.32	1.75	3.42	2.51	1.13	0.57	**73.60**	**21.84**	**95.88**	**95.48**
90	1	**5.39**				**18.29**	**12.58**	3.75	1.48	**5.58**	1.96	1.65	0.99	**56.14**	7.75	**92.30**	**94.90**
	2	4.27	0.82			**18.47**	**12.25**	4.14	2.37	**5.48**	2.02	1.12	0.89	**59.09**	**8.28**	**99.70**	**94.48**
120	1	**6.21**				**19.19**	**10.82**	3.85	2.45	**5.40**	0.40	1.05	0.83	**51.92**	3.56	**92.34**	**94.35**
	2	**5.17**				**18.28**	**10.25**	3.34	1.73	**5.37**	0.75	1.62	1.10	**54.09**	3.91	**93.12**	**94.13**

TI: test item; blank cells = ND: radioactivity not detected; bold values are ≥ 5% of total radioactivity.
M: mouse; R: rat; D: dog; H: Human

Color code: percentage Color code: percentage of total radioactivity

- < 5%
- ≥ 5% and < 10%
- ≥ 10% and < 50%
- ≥50%

Table B.6.1.3.1-6: Comparison of significant peaks (containing at least one value ≥ 5% of total radioactivity in at least one species) across species: Major metabolites detected in hepatocyte samples of mouse, rat, dog and human, and their associated metabolic reaction.

Metabolite code	Metabolic reaction	Structure proposed
M24	Oxidation + dehydrogenation=IN-KE121 or isomer	See proposed metabolic pathway
M25	Oxidation on cyclohexane ring	See proposed metabolic pathway
M26	Oxidation on cyclohexane ring	See proposed metabolic pathway
	Oxidation, dehydrogenation and glutathione conjugation	
M32	Dehydrogenation on cyclopentene ring	See proposed metabolic pathway
M33	Oxidation on cyclopentene ring	See proposed metabolic pathway
M35	**Oxidation on cyclopentene ring**	See proposed metabolic pathway
	Oxidation + glucuronic acid conjugation	See proposed metabolic pathway
M39	Dehydrogenation on cyclopentene ring	See proposed metabolic pathway
Lenacil	Parent compound	

(bold indication corresponds with the major metabolite both *in-vivo* and *in-vitro*).

CONCLUSION of the comparative *in-vitro* metabolism study

[pyrimidine-2-^{14}C]Lenacil showed high metabolic stability in human and dog hepatocytes (96% and 103%, respectively, remaining after 120 min incubation).
The extent of conversion of [pyrimidine-2-^{14}C]Lenacil in the hepatocytes was ~50% in mouse, ~96% in rat, ~7% in dog , and ~6% in human after 120 minutes of incubation.
Thus the metabolism in isolated hepatocytes appears to be much more extensive in rodents as compared to that in the human and the dog.

The calculated *in vitro* $T_{1/2}$ values were 115 minutes for mouse hepatocytes, 26 minutes for rat hepatocytes and were above 120 min for dog and human hepatocytes: rat < mouse < dog ~ human.

A total of 42 radioactive peaks were found in the different incubations, of which 7 metabolites (M24, M25, M26, M32, M33, M35, and M39) represented above 5% of total radioactivity of the chromatogram in at least one species.

All of the major metabolites (≥ 5% of total radioactivity of the chromatogram) could be identified and structures are proposed. Metabolic reactions observed included oxidation, dehydrogenation and combinations with glutathione conjugation and glucuronidation.

The recovered and identified metabolites *in-vitro* are in line with those found *in-vivo* in the rat. No human-specific metabolite was detected.

Figure B.6.1-2: Proposed metabolic reactions for [pyrimidine-2-^{14}C]Lenacil in incubations with mouse, rat, dog and human hepatocytes

B.6.1.4. Summary of the absorption, distribution, metabolism and excretion of Lenacil

Absorption

Based upon the urinary excreted radioactivity after a single dose (10 mg/kg b.w.), oral absorption represents 59% of the administered low dose. Taking into account the metabolite identification in urine, it appears clearly that oral absorption is more important as also suggested by the results of repeated dosing (7 doses) where urine radio-activity amounts to 72 (♂)-86(♀)% at 144h. In contrast, at high dose (1000 mg/kg b.w.), urinary excretion is limited to about 8%, indicating a saturation effect.

In the repeated dose toxicity studies in rats and dogs and in the carcinogenicity study in rodents, the liver was identified to be at target. For this reason, the excretion via the bile (7% in ♂ and 17% in ♀ which was determined at 12 and 24 hours after oral administration could potentially be taken into account in the determination of the total absorption.

Therefore, the total gastrointestinal absorption, after repeated low-dose administration (the most relevant), taking into account urine excretion, tissues and residual carcass levels, would amount to 77+6 (bile)=**83%** in the ♂, and 89 (17% bile)~**100%** in the ♀.

Distribution

Maximal lenacil-related tissue concentration is observed in the gastrointestinal tract as well as in its content. High concentrations are also identified in excretory organs (liver, kidneys). Six days after oral administration, radioactivity is still detected in a large part of tissues at a very low concentration, with a higher concentration in excretory organs. At that time, at 10 mg/kg bw, ♂ rat carcass retained the highest concentration representing 0.78% of the administered dose. The increase of total residue concentration between the low and high dose groups was less than the 100-fold increase in the dose. There was no evidence of tissue accumulation after single dose administration.

Metabolism

The *in-vivo* metabolism of the absorbed lenacil is extensive. Up to about 58% of the administered dose was characterised in urine after a single oral low dose, and up to 81% after repeated dosing. The major biotransformation pathway is hydroxylation of either the cyclohexyl or cyclopentenyl ring, or both rings. The major component in urine was a hydroxylated metabolite of lenacil with the hydroxyl group on C5 or C6. No glucuronide or sulfate conjugates is released by glucuronidase or sulfatase.

An *in-vitro* experiment was conducted in order to investigate the comparative metabolism in rat, mouse, dog and human hepatocytes. The findings suggest that the metabolism is much more extensive in rodents as compared to that in the human and the dog. All of the major metabolites (≥5% of total radioactivity) could be identified and structures are proposed. Metabolic reactions observed included oxidation, dehydrogenation and combinations with glutathione conjugation and glucuronidation. The recovered and identified metabolites *in-vitro* are in line with those found *in-vivo* in the rat. No human-specific metabolite was detected.

Excretion

Radioactivity is mainly excreted into urine within 12-24h. Urine represents the main excretion route after low single dose reaching about 60% of the dose (as compared to about 32% excreted in the faeces). There is no important quantitative differences between ♂ and ♀ rats.

When the oral dose is repeatedly administered, urinary excretion is increased to 72-86% (as compared to about 12-19% faecal excretion) and a slight delay in excretion occurs as well, suggesting an increase in oral absorption and/or an induction of biotransformation of lenacil.

After oral high dose administration, urinary excretion is strongly reduced to 5-8% of the dose (while faecal excretion amounts to 81-87% of the dose), suggesting saturation of intestinal absorption.

Recovery of radioactivity ranges between 92 and 100%.

The metabolic profile identified in bile, was similar to that seen in faeces and urine. These results could suggest that enterohepatic circulation may occur.

B.6.2 (CA 5.2) Acute toxicity

B.6.2.1 (CA 5.2.1) Oral toxicity

Lenacil technical - Acute oral toxicity to the rat (acute toxic class method) ([redacted] 2001a)

DuPont Report No.: ACD 004/013224/AC

Guidelines: Dir 96/54/EEC Method B.1 tris, equivalent to OECD 423 (1996).

GLP status: yes

Materials and Methods

Five (5) fasted ♀rats (Sprague Dawley) received a single oral gavage dose of Lenacil technical (Lot/Batch #: 141712003, Purity: 98.6%), formulated in 1% w/v aqueous methylcellulose, at a dose level of 5000 mg/kg bodyweight. As results at this dosage indicated the acute lethal oral dose of the test material to be greater than 5000 mg/kg bodyweight, in compliance with the study guidelines, a group of five fasted ♂ was dosed at 5000 mg/kg to confirm results at this dosage and complete the study. No control animals were included in this study.

The study is accepted

Findings

Mortality: There were no deaths during the study.

Clinical signs: Clinical signs of reaction to treatment were confined to piloerection, seen in all females (5/5) only approximately one hour after dosing. Recovery of rats, as judged by external appearance and behaviour, was complete by Day 2. No clinical signs of reaction to treatment were observed in any of the males throughout the study.

Body weight: All animals were considered to have achieved satisfactory bodyweight gains throughout the study.

Macroscopic examination and pathology: No abnormalities were revealed at the macroscopic examination at study termination on Day 15.

Conclusion

The acute oral LD_{50} value of lenacil technical was determined to be greater than 5000 mg/kg bw in rats.

A classification and labelling of lenacil with respect to acute oral toxicity is not required.

- Approximate lethal dose (ALD) of IN E1512-2 in rats, HLR564-89 [HLR 564-89] ([redacted], 1989) ([redacted])

Transferred to the confidential DAR (vol. 4)

B.6.2.2 (CA 5.2.2) Dermal toxicity

Lenacil technical - Acute dermal toxicity to the rat ([redacted], 2001b)

DuPont Report No.: ACD 005/013220/AC

Guidelines: EEC Directive 92/69/EEC Method B.3, equivalent to OECD 402 (1987).

GLP status: yes (except for stability/homogeneity/concentration of the formulation)

Materials and Methods

Five (5) rats (Hsd:Sprague-Dawley strain) /sex were treated at 5000 mg/kg bodyweight with lenacil technical (Batch No. 141712003, purity 98.6%)

One day prior to treatment, hair was removed from the dorso-lumbar region of each rat with electric clippers and an area equivalent to approximately 10% of the total body surface area was exposed. The treatment area (approximately 50 mm x 50 mm) was covered with porous gauze held in place with a non-irritating dressing, and further covered by a waterproof dressing encircled firmly around the trunk of the animal.

Treatment in this manner was performed on Day 1 (day of dosing) of the study only.

At the end of the 24 hours exposure period, skin was washed with warm water (30 - 40 C) to remove any residual test substance. The treated area was blotted dry with absorbent paper. No control animals were included in this study.

The study is accepted.

Findings

Mortality and clinical signs: There were no deaths and no systemic response to treatment following a single dermal application of Lenacil Technical to a group of ten rats (five males and five females) at a dose level of 5000 mg/kg bodyweight.

Dermal response: No dermal responses were observed for any animal throughout the study.

Body weight loss was recorded for one female and a low bodyweight gain was recorded for one further female on Day 8. All remaining animals were considered to have achieved satisfactory bodyweight gains throughout the study.

Macroscopy: No abnormalities were recorded at the macroscopic examination at study termination on Day 15.

Conclusions

The acute dermal LD_{50} value of lenacil technical was determined to be greater than 5000 mg/kg bw in rats.

A classification and labelling of lenacil with respect to acute dermal toxicity is not required.

B.6.2.3 (CA 5.2.3) Inhalation toxicity

Lenacil technical - Acute (four-hour) inhalation study in rats ([redacted], 2001)

DuPont Report No.: ACD 021/013229

Guidelines: EEC Directive 92/96/EEC Method B.2, equivalent to OECD 403 (1987).

GLP status: yes

Materials and Methods

Five (5) rats (Crl: CD (SD) IGS BR, Sprague-Dawley in origin) /sex were exposed snout-only to a mean concentration of 5.12 mg/L particulate aerosol atmosphere of Lenacil technical (Batch No. 141712003, purity 98.6%) for 4 hours. The test substance was generated using a Wright Dust Feed Mechanism, and during exposure 10 samples were taken for total Lenacil technical concentration and 2 samples for particle size determination. The Mass Median Aerodynamic Diameter (MMAD) of the Lenacil technical atmosphere was 5.2 µm and the proportion considered respirable (< than 7µm) was 62%. A similar sized control group of rats was run concurrently with the test animals but were only 'exposed' to air.

The study is accepted.

Findings

Mortality: There were no unscheduled deaths.

Clinical signs:
- During the exposure - Exaggerated breathing was evident in a proportion of test rats from 30 minutes, and all test rats from 4 hours into exposure.
Soiling of the fur with excreta was observed in all control and test group rats from 1 and 2 hours into exposure respectively and was considered to be associated with the method of restraint.
- During the observation period - Exaggerated breathing was evident in all test rats immediately following exposure, persisting to at least 2 hours post exposure. Brown staining around snout/jaws was noted for a female test rat on Day 1.
Soiling of the fur with excreta was noted in all control and test rats immediately following exposure. This sign was considered to be associated with the method of restraint used for exposure.
All test rats were normal in appearance and behaviour from Day 2 of the observation period.

Bodyweight:
Slightly increased mean bodyweight gains were evident compared with control males for male test rats throughout the 14-day observation period.

Water consumption:
There were no treatment-related effects.

A visual appraisal of the water bottles indicated that the amount of water consumed by test rats was similar to that of the control rats.

Necropsy findings:
There were no treatment-related findings noted at necropsy. Lung weights were normal.

Conclusion

The LC_{50} (4-hour) for Lenacil technical is > 5.12 mg/L in air.

A classification and labelling of lenacil with respect to acute inhalation toxicity is not required.

B.6.2.4 (CA 5.2.4) Skin irritation

Skin irritation to the rabbit ([redacted] E.L., 2001c)

DuPont Report No.: ACD 006/013201/SE

Guidelines: EEC Directive 92/69/EEC Method B.4, equivalent to OECD 404 (1992)

GLP status: yes

Materials and Methods

Approximately 24 hours prior to application of the test substance, hair was removed with electric clippers from the dorso-lumbar region of 3 female New Zealand rabbit exposing an area of skin approximately 100 mm x 100 mm. Approximately 0.5 g of Lenacil technical (Batch No. 141712003, purity 98.6%) was applied under a 2-ply 25 mm x 25 mm porous gauze pad, which had been moistened with 0.5 ml distilled water, to one intact skin site on each animal. Each treatment site was covered with elastic adhesive dressing for four hours. The animals were not restrained during the exposure period and were returned to their cages immediately after treatment. At the end of the exposure period, the semi-occlusive dressing and gauze pad were removed and the treatment site was washed with warm water (35°C) to remove any residual test substance. The treated area was blotted dry with absorbent paper.

The study is accepted

Findings

No erythema or edema was observed in any application sites of the animals at any observation time.

<Score erythema>24+48+72h =0

<Score oedema >24+48+72h =0

Table B.6.4.1-1 Skin irritation to the rabbit ([redacted] 2001): individual scores

Animal number +sex	Clinical sign	Day			
		1*	2	3	4
2562 Female	Erythema	0	0	0	0
	Oedema	0	0	0	0
2563 Female	Erythema	0	0	0	0
	Oedema	0	0	0	0
2564 Female	Erythema	0	0	0	0
	Oedema	0	0	0	0

*Approximately 60 minutes after removal of the dressing.

Conclusion

Lenacil technical elicited no dermal irritation.

A classification and labelling of lenacil as a potential skin irritant is not required.

B.6.2.5 (CA 5.2.5) Eye irritation

Eye irritation to the rabbit ([redacted]., 2001d)

DuPont Report No.: ACD 007/013273/SE

Guidelines: EEC 92/69/EEC Method B.5, equivalent to OECD 405 (1987).

GLP status: yes

Materials and Methods

The eyes of 3 female rabbits New Zealand White were examined prior to instillation of the test substance to ensure that there was no pre-existing corneal damage, iridial or conjunctival inflammation.

Screen study - one animal - rinsed eye

One animal was treated in advance of the others, to ensure that if a severe response was produced, no further animals would be exposed. The treated eye of this animal was rinsed with distilled water approximately 30 seconds after instillation for duration of approximately 30 seconds.

Main study - three animals - unrinsed eyes

One animal was treated in advance of the other two, again to ensure that if a severe response was produced, no further animals would be exposed. A volume of 0.1 ml of lenacil technical (Batch No. 141712003, purity 98.6%) (Mean weight 70 mg) was placed in the lower reverted lid of one eye of each animal. The eyelids were then gently held together for one second before releasing. The contra lateral eye remained untreated.

The study is accepted.

Findings

In unwashed eyes:

<Score cornea opacity>$_{24+48+72h}$	= 0	/0	/0
<Score iris>$_{24+48+72h}$	= 0	/0	/0
<Score erythema>$_{24+48+72h}$	= 0.3	/0.3	/0
<Score chemosis>$_{24+48+72h}$	= 0	/0	/0

Conclusion

Lenacil is not irritating to eyes under these experimental conditions.

A classification and labelling of lenacil as a potential eye irritant is not required.

B.6.2.6 (CA 5.2.6) Skin sensitisation

Closed-patch repeated insult dermal sensitisation study (maximisation method) with DPX-B634-91 in guinea pigs ([redacted], 1992)

DuPont Report No.: HLO 34-92

Guidelines: not fully in compliance with Dir EEC 96/54/EEC, Annex IV C or 92/69-84/449 or OECD test guideline n 406 (1981-92).

Deviation from official protocol:

intradermal induction is performed with a too low concentration. It is considered (Notifier) that "*this deviation does not affect the validity of the study. Hence, the study is considered to be valid*".

GLP status: yes (no attest of national authority)

Materials and Methods

A preliminary range finding test was performed to determine the intradermal and topical irritation potential.

The test was performed in adult ♂ and ♀ Duncan Hartley albino Guinea pigs.

For the main study, the intradermal induction phase was conducted in 20 guinea pigs by intradermally injecting 0.1 mL of a 1.5% (w/v) suspension of lenacil technical (Batch No. 9038, purity 98.2% (reanalysed 98.5%) with or without Freunds Complete Adjuvant. Seven days after the intradermal induction phase a topical induction was performed using patches with 0.3 mL of control, test article or positive control article. Two weeks later, a topical challenge was performed. For both, intradermal and topical induction phases, the test article was dosed at a 25% concentration.

1-chloro-2, 4-dinitrobenzene was used as positive control

The study is accepted.

Findings

Based on the results of the range finding study performed with intradermal injections of 0.1 mL at 0.5, 1.5, 3.0 and 5% suspensions of lenacil in 0.9% saline, the test article was dosed at 1.5% concentration.

In the topical range finding test, no signs of irritation were observed at 1.0, 5.0, 10 or 25% concentration in petrolatum. The test article was dosed at a 25% concentration for the topical induction and challenge.

During the challenge phase, slightly patchy mild redness was observed in one animal each in both the test and vehicle control groups. Slightly patchy mild to severe redness and swelling was observed in the positive control animals.

Conclusion

Lenacil was found to be not a potential skin sensitiser in guinea pigs.

A classification and labelling of lenacil with respect to skin sensitisation is not required.

B.6.2.7 (CA 5.2.7) Phototoxicity

Evaluation of in vitro phototoxicity of lenacil TGAI in 3T3 fibroblasts using the Neutral Red Uptake (NRU) assay (Westerink WMA, 2016)

Guideline(s): compliant with Regulation (EC) No. 440/2008, Part B.41 (31 May 2008) and OECD TG432 as of 13 April 2004.

GLP: yes OECD -*Principles of Good Laboratory Practice concerning Mutual Acceptance of Data in the Assessment of Chemicals , 26 November 1997*- and EC -*directive 2004/10/EC on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances*- with deviation.

Deviation : analysis of test item in vehicle for concentration, homogeneity was not performed, however, to limit the impact, the test item preparation was performed with approved procedures and documented in detail. Preparations were visually inspected for homogeneity prior to use.

Material and Methods:

Cultures of Balb/c 3T3 fibroblasts (clone 31) were seeded in 96-well plates at a density of 10000 cells per well in a total volume of 100 µL. Plates were then incubated for 40-48 h in an incubator to obtain approximately 40-60% confluence. No cells were seeded in columns 11 and 12 (blanks) of the 96-well plate. Plates were then incubated for 40-48 h in an incubator to obtain approximately 40-60% confluence. The culture medium was removed from the plates and the wells were washed with DPBS. Aliquots (200 µL) of EBSS medium containing concentrations of the:

- test item (Lenacil TGAI, batch FEB12HE004, purity: 98.6% according to the CoA,
- vehicle control (1% dimethyl sulphoxide of spectroscopic quality (DMSO; SeccoSolv, Merck, Darmstadt, Germany) in Earle's Balanced Salt Solution (EBSS; Invitrogen, Breda, The Netherlands),
- positive control (Anthracene) and
- negative control (Sodium dodecyl sulphate (SDS)

were applied to duplicate plates. For all compounds, 8 test concentrations were tested in 8 independent replicates. Columns 9-12 contained incubation medium. Plates were incubated in the dark at 37 C in a humidified atmosphere (80-100%) of 5% (v/v) CO2 in air for 60 minutes. One plate for the test item, negative control, and positive control (designated "+Irr" plates) was then irradiated using the UVA light source. Plates received a concentration of 5 J/cm^2 UV-A. The remaining plates (designated "-Irr" plates) were kept in the dark at room temperature for the same period of time. The test solutions were then removed from the plates, and the cells were washed once with DPBS. Two hundred µL of supplemented DMEM per well were added and the plates were incubated for 20-24 h in an incubator.

Microscopic evaluation of cytotoxicity

Following incubation, the control wells on each plate were inspected for confluency. All cells were examined for growth, morphology, and integrity of the monolayer using a phase contrast microscope. No changes in cell morphology and effects on cell growth were observed.

Evaluation of cytotoxic effects with the Neutral Red Uptake test

The cytotoxicity was expressed as a concentration-dependent reduction of the uptake of the Neutral Red dye approximately 20-24 h after treatment with the test item, in the presence and absence of UV-A irradiation.

Immediately following the visual assessment, medium was removed, and 100 µL of Neutral Red dye (50 µg/mL in DMEM) was added. The plates were then incubated at 37 ± 1 C in a humidified atmosphere (80-100%) of 5% (v/v) CO_2 in air for approximately 3.5 hours. Following the incubation, the Neutral Red solution was removed and the cells were washed at least once with 150 µL of DPBS which was removed before adding 150 µL of Neutral Red destain solution (ethanol:acetic acid:distilled water, 50:1:49). Plates were shaken on a plate shaker for approximately 20-40 minutes until all Neutral Red was extracted from the cells.

Analysis was performed using a TECAN Infinite® M200 Pro Plate Reader at a wavelength setting of 540 nm. Neutral Red absorbance was expressed in terms of absolute optical density (OD_{540}).

The OD540 was measured for each concentration of the test item, negative control, and positive control on the "+UVA" and "-UVA" plates and were compared to the OD_{540} of the vehicle control(s), as appropriate.

Findings

Lenacil TGAI was evaluated for phototoxicity in the *in vitro* 3T3 NRU at concentrations of 0.01, 0.316, 0.1, 0.316, 1.0, 3.16, 10.0 and 31.6 µg/mL.
An overview of the cytotoxicity, IC_{50} and PIF values of Lenacil TGAI are presented in table B.6.2.7-1
Results of the controls are tabulated in tables B.6.2.7-2 (negative control) and B.6.2.7-3 (positive control).

Table B.6.2.7-1 *in vitro* phototoxicity of lenacil in 3T3 fibroblasts (Westerink, 2016): Overview of the cytotoxicity of Lenacil TGAI and calculated IC_{50} and PIF values

Concentration (µg/mL)	31.62	10.00	3.16	1.00	0.32	0.10	0.03	0.01	Vehicle control	IC_{50} (µg/mL)	PIF
- UV	1.28 ± 0.04	1.34 ± 0.02	1.32 ± 0.02	1.32 ± 0.02	1.33 ± 0.01	1.32 ± 0.02	1.36 ± 0.03	1.34 ± 0.02	1.38 ± 0.02	No value	No PIF
+ UV	1.33 ± 0.02	1.34 ± 0.01	1.30 ± 0.03	1.32 ± 0.03	1.30 ± 0.02	1.30 ± 0.01	1.36 ± 0.02	1.32 ± 0.01	1.34 ± 0.01	No value	

No significant cytotoxicity was observed at any of the test concentrations in the absence and presence of UV-A. Therefore no IC_{50} values could be calculated for Lenacil. The highest concentration of Lenacil TGAI tested was the limit of solubility (31.6 µg/mL).

Table B.6.2.7-2 *in vitro* phototoxicity of lenacil in 3T3 fibroblasts (Westerink, 2016): Overview of the cytotoxicity of Sodium dodecyl sulphate and calculated IC_{50} and PIF values

Concentration (µg/mL)	316.2	100.0	31.62	10.00	3.16	1.00	0.32	0.10	Vehicle control	IC_{50} (µg/mL)	PIF
- UV	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	1.18 ± 0.03	1.32 ± 0.03	1.33 ± 0.02	1.37 ± 0.02	1.36 ± 0.01	1.39 ± 0.02	12.36	0.66
+ UV	0.02 ± 0.01	0.05 ± 0.01	0.12 ± 0.02	1.23 ± 0.01	1.26 ± 0.02	1.28 ± 0.01	1.28 ± 0.01	1.30 ± 0.01	1.32 ± 0.01	18.66	

The negative control SDS showed an IC_{50} value of 12.36 µg/mL and 18.66 µg/mL in the absence and presence of UV-A treatment, respectively, resulting in a PIF value of 0.66.

Table B.6.2.7-3 *in vitro* phototoxicity of lenacil in 3T3 fibroblasts (Westerink, 2016): Overview of the cytotoxicity of Anthracene and calculated IC_{50} and PIF values

Concentration (µg/mL)	31.62	10.00	3.16	1.00	0.32	0.10	0.03	0.01	Vehicle control	IC_{50} (µg/mL)	PIF
- UV	1.59 ± 0.03	1.51 ± 0.01	1.45 ± 0.03	1.45 ± 0.02	1.46 ± 0.03	1.44 ± 0.02	1.43 ± 0.02	1.42 ± 0.01	1.41 ± 0.01	> 31.62	>3162
+ UV	0.11 ± 0.02	0.07 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	0.07 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	1.37 ± 0.01	< 0.01	

The positive control Anthracene showed an IC_{50} value of >31.6 and <0.01 µg/mL in the absence and presence of UV-A treatment, respectively, resulting in a PIF value of >3162.

The mean OD_{540} value (corrected for the blank) of UV-A treated and non-treated control cells was 1.34 (individual values 1.37, 1.32, 1.34) and 1.39 (individual values 1.41, 1.39, 1.38) respectively resulting in a cell viability of 96% after UV-A treatment.

Conclusion:

Since no toxicity was observed after treatment with Lenacil both after exposure with and without UV-A treatment, no IC_{50} values and Photo Irritation Factor (PIF) could be calculated leading to the classification 'non-phototoxic'. The highest concentration of Lenacil tested was the limit of solubility (31.6 µg/mL).

It is thus concluded that **Lenacil TGAI is classified as non-phototoxic** under the experimental conditions described in this report.

B.6.2.8 (CA 5.2.8) Summary of the acute toxicity studies

Results of studies assessing acute toxicity, primary irritation, sensitisation and phototoxicity with lenacil are summarised in table 6.2-1.

It was concluded that lenacil has a low acute toxicity for all routes evaluated (oral, dermal, inhalation) after single exposure. It was not irritating to the skin and eye.

It did not show any allergenic or phototoxic potential. No classification was required.

Table B.6.2-1 Summary of acute toxicity

Type of test, test species	Batch n°, purity	Results	References
Acute oral toxicity, rat	Batch n° 141712003, Purity 98.6%	LD_{50} > 5000 mg/kg	[redacted] 2001a
Acute dermal toxicity, rat	Batch n° 141712003, Purity 98.6%	LD_{50} > 2000 mg/kg	[redacted] 2001b
Acute inhalation toxicity, rat	Batch n° 141712003, Purity 98.6%	LC_{50} (4 hours) > 5.12 mg/L	[redacted] 2001
Skin irritation, rabbit	Batch n° 141712003, Purity 98.6%	Not irritating	[redacted] 2001c
Eye irritation, rabbit	Batch n° 141712003, Purity 98.6%	Not irritating	[redacted] 2001d
Skin sensitisation study, maximisation method	Batch n°9038, Purity 98.2%	Not sensitising	[redacted], 1992
Phototoxicity	Batch n°FEB12HE004, Purity 98.6%	Not phototoxic	Westerink, 2016

B.6.3 (CA 5.3) Short-term toxicity

The following short-term toxicity studies were conducted with lenacil: 28-day feeding studies in rats and dogs and 90-day feeding studies in rats, mice and dogs, respectively.

B.6.3.1 (CA 5.3.1) Oral 28-day study

It is not mandatory to conduct either 14-day or 28-day oral studies if subchronic studies are available. The 28-day oral studies (Point IIA 5.3.1) provided useful toxicity data for follow-up studies of longer study duration and are thus summarised below.

B.6.3.1.1

Lenacil technical: preliminary study by dietary administration to Han Wistar rats for 4 weeks ([redacted] 2002) DuPont Report No.: ACD 001/010098

Guidelines: study is not fully in compliance with Dir. EEC 96/54/EEC Annex IV D or 92/69-84/449 or OECD test guideline n° 407 (1995-81).

Deviation from official protocol: no blood tests were performed. Tissues are stored but not examined for histopathology.

GLP status: yes (no attest of QAU and national authority)

Materials and Methods

Lenacil technical (Batch No. 141712003, purity 98.6%) was incorporated into the ground diet to provide the required concentrations. The mean concentrations for Lenacil technical as analysed from feed samples of dosing weeks 1 and 3 ranged from 97.5% to 106% of nominal concentrations and were considered satisfactory.

Three groups of five male and five female Han Wistar rats received Lenacil technical in the diet for four weeks. One group received 5000 ppm throughout the treatment period. The other groups received 10000 or 20000 ppm for the first two weeks and, following an absence of any treatment related effect, these concentrations were raised to 30000 or 50000 ppm, respectively, during weeks 3 and 4. A similarly constituted Control group received the basal diet only.

Week 1 and 2	0		5000 ppm		10000 ppm		20000 ppm	
Week 3 and 4	0		5000 ppm		30000 ppm		50000 ppm	
Achieved dose :	M	F	M	F	M	F	M	F
mg/kg bw/d wk 1-2	0	0	571	631	1269	1288	2545	2643
mg/kg bw/d wk 3-4	0	0	571	631	2978	3576	5029	5913

Statistical analysis: organ weight, body weight changes, homogeneity of variance was tested using the Bartlett's test. When statistically significant, a Behrens-Fischer test was used to perform comparisons, otherwise a Dunnett's test was used.

The study is accepted.

Findings

Notifier asserted: "The oral 28-day feeding study in male and female Han Wistar rats (DuPont Report ACD 001/010098) was originally submitted under EU Rev8 Point IIA 5.3.1 and has been conducted with lenacil technical. No information on the guidelines used was reported. A review of this study indicates that it partially meets the current OECD Test Guideline 407; deviations includes that no blood tests were performed and tissues were stored but not examined for histopathology. However, when assessed in conjunction with the dietary subchronic toxicity study in beagle dogs (DuPont Report ACD 003/013230, see below) and when applying a weight-of-the-evidence approach, it adequately completes the requirements of an oral 28-day study.

Mortality: no animals died during the study.

Clinical signs: brown staining was observed in some animals at the top-dose. However, the incidences were low (single animal findings), indicating a doubtful toxicological significance.

Table B.6.3.1.1-1 Lenacil technical: preliminary study by dietary administration to Han Wistar rats for 4 weeks (Thirlwell P.M., 2002): clinical signs

Week 1 and 2	**0**		**5000 ppm**		**10000 ppm**		**20000 ppm**	
Week 3 and 4	**0**		**5000 ppm**		**30000ppm**		**50000ppm**	
Achieved dose :	M	F	M	F	M	F	M	F
mg/kg bw/d wk 1-2	0	0	571	631	1269	1288	2545	2643
mg/kg bw/d wk 3-4	0	0	571	631	2978	3576	5029	5913
Coat, hairloss, head	1/5 (wk 3-4)	1/5 (wk 1)	1/5 (wk 1)		1/5 (wk 1-5)			
Coat, hairloss, dorsal body surface	2/5 (wk 1, 3) (wk 1-4)				2/5 (wk 1) (wk 3-5)			
Coat, hairloss, right forelimb								1/5 (wk 3-4)
Skin, encrustation(s), dorsal body surface	1/5 (wk 1)							
Skin, exfoliation, tail			1/5 (wk 2-5)				1/5 (wk 3-5)	
Staining, brown, head							1/5 (wk 5)	
Staining, brown, upper dorsal thorax							1/5 (wk 2)	

Body weight: was not affected.

Food consumption: there was no effect and food conversion efficiency was unaffected.

Organ weight: absolute and relative liver weights were slightly high, compared with the controls in females receiving 20000/50000ppm. This was not considered to be adverse by the notifier.
In the study report, it was noted that "*the cause of this* (effect) *was not established in this study, since no histopathological or clinical pathology investigations were performed. Since the effect on liver weight was small and confined to one sex, dietary concentrations up to 50000 ppm are considered suitable for use in a 13 week toxicity study in this strain of rat*". However, according to **RMS** 1) the increase in liver weight in top-dose ♀ was ≥ 10% compared to control, thus not considered small, and 2) the apparent dose-response relationship for the relative liver weight in ♀ (**table B.6.3.1.1-2**) indicates that the organ is a target of lenacil in this study, with a LOAEL set at the top-dose.
The table also mentions the weights of thyroids (+ paras) and uteri, for which no clear picture may be seen.

Table B.6.3.1.1-2 Lenacil technical: preliminary study by dietary administration to Han Wistar rats for 4 weeks ([redacted] 2002): organ weight.

Week 1 and 2		0		5000 ppm		10000 ppm		20000 ppm	
Week 3 and 4		0		5000 ppm		30000ppm		50000ppm	
Achieved dose :		M	F	M	F	M	F	M	F
mg/kg bw/d wk 1-2		0	0	571	631	1269	1288	2545	2643
mg/kg bw/d wk 3-4		0	0	571	631	2978	3576	5029	5913
Liver	A	11.64 ± 0.51	6.62 ± 0.67	10.70 ± 1.43	**7.65*** ± 0.50 (↑ 16%)	12.02 ± 0.56	7.20 ± 0.34 (↑ 9%)	11.61 ± 1.38	**8.45**** ± 0.77 (↑ 28%)
	R	4.678 ± 0.227	4.295 ± 0.270	4.405 ± 0.348	4.507 ± 0.418 (↑5%)	4.836 ± 0.205	4.627 ± 0.129 (↑8%)	4.760 ± 0.372	**4.855*** ± 0.058 (↑13%)
Thyroids + Paras	A	0.014 ± 0.001	**0.011 ± 0.002** (↓21%)	0.014 ± 0.003	0.015 ± 0.003 (↑7%)	0.010 ± 0.002	**0.009 ± 0.002** (↓10%)	0.010 ± 0.003	**0.011 ± 0.003** (↑10%)
	R	0.0056 ±0.0005	0.0045 ±0.0007	0.0056 ± 0.0011	0.0063 ± 0.0012	0.0066 ± 0.0014	0.0055 ± 0.0009	0.0066 ± 0.0017	0.0062 ± 0.0019
Uterus	A					0.446 ± 0.157	0.395 ± 0.102 (↓11%)	**0.588 ± 0.319** (32%)	**0.527 ± 0.219** (18%)
	R					0.2890 ± 0.0954	0.2330 ± 0.0610 (↓19%)	0.3788 ± 0.2046 (↑31%)	0.2967 ± 0.1066 (↑3%)

* statistically significantly different from control p<0.05: p<0.01; A: absolute; R: relative weight; N=5/sex/dose

Macroscopy:

According to the notifier, no macroscopic changes were observed. However, according to **RMS**, there was an increase in the rate of females with uterine fluid distention at the two higher doses (**table B.6.3.1.1-3**), suggesting that uterus could be a target of lenacil, with a LOAEL at 10000/30000 ppm. Dark areas (punctate foci) were also occasionally observed in the thymus, without a clear dose-response.

No macropathology change was seen for thyroids. Masses were occasionally observed in the liver (0-1/5 animals), but without any dose-response relationship.

Table B.6.3.1.1-3 Lenacil technical: preliminary study by dietary administration to Han Wistar rats for 4 weeks ([redacted] 2002): macroscopy data uterus.

Week 1 and 2	0	5000 ppm	10000 ppm	20000 ppm
Week 3 and 4	0	5000 ppm	30000ppm	50000ppm
Achieved dose :	F	F	F	F
mg/kg bw/d wk 1-2	0	631	1288	2643
mg/kg bw/d wk 3-4	0	631	3576	5913
Uterus Fluid distention	1/5 (<3mm dia.)	1/5 (<3mm dia.)	**3/5** (2 cases <5mm dia., 1 case <4mm dia.)	**3/5** (1 case <5mm dia., 2 cases <4mm dia.)

Conclusion (28d rat):

During the first evaluation, the only treatment-related effect was considered being a slight increase in liver weights in females given 50000 ppm.

During renewal, RMS BE revised the NOAEL of the 28d-rat study downwards:

NOAEL = 5000 ppm = **571** mg/kg bw/day.

LOAEL = 10000/30000 ppm = 2978 mg/kg bw/day, based on ↑uterine fluid distention.

At top-dose (20000/50000 ppm), ↑absolute and relative liver weight in ♀.

Notifier considered the 28d rat study NOAEL = 50000 ppm = 5029 mg/kg bw/day

B.6.3.1.2

Preliminary study by dietary administration to beagle dogs for 4 weeks ([redacted] 2001)

DuPont Report No.: ACD 003/013230

Guidelines: EEC Directive 92/69/EEC Method B.4, equivalent to OECD 404 (1992)

Deviation from official protocol: only 1 animal/sex/dose; tissues were prepared for histopathology but not examined

Notifier considered:

"*The oral 28-day feeding study in male and female Beagle dogs (ACD 003/013230) was originally submitted under EU Rev8 Point IIA 5.3.1 and has been conducted with lenacil technical. The study was conducted according to OPPTS 870.2500 (1998), EEC Directive 92/69/EEC Method B.4, OECD 404 (1992), JMAFF 12 Nohsan No. 8147 (2000). A review of this study indicates that it partially meets the current OECD Test guideline 407; deviations include that tissues for histopathology were prepared from only 1 animal/sex/dose but were not examined. However, in conjunction with the 90-day feeding study in dogs (ACD 022/014297), it adequately completes the the toxicity profile in dogs.* "

GLP status: yes

Materials and Methods

Lenacil technical (Batch No. 141712003, purity 98.6%) was incorporated into the ground diet to provide the required concentrations. The dosages used in this study were 5000, 20000 and 50000 ppm. Prior to the commencement of the study, the proposed formulation procedure was checked to confirm that the proposed procedures produced homogeneous diet. Results of homogeneity and stability analyses are included in the 13 week report (DuPont Report ACD/022).

A total of 3 male and 3 female pure-bred beagle dogs were used for the study. Their age at the start of treatment was 22 to 26 weeks and their bodyweights were in the range 8.0 to 8.7 kg for males and 7.5 to 7.9 kg for females. Clinical signs and mortality were assessed daily. Body weights were recorded twice weekly and food consumption once a week. Haematology and clinical chemistry investigations were performed at the beginning and at the end of the study. At terminal autopsy, macroscopic changes were recorded and organs preserved for possible future examinations

Dietary level (ppm)	0		5000		20000		50000	
Achieved dose :	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
mg/kg bw/d	0	0	219	242	807	967	1941	2331

The study is accepted.

Findings

Body weight (gain), food consumption: body weight change was decreased more than 10% were observed in males at the two top doses and in the female at top dose. Food consumption was also decreased in males at the two top doses.

Table B.6.3.1.2-1 Preliminary study by dietary administration to beagle dogs for 4 weeks ([redacted] 2001): body weight (change), food consumtion.

Endpoints/Dose (ppm)	5000		20000		50000	
	M	**F**	**M**	**F**	**M**	**F**
Body weight terminal: kg	9.5	8.2	9	8.4	9.5	8.8
Body weight change kg	1.3	0.9	**1.0** (↓23%)	0.9	**0.8** (↓38%)	**0.7** (↓22%)
Food consumption (g)	2790	2700	**2455** (↓12%)	2743	**2480** (↓11%)	2718

Haematology:

Individual parameters were variable but were generally comparable between treatment groups and with pre-treatment values.

RMS: noted a dose-dependent decrease in WBC / neutrophils counts in ♂ at the mid and top-doses. Taking into account that only 1 animal/sex/dose was investigated, no firm conclusions can be drawn from the observed modifications.

Clinical chemistry:

Slightly increased urea values were noted for all treated animals in comparison with pre-treatment values. Increased creatinine values were noted for all treated males and for females at 20000ppm or 50000 ppm. Increased alkaline phosphatase values were noted for the female receiving 50000 ppm.

RMS: noted an increase in alkaline phosphatase level in ♀ at the mid- and top-doses, an increase in urea in both sexes at top-dose, and an increase in creatinine level in ♂ at top-dose.

Table B.6.3.1.2-2 Preliminary study by dietary administration to beagle dogs for 4 weeks [redacted]., 2001): selected haematology data.

Dose (ppm)		5000		20000		50000	
		M	F	M	F	M	F
WBC (x 10^{-9}/L)	wk -4					13.66	11.82
	wk -3			12.47	11.33		
	wk -2	13.78	9.28				
	wk 4	13.18	8.78	11.19	11.20	9.83	12.10
	vs. predose	(↓4%)	(↓5%)	**(↓10%)**	(↓1%)	**(↓28%)**	(↑2%)
	vs. low-dose	-	-	**(↓15%)**	(↑28%)	**(↓25%)**	(↑38%)
Lymphocytes (x 10^{-9}/L)	wk -4					6.02	4.06
	wk -3			4.46	3.83		
	wk -2	5.76	3.28				
	wk 4	5.70	3.88	4.59	4.20	4.81	4.26
	vs. predose	(↓1%)	(↑18%)	(↑3%)	(↑10%)	**(↓20%)**	(↑5%)
	vs. low-dose	-	-	(↓21%)	(↑8%)	(↓16%)	(↑10%)
Monocytes (x 10-9/L)	wk -4					0.90	0.30
	wk -3			0.48	0.50		
	wk -2	0.71	0.67				
	wk 4	0.51	0.43	0.45	0.76	0.62	0.74
	vs. predose	(↑28%)	(↓36%)	(↓6%)	(↑52%)	(↓31%)	(↑147%)
	vs. low-dose	-	-	(↓12%)	(↑ 77%)	(↑22%)	(↑ 72%)
Neutrophils (x 10-9/L)	wk -4					6.36	6.52
	wk -3			7.26	6.86		
	wk -2	7.02	4.99				
	wk 4	6.64	4.29	5.91	6.09	4.14	6.73
	vs. predose	(↓5%)	(↓14%)	**(↓19%)**	(↓11%)	**(↓35%)**	(↑3%)
	vs. low-dose	-	-	**↓11%**	↑42%	**↓38%**	↑57%

Table B.6.3.1.2-3 Preliminary study by dietary administration to beagle dogs for 4 weeks ([redacted]., 2001): selected clinical chemistry data.

Dose (ppm)		5000		20000		50000	
		M	F	M	F	M	F
Alkaline phosphatase	wk -4					290	314
	wk -3			257	370		
	wk -2	324	273				
	wk 4	325	256	268	412	326	406
	vs. predose	(↑0.3%)	(↓6.2%)	(↑4.3%)	**(↑11.4%)**	(↑12.4%)	**(↑29.3%)**
	vs. low-dose	-	-	(↓18%)	**(↑61%)**	(↑0.3%)	**(↑59%)**
Urea	wk -4					5.59	3.40
	wk -3			3.01	3.15		
	wk -2	3.05	2.87				
	wk 4	4.47	4.02	4.78	4.26	8.54	4.59
	vs. predose	(↑47%)	(↑40%)	(↑59%)	(↑35%)	(↑53%)	(↑35%)
	vs. low-dose	-	-	(↑7%)	(↑6%)	**(↑91%)**	**(↑14%)**
Creatinine	wk -4					69	62
	wk -3			56	64		
	wk -2	59	73				
	wk 4	70	72	75	77	85.5	70
	vs. predose	(↑19%)	(↓1%)	(↑38%)	(↑20%)	**(↑24%)**	(↑13%)
	vs. low-dose	-	-	(↑7%)	(↑7%)	**(↑22%)**	(↓3%)

Organ weight: liver weight was increased, while kidneys and thymus weights were decreased at mid- and top-doses.

Table B.6.3.1.2-4 Preliminary study by dietary administration to beagle dogs for 4 weeks ([redacted] 2001): selected organ weight data.

Dose	5000 ppm		20000 ppm		50000 ppm	
	M	F	M	F	M	F
Liver (absolute)	362	269	370	329 ([a]↑22%)	383	350 ([a]↑30%)
Liver (relative, %)	3.8	3.3	4.1	**3.9** ([a]↑18%)	4.0	**4.0** ([a]↑21%)
Kidneys (absolute)	53.1	48.4	43.1 ([a]↓19%)	43.5	40 ([a]↓25%)	45.5
Kidneys (relative, %)	0.56	0.59	**0.48** ([a]↓14%)	**0.52** ([a]↓12%)	**0.42** ([a]↓25%)	**0.52** ([a]↓12%)
Thymus (absolute)	26.67	0.98	18.98 ([a]↓28%)	1.011 ([a]↑13%)	17.05 ([a]↓36%)	1.12 ([a]↑14%)
Thymus (relative, %)	0.281	0.202	**0.211** ([a]↓25%)	0.171	**0.179** ([a]↓36%)	0.255 ([a]↑26%)

[a]: (liver, kidneys, thymus) relative weight variations compared to low-dose (5000 ppm)

Macroscopy: according to the study, there were no findings noted at necropsy that were considered to be related to treatment.

Comment from RMS (previous DAR): Renal function is often tested by measuring the concentration of urea in the blood and this concentration is increased if renal function is impaired. The absence of dose effect relationship could result from the fact that oral absorption of lenacil saturated at doses as high as 1000 mg/kg, but it was not demonstrated at which level saturation started.

Comment from the notifier (previous DAR):

"The study, as a range-finding investigation, does not claim GLP compliance.
The RMS comment relates to results from a limited number of animals with no control group comparator. The notifier is of the opinion that any conclusions relating to renal dysfunction should be based on results from longer term and fully guideline compliant studies rather than extrapolated from range-finding preliminary investigations and requests this point to be reflected in the comment from RMS."

Although this non-guidance, non-GLP study is considered preliminary, the variations of liver (↑), kidneys (↓) and thymus (↓) weight, which are frequently >10%, the ↓ WBC and ↓ neutrophil counts, and the ↑ in alkaline phosphatase activity at the mid- and top-dose, compared to the low-dose, should lead to the setting of a NOAEL to this low-dose (5000 ppm, equivalent to 219 mg/kg bw/day in ♂).

Conclusion (28d dog)

Under the conditions of the study and at the dose levels administered, lenacil was well tolerated.

The **NOAEL** in the 28-day study in dogs was determined to be = 5000 ppm = **219 mg/kg bw/d**
on the basis of ↓body weight change, ↑liver, ↓kidneys and ↓thymus weight, ↓WBC and ↓neutrophil counts, and ↑alkaline phosphatase activity.

The relevance of the established NOAEL's should be considered with caution, taking into account the involvement of only 1 dog/sex/dose.

Notifier considered the 28d dog study NOAEL = 50000 ppm = 1941 mg/kg bw/d

B.6.3.2 (CA 5.3.2) Oral 90-day study

B.6.3.2.1 Oral 90-day toxicity in the rat

B.6.3.2.1.1

Toxicity study by dietary administration to rats for 13 weeks followed by a 4 week recovery period ([redacted] 2002) DuPont Report No.: ACD 002/013903

Guidelines: EEC Directive 88/302/EEC, EEC Directive 92/69/EEC, EEC Directive 96/54/EEC equivalent to OECD 408.

GLP status: yes (no attest of competent authority)

Materials and Methods

Lenacil technical (Batch No. 141712003, purity 98.6%) was incorporated into the ground diet to provide the required concentrations.

Before the commencement of treatment, homogeneity and stability investigations were carried out and confirmed for dietary concentrations at 50 and 50000 ppm. Concentration analyses were performed in weeks 1, 6 and 12 of treatment. The actual concentration average range was 97.2% (101-92.8%).

Groups of 10♂ and 10♀ Han Wistar rats received lenacil technical orally, via the diet, at concentrations of 500, 5000 or 50000 ppm for 13 weeks. A similarly constituted Control group received the basal diet only. A further 5♀and 5♂were assigned to the group receiving 50000 ppm and also to the Control group. These animals were treated for 13 weeks, followed by a four week period without treatment to assess recovery from any treatment related effects.

Dietary level (ppm)	0		500		5000		50000	
Achieved dose :	♂	♀	♂	♀	♂	♀	♂	♀
mg/kg bw/d	0	0	40.6	44.7	412.0	467.6	4356.9	4892.9

Statistical analysis: Mantel test and Pair wise Fishers exact tests for comparison of control and treated groups. When Bartlett's test for variance homogeneity was not significant, then parametric analysis was applied. William test for a monotonic trend was applied. Dunnetts test was performed if F-test was significant. For organ weight, homogeneity of variance was tested using Bartletts test. For the functional observation battery of tests, statistical analysis was performed for rearing, activity counts, grip strength and Coulbourne activity data. One way analysis was performed by Williams test. Macroscopy and microscopy were analyzed with the Fisher exact test.

The study is accepted

Findings

Mortality: one top-dose ♂rat was killed *in extremis* during week 11. The death of this animal is considered not to be related to treatment.

Clinical signs: brown staining of the tail was observed from week 7 in ♂ and from week 8 in ♀ at top dose. The origin of this effect was not established as there was no evidence of any change in the color of the urine in these animals. Following cessation of treatment, the incidence of this sign declined in both sexes (but did not disappear completely in ♂), indicating only partial recovery (**Table B.6.3.2.1-1**).

Body weight: Although ♂ receiving 5000 or 50000 ppm gained significantly less weight than controls, there was limited evidence of dosage relationship, perhaps because of saturation phenomena after oral absorption of high doses. In the original evaluation, the difference in weight gain in treated males was not considered related to treatment (**table B.6.3.2.1-1**) but in the absence of a more consistent explanation, the effect is considered adverse. In addition, body weight remains low at recovery phase, clearly indicating adversity.

Notifier: As noted in the 90-day report, 2 males in the control (Animal Nos. 47 and No. 50) gained an enormous amount

of weight, and therefore, skewed the final body weight and body weight gain of the control group. The study reported that concurrent controls had a mean weight gain of 214 g (range 157 to 290 g) versus 262 ± 40 g in the current study. It is interesting to note that the dosed groups in this study all had an average gains around 230 g, which are closer to the concurrent controls.

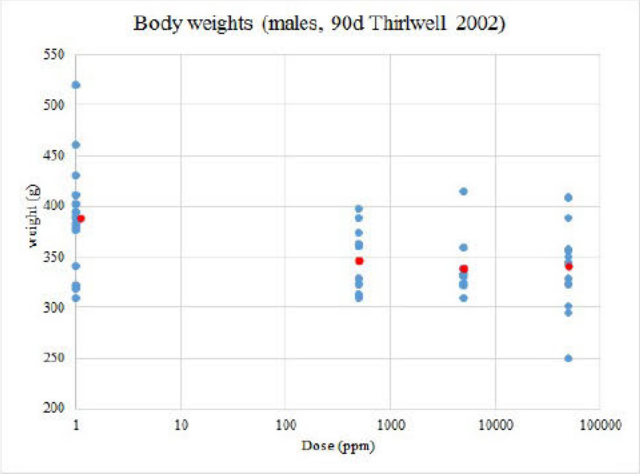
RMS: withdrawing "outlying" animals #47 (or #47 and #50) leads to a control mean body weight of 379 ± 43.4 (ot 373 ± 37.9), thus reducing (although not suppressing) the gap with treated animals. However, some animals in treated groups could also be considered as high-value outliers, such as animal #28 treated at 5000 ppm (bodyweight at week 13: 414 g) or animals #6 and #9 at the top-dose (bodyweights at week 13: 409 g and 408 g), and also withdrawn, leading to the reduction of their corresponding mean. Thus, some doubt may persist. However, taking into account the statistically significantly relevant b.w. *change* at 5000 ppm and above, it is more likely that the NOAEL for body weight effects may be set at the lowest dose. **Co-RMS** agreed with the conclusion of the RMS that the finding should be considered treatment –related and adverse in the two higher dose groups. Although there is no real dose – response (which might indeed be due to a saturation effect), the finding is statistically significant in the two higher dose groups, and is also >10 % which should not be disregarded. It is questionable whether the slightly decreased food consumption in ♂ is responsible for the decrease in body weight gain, as food consumption in ♀ was also decreased (in the two lower dose groups) without any effect on body weight gain.

The issue is illustrated in Figure B.6.3.2.1 – 1

(average values represented by red dots).

Figure B.6.3.2.1.1 – 1

Rat 13 weeks study ([redacted] 2002): body weight (individual data).



Food consumption: was slightly less in ♂ at 500 or 5000 ppm but in the absence of similar differences in ♂receiving 50000ppm, these were originally attributed to normal biological variation (**Table B.6.3.2.1-1**). It is questionable whether the slightly decreased food consumption in ♂ is responsible for the decrease in body weight gain, as food consumption in ♀was also decreased (in the two lower dose groups) without any effect on body weight gain.

Table B.6.3.2.1.1-1 Rat 13 weeks study ([redacted] 2002): clinical signs, body weight and food consumption.

	Male				Female			
	1M	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
ppm	**0**	**500**	**5000**	**50000**	**0**	**500**	**5000**	**50000**
Clinical signs:								
Brown stain on tail, wk 13	0/15	0/10	0/10	**5/14**	0/15			**4/15**
Brown stain on tail, Recovery week 4	0/5			**2/5**	0/5			1/5
Brown stain on tail, Recovery week 5	0/5			**1/5**	0/5			0/5
Body weight:								
wk 13	388 ± 55.4	347 ± 33.6 (↓11%)	339 ± 29.5 (↓13%)	341± 43.6 (↓12%)	212 ± 19.9	215 ± 21.2	211 ± 16.9	208 ± 16.8
Recovery wk 5	418 ± 88.5			**347 ± 50.8 (↓17%)**	223 ± 15.6			210 ± 18.3 (↓6%)

	Male				Female			
	1M	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
ppm	**0**	**500**	**5000**	**50000**	**0**	**500**	**5000**	**50000**
Body weight gain:								
wk 0-13	262 ± 40.1	234 ± 32.6 (↓14%)	**224*±26.8 (↓17%)**	**228*±40.1 (↓16%)**	112 ± 16.3	113 ± 15.2	114 ± 14.0	108 ± 14.2 (↓4%)
recovery	8 ± 2.6			19** ± 2.1 (↑138%)	5 ± 4.9			8 ± 2.5 (↑60%)
Food consumption:								
Week 13		↓8%	↓8%	↓4%		↓5%	↓14%	
Recovery wk5				↓5%				↓8%

Statistically significant modification: *: p<0.05; **: p<0.01

Behavioural investigations:

The notifier was of the opinion that there were no findings at the in the hand and in the arena investigations performed during the treatment period that were attributable to treatment with lenacil. There was a slight increase in the number of ♂rats given 50000 ppm that were seen to be walking on the toes. The number of animals showing this sign was generally low and the trend was not observed at all investigation periods. Originally, this sign was not attributed to treatment.

According to the **notifier**, this type of data is highly variable. If this was a treatment related finding, a pattern similar to what was seen in Week 1 ♂, but with incidence levels more closely resembling the higher responses seen in the ♀ (values as high as 7, 8 or 9) would be expected. Instead, note how in Week 4 the control has 4 ♂ walking on their toes versus 3 in the highest dose group. The Week 4 control is not much different than the high dose group on week 1. Note for Week 5 there are 2 walking on toes in the control versus 3 in the high dose group. Week 9 and 13 also show no consistent response. If this was a true test substance-related neurobehavioral finding, it would co-occur with abnormal gait; however, no related findings were observed.

RMS observed that the number of ♂ walking on toes is higher at top-dose compared to controls, and a certain trend is observed across the doses. The finding was unremarkable in the ♀. The RMS is of the opinion that the top-dose increase in the ♂ is not negligeable (table B.6.3.2.1-3). The mere fact that the same trend was not observed in the ♀ does not invalidate the potential substance-related finding in the ♂.

At week 12, motor activity was apparently increased in treated ♀, though the magnitude of this increase was generally slight and did not follow a trend with dosage. No similar finding was observed in ♂. Consequently, the inter-group differences in ♀were attributed to normal biological variations.

At the end of the recovery period the locomotor activity of previously treated ♀was also higher than that of controls.

RMS notes that there was a slight decrease in the proportion of ♀ rats manifesting vocalisation in function of the dose (table B.6.3.2.1-2). Compared to controls, the average incidence of sounds ↓30%, ↓48% and ↓70% at 500, 5000 and 50000 ppm, respectively. Although the adversity seems not directly evident (the finding is not , the finding could reflect some behavioural or communicative depression, possibly secondary to systemic toxicity, which should be commented by the notifier.

Notifier was of the opinion that an increased incidence of vocalisations normally indicates stress and a decrease means less stress. It is unclear why there is a tendency for decreased vocalisations in the higher dose groups, but note that even at the pre-treatment timepoint, the control group had a higher incidence of vocalisations than any of the treated groups (6, 4, 1, 3 in the control, low, middle and high dose groups respectively, see Report No. ACD 002/013903, Tinwell, 2002, p.170). It appears that the quieter animals just happened to be in the higher dose groups.

RMS in unsure that the hypothesis of the notifier is explaining the phenomenon. In the absence of any explanation the finding at the two highest doses is considered potentially adverse.

Table B.6.3.2.1.1-2 Rat 13 weeks study ([redacted] 2002): behavioural investigations (vocalisation)

		Males				Females			
		1M	2M	3M	4M	1F	2F	3F	4F
ppm		0	500	5000	50000	0	500	5000	50000
N° of animals	Wk	10	10	10	10£	10	10	10	10
Pre treatment		1	0	0	0	6	4	1	3
	1	2	0	1	1	4	2	3	3
	2	0	0	1	0	4	2	**1**	**1**
	3	0	0	0	0	6	3	**2**	**1**
	4	0	1	0	1	4	5	**4**	**2**
	5	0	0	0	0	4	5	**2**	**1**
	6	0	0	1	0	4	4	**2**	**0**
	7	0	1	2	1	4	4	1	2
	8	2	0	1	0	3	1	2	**0**
	9	0	0	0	0	4	3	3	3
	10	0	0	0	0	2	2	**1**	**0**
	11	0	0	1	0	3	1	2	1
	12	0	0	0	0	4	1	1	**0**
	13	0	0	1	0	4	3	**2**	**1**
Wks 1-13		4	2	8	3	50	36	**26**	**15**
Mean ± SD per week		0.31 ±0.75	0.15±0.38 (↓52%)	0.62±0.65 (↑50%)	0.23±0.44 (↓26%)	3.85 ±0.90	2.77±1.42 (↓30%)	**2.00±0.91 (↓48%)**	**1.15±1.07 (↓70%)**

£:9 from wk 12 onwards

Table B.6.3.2.1.1-3 Rat 13 weeks study ([redacted] 2002): behavioural investigations (tiptoe gait).

		Males				Females			
		1M	2M	3M	4M	1F	2F	3F	4F
Ppm		0	500	5000	50000	0	500	5000	50000
N° of animals	Wk	10	10	10	10£	10	10	10	10
Pre treatment		1	0	0	1	0	2	2	3
	1	1	2	2	**5**	6	6	4	4
	2	0	0	2	**3**	8	6	7	7
	3	1	0	3	**4**	5	4	3	4
	4	4	1	1	3	4	5	4	5
	5	2	1	2	**3**	9	6	5	8
	6	1	1	1	**4**	7	3	3	8
	7	1	1	**2**	**3**	5	6	6	4
	8	1	3	3	**4**	4	4	2	8
	9	0	3	2	2	6	6	5	4
	10	1	2	0	**4**	4	3	6	6
	11	1	1	0	**4**	5	8	5	4
	12	1	2	0	**6**	9	7	6	5
	13	2	2	2	**3**	7	6	4	7
Wks 1-13		16	19	20	**48**	79	70	60	74
Mean ± SD per week		1.23 ±1.01	1.46 ±0.97 (↑19%)	1.54 ±1.05 (↑25%)	**3.69 ±1.03 (↑200%)**	6.08 ±1.80	5.39±1.50 (↓11%)	4.62±1.45 (↓24%)	5.69±1.70 (↓6%)

£:9 from wk 12 onwards

Ophthalmoscopy: no abnormalities were identified.

Haematology: lymphocyte counts in ♀at 5000 ppm and in ♂and ♀at 50000 ppm were low. Monocyte count was reduced in ♀at 5000 or 50000 ppm. These differences resulted in a reduction of total leukocyte count in ♂ and ♀ at 5000 and 50000 ppm, though in ♂at 5000 ppm this difference was not statistically significant. The cause of reduced lymphocyte numbers at 5000 and 50000 ppm in both sexes and reduced monocytes in ♀was not established in this study. As regards the question of potential adversity, there was no evidence of inflammatory change in any tissue, nor was there any effect of treatment upon lymphoid tissue. The notifier considered these effects as of uncertain toxicological significance. Monocyte counts were still slightly low at the end of the recovery period in top-dose ♀, although the difference was not as high as seen at the end of the treatment period, indicating some recovery occurred. However, RMS considers that here was incomplete recovery in respect of the decreases in WBC counts overall. While differences were attributed to normal biological variations, the mode of action of the modification in the white blood compartment remains elusive.

Although not statistically significant, an effect of lenacil >10% on various haematological cell counts was frequently seen sometimes apparent from low-dose onwards (**Table B.6.3.2.1.1-4**). With respect to the changes in lymphocyte counts, the recovery was not complete in ♀, suggesting some persistence. While the effects are "*of uncertain toxicological significance*" (as suggested by the notifier), it should be stressed that the lenacil haematomodulation is observed in numerous studies in this DRAR, and deserves some exploration of the underlying mechanism(s) and (potential) adversity. **Co-RMS** highlighted that haematology findings at the lowest dose are not statistically significant and there seems to be high inter-individual variability. There were no other findings at this dose level. Therefore, the low dose of 500 ppm should be considered as the NOAEL, based on a decrease in BW gain (♂), leukopenia (♀), ↑proteinuria (♂),↑liver weight (♀) at 5000 ppm.

Notifier indicated that haematology parameters can be highly variable. For example, note the standard deviation for the WBCs for ♂is very high in this study (e.g. ♂ 8.18 ± 2.18) and could indicate outliers. For this reason, it is helpful to compare the same sampling (13 weeks) in the 2-year rat study; however, the different dietary concentrations should be noted between the studies. The reference range for haematology parameters can help to interpret such findings, and therefore have been requested from Envigo. That having been said, the findings in ♂ at 50000 ppm and in ♀ at 5000 and 50000 ppm appear to be test substance-related.

RMS: regarding the example proposed by the notifier, we have recalculated the ♂WBC values when withdrawing the two extreme values in each group (for controls, these extreme values are 12.89 and 4.98). The resulting data are 7.99 ± 1.20; 8.21 ± 0.89 (↑3%); 7.37 ± 1.27 (↓8%); 6.68 ± 0.98 **(↓17%)** for groups 1M (controls), 2M, 3M and 4M. Thus, a dose-response trend in the ♂ subsists, and it is not considered that HCD are relevant for this kind of parameter, and will be very helpful to write away a dose-responsive finding.

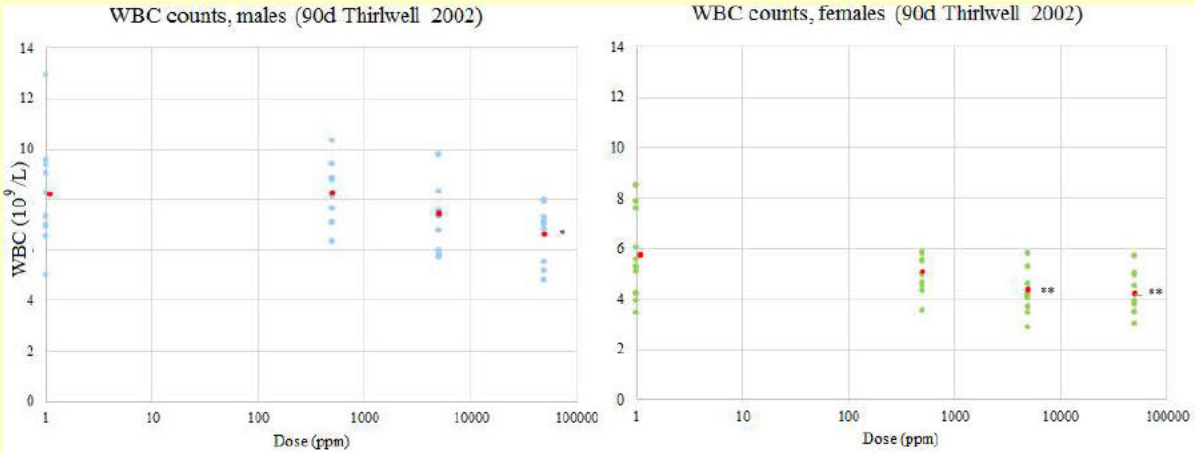
Table B.6.3.2.1.1-4 Rat 13 weeks study (2002): Haematology

	Male				Female			
Treatment	**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
WBC (x10^9/L)	8.18 ± 2.182	8.23 ±1.216	7.45 ±1.482 (↓9%)	**6.61[a] ±1.175 (↓19%)**	5.74 ±1.745	4.96 ±0.732 (↓14%)	**4.22[b] ±0.860 (↓26%)**	**4.22[b] ±0.842 (↓26%)**
L (x10^9/L)	6.55 ± 1.773	6.72±0.940	5.51 ±1.282 (↓16%)	**4.87[b] ±0.823 (↓26%)**	4.75 ±1.579	3.98 ±0.654 (↓18%)	**3.27[b] ±0.627 (↓31%)**	**3.45[b] ±0.752 (↓27%)**
E (x10^9/L)	0.08 ±0.027	0.10 ±0.051 (↑25%)	0.08 ± 0.020 (=)	0.09 ± 0.023 (↑13%)	0.08 ±0.024	0.06 ±0.017 (↓25%)	0.06 ±0.019 (↓25%)	**0.06[a] ±0.024 (↓25%)**
M (x10^9/L)	0.15 ±0.040	0.13 ±0.044 (↓13%)	0.14 ±0.041 (↓7%)	0.14 ±0.066 (↓7%)	0.11 ±0.044	0.09 ±0.024 (↓18%)	**0.07[a] ±0.017 (↓36%)**	**0.06[b] ±0.015 (↓45%)**
N (x10^9/L)	1.29 ±0.525	1.16 ±0.412 (↓10%)	1.61 ±0.941 (↑25%)	1.42 ±0.515 (↑10%)	0.74 ±0.272	0.77 ±0.313 (↑4%)	0.76 ±0.425 (↑3%)	0.61 ±0.257 (↓18%)
LUC (x10^9/L)	0.09 ±0.040	0.10 ±0.037 (↑11%)	0.09 ±0.030	0.09 ±0.027	0.06 ±0.020	0.06 ±0.012	**0.04[a] ±0.014 (↓33%)**	**0.04[a] ±0.016 (↓33%)**
Recovery	**1M**			**4M**	**1F**			**4F**
WBC (x10^9/L)	5.68 ± 1.143			**6.37 ± 0.866 (↑12%)**	4.48 ± 0.660			**3.76 ± 0.980 (↓16%)**
L (x10^9/L)	4.24 ± 1.128			**4.88 ± 0.667 (↑15%)**	3.46 ± 0.549			**3.02 ± 0.923 (↓13%)**
E (x10^9/L)	0.08 ± 0.043			0.09 ± 0.014	0.07 ± 0.030			**0.05 ± 0.009 (↓28%)**
M (x10^9/L)	0.16 ± 0.066			0.13 ± 0.045	0.13 ± 0.035			**0.08[a] ± 0.026 (↓38%)**
N (x10^9/L)	1.11 ± 0.203			1.16 ± 0.333	0.75 ± 0.188			**0.57 ± 0.092 (↓24%)**
LUC (x10^9/L)	0.08 ± 0.039			0.09 ± 0.016	0.06 ± 0.005			**0.03[b] ± 0.010 (↓50%)**

Statistically significant modification: [a]: p<0.05; [b]: p<0.01; selected group mean values during week 13 of treatment and during week 4 of recovery; 9-10 animals/dose/sex at wk 13 and 5 animals/dose/sex at wk 4 of recovery; WBC: white blood cells; L: lymphocytes; E: eosinophils; M: monocytes; N:neutrophils; LUC: large unstained cells

The issue is illustrated in Figure B.6.3.2.1 – 2 (average values represented by red dots).

Figure B.6.3.2.1.1-2: Rat 13 weeks study ([redacted] 2002): white blood cell counts in ♂ and ♀ (individual data).



Statistically significant modification: *p<0.05, **p<0.01

Overall, RMS considers that, in the light of the significant WBC count reductions at 5000 ppm and above, the lowest dose may prcautionously be considered a NOAEL for this endpoint.

Clinical chemistry:

Low phosphorus concentrations at 5000 ppm (♀) and above (♂,♀) and slightly low K^+ and high creatinine in top-dose ♀ were observed. These changes showed full recovery by the end of the period of recovery. BUN was unchanged (see table B.6.3.2.1.1-5).

An effect upon renal function is suggested by variations of plasma electrolyte concentrations, the increased plasma creatinine concentrations and urinary specific gravity and protein content in ♀. There was, however, no effect upon the weight or histopathological appearance of the kidneys and these changes are considered most likely to represent an adaptive response to the excretion of the compound and/or metabolites and are not considered of toxicological significance by the notifier, however, the pattern of effects suggest a treatment-related and adverse outcome.

The slightly increased ALT activity (top-dose ♀) could point to an effect on the liver. However, the analysis of most liver enzymes did not predict a particular adverse effect on the liver (although top-dose animals exhibited some hepatocellular hypertrophy, see below).

Table B.6.3.2.1.1-5. Rat 13 weeks study ([redacted] 2002): clinical chemistry.

	Male				Female			
Treatment	**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
ALP (u/L)	142 ±34.6	167 ±46.2 (↑18%)	172 ±24.5 (↑21%)	154 ±18.3	66 ±10.3	69 ±11.8	75 ±20.4 (↑14%)	66 ±20.3
ALT (u/L)	29 ± 4.0	29 ± 5.4	30 ± 4.5	30 ± 4.5	30 ± 5.1	32 ± 8.3	32 ± 5.3	**36 ±22.5 (↑20%)**
AST (u/L)	72 ±7.7	72 ±7.9	69 ±8.1	62[a] ±8.5 (↓14%)	66 ±13.4	64 ±7.4	72 ±11.5	53[b] ±9.9 (↓20%)
gGT (u/L)	1 ± 0.5	1[a] ±0.6	1[a] ±0.6	0 ±0.7	1 ±0.8	2 ±0.5	1 ±0.3	2 ±0.5
Bili (µmol/L)	1 ±0.3	1 ±0.5	1 ±05	1 ±0.5	2 ±0.5	1 ±0.3	1 ±0.6	1[b] ±0.4
Urea (mmol/L)	6.04 ±0.719	6.20 ±0.888	6.79±0.666 (↑12%)	6.25±1.003	6.59 ±0.896	6.89±0.697	7.26±0.951	7.20±0.736
Creat (µmol/L)	47 ± 3.9	48 ± 4.4	49 ± 3.9	50 ± 4.7 (↑6%)	52 ± 4.1	54 ± 3.2	55 ± 4.5	**57[b] ± 3.7 (↑10%)**
Gluc (mmol/L)	7.28 ±0.877	6.63 ±0.832	7.17 ±0.595	6.62 ±0.927	5.61 ±1.146	5.34 ±0.629	4.91±0.720 (↓12%)	5.22 ±0.842
Chol (mmol/L)	1.65 ±0.292	1.50±0.261 (↓10%)	1.73±0.388	1.72±0.267	1.52±0.274	1.73±0.314 (↑14%)	1.76±0.261 (↑16%)	1.56±0.264 (↑3%)
Trig (mmol/L)	1.09 ±0.443	1.08±0.408	1.09±0.493	0.93±0.359 (↓15%)	0.54 ±0.205	0.65±0.586 (↑20%)	0.58±0.349	0.51±0.253
Na (mmol/L)	144 ± 0.9	144 ± 1.6	143 ± 0.9	143 ± 0.5	141 ± 1.6	142 ± 1.7	142 ± 1.7	142 ± 1.8
K (mmol/L)	3.9 ± 0.27	3.9 ± 0.20	3.9 ± 0.21	4.0 ± 0.19	3.7 ± 0.34	3.5 ± 0.26	3.6 ± 0.27	**3.3[b] ±0.30 (↓11%)**
Cl (mmol/L)	106 ± 1.0	107 ± 1.4	106 ± 1.4	107 ± 1.3	105 ± 1.8	107 ± 2.7	106 ± 2.0	106 ± 1.3
Ca (mmol/L)	2.64 ±0.040	2.56[b]±0.052	2.54[b]±0.054	2.61 ±0.045	2.79 ±0.070	2.79±0.066	2.77±0.079	2.75±0.091
Phos (mmol/L)	1.85 ±0.141	1.72 ±0.116	1.80 ±0.102	**1.73[a]±0.118 (↓6%)**	1.55 ±0.134	1.43±0.200	**1.22[b]±0.160 (↓21%)**	**1.27[b]±0.292 (↓18%)**
Total Prot (g/L)	63 ± 2.0	61 ± 2.5	61 ± 1.4	63 ± 1.9	69 ± 2.4	70 ± 2.8	70 ± 3.5	69 ± 4.2
Alb (g/L)	34 ± 1.4	33 ± 1.5	33 ± 1.0	35 ± 1.1	40 ± 1.6	39 ± 1.9	39 ± 1.9	39 ± 1.9
A/G (ratio)	1.18 ± 0.116	1.21 ±0.102	1.18 ±0.107	1.21 ±0.106	1.33 ± 0.081	1.30±0.049	1.29±0.080	1.27±0.068
Recovery	**1M**			**4M**	**1F**			**4F**
Creat (µmol/L)	48 ±2.3			**52±3.1 (↑8%)**	55 ± 3.0			**59±4.2 (↑7%)**
K (mmol/L)	4.4 ±0.19			**4.2±0.05 (↓5%)**	3.8 ± 0.18			**3.7±0.31 (↓3%)**
Phos (mmol/L)	1.81 ± 0.177			1.82 ± 0.057	1.37 ± 0.133			1.37 ±0.107

Statistically significant modification: [a]: $p<0.05$; [b]: $p<0.01$; selected group mean values during week 13 of treatment and during week 4 of recovery; 9-10 animals/dose/sex at wk 13 and 5 animals/dose/sex at wk 4 of recovery.

Overall, RMS considers that, in the light of the significant modifications indicating kidney function adversity in top-dose animals, the mid dose may cautionously be considered a NOAEL for these endpoints.

Urinalysis: increased specific gravity of males at top dose and urinary proteins were identified in ♂at 5000 ppm and above (table B.6.3.2.1.1-6).

Urine volume was slightly decreased, however without dose-responsiveness. It is of note that decreased urinary volume was also observed in the follow-up 2yr rat study ([redacted], 2003), but again the effect lacked a dose-related response. There was no aggravation of urinary volume modification with time either, as terminal animals (both ♂ and ♀, wk 103) were unremarkable .

Regarding proteinuria, the findings were significant in the ♂ at 5000 ppm and above, and a slight dose-response was observed. The values were slightly high, but insignificant, in all treated ♀. It is of note that in the follow-up 2yr study, urinary proteins were observed in the top-dose ♂on week 12 and 51, but not in the ♂ animals sampled at other times (wk 25, 77, 103). Proteinuria was observed in the ♀at week 51, but not at the other sampling times (week 12, 25, 71, 103). In

conclusion, whereas some effects could be observed on occasions, both the subchronic and chronic study failed to reveal a consistent pattern, both in terms of dose-response and time-relationship.

Thus, whereas some effect of the substance on the fluid homeostasis cannot entirely be excluded, the measured parameters show a disparate response obscuring the adversity of the finding. It is of note that no kidney alteration is suggested by organ weight variation, morphology and histopathology. The significance of this proteinuria remains thus unclear, in absence of a consistent pattern (dose, duration), a plausible mechanism of action for lenacil in rat and, more generally, on animals.

Table B.6.3.2.1.1-6 Rat 13 weeks study (2002): Urinalysis

Treatment	Male				Female			
	1M	2M	3M	4M	1F	2F	3F	4F
Volume (mL)	4.7 ±2.06	3.4 ±0.82 (↓28%)	3.5 ±0.62 (↓26%)	4.3 ± 1.12 (9%)	2.5 ± 0.76	2.3 ±1.28	1.4 ±0.52 (↓44%)	1.9 ±0.91 (↓24%)
SG (g/L)	1034 ±8.3	1038 ±6.8	1030 ±8.8	**1041** [a] ±5.2	1040 ±7.1	1033 ±12.7	1044 ±15.5	1045 ±8.4
Prot (g/L)	1.09 ± 0.129	1.17 ±0.248 (↑7%)	**1.35**[a] **±0.277 (↑24%)**	**1.60** [b] **±0.161 (↑47%)**	0.19 ± 0.056	0.22 ±0.053 (↑16%)	0.22 ±0.056 (↑16%)	0.22 ±0.033 (↑16%)

Statistically significant modification: [a]: $p<0.05$; [b]: $p<0.01$; selected group mean values during week 13 of treatment and during week 4 of recovery; 9-10 animals/dose/sex at wk 13 and 5 animals/dose/sex at wk 4 of recovery.

Overall, RMS considers that, in the light of the significant modifications indicating kidney function adversity in top-dose animals, the mid dose may cautionously be considered a NOAEL for these endpoints.

Organ weight:
Liver weight of ♀rats was increased at 5000 and 50000 ppm; relative liver weight was also increased in ♂ (**table B.6.3.2.1.1**-7).
Increased (non-statistically significant but >10%) absolute and relative thyroid (+ parathyroids) weight was seen at top-dose. In ♀, relative thyroid (+ parathyroids) weight was still increased at top-dose (↑16%, although not statistically significant) after a 4 week-recovery.
Spleen relative weight was increased at top-dose in ♂ and seemed persistent. Of note, after a 4-week recovery, this change was also observed in ♀.
A –seemingly persistent– relative weight increase was observed for uterus + cervix. However, in the absence of meaningful modification in uterus weight after the treatment period of 13 weeks, the relevance of this observation remains unexplained.
Except for the increased liver weight, the modifications were all statistically non-significant, and in the absence of corroborating necropsy and histopatological findings, the toxicological relevance is doubtful.

Table B.6.3.2.1.1-7a Rat 13 weeks study ([redacted] 2002): Organ weight (13 wk findings)

Treatment	Male				Female			
	1M	2M	3M	4M	1F	2F	3F	4F
Terminal body weight	376.3 ±29.9	344.2 ±32.8 (↓9%)	338.5[a] ±29.0 (↓10%)	347.7 ±40.6 (↓8%)	210.3 ±22.5	217.9 ±18.2	214.6 ±17.8	212.2 ±17.9
Adrenals (a)	0.061 ± 0.006	0.061 ± 0.008	0.056 ± 0.006	0.060 ±0.006	0.076 ± 0.009	0.071 ± 0.008	0.070 ± 0.008	0.080 ± 0.009
Adrenals (r)	0.0163 ± 0.0016	0.0177 ± 0.0026	0.0167 ± 0.0020	0.0175 ±0.0018	0.0361 ± 0.004	0.0328 ± 0.003	0.0325[a] ± 0.002(↓10%)	0.0378 ± 0.003
Epididymides (a)	1.216 ± 0.170	1.195 ± 0.093	1.224 ± 0.081	1.228 ±0.196				
Epididymides (r)	0.3242 ± 0.0441	0.3505 ± 0.0469 (↑8%)	0.3642 ± 0.0398 (↑12%)	0.3546 ±0.0507 (↑9%)				
Liver (a)	14.80 ± 1.64	13.11 ± 2.05	13.40 ± 1.61	15.02 ± 1.87	7.88 ± 1.01	8.35 ± 0.77	**9.77 ± 2.61 (↑24%)**	**9.60[b] ± 0.87 (↑22%)**
Liver (r)	3.933 ± 0.302	3.796 ± 0.336	3.954 ± 0.429	**4.333 ± 0.376 (↑10%)**	3.747 ± 0.307	3.837 ± 0.244	**4.556 ± 1.165 (↑22%)**	**4.530[b] ± 0.271 (↑21%)**
Kidneys (a)	2.24 ± 0.22	2.02 ± 0.21 (↓10%)	2.05 ± 0.18 (↓8%)	2.18 ±0.22 (↓3%)	1.55 ±0.16	1.50 ± 0.15	1.56 ± 0.17	1.58 ± 0.12
Kidneys (r)	0.595 ± 0.043	0.589 ± 0.039	0.608 ± 0.032	0.629 ±0.050	0.736 ±0.028	0.589 ±0.047	0.725 ±0.050	0.749 ± 0.052
Ovaries (a)					0.080 ±0.010	0.076 ± 0.009	0.082 ± 0.012	0.086 ± 0.016 (↑8%)
Ovaries (r)					0.0384 ±0059	0.0350 ±0.0039	0.0384 ±0.0049	0.0407 ± 0.0066 (↑6%)
Spleen (a)	0.599 ± 0.069	0.584 ± 091	0.562 ± 0.090	0.610 ± 0.107	0.481 ± 0.076	0.528 ± 0.064 (↑10%)	0.517 ± 0.086	0.496 ± 0.093
Spleen (r)	0.1592± 0.0157	0.1694± 0.0189	0.1659 ± 0.0195	**0.1747 ± 0.0150 (↑10%)**	0.2285 ± 0.0267	0.2422 ± 0.0218	0.2407 ± 0.0320	0.2337 ± 0.0389
Testes (a)	3.51 ± 0.25	3.57 ± 0.18	3.45 ± 0.12	3.45 ± 0.59				
Testes (r)	0.938 ± 0.094	1.045 ± 0.087 (↑11%)	1.026 ± 0.085 (↑9%)	0.994 ± 0.149 (↑6%)				
Thymus (a)	0.387 ±0.088	0.380 ± 0.098	0.324 ± 0.040 (↓16%)	0.361 ± 0.121	0.313 ± 0.060	0.299 ± 0.083	0.292 ± 0.078	0.333 ± 0.048
Thymus (r)	0.1033±0.0236	0.1095 ± 0.0211	0.0962 ± 0.0121	0.1028 ± 0.0286	0.1483 ± 0.0204	0.1361 ± 0.0328 (↓8%)	0.1351 ± 0.0299 (↓9%)	0.1567 ± 0.0161 (↑6%)
Thyroid +Paras (a)	0.021 ± 0.004	0.021 ± 0.006	0.020 ± 0.004	**0.023 ±0.005 (↑10%)**	0.015 ± 0.003	0.015 ± 0.003	0.016 ± 0.003	**0.017 ± 0.002 (↑13%)**
Thyroid +Paras (r)	0.0056 ± 0.0015	0.0061 ± 0.0012 (↑9%)	0.0060 ± 0.0013 (↑7%)	**0.0068 ± 0.0017 (↑21%)**	0.0072 ± 0.0010	0.0067 ± 0.0012	0.0075 ± 0.0014	**0.0081 ± 0.0015 (↑13%)**
Uterus +Cervix (a)					0.505 ± 0.124	0.705 ± 0.317 (↑40%)	0.519 ± 0.126	0.572 ± 0.189 (↑13%)
Uterus +Cervix (r)					0.2416 ± 0.0576	0.3305 ± 0.1625 (↑37%)	0.2439 ± 0.0656	0.2726 ± 0.0966 (↑13%)

Absolute (a) in gram or relative (r) mean organ weight; Statistically significant modification: [a]: $p<0.05$; [b]: $p<0.01$; selected group mean values during week 13 of treatment; 9-10 animals/dose/sex.

Table B.6.3.2.1.1-7b Rat 13 weeks study ([redacted], 2002): Organ weight (recovery period findings)

Recovery	1M			4M	1F			4F
Terminal body weight	418.2 ± 88.7			345.7 ± 49.8 (↓17%)	222.9 ± 16.7			208.8 ± 18.1
Adrenals (a)	0.059 ± 0.008			0.055 ± 0.009	0.069 ± 0.014			0.069 ± 0.010
Adrenals (r)	0.0143 ± 0.0020			0.0160 ± 0.0028 (↑12%)	0.0310 ± 0.0052			0.0329 ± 0.0041 (↑6%)
Epididymides (a)	1.299 ± 0.088			1.195 ± 0.109 (↓7%)				
Epididymides (r)	0.3191 ± 0.0525			0.3494 ± 0.0368 (↑9%)				
Liver (a)	16.31 ± 2.67			14.30 ± 2.41 (↓12%)	8.72 ± 1.13			7.90 ± 1.09 (↓9%)
Liver (r)	3.967 ± 0.574			4.128 ± 0.152 (↓4%)	3.900 ± 0.238			3.778 ± 0.307 (↓3%)
Kidneys (a)	2.37 ± 0.25			2.15 ± 0.34 (↓9%)	1.51 ± 0.10			1.45 ± 10.21 (↓4%)
Kidneys (r)	0.579 ± 0.076			0.624 ± 0.065 (↓8%)	0.680 ± 0.021			0.692 ± 0.046 (↑2%)
Ovaries (a)					0.085 ± 0.010			0.085 ± 0.012
Ovaries (r)					0.0380 ± 0.0028			0.0408 ± 0.0053 (↑7%)
Spleen (a)	0.619 ± 0.099			0.573 ± 0.128 (↓7%)	0.440 ± 0.062			0.453 ± 0.067 (↑3%)
Spleen (r)	0.1500 ± 0.0158			0.1647 ± 0.0192 (↑10%)	0.1968 ± 0.0158			0.2165 ± 0.0203 (↑10%)
Testes (a)	3.57 ± 0.23			3.61 ± 0.33				
Testes (r)	0.877 ± 0.147			1.054 ± 0.104 (↑20%)				
Thymus (a)	0.331 ± 0.145			0.300 ± 0.053 (↓9%)	0.310 ± 0.042			0.259 ± 0.044 (↓16%)
Thymus (r)	0.0778 0.0249			0.0888 0.0227 (↑14%)	0.1386 ± 0.0089			0.1256 ± 0.0278 (↓9%)
Thyroid +Paras (a)	0.022 ± 0.004			0.018 ± 0.004 (↓18%)	0.017 ± 0.002			0.017 ± 0.005 (=)
Thyroid +Paras (r)	0.0054 ± 0.0007			0.0051 ± 0.0006 (↓6%)	0.0077 ± 0.0010			**0.0085 ± 0.0030 (↑16%)**
Uterus +Cervix (a)					0.519 ±0.140			0.592 ±0.188 (↑14%)
Uterus +Cervix (r)					0.2333 ±0.0629			0.2888 ±0.1150 (↑24%)

Absolute (a) in gram or relative (r) mean organ weight; Statistically significant modification: [a]: $p<0.05$; [b]: $p<0.01$; selected group mean values during week 4 of recovery; 5 animals/dose.

Macropathology: (**Table B.6.3.2.1.1-8)**
The incidence of fluid filled dilated uterus was unremarkable at week 13 and occurred at singularity after the recovery period. Toghether with all other single observations, this finding was not considered toxicologically relevant.

B.6.3.2.1.1-8 Rat 13 weeks study [redacted] 2002): Macropathology

week 13	Male				Female			
	1M	2M	3M	4M	1F	2F	3F	4F
Liver (enlarged)	0	0	0	0	0	0	1	0
Epididymides (unilaterally small)	0	0	0	1	-	-	-	-
Ovaries (periovarian sac distention)	-	-	-	-	0	0	1	0
Testes (unilaterally small)	0	0	0	1	-	-	-	-
Uterus (fluid distension)	-	-	-	-	2	4	1	3
Recovery	**1M**			**4M**	**1F**			**4F**
Ovaries (periovarian sac distention)	-			-	0			1
Uterus (fluid distension)	-			-	0			1

Statistically significant modification: [a]: p<0.05; [b]: p<0.01; selected group mean values during week 13 of treatment and during week 4 of recovery; 9-10 animals/dose at wk 13 and 5 animals/dose/sex at wk 4 of recovery.

Histopathology:
There was an increased incidence of centrilobular hepatocyte hypertrophy in all top-dose animals. This effect disappeared at the end of the 4 week recovery period. This change was considered by the notifier to represent enzyme induction and, as such, is considered an adaptive response to treatment. However, liver enzyme induction was not measured (**table B.6.3.2.1.1-9**).
The number of changes affecting lymphoid organs (spleen, thymus, lymph nodes) is of interest, as it suggests some immunomodulatory effects of lenacil. As bone marrow is another lymphoid tissue, it is unfortunate that this tissue was (according to the study report) not preserved for histopathology. Any change in this tissue could have been a verification whether it has been reached by lenacil, which may be informative also for the genotoxicity section.

The potential immunomodulation of lenacil should at least trigger a thorough discussion on a possible mechanism of action on the immune system and how to further conclude on this point. RMS is of the opinion that an immunotoxicity sytudy should be undertaken in order to exclude any adverse effect of the substance on the immune system.

Table B.6.3.2.1.1-9 Rat 13 weeks study ([redacted] 2002): Histopathology

Treatment	Male				Female			
	1M	2M	3M	4M	1F	2F	3F	4F
Epididymides (spermatozoa absent, unilateral)	0	0	0	1	-	-	-	
Liver (hepatocyte hypertrophy, centrilobular)	0	0	0	5[a]	0	0	0	**4**
Mesenteric Lymph nodes (increased cellularity, generalised)	0	0	0	0	1	0	0	**3**
Pancreas (acinar atrophy, focal)	0	0	0	1	0	0	0	0
Pancreas (lymphoid aggregates	0	0	0	1	0	0	0	0
Spleen (haemosiderosis)	6	0	0	6	2	0	0	3
Spleen (extramedullary haemopoiesis)	0	0	0	0	4	0	0	5
Testes (seminiferous, tubular atrophy)	0	0	0	1	-	-	-	-
Thymus (apoptosis – cortex)	3	0	0	3	3	0	0	4
Uterus (luminal dilatation)	-	-	-	-	2	4	4	3
Mediastinal LN (sinus, erythrocytosis /erythrophagocytosis)	0	0	0	1	0	0	0	0
Mediastinal LN (dilated/cystic sinuses)	0	0	0	1	0	0	0	0
Recovery	**1M**			**4M**	**1F**			**4F**
Liver (hepatocyte hypertrophy, centrilobular)	0	0	0	0	0	0	0	0
Uterus (luminal dilatation)	-	-	-	-	0	-	-	**1**

Statistically significant modification: [a]: p<0.05; [b]: p<0.01; selected group mean values during week 13 of treatment and during week 4 of recovery; 10 animals/dose (except top-dose ♂, N=9) at wk 13 and 5 animals/dose/sex at wk 4 of recovery.

Conclusion (90d rat):

From the results reported in this study, at the highest dose of 50000 ppm, target organ in rats seems to be the liver as suggested by the weight increase and the centrilobular hepatocyte hypertrophy (reported at top dose). Renal dysfunction seems to occur as suggested by the alteration of electrolytes excretion as well as the increased urinary protein at 5000 ppm onwards.
Effects on white blood cells which were not explained were observed at the two high doses. RMS considers that there is no reason to disregard these different effects.
Whether the lowest dose of 500 ppm is a NOAEL or should be considered a LOAEL based on haematology data is questioned.

NOAEL = 500 ppm = **40.6 mg/kg bw/d**
LOAEL = 5000 ppm = 412 mg/kg bw/d
based on:
↓body weight change, clinical signs, leukopenia, ↑proteinuria (♂),↑liver weight (21-24%).

According to the notifier, oral administration, via the diet, to Han Wistar rats of Lenacil technical at concentrations up to 50000 ppm for 13 weeks did not produce any significant toxic effect. Adaptive changes in the liver occurred at 50000 ppm and reduced lymphocyte and monocyte numbers occurred at 5000 and 50000 ppm, the latter findings being of uncertain toxicological significance. All changes were shown to be fully reversible during the four week recovery period. There were no changes at 500 ppm (equivalent to 40.6 mg/kg/day in males and 44.7 mg/kg/day in females) and this is considered to represent the No-Observed-Effect Level (NOEL) in this study.

"Notifier comment: Additional histopathological examinations were completed for this study.

Following observation of thyroid changes in the multi-generation reproductive toxicity study additional histopathological examinations of thyroid tissue preserved from a 13 week dietary study in rats were instigated. In the original study (Point 5.3.2.1) thyroids from the control and high dose (50000 ppm) groups were examined. The additional investigation extended the examination to the low and intermediate groups.

The study authors concluded that examination of sections stained with haematoxylin and eosin revealed no changes indicative of any accumulation of pigment in the follicular epithelium or any other change indicative of a response to treatment. Schmorl's staining of the thyroids, however, revealed a background level of Schmorl's positive staining in all groups, particularly in males. Schmorl's positive staining is indicative of lipofuscin in the follicular epithelium. There was a treatment-related increase in the incidence and severity of Schmorl's-positive staining in females given lenacil technical at 50000 ppm, and a slight increase in the severity of this finding in males given 50000 ppm. The slightly increased incidence of Schmorl's-positive staining in females given 5000 ppm was within the background incidence and was, therefore, not attributed to treatment. Following a recovery period of four weeks there were no significant differences in incidence of Schmorl's-positive staining between control and high dose group males or females.

Further thyroid function tests were also completed in female rats dosed for 20 weeks at 250 or 50000 ppm lenacil. Investigations included assessment of T3 and T4 levels, thyroid weights, ^{125}Iodide uptake and displacement. The study concluded that there was no evidence to suggest that lenacil technical at doses of up to 50000 ppm affected the ability of the thyroid to take-up and organify ^{125}Iodide. Measurements of T3 during the study also indicated that lenacil does not act as an inhibitor of the deiodinase which converts T4 to T3. Overall, the results of the study showed that lenacil technical was not directly toxic to the thyroid.

The conclusion to this summary states 500 ppm to be a NOEL. It appears that the RMS has also concluded 500 ppm to be the NOAEL also. From the results presented it is apparent that changes in the two higher dose levels were inconsistent and generally showed no clear dose relationship. While an effect of treatment is clearly apparent at 5000 ppm, this is not the case at the intermediate dose level where reduced monocytes and a slight increase in urinary protein were the only changes of note, both showing recovery after removal of treatment, indicating no adverse long term effects of lenacil administration. There was no corroborative evidence from macroscopic or microscopic findings to confirm any adverse effects of treatment at 5000 ppm.

The lowest NOEL derived from short-term toxicity studies in rat, mouse and dog was based on the results of the 90-day rat study and set at 40.6 mg/kg/day (500 ppm). The lowest appropriate NOAEL value was derived from the same study as the intermediate dose level of 412 mg/kg/day (5000 ppm). This was based on adaptive liver changes at the highest dose of 50000 ppm, which constituted the LOAEL. The NOAEL was defined by reduced white blood cell numbers at 5000 ppm,

considered of uncertain toxicological significance, in that the findings were not consistently seen in the long-term rat study. There were no bodyweight effects at any dosage.

*In the opinion of the notifier, the data support the conclusion indicating an **NOAEL** in the rat 90 day study of **5000 ppm** and a NOEL of 500 ppm."*

RMS disagreed with the notifier and maintained its proposal reported in the conclusion of the study. The NOAEL proposed by the RMS was accepted during the previous peer review.

B.6.3.2.1.2

Lenacil technical additional histopathological investigations to a toxicity study by dietary administration to han wistar rats for 13 weeks followed by a 4-week recovery period ([redacted]., 2004). DuPont Report No.: ACD 055/024499

Guidelines: EEC Directive 88/302/EEC, EEC Directive 92/69/EEC, EEC Directive 96/54/EEC, OECD 408.
Notifier "The oral 90-day feeding study in male and female Han Wistar rats (ACD 055/924499) was originally submitted under EU Rev8 Point IIA 5.3.2, 5.3.3 and has been conducted with lenacil technical. The study was conducted according to OPPTS 870.3100, OECD 408, EEC Directive 88/302/EEC, EEC Directive 92/69/EEC, and EEC Directive 96/54/EEC. A review of this study indicates that it fully meets the current OECD Test Guideline 408 or OPPTS guideline 870.3100. The study is considered valid."

GLP status: unclear

Materials and Methods
The thyroids of all animals of groups 1, 2, 3 and 4, sacrificed after completion of the 13-week treatment period, and five ♂and 5 ♀rats of Group 1 and 4 sacrificed on completion of the 4 week recovery period, were subjected to histopathological evaluation. In the original study (ACD/002, see under Point 5.3.2.1 (**RMS**: B.6.3.2.1.1)), the thyroids of all males and females from Group 1 (control) and 4 (high dose), killed after completing the 13 weeks of treatment were examined.
This additional study was intended to re-assess these tissues in all animals of these groups, together with those from females of the low and intermediate dose groups and recovery phase animals, in the light of changes seen in other toxicity studies performed for Lenacil technical.
The thyroids of all animals were originally fixed in the original study (ACD/022) in 10% neutral buffered formalin.
Appropriate samples of the thyroid including, where possible, the parathyroid sections, were dehydrated, embedded in paraffin wax, sectioned at approximately four to five micron thickness. The section were stained with haematoxylin and eosin and Schmorl's stain (Schmorl's positive staining can indicate the presence of a variety of materials including thyroid colloid, bile pigments, melanin or lipofucscin specific stain for lipofuscin) and subjected to light microscopy examination.
(The effect of Lenacil technical on the thyroid function to female rats, as reflected in the capacity of the thyroid to take up and "organify" ^{125}Iodide was assessed over a period of 20 weeks. Previous studies with the test material had revealed a darkening of the thyroid gland and the purpose of this study was to specifically investigate the action of Lenacil technical on thyroid function.)

Findings
The objective of this study was to perform an additional histopathological examination of the thyroid from a 13-week study in order to assist in the further interpretation of thyroid changes reported in other studies.
In the multi generation study, thyroids in some treated animals were macroscopically dark and microscopically demonstrated increased pigmentation when stained with haematoxylin and eosin. The thyroids were examined further on the reproduction study. Those thyroids stained with Schmorls reagent showed an increased incidence of Schmorls positive reaction, even in animals where no pigment deposition has been detected with haematoxylin and eosin staining procedure. In view of these observations the decision was taken to perform additional histopathological investigations, by the application of Schmorls stain, on the thyroids taken from the 13 week rat study.
Schmorls staining of the thyroids revealed a background level of positive staining in all groups, particularly in males. Positive staining is indicative of lipofuscin in the follicular epithelium. Lipofuscin pigment is associated with the degradation of the cell membrane and could suggest the presence of persistent chronic injury. Lipofuscin is reported where there is atrophic change, though in this study, examination of haematoxylin and eosin stained sections of the thyroid did not identify any evidence of atrophy. This change may be related to an increased rate of thyroid metabolism as a consequence of hypertrophic change in the liver which was reported in the original study at 50000 ppm and was attributed to the induction of hepatic enzymes.

In females, there was a treatment-related increase in the incidence and severity of Schmorls positive staining at 50000ppm and a slight increase in the severity of this finding in males at 50000ppm. The slightly increased incidence of staining in females at 5000 ppm was within the background incidence.
At the end of the recovery period, the incidence and severity of staining was higher than controls in females at top dose and in males the severity was marginally higher than controls.

Lipofuscin is an insoluble endogenous formed pigment which represents the indigestible residue of autophagic vacuoles within cells formed during aging or atrophy. The pigment appears to be composed of polymers of lipids and phospholipids complexed with protein.
The following is the manner in which lipofuscin is formed.
During atrophy and aging, degenerating cellular organelles are enclosed in autophagic vacuoles. Subsequently, lysosomes discharge their hydrolytic enzymes into these membrane bound vacuoles and the cellular organelles are digested by autophagy. However, some of the organelle components may resist digestion or be incompletely digested. Lipoproteins and other lipids make up most of the indigestible debris and their accumulation reflects the lack of sufficient quantities of lipase in most lysosomes. When organelles are not digested completely, the debris persists as membrane-bounded residual bodies. Some of these residual bodies may be extruded from the cytoplasm, or may be eventually digested. However, in some instances, the residual bodies persist in the cytoplasm of atrophic or aging cells. Microscopically, lipofuscin pigment appears as minute yellow-brown granules. Grossly, the lipofuscin pigment may impart a brownish discoloration to tissues when present in sufficient amounts (brown atrophy). Lipofuscin itself is not injurious to the cell or to its function. Lipofuscin occurs in a variety of organs and tissues, but it is especially prominent in the brain neurons, myocardial cells and in the adrenal and thyroid glands.
RMS: accumulation of lipofuscin in thyroid could then suggest that atrophy occurred and that membranes of destroyed organelles are converted within the lysosomes to lipid-containing lipofuscin. Lipofuscin in itself is not injurious to the cell but its presence could suggest a potential adverse occurrence.
While a Schmorl's positive staining is seen in the thyroids, it is not clearly demonstrated that lipofuscin is involved. The involvement of melanin is not excluded. Notifier further pointed out that any reducing agent can react with Schmorl's stain including lipofuscin, melanin, some test substances, and even colloid itself. Lipofuscin would be the most common reason for the Schmorl positive stain. Some chemicals or their metabolites can cause deposition of pigment into the thyroid follicular epithelium (*e.g.* the antibiotic minocycline).

Table B.6.3.2.1.2-1: Additional rat 13 weeks study ([redacted] 2004): thyroid changes

Endpoints/dose	**0**		**500**		**5000**		**50000 ppm**	
Achieved dose	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
mg/kg bw/d	0	0	40.6	44.7	412	467.6	4356.9	4892.9
Schmorl's positive pigment: 13 week								
Minimal	7	1	8	1	6	**3**	2	**4**
Slight	2	0	1	0	2	0	**5**	3
Moderate	0	0	0	0	0	0	**3**	0
Total	9	1	9	1	8	**3**	10	**7***
Schmorl's positive pigment: 4 week recovery								
Minimal	1	2					1	2
Slight	3	0					3	3
Moderate	0	0					1	0
Total	4	2					5	**5**

Statistical significant modification: *: $p<0.05$ (Fishers exact test)

Conclusion:
RMS considers that the NOAEL is the lowest dose taking into account the slight increased incidence of staining of lipofuscin in the follicular epithelium of thyroids of females at 5000 ppm and above. The effects of lenacil on the thyroid are not clear and could result

1) from an effect on hypothalamic/thyroid axis resulting from the enzyme inducing effect; however this was not demonstrated,

but could also result

2) from an atrophic change, which was not evident from this study. Black thyroid is rare and pigment accumulation in normal tissue is thought to occur by inhibition of thyroid peroxidase.

Thyroid accumulation of lipofuscin: accumulation of lipofuscin in thyroid could suggest that atrophy occurred and that membranes of destroyed organelles were converted within the lysosomes to lipid containing lipofuscin. Lipofuscin is itself not injurious to the cell, but its presence suggests that something adverse has occurred. Moreover, RMS considered that no sufficient information is provided for complete interpretation of the observed changes. However, the issue is not compromising the determination of the reference doses. In the meanwhile, it was demonstrated that lenacil has enzyme inducing capacities, possibly addressing point 1 of the discussion.

According to the notifier:
"It is concluded that oral administration, via the diet, to Han Wistar rats of Lenacil technical at a concentration of 50000 ppm caused an increase in the incidence and severity of Schmorl's-positive staining in ♀ and a slight increase in the severity of this finding in ♂. In view of the nature of the staining reaction applied in this highly specific study, it was not possible to establish evidence for any significant recovery after four weeks respite from treatment. The no-observed-effect level (NOEL) for changes in the thyroid as identified by this study was 5000 ppm."

"The notifier disagrees with RMS in relation to the interpretation of the effect of lenacil on the thyroid. While it is possible that the effects of lenacil at 50000 ppm were evident in terms of lipofuscin staining, there are no findings in the study to support the postulated causes of minor thyroid changes. The report author and the notifier consider it is reasonable to assume, in the absence of any such evidence, that the slight changes noted at 5000 ppm were not adverse and that 5000 ppm is a valid choice of NOAEL."

Conclusion (90d rat, thyroid re-assessment)
NOAEL = 500 ppm, equivalent to **41 mg/kg bw/day**

LOAEL = 5000 ppm, equivalent to 412 mg/kg bw/day, based on ↑ Schmorl's positive pigment in thyroid cells

The establishment of this NOAEL does not influence the overall assessment of the subchronic rat study, where the NOAEL was identified at the same dose as in this mechanistic study. However, the endpoint should be added to the 90d findings in the rat.

B.6.3.2.2 Oral 90-day toxicity in the mouse

B.6.3.2.2.1
Subchronic oral toxicity: 90-day study with DPX-B634-91 (lenacil) feeding study in mice ([redacted] 1991)
DuPont Report No.: HLR 293-91

Guidelines: study is not fully in compliance with Dir 2001/59/EC or 87/302 or OECD test guideline n° 408 (1998-81).
Deviations from OECD TG 408 : the coagulation time was not measured; epididimydes, thymus, uterus and ovaries were not weighed; salivary gland, stomach and urinary bladder were not examined for histopathology; blood chemistry was limited to proteins; and duration of treatment and sacrifice time was not clearly reported.
According to the Notifier: despite the deviations, when assessed in conjunction with the subchronic feeding study in rats (ACD 002/013903), it adequately completes the subchronic toxicity profile in rodents when administered via the diets. The study is considered valid.
GLP status: yes (no attest of competent authority)
Materials and Methods
Lenacil technical (Code DPX-B634-91, batch no. 9038, purity 98.2%) was incorporated into the ground diet to provide the required concentrations.
Before the commencement of treatment, concentration, homogeneity and stability investigations were carried out. A repeat homogeneity analysis was carried out from samples collected on day 46. The actual concentration average range was between 103 and 116 % from nominal values. The stability in diet was confirmed over 14 days. Ten (10) CrL: CD-1(ICR) BR mice/sex/dose received lenacil technical orally, via the diet, at concentrations of 0, 100, 1000, 5000 and 10000 ppm. Body weight and food consumption were determined weekly. Evaluation of haematology parameters was performed at 45 and 90 days. At termination, all mice were sacrificed, selected organs were weighed and tissues examined microscopically.

Dose (ppm)	0		100		1000		5000		10000	
Achieved dose :	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
mg/kg bw/d	0	0	15.5	20.2	157	207	787	1127	1616	2150

Statistical analysis: one way analysis of variance for bw, bw gain, organ weight, clinical laboratory; Dunnetts test for comparison between test and control; incidence of clinical signs was evaluated by the Fisher exact test with a Bonferroni correction and Cochran-Armitage test for trend. The Bartletts test for homogeneity of variance was performed on organ weight and clinical laboratory data if significant.

The study is accepted.

Findings
Mortality: did not occur during the course of the study.
Body weight: no effects were reported on body weight or body weight gain
Food consumption: was not affected and food efficiency was not altered.
Clinical signs: a compound-related effect on the incidence of clinical signs was not evident. A few signs are reported in table **B.6.3.2.2.1-1.**
Ophthalmoscopy: all of the mice examined were normal.

B.6.3.2.2.1-1 90-day mouse study ([redacted] 1991): clinical signs, body weight, body weight gain & food consumption.

	Male					Female				
Dose (ppm)	**0**	**100**	**1000**	**5000**	**10000**	**0**	**100**	**1000**	**5000**	**10000**
Clinical signs										
Alopecia	1	0	0	4	2	0	1	1	1	1
Colored discharge eye(s)	-	-	-	-	-	2	0	1	0	0
Ruffled fur	0	1	**2**	**2**	2	0	0	0	**1**	**1**
Sore	1	4	**6**	2	0	0	0	0	**1**	**3**
Body weight (g)	36.6 ± 2.8	37.2 ± 4.1	38.9 ± 2.8	37.8 ± 3.5	38.9 ± 3.1	25.8 ± 2.6	26.9 ± 2.7	26.0 ± 2.0	26.9 ± 3.2	26.4 ± 1.8
Body weight gain d0-91 (g)	6.2 ±1.6	6.5 ±1.9 (↑5%)	8.3 ±1.5 (↑34%)	7.2 ±1.6 (↑16%)	7.9 ±1.5 (↑27%)	2.8 ±1.6	3.8 ±2.1 (↑36%)	2.9 ±1.1 (↑4%)	4.4 ±3.2 (↑57%)	3.8 ±1.2 (↑36%)
Food consumption d0-91 (g)	5.3	5.4	5.6	5.5	5.8	5.2	5.1	5.1	5.6	5.3

N° of animals examined: 10/dose/sex.

Haematology:

Male mice had meaningfully decreased mean total leucocytes at 1000 ppm onwards and this effect was related to decreased neutrophils, lymphocytes and monocytes (affected at 45-day sampling). A similar trend was observed in 1000, 5000 and 10000 ppm ♀at 45-day sampling period, although differences were not statistically significant at all doses. WBC, L, M and N counts were depressed (>10%) at all doses in ♂, but this was statistically significant at 1000 ppm and above.

At the 45-day evaluation period, ♂ mice administered 1000 ppm onwards had significantly increased RBC counts. In addition, Hb was significantly higher at 1000 and 10000 ppm ♂. All treated ♂ had higher hematocrit values compared to controls.

A depression was also seen for the platelet count. All the decreases were still observed after 90 days, but only at 1000 ppm and higher, suggesting some attenuation, at least at low-dose (100 ppm). While similar modulations were seen in ♀ after 45 days, they were not generally observed after 90 days, suggesting that the effect of lenacil on these haematological parameters has a lesser impact in ♀.

At the 90-day sampling period, the neutrophil count was lower for the top-dose ♀. The leucopenia observed in ♂and ♀ at 1000ppm and higher was considered to be compound related. At the 90-day evaluation, 1000, 5000, 10000 ppm ♀ had significantly higher hematocrit values and mean corpuscular Hb values, which were however within the range of biological variations and not considered to be biologically significant.

Of note, the haematological effects observed seem to reach a 'plateau' at doses ≥1000 ppm for many parameters, and a proper dose-response was in many cases not evident. However, as haematologic perturbations are observed throughout this dossier in different species, they deserve a further discussion from the notifier.

Notifier:
"This type of data is normally quite variable (untreated animals have a broad range of values), and thus, it is best to look at multiple related parameters over different sampling times. In the 90-day study WBC and NEU were affected in males at the Day 45 sampling at ≥1000 ppm, and WBC, NEU and LYM were affected at the Day 90 sampling. None of the changes in females were graded as adverse in the 90-day study. Interestingly, there were no consistently marked findings in males at any sampling in the 18-month study (over 3 to 18 months), tested up to 7000 ppm. The transient nature and lack of severity for these findings suggests that they are of borderline adversity in the mouse. This position is supported by the fact that most of the changes were within the laboratories reference range (the reference range for the 13-week study will be provided)."

B.6.3.2.2.1-2 90-day mouse study ([redacted] 1991): Haematology, after 45 and 90-days.

	Male					Female				
Dose (ppm)	**0**	**100**	**1000**	**5000**	**10000**	**0**	**100**	**1000**	**5000**	**10000**
Dose (mg/kg bw/d)	**0**	**15.5**	**157**	**787**	**1613**	**0**	**20.2**	**207**	**1127**	**2150**
45-day										
RBC (x 10^6/µL)	8.92 ±0.45	9.50 ±0.64 (7%)	**10.09* ±0.98 (↑13%)**	**9.77* ±0.53 (↑10%)**	**9.99* ±0.66 (↑12%)**	9.31 ±0.41	9.52 ±0.37	9.70 ±0.61	9.39 ±0.37	9.24 ±0.54
Hb (g/dL)	15.0 ±0.6	15.7 ± 0.6	16.7*±1.7 (↑11%)	16.0 ± 0.9	16.7*±1.3 (↑11%)	14.9 ±0.4	15.1 ±0.4	15.9 ±1.0	15.5 ±0.5	14.8 ±0.6
Ht (%)	44 ±2	47# ± 2 (↑7%)	49# ± 5 (↑11%)	47# ± 2 (↑7%)	49# ± 3 (↑11%)	44 ±1	45 ± 1	48* ±3 (↑9%)	46 ±2	45 ± 2
MCV (fL)	50 ±1	49 ± 2	49 ± 2	48 ± 1	49 ± 1	48 ±2	47 ± 2	49 ± 2	49 ± 2	49 ± 2
MCH (pg)	17 ±0	17 ± 1	17 ± 0	16 ± 0	17 ± 1	16 ±1	16 ± 1	16 ± 1	17 ± 1	16 ± 1
MCHC (g/dL)	34 ±1	34 ± 1	34 ± 0	34 ± 1	34 ± 1	33 ±0	33 ± 0	33 ± 0	33 ± 1	33 ±1
PLAT (x 10^3/µL)	1412 ±237	1334 ±300 (↓6%)	918* ±380 (↓35%)	1262 ±188 (↓11%)	987* ±326 (↓30%)	1350 ±321	1306 ±207 (↓3%)	1105 ±333 (↓18%)	1138 ±187 (↓16%)	1146 ±178 (↓15%)
WBC (x 10^3/µL)	9.3 ±2.3	7.9 ± 2.4 (↓15%)	**6.8* ±2.1 (↓27%)**	**6.4* ±1.9 (↓31%)**	**6.6* ± 1.9 (↓29%)**	7.5 ±2.6	7.4 ± 1.4 (1%)	**5.9 ± 1.5 (↓21%)**	**5.4 ± 1.4 (↓28%)**	**5.4 ± 2.4 (↓28%)**
Neut	1247 ±476	906±295 (↓27%)	**609* ±233 (↓51%)**	**752*±388 (↓40%)**	**557*±236 (↓39%)**	915 ±442	834±460 (↓9%)	**567 ±241 (↓38%)**	**551 ±292 (↓40%)**	**517 ±229 (↓43%)**

	Male					Female				
Dose (ppm)	**0**	**100**	**1000**	**5000**	**10000**	**0**	**100**	**1000**	**5000**	**10000**
Dose (mg/kg bw/d)	**0**	**15.5**	**157**	**787**	**1613**	**0**	**20.2**	**207**	**1127**	**2150**
Band (WBC x %)	0 ±0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	6 ± 20	6 ± 18	7 ± 22	0 ± 0
Lymph (WBC x%)	6969 ±179 7	6172 ±2106 (↓14%)	5539 ±1786 (↓21%)	4998 ±1478 (↓28%)	5408 ±1605 (↓23%)	6105 ±2126	6029 ±1053 (↓1%)	4937 ±1335 (↓19%)	4566 ±1196 (↓25%)	4475 ±2166 (↓27%)
Alym (WBC x %)	0 ± 0	5 ± 16	17 ± 36	50* ± 36	26 ± 36	25±31	22 ± 36	5 ± 16	5 ± 17	15 ± 25
Mono (WBC x %)	1022 ±344	803 ±377 (↓21%)	**581* ±219 (↓43%)**	**527*±334 (↓48%)**	**521*±242 (↓49%)**	408 ±225	449 118	**302 ±76 (↓26%)**	**242 ±117 (↓41%)**	**302 ±215 (26%)**
Eosin (WBC x %)	51 ±56	53 ± 69	54 ± 57	43 ± 48 (↓16%)	**88 ± 100 (↑73%)**	58 ± 51	49 ± 55 (↓16%)	43 ± 53 (↓26%)	58 ± 87	61 ± 78
Baso (WBC x %)	0 ±0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
90-day										
RBC (x10^6/µL)	9.13 ±0.60	9.31 ±0.59	9.62 ±0.56	9.52 ±0.31	9.63 ±0.61	9.22 ±043	9.60 ±0.49	9.57 ±0.45	9.56 ±0.58	9.52 ±0.44
Hb (g/dL)	14.3± 0.8	14.6±0.7	14.8 ±1.0	14.7 ±0.5	15.1 ±1.0	14.9± 0.5	15.3±0.7	15.7 ±0.6	15.6 ±0.6	15.3 ±0.7
Ht (%)	49 ±3	50 ± 2	51 ± 4	51 ± 2	52 ± 3	49 ±1	51 ± 2	**54* ± 2 (↑10%)**	**54* ± 2 (↑10%)**	**53* ± 2 (↑8%)**
MCV (fL)	54 ±1	54 ± 3	53 ± 2	54 ± 1	54 ± 2	54 ±2	53 ± 2	56 ± 2	56* ± 2	55 ± 2
MCH (pg)	16 ±0	16 ± 1	15 ± 0	15 ± 0	16 ± 1	16 ±1	16 ± 0	16 ± 1	16 ± 1	16 ± 1
MCHC (g/dL)	29 ±0	29 ± 0	29 ± 0	29 ± 0	29 ± 1	30 ±1	30 ± 0	29* ± 0	29* ± 0	29* ± 0
PLAT (x10^3/µL)	1176 ±308	1161 ±191	1060 ±206 (↓10%)	1146±149 (↓3%)	**945*±326 (↓20%)**	1023 ±367	1009±20	986 ±316 (↓4%)	**891 ±266 (↓13%)**	**870 ±212 (↓15%)**
WBC (x 10^3/µL)	5.6 ±1.2	5.2 ±1.7 (↓7%)	**3.9# ±0.7 (↓30%)**	**3.5# ±0.9 (↓38%)**	**3.7# ± 0.6 (↓34%)**	3.8 ±1.5	5.1 ± 1.8 (↑34%)	3.8 ± 1.0	4.0 ± 0.7	3.7 ± 1.2
Neut	1057 ±545	1001±93 9 (↓5%)	638# ±290 (↓40%)	667# ±309 (↓37%)	**384# ±153 (↓64%)**	564 ±207	744±734 (↑32%)	533 ±191	**501 ±261 (↓11%)**	**392 ±208 (↓30%)**
Band (WBC x%)	5 ±17	10 ± 21	0 ± 0	0 ± 0	0 ± 0	11±24	7 ± 21	0 ± 0	4 ± 13	4 ± 14
Lymph (WBC x %)	4107 ±910	3831 ±1485 (↓7%)	**2896* ±615 (↓29%)**	**2600* ±653 (↓37%)**	**3027* ±592 (↓26%)**	3032 ±1272	3819 ±933	2989 ±890	3221 ±741	3101 ±964
Alym (WBC x%)	17 ±28	12 ± 26	9 ± 19	33 ± 22	20 ± 19	5±14	7 ± 21	8 ± 17	6 ± 13	6 ± 13
Mono (WBC x%)	373 ±259	339 ±189 (↓9%)	339 ±180 (↓9%)	179 ±134 (↓52%)	227 ±179 (↓39%)	138 ±117	463 ±661	230 ±167	182 ±113	167 ±156
Eosin (WBC x%)	61 ±51	48 ± 49 (↓21%)	49 ± 42 (↓20%)	21 ± 32 (↓66%)	42 ± 29 (↓31%)	84 ±86	82 ± 44	20* ± 26 (↓76%)	56 ± 58 (↓33%)	39 ± 34 (↓54%)
Baso (WBC x%)	0 ±0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ±0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

N° of animals examined: 10/dose/sex.
*: significantly different from control at 5% level by Dunnett criteria;
#: significantly different from control at 5% level by Mann-Whitney U
RBC: erythrocytes; Hb: hemoglobin concentration; Ht: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration: PLAT: platelets; WBC: white blood cells (leukocytes); Neut: neutrophils; Band: band neutrophils; Lymph: lymphocytes; Alym: atypical lymphocytes; Mono: monocytes; Eosin: eosinophils; Baso: basophils.

Clinical chemistry:

Plasma proteins were slightly increased in ♂at 5000 and 10000 ppm. Other parameters were not measured (**table B.6.3.2.2.1-3**).

B.6.3.2.2.1-3 90-day mouse study ([redacted] 1991): Plasma protein, after 45 and 90-days.

Dose	Male					Female				
(ppm)	0	100	1000	5000	10000	0	100	1000	5000	10000
mg/kg bw/d	0	15.5	157	787	1613	0	20.2	207	1127	2150
45-day	6.1 ±0.4	6.3 ±0.3 (↑3%)	6.6* ±0.5 (↑8%)	6.4 ±0.2 (5%)	**6.7* ±0.4 (↑10%)**	5.8 ±0.3	5.9 ±0.2	6.2* ±0.2	5.9 ±0.3	6.0 ±0.3
90-day	5.9 ±0.3	6.1 ±0.2 (↑3%)	6.2 ±0.3 (↑5%)	**6.3* ±0.3 (↑7%)**	**6.3* ±0.3 (↑7%)**	5.7 ±0.3	5.9 ±0.3	5.9 ±0.2	5.8 ±0.2	5.9 ±0.3

*: significantly different from control at 5% level by Dunnett criteria; #: significantly different from control at 5% level by Mann-Whitney U; N° of animals examined 10/dose/sex

Organ weight: the relative liver weight was increased in ♀ at 5000 and 10000 ppm. Absolute liver weight was increased in ♂at top-dose (10000 ppm). Spleen weight was increased in ♀ at top-dose.
Remark: thyroid, thymus, uterus, ovaries, prostate, epididymides were not weighed.

B.6.3.2.2.1-4 90-day mouse study ([redacted] 1991): Absolute and relative organ weight (g)

Dose	Male					Female				
ppm	0	100	1000	5000	10000	0	100	1000	5000	10000
mg/kg bw/d	0	15.5	157	787	1613	0	20.2	207	1127	2150
Liver (a)	1.901 ±0.148	1.965 ±0.202	1.964 ±0.107	2.001 ±0.193 (↑5%)	**2.148 ±0.259 (↑13%)**	1.240 ±0.185	1.335 ±0.123	1.327 ±0.166	**1.501# ±0.174 (↑21%)**	**1.514# ±0.332 (↑22%)**
Liver (r)	5.0845±0.2605	5.1496± 0.4206	4.9603± 0.2454	5.1947 ±0.4165	5.4076 ±0.5530 (↑6%)	4.6571 ±0.4129	4.7794 ±0.4416	4.8762 ±0.4588	**5.3202 ±0.4202 (↑14%)**	**5.4338# ±1.005 (↑17%)**
Kidney (a)	0.628 ±0.043	0.668 ±0.039	0.625 ±0.172	0.661 ±0.105	0.696 ±0.109	0.410 ±0.053	0.418 ±0.038	0.393 ±0.046	0.410 ±0.052	0.412 ±0.036
Kidney (r)	1.6841 ±0.1303	1.7588 ±0.1632	1.5832 ±0.4494	1.7091 ±0.1642	1.7600 ±0.3079	1.5420 ±0.1419	1.5002 ±0.1690	1.4482 ±0.1761	1.4566 ±0.1926	1.5056 ±0.1801
Heart (a)	0.205 ±0.018	0.197 ±0.025	0.210 ±0.024	0.200 ±0.033	0.212 ±0.028	0.143 ±0.014	0.151 ±0.023	0.142 ±0.013	0.159 ±0.012	0.158 ±0.025
Heart (r)	0.5489 ±0.0504	0.5148 ±0.0459	0.5323 ±0.0687	0.5176 ±0.0693	0.5341 ± 0.0726	0.5378 ±0.0327	0.5392 ±0.744	0.5241 ±0.0461	0.5684 ±0.0592	0.5774 ±0.1030
Spleen (a)	0.102 ±0.017	0.095 ±0.018	0.101 ±0.015	0.102 ±0.020	0.098 ±0.023	0.103 ±0.019	0.095 ±0.016	0.100 ±0.008	0.105 ±0.021	**0.147 ±0.128 (↑43%)**
Spleen (r)	0.2738 ±0.0509	0.2464 ±0.0327 (↓10%)	0.2541 ±0.0326	0.2335 ±0.0513 (↓15%)	0.2469 ±0.0614 (↓10%)	0.3871 ±0.0562	0.3374 ±0.0361 (↓13%)	0.3679 ±0.0337	0.3721 ±0.0660	**0.5262 ±0.4424 (↑36%)**
Brain (a)	0.490 ±0.028	0.484 ±0.016	0.489 ± 0.023	0.486 ± 0.019	0.487 ± 0.014	0.495 ±0.020	0.484 ±0.019	0.476 ±0.026	0.484 ±0.025	0.440 ±0.153
Brain (r)	1.3155 ±0.1179	1.2760 ±0.1375	1.2562 ± 0.1062	1.2696 ± 0.1436	1.2324 ± 0.1183	1.8746 ±0.1555	1.7404 ±0.1862	1.7558 ±0.0893	1.7254 ±0.1309	1.6141 ±0.5734
Adrenals (a)	0.010 ±0.004	0.006 ±0.004 (↓40%)	0.007 ±0.003 (↓30%)	0.008 ±0.005 (↓20%)	0.007 ±0.003 (↓30%)	0.012 ±0.004	0.012 ±0.002	0.011 ±0.003	0.012 ±0.003	0.012 ±0.003
Adrenals (r)	0.0264 ±0.0118	0.0166 ±0.0108 (↓37%)	0.0168 ±0.0067 (↓37%)	0.0220 ±0.0144 (↓17%)	0.0169 ±0.0092 (↓36%)	0.0456 ±0.0132	0.0418 ±0.0081	0.0395 ±0.0097	0.0417 ±0.0116	0.0457 ±0.0127
Testes (a)	0.243 ±0.039	0.253 ±0.041	0.252 ±0.033	0.227±0.044 (↓9%)	0.245 ±0.035					
Testes (r)	0.6494 ±0.0991	0.6699 ±0.1434	0.6347 ±0.1048	0.5733 ±0.0933	0.6204 ±0.1039					

Absolute (a) and relative (r) mean organ weight #: significantly different from control at 5% level by Dunnett's test; N° of animals examined 10/dose/sex

B.6.3.2.2.1-5 90-day mouse study ([redacted] 1991): Macropathology

Dose	Male					Female				
ppm	0	100	1000	5000	10000	0	100	1000	5000	10000
mg/kg bw/d	0	15.5	157	787	1613	0	20.2	207	1127	2150
Skin (stain)	0	0	0	0	1					
Kidneys (discolouration)	0	0	1	0	0					
Kidneys (cyst)						0	0	0	0	1
Ovaries (cyst)						1	1	0	1	2
Spleen (large)						0	0	0	0	1
Mesenteric LN (discolouration)						0	1	0	0	0
Pinna (deformity)	0	3	4	1	0	0	0	0	0	1

N° of animals examined 10/dose/sex

Histopathological findings:

A higher incidence of extramedullary haematopoiesis was seen in ♀ at top dose in liver and spleen **(table B.6.3.2.2.1-6).** Other notable events observed at top-dose include lymphoid cell foci/hyperplasia and focal inflammation, suggestive of potential slight immunomodulation.

B.6.3.2.2.1-6 90-day mouse study ([redacted] 1991): Histopathology

Dose groups	Effect	Male					Female				
ppm		0	100	1000	5000	10000	0	100	1000	5000	10000
mg/kg bw/d		0	15.5	157	787	1613	0	20.2	207	1127	2150
Liver	Extramedullary haematopoiesis	0	0	0	0	2	0	0	0	0	4
	Necrosis, focal	1	0	0	0	0					
	Inflammation, focal						1	2	0	0	1
	Kupffer cell, hyperplasia						0	0	0	0	1
	Mitosis, hepatocellular						0	0	0	0	1
	Single cell necrosis, hepatocellular						0	1	1	1	1
Kidneys	Proximal tubule, vacuolation	0	0	1	0	0					
Kidneys	Cyst						0	0	0	0	1
Lungs	Alveolitis, focal	0	0	0	0	1					
Spleen	Extramedullary haematopoiesis	0	0	0	0	1	2	0	0	0	5
Spleen	Lymphoid cell hyperplasia	0	0	0	0	2					
Stomach	Inflammation, focal	0	0	0	0	1					
Mesenteric LN	Hyperplasia	0	0	0	0	2	0	1	0	0	0
Salivary glands	Submaxillary, lymphoid cell foci	0	0	0	0	3					
Mandibular lymph node	Hyperplasia	1	0	0	0	1	0	0	0	0	1
Exorbital lacrimal gland	Lymphoid cell foci	2	0	0	0	3					
Harderian gland	Inflammation, focal[U]	5	0	0	0	3	3	0	0	0	3
Eyes	Optic nerve, necrosis[U]	0	0	0	0	1					
Eyes	Retina, degeneration, diffuse[U]	1	0	0	0	0					
Eyes	Retina, outer nuclear layer, atrophy, focal[U]						4	0	0	0	0
Ovaries	Cyst[B]						0	0	0	0	1
Ovaries	Cyst[U]						0	1	0	1	1
Other											
Ear,	pinna, dermatitis[B]	1	0	1	1	0	0	0	0	0	1
Ear,	pinna, dermatitis[U]	0	3	4	0	0					
Eye,	nictiting membrane, malformation[U]	0	1	0	0	0					
Leg	LN, hyperplasia	0	0	0	0	1					
Tail	dermatitis	0	0	1	0	0					

N° of animals examined 10/dose/sex; [U]: unilateral; [B]: bilateral; N=10 animals/sex/dose (except ♀ control group: N=9)

Conclusion (90d mouse)

Whereas, originally, the lowest dose was proposed as a NOAEL,
NOAEL = 100 ppm = 15.5 mg/kg bw/d,
LOAEL = 1000 ppm = 157 mg/kg bw/d,
based on blood toxicity in ♂ and ♀ mice

It was commented that it was difficult to assess whether the effects on blood (in particular leucopenia) im mice at dose levels of 1000 ppm and above are toxicological relevant since a dose response is lacking. The NOAEL is rather seen at 1000 ppm (157 mg/kg bw/d) than at 100 ppm (15.5 mg/kg bw/d). At least, because of this uncertainty and also the wide dose spacing, it is doubtful whether this study may in fact provide the most suitable basis to derive the AOEL. Instead, the 90-day study in rats might be used.
RMS agreed that a dose response is lacking but is probably related to a saturation process of oral absorption at high doses as suggested in the ADME part of the DAR: at single dose as high as 1000 mg/kg bw (corresponding to 5000 ppm in mice) oral absorption is very low (0-7%). Repeated dosing increased the absorption of the low dose but it is unknown if this applies to a high dose.
Therefore, **RMS** considered that the lack of dose effect results from a low oral absorption with a plateau in the toxic effects.
During the original peer review (first inclusion of lenacil) the relevant NOAEL from the 90-day mice study was discussed and agreed to be 157 mg/kg bw/d, based on increase liver weight in ♀ treated at dose level of 787 mg/kg bw/d.

NOAEL = 1000 ppm = **157 mg/kg bw/d**,
LOAEL = 5000 ppm = **787 mg/kg bw/d,**
based on increased liver weight.
At top dose : ↑proteinuria, ↑liver and spleen weight, ↑hyperplasia in LN, ↑extramedullary haematopoiesis in liver and spleen.

RMS is still uncertain on the relevance of the consistent effect of the a.s. on WBC, including at doses <157 mg/kg b.w./d in the subchronic mouse study, and considers that a further re-discussion may be appropriate.

First comment from the notifier:
"Oral administration, via the diet, to CrL: CD-1 mice of lenacil technical at concentrations up to 10000 ppm for 13 weeks did not produce any significant toxic effect. Adaptive changes in the liver occurred at 5000 and 10000 ppm (increased organ weight without concomitant histopathological changes) and reduced neutrophilic granulocytes, lymphocyte and monocyte numbers occurred from and including 1000ppm onwards, the latter findings being of uncertain toxicological significance, because these findings were not dose-related. Therefore these haematological findings are not considered to be of toxicological importance. Due to liver weight changes, the NOEL can be set at 1000ppm, (equivalent to 157 mg/kg/day in males and 207 mg/kg/day in females).
Since all statistically significant changes in haematology and organ weight determinations were considered due to an adaptive effect rather than a significant toxicological effect, 10000ppm could be classified as the highest NOAEL in this study, equivalent to 1616 mg/kg/bw/day for males and 2150 mg/kg/bw/day for the females, respectively."

Further comment from the notifier (renewal):
*"The notifier conclusions presented above are based on a lack of dose relationship for the majority of haematological findings, combined with an absence of consistency between weeks 6 and 13 and the absence of any increasing effect with repeated administration of lenacil. This combined with the adaptive response in liver weight at the 1000 ppm and 5000 ppm level, demonstrates that 100 ppm can be clearly stated to be an NOEL but the level at which non-adverse findings are detected is clearly higher than the NOEL. The toxicological and biological significance of the high dose findings in mice, when extrapolated to man may be debated, particularly since similar effects were not recorded in the rat when similarly exposed, at doses of less than 5000 ppm. In the opinion of the notifier, 1000 ppm is the NOAEL for this study. Effects on haematology were not considered to be adverse due to the absence of consistent findings in relation to exposure duration and considering the lack of a dose-response relationship. No noteworthy histopathological findings were observable. The NOAEL of the 90-day feeding study in male and female mice was determined to be **157 mg/kg bw/day**, based on leucopenia observed at the next higher dose level of 787 mg/kg bw/day."*

B.6.3.2.3 Oral 90-day toxicity (dog)

B.6.3.2.3.1 Toxicity study by dietary administration to beagle dogs for 13 weeks ([redacted] 2002) DuPont Report No.: ACD 022/014297

Guidelines: EEC Directive 96/54/EEC Method B.27, equivalent to OECD 409.
GLP status: yes
Materials and Methods
Lenacil technical (Batch No. 141712003, purity 98.6%) was incorporated into the ground diet to provide the required concentrations. The homogeneity and stability of Lenacil technical in diet formulations were assessed analytically in trial formulations, at concentrations of 50 and 50000 ppm. Each formulation achieved an accuracy within 3% of the nominal concentration and a precision, measured by the coefficient of variation, of <1.5%. The mean analysed concentrations remained very close to the Day 0 values (±1%) after ambient temperature storage for 22 days. The mean concentrations of Lenacil technical in formulations, prepared for dosing during Weeks 1, 6 and 12 of treatment of the study ranged from 98.4% to 99.9% of nominal concentrations and were considered satisfactory. Three groups of pure-bred beagle dogs (4 ♂and 4♀ animals per group) received Lenacil technical, by dietary administration at dosages of 1000, 5000, or 25000 ppm for 13 weeks. A further group of pure-bred beagle dogs (4♂+4♀ animals) was held as concurrent control receiving basal diet alone. Laboratory examinations were performed prior to the start of the study and at weeks 6 and 13. At terminal autopsy, macroscopic findings and organ weights were recorded and a broad spectrum of organs was subjected to histopathological examination from all animals.

Dose (ppm)	0		1000		5000		25000	
Achieved dose :	1M	1F	2M	2F	3M	3F	4M	4F
mg/kg bw/d	0	0	44.07	45.76	221.19	224.85	1120.67	1101.92

Statistical analysis:
All statistical analyses were carried out separately for males and females. The individual animals are the basic experimental unit. Bodyweight data were analysed using weight gains. Food consumption data could not be analysed statistically due to the small group size (1 cage/sex/group). Organ weight data were analysed as absolute and adjusted for terminal bodyweight, where appropriate.
Bodyweight, haematology, blood chemistry, urinalysis and organ weight data: frequency analysis was applied. Treatment groups were compared using a Mantel test for a trend in proportions and also pairwise Fisher's Exact tests for each dose group against the control. If Bartlett's test for variance homogeneity was not significant at the 1% level, then parametric analysis was applied. If the F1 test for monotonicity of dose-response was not significant at the 1% level, Williams' test for a monotonic trend was applied. If the F1 test was significant, suggesting that the dose-response was not monotone, Dunnett's test (Dunnett 1955, 1964) was performed instead.
If Bartlett's test was significant at the 1% level, then logarithmic and square-root transformations were tried. If Bartlett's test was still significant, then non-parametric tests were applied. If the H1 test for monotonicity of dose-response was not significant at the 1% level, Shirley's test for a monotonic trend was applied. If the H1 test was significant, suggesting that the dose-response was not monotone, Dunn's test was performed instead.
Where appropriate, analysis of covariance was used in place of analysis of variance.
For organ weight data, analysis of variance was performed using terminal bodyweight as covariate when the within group relationship between organ weight and bodyweight was significant at the 10% level in an attempt to allow for differences in bodyweight which might influence the organ weights.
Significant differences between control and treated groups were expressed at the 5% ($p<0.05$), or 1% ($p<0.01$) level.
The study is accepted.

Findings

Mortality:
there were no unscheduled deaths.
Clinical signs:
there were no signs of ill health, behavioural change or reaction to treatment.
Body weight:
bw gain was slightly reduced at top dose and in ♂ at 5000ppm. However, the statistical significance was not attained and the differences from controls were not considered to represent an effect of treatment. Lower mean bw gain was also noted for ♀at 1000ppm and this effect was attributable to 1 ♀ (**table B.6.3.2.3.1-1**).
Food consumption:
was near maximal and was similar to that of the controls for all groups (**table B.6.3.2.3.1-1**).

Table B.6.3.2.3.1-1 90-day dog study ([redacted] 2002): body weight gain and food consumption.

	Male				Female			
	1M	2M	3M	4M	1F	2F	3F	4F
Dose (ppm)	0	1000	5000	25000	0	1000	5000	25000
mg/kg bw/d	0	44	221	1121	0	46	225	1102
Bw gain wk 0-13 (kg)	3.7	3.5 (↓5%)	3.4 (↓8%)	**3.2 (↓13%)**	3.3	2.5 (↓25%)	3.0 (↓9%)	2.8 (↓15%)
Food consumption	No compound related effect							

N° of animals examined 4/dose/sex

Behavioural investigations: there were no treatment-related changes.

Ophthalmoscopy: there were no treatment-related changes.

Haematology:

Numerous variations > 10% were found, affecting reticulocytes, but also WBC, neutrophils (N), lymphocytes (L) and monocytes (M), as frequently observed in rodents. As also observed in rodents, many parameters were significantly modified in a non-dose-responsive way. Meaningful changes were restricted to top-dose animals (↑APTT, ♂) or at 5000 ppm and above (↓reticulocytes, ♂).

The evaluation of other parameters indicated that there were no differences from controls thought to be related to treatment (**table B.6.3.2.3.1-2**).

Table B.6.3.2.3.1-2 90-day dog study ([redacted] 2002): Haematology, predose, week 6 and week 13.

		Male				Female			
	wk	1M	2M	3M	4M	1F	2F	3F	4F
ppm		0	1000	5000	25000	0	1000	5000	25000
mg/kg bw/d		0	44	221	1121	0	46	225	1102
Hct (L/L)	-1	0.409 ± 0.0342	0.399 ± 0.0330	0.384 ± 0.0215	0.398 ± 0.0360	0.384 ± 0.0039	0.427** ±0.0155	0.410* ±0.0167	0.409* ±0.0166
	6	0.382 ± 0.0530	0.381 ± 0.0250	0.358 ± 0.0248	0.370 ± 0.0291	0.395 ± 0.0144	0.414 ± 0.0175	0.376 ± 0.0151	0.422 ± 0.0222
	13	0.398 ± 0.0285	0.395 ± 0.0223	0.397 ± 0.0191	0.394 ± 0.0280	0.409 ± 0.0350	0.419 ± 0.0255	0.404 ± 0.0136	0.414 ± 0.0143
Hb (g/dL)	-1	12.6 ± 0.92	12.4 ± 1.02	11.9 ± 0.54	12.4 ± 0.97	11.8 ± 0.31	13.3** ±0.66	12.7* ±0.41	12.6 ±0.45
	6	13.0 ± 1.83	13.0 ± 0.81	12.3 ± 0.74	12.6 ± 0.97	13.5 ± 0.55	14.2 ± 0.49	12.8 ± 0.49	14.3 ± 0.77
	13	13.6 ± 1.09	13.5 ± 0.79	13.6 ± 0.72	13.4 ± 0.96	13.8 ± 1.04	14.3 ± 0.68	13.85 ± 0.43	14.3 ± 0.48
RBC (x 10^{12}/L)	-1	5.68 ± 0.495	5.65 ± 0.345	5.41 ± 0.353	5.44 ± 0.570	5.27 ± 0.167	5.88** ±0.239	5.64* ±0.289	5.66* ±0.204
	6	5.52 ±0.762	5.59±0.328	5.27 ±0.407	5.31 ±0.466	5.65 ± 0.173	5.94 ± 0.183	5.37 ± 0.316	6.04 ± 0.228
	13	5.90 ± 0.564	5.86 ± 0.217	6.00 ± 0.297	5.80 ± 0.526	5.95 ± 0.486	6.11 ± 0.280	5.95 ± 0.326	6.17 ± 0.222
Retic (%)	-1	0.92 ± 0.396	0.88 ± 0.328	0.74 ± 0.148	0.78 ± 0.184	0.67 ± 0.182	0.91 ± 0.323	0.67 ± 0.261	1.00 ± 0.437
	6	1.14 ± 0.36	0.94±0.370 (↓18%)	**0.61*±0.13 (↓46%)**	**0.80*±0.10 (↓30%)**	0.63 ± 0.137	0.76 ± 0.267 (↑21%)	0.73 ± 0.162 (↑16%)	0.81 ± 0.381 (↑29%)
	13	0.87 ± 0.293	0.86 ±0.280	**0.57±0.125 (↓34%)**	**0.51±0.164 (↓41%)**	0.45 ± 0.121	0.56 ±0.181 (↑24%)	0.54 ± 0.187 (↑20%)	0.57 ± 0.421 (↑27%)
MCH (pg)	-1	22.3 ± 0.53	22.0 ± 0.99	22.0 ± 0.89	22.8 ± 0.68	22.5 ± 0.31	22.7 ± 0.48	22.5 ± 0.63	22.2 ± 0.34
	6	23.5 ± 0.72	23.4 ± 0.53	23.4 ± 1.01	23.7 ± 0.82	23.9 ± 0.58	23.9 ± 0.13	23.8 ± 0.84	23.6 ± 0.38
	13	23.1 ± 0.62	23.1 ± 0.62	22.8 ± 1.09	23.2 ± 0.73	23.3 ± 0.42	23.4 ± 0.13	23.3 ± 0.86	23.1 ± 0.16
MCHC (g/dL)	-1	30.9 ± 0.44	31.2 ± 0.50	31.0 ± 0.37	31.1 ± 0.43	30.8 ± 0.59	31.2 ± 0.39	30.9 ± 0.48	30.8 ± 0.43

		Male				Female			
	wk	1M	2M	3M	4M	1F	2F	3F	4F
ppm		0	1000	5000	25000	0	1000	5000	25000
mg/kg bw/d		0	44	221	1121	0	46	225	1102
	6	33.9 ± 0.53	34.3 ± 0.42	34.4 ± 0.25	33.9 ± 0.35	34.2 ± 0.25	34.2 ± 0.40	33.9 ± 0.49	33.8 ± 0.10
	13	34.2 ± 0.54	34.3 ± 0.29	34.4 ± 0.27	34.1 ± 0.13	33.9 ± 0.54	34.1 ± 0.63	34.3 ± 0.48	34.4 ± 0.29
MCV (fL)	-1	72.1 ± 1.50	70.6 ± 2.34	71.0 ± 2.55	73.2 ± 1.47	72.9 ± 2.13	72.6 ± 1.33	72.8 ± 0.97	72.2 ± 0.62
	6	69.3 ± 1.35	68.2 ± 2.35	68.0 ± 2.89	69.9 ± 1.76	70.0 ± 2.03	69.7 ± 0.91	70.2 ± 1.49	69.8 ± 1.16
	13	67.6 ± 1.72	67.4 ± 1.92	66.2 ± 3.14	68.1 ± 2.36	68.5 ± 1.96	68.5 ± 1.22	68.0 ± 1.64	67.2 ± 0.76
WBC (x 10^9/L)	-1	14.46 ± 4.384	13.24 ± 1.953	15.14 ± 6.281	14.49 ± 3.401	10.79 ± 1.312	11.56 ± 1.560	11.29 ± 1.958	13.15 ± 1.877
	6	12.68 ± 3.726	12.03±2.55 2 (↓5%)	11.69±1.98 1 (↓8%)	13.07±2.86 2 (↑3%)	9.70 ± 2.193	12.19 ± 3.057 (↑26%)	11.42 ± 1.805 (↑18%)	11.04 ± 1.446 (↑14%)
	13	11.47 ±1.532	13.26±3.43 4 (↑16%)	16.50±5.63 3 (↑44%)	11.88±1.71 6 (↑4%)	10.21 ±1.084	11.07 ± 2.046 (↑8%)	11.87 ± 2.117 (↑16%)	10.49 ± 0.489 (3%)
Neutrophils (x 10^9/L)	-1	9.01 ± 3.704	7.62 ± 1.291	9.76 ± 5.685	8.26 ± 2.032	6.15 ± 1.207	6.03 ± 0.981	6.27 ± 1.211	7.81 ± 0.994
	6	7.23 ± 2.762	7.11±1.641 (↓2%)	6.99±1.823 (↓3%)	7.39±2.473 (↑2%)	5.61 ± 1.596	6.97 ±2.548 (↑24%)	6.43 ±1.218 (↑15%)	6.34 ±1.1365 (↑13%)
	13	6.62 ± 1.541	8.28±3.236 (↑25%)	11.14±5.40 6 (↑68%)	6.57±0.914 (↓1%)	6.00 ± 1.011	6.28 ± 1.283 (↑5%)	6.88 ± 1.311 (↑15%)	5.93 ± 0.657 (↓1%)
Lymphocyte (x 10^9/L)	-1	4.20 ± 0.264	4.53 ± 1.400	3.95 ± 1.053	4.87 ± 0.980	3.65 ± 0.528	4.30 ± 0.785	3.95 ± 1.130	3.90 ± 1.484
	6	4.52 ±0.959	4.16±1.234 (↓8%)	3.78±0.866 (↓16%)	4.78±0.944 (↑6%)	3.43 ± 0.578	4.12 ± 0.512 (↑20%)	4.10 ± 0.731 (↑20%)	3.71 ± 0.763 (↑8%)
	13	3.98 ±0.776	4.02±1.013 (↑1%)	3.96±1.085 (↓1%)	4.33±0.992 (↑9%)	3.40 ± 0.241	3.76 ± 0.699 (↑11%)	3.95 ± 0.840 (↑16%)	3.57 ± 0.599 (↑5%)
Eosinophil (x 10^9/L)	-1	0.12 ± 0.064	0.17 ± 0.077	0.20 ± 0.035	0.19 ± 0.101	0.16 ± 0.026	0.23 ± 0.102	0.22* ± 0.037	0.27 ± 0.044
	6	0.17 ±0.054	0.16 ±0.066	0.19 ±0.039	0.16 ±0.030	0.13 ± 0.026	**0.27** ±0.092** (↑108%)	**0.27** ±0.036** (↑108%)	**0.21** ±0.018** (↑62%)
	13	0.13 ± 0.068	0.23 ± 0.143	0.17 ± 0.036	0.13 ± 0.034	0.16 ± 0.030	0.16 ± 0.049	**0.30** ±0.104** (↑88%)	**0.29** ±0.031** (↑81%)
Basophil (x 10^9/L)	-1	0.06 ±0.010	0.06 ±0.032	0.09*±0.02 6	0.14 ±0.078	0.06 ± 0.013	0.07 ± 0.015	0.06 ± 0.035	0.06 ± 0.005
	6	0.05 ±0.024	0.04 ±0.017	0.04 ±0.013	0.06 ±0.026	0.03 ± 0.010	0.04 ± 0.010	0.03 ± 0.010	0.04 ± 0.024
	13	0.03 ±0.019	0.04 ±0.010	0.05 ±0.010	0.05 ±0.022	0.04 ± 0.013	0.04 ± 0.013	0.04 ± 0.022	0.03 ± 0.010
Monocyte (x 10^9/L)	-1	0.87 ±0.317	0.72 ±0.270	0.91 ±0.334	0.79 ±0.245	0.61 ± 0.143	0.73 ± 0.140	**0.65* ±0.128**	0.92 ±0.178
	6	0.66 ± 0.165	0.53±0.142 (↓20%)	0.64±0.399 (↓3%)	0.63±0.131 (↓5%)	0.47 ± 0.112	0.73 ±0.186 (↑55%)	0.55 ±0.110 (↑17%)	**0.70* ±0.099 (↑49%)**
	13	0.64 ±0.128	0.63±0.345 (↓2%)	1.07±0.594 (↑67%)	0.70±0.171 (↑9%)	0.56 ± 0.070	0.75 ± 0.190 (↑34%)	0.63 ± 0.114 (↑13%)	0.62 ± 0.201 (↑11%)
LUC (x 10^9/L)	-1	0.20 ±0.094	0.16 ±0.082	0.23 ±0.070	0.24 ±0.100	0.16 ± 0.036	0.19 ± 0.049	0.14 ± 0.048	0.20 ± 0.039
	6	0.06 ±0.023	0.05 ±0.022	0.06 ±0.025	0.06 ±0.022	0.04 ± 0.019	0.07 ± 0.019	0.04 ± 0.005	0.04 ± 0.005
	13	0.07 ±0.031	0.07±0.033	0.13 ±0.059 (↑86%)	0.10±0.036 (↑43%)	0.07 ± 0.017	0.07 ± 0.033	0.08 ± 0.042	0.07 ± 0.017
Plt (x 10^9/L)	-1	357 ±93.6	350 ±110.1	391 ± 43.1	371 ± 50.3	339 ±30.7	361 ± 33.5	296 ± 108.9	310 ± 48.8
	6	326 ±75.4	303 ± 64.4	355 ± 50.8	351 ± 37.1	288 ±42.4	305 ± 26.8	256 ± 78.8	301 ± 60.7

		Male				Female			
	wk	**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
ppm		**0**	**1000**	**5000**	**25000**	**0**	**1000**	**5000**	**25000**
mg/kg bw/d		**0**	**44**	**221**	**1121**	**0**	**46**	**225**	**1102**
	13	315 ±46.5	305 ± 48.0	358 ± 29.4	348 ± 17.9	292 ±49.5	303 ± 8.1	258 ± 75.4	301 ± 57.8
PT (sec)	-1	6.3 ±0.49	7.1 ± 1.989	6.0 ± 0.08	6.2 ± 0.17	6.3 ±0.41	6.4 ± 0.52	6.9 ± 1.72	6.1 ± 0.06
	6	6.3 ±0.54	7.1 ± 1.90	6.1 ± 0.10	6.2 ± 0.23	6.4 ±0.44	6.4 ± 0.45	6.9 ± 1.55	6.1 ± 0.14
	13	6.3 ±0.38	7.1 ± 1.87	6.0 ± 0.08	6.2 ± 0.18	6.4 ±0.43	6.5 ± 0.51	6.8 ± 1.52	6.2 ± 0.17
APTT (sec)	-1	21.6 ±1.74	22.2 ± 2.09	21.5 ± 2.88	22.7 ± 3.28	22.9 ±2.62	22.7 ± 1.27	23.2 ± 1.59	24.3 ± 0.78
	6	22.2 ±0.97	22.9 ± 3.00	20.1 ± 4.76	24.4 ± 3.40	23.8 ±1.58	24.0 ± 1.19	23.9 ± 1.42	26.1 ± 1.65
	13	18.3 ±1.88	22.9 ± 2.08	20.2 ±2.08	**22.3*±2.28 (↑22%)**	24.2 ±2.35	22.2 ± 1.15	22.6 ± 1.03	25.3 ± 1.27

N° of animals examined 4/dose/sex; *:↑↓ statistically significant modification: $p<0.05$;
Hct: haematocrit; Hb: haemoglobin concentration; RBC: erythrocytes; Retic: reticulocytes; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; WBC: white blood cells (leukocytes); LUC: large unstained cells ; Plt: platelets; PT: prothrombin time ; APTT: activated partial thromboplastin time.

Clinical chemistry:

During week 6 and 13, higher mean alkaline phosphatase was seen at top dose for both sexes. Alkaline phosphatase was increased in the 28-day study ([redacted], 2001) and probably reflects lenacil liver toxicity, at least at top-dose. The latter may also be reflected by the slightly increased gamma-glutamyl transpeptidase activity at 5000 ppm and above (♂).

Cholesterol level was increased >10%, in both ♂ and ♀ top-dose animals, although not s.s., at mid- and top-dose. Another notable modification was aslight decrease of phosphorous in the top-dose ♀.

There were no other differences from controls thought to be related to treatment as they tended to reflect pre-dose trends and/or were minor in magnitude and did not follow dosage relationships when noted in more than one treatment group (**table B.6.3.2.3.1-3**). Clearly isolated statistically significant modifications at intermediate or low doses without time- and/or dose-dependency were considered fortuitous changes without toxicological relevance.

Urinalysis:

Urinary pH was unremarkable across doses. Top-dose ♀ exbibited significantly more proteins in their urine than control animals. Other parameters showed no differences from controls.

Table B.6.3.2.3.1-3 90-day dog study (2002): Clinical chemistry, predose, week 6 and week 13.

		Male				Female			
	wk	1M	2M	3M	4M	1F	2F	3F	4F
ppm		0	1000	5000	25000	0	1000	5000	25000
mg/kg bw/d		0	44	221	1121	0	46	225	1102
Alk. Phos (U/L)	-1	342 ± 55.5	371 ± 72.1	338 ± 56.4	364 ± 57.2 (↑6%)	331 ± 49.8	321 ± 75.4	301 ± 44.5	297 ± 33.2 (↓10%)
	6	300 ± 70.6	319 ± 27.6	283 ± 54.3	**386*±45.7 (↑29%)**	280 ± 36.1	264 ± 40.6	271 ± 38.3	**341 ±49.1 (↑22%)**
	13	247 ± 66.4	271 ± 30.9	266 ± 60.5	**380**±33.2 (↑54%)**	246 ± 20.3	235 ± 40.2	278 ± 40.8	**322* ± 51.7 (↑31%)**
ALT (U/L)	-1	30 ± 6.6	27 ± 3.5	32 ± 1.7	32 ± 5.1	27 ± 0.6	26 ± 3.7	29 ± 2.2	26 ± 5.9
	6	34 ± 6.2	33 ± 5.7	37 ± 5.9	35 ± 3.2	32 ± 2.5	28 ± 6.7	35 ± 5.1	33 ± 8.7
	13	34 ± 4.7	33 ± 3.4	37 ± 5.5	33 ± 5.4	34 ± 3.5	30 ± 7.0	40 ± 6.4	35 ± 13.2
AST (U/L)	-1	31 ± 3.7	33 ± 7.0	**39** ± 3.8	37 ± 3.9	34 ± 8.8	32 ± 4.7	38 ± 2.7	35 ± 2.9
	6	37 ± 5.6	33 ± 4.8	43 ± 8.0	36 ± 5.1	41 ± 6.9	35 ± 5.1	38 ± 3.5	37 ± 5.1
	13	42 ± 4.3	50 ± 2.1	44 ± 11.2	39 ± 8.2	45 ± 7.9	44 ± 7.8	44 ± 7.3	38 ±9.7
γGT (U/L)	-1	4 ± 0.6	3 ± 0.5	4 ± 1.0	3 ± 1.0	4 ± 1.3	2 ± 1.3	4 ± 1.3	4 ± 1.4
	6	4 ± 0.8	5 ± 1.0	5 ± 0.8	4 ± 0.5	4 ± 1.7	3 ± 2.2	5 ± 1.0	4 ± 1.5
	13	2 ± 1.7	2 ± 1.7	**4 ± 0.6**	**4 ± 1.0**	2 ± 0.5	1 ± 1.3	3 ± 1.4	2 ± 0.5
Bili. Total (µmol/L)	-1	1 ± 0.0	2* ± 0.8	1 ± 0.5	2 ± 0.6	1 ± 0.5	1 ± 0.5	2 ± 0.6	2 ± 0.6
	6	1 ± 0.8	2 ± 0.8	2 ± 0.0	1 ± 0.5	2 ± 1.0	2 ± 0.6	2 ± 0.5	1 ± 0.0
	13	1 ± 0.5	1 ± 0.0	2 ± 1.0	1 ± 0.0	2 ± 1.3	1 ± 0.5	1 ± 0.5	1 ± 0.5
Urea (mmol/L)	-1	2.37±0.618	3.37*±0.269	2.62 ±0.561	2.99 ±0.755	3.17±0.292	3.06 ±0.677	3.73 ± 0.498	3.30 ± 0.303
	6	3.78 ±0.398	4.03 ± 0.414	3.58 ±0.552	3.72 ±0.863	4.34 ± 0.388	3.60 ± 0.611	3.64 ± 0.461	3.78 ± 0.511
	13	4.79 ±1.206	5.24 ± 0.968	5.09 ±0.868	4.64 ±0.410	5.04 ±0.415	4.59 ±0.583	4.74 ±0.911	5.08 ± 0.495
Creat. (µmol/L)	-1	52 ± 5.9	56 ± 3.1	55 ± 3.4	58 ± 5.1	62 ± 6.4	59 ± 3.1	63 ± 2.6	62 ± 4.9
	6	64 ± 7.2	68 ± 3.7	67 ± 3.4	64 ± 4.3	74 ± 6.2	68 ± 3.9	70 ± 4.0	74 ± 4.2
	13	77 ± 8.8	81 ± 6.7	76 ± 4.2	78 ± 1.3	86 ± 902	78 ± 6.8	79 ± 3.3	84 ± 5.1
Gluc (mmol/L)	-1	4.99 ±0.314	5.38 ± 0.584	5.37 ±0.543	5.46 ±0.914	5.70 ±0.378	5.51 ±0.390	5.45 ±0.248	5.36 ±0.434
	6	5.14 ±0.430	5.38 ± 0.440	5.20 ±0.408	5.57 ± 0.661	5.30 ±0.306	5.29 ±0.268	5.62 ±0.201	5.55 ± 0.155
	13	5.29 ± 0.628	5.29 ± 0.306	5.26 ± 0.162	5.70 ± 0.347	5.42 ± 0.236	5.31 ± 0.371	5.29 ± 0.224	5.31 ± 0.172
Chol. Total (nmol/L)	-1	3.79 ± 0.819	3.34 ± 0.465	4.11 ±0.958 (↑8%)	3.92 ±0.853 (↑3%)	3.58 ± 0.743	4.17 ± 0.644	4.35 ± 0.581	3.78 ± 0.354
	6	3.40 ± 0.719	3.48 ±0.352	**4.06 ±1.374 (↑19%)**	**4.26 ±1.192 (↑25%)**	3.35 ±0.530	3.75±0.356 (↑12%)	**4.22±0.593 (↑26%)**	**4.10 ±0.636 (↑22%)**
	13	3.31 ± 0.529	3.33 ±0.580	**3.80 ±1.135 (↑15%)**	**3.84 ±1.043 (↑16%)**	3.21 ±0.588	3.45±0.543 (↑7%)	**4.02±0.535 (↑20%)**	**3.70 ±0.563 (↑15%)**
Trig. (mmol/L)	-1	0.33 ±0.035	0.32 ±0.046	0.34 ±0.154	0.37 ±0.063	0.31 ±0.087	0.34 ±0.038	0.31 ±0.079	0.30 ±0.087
	6	0.32 ±0.107	0.30 ±0.074	0.30 ±0.043	0.31 ±0.060	0.27 ±0.091	0.30 ±0.043	0.24 ±0.073	0.26 ±0.089
	13	0.31 ±0.094	0.33 ±0.100	0.31 ±0.086	0.33 ±0.077	0.32 ±0.097	0.25 ±0.049	0.28 ±0.055	0.34 ± 0.095
Na (mmol/L)	-1	146 ± 1.9	145 ± 0.8	145 ± 1.0	146 ± 1.9	146 ± 1.0	146 ± 1.7	145 ± 1.3	146 ± 1.0
	6	143 ± 1.2	144 ± 1.3	144 ± 1.3	143 ± 1.4	145 ± 1.3	143 ± 0.8	143 ± 1.3	143 ± 0.8
	13	147 ± 1.9	148 ± 1.4	148 ± 1.9	**149* ±0.5**	149 ± 2.2	147 ± 1.5	148 ± 2.2	147 ± 1.0
K (mmol/L)	-1	4.3 ± 0.29	4.9 ± 0.65	4.4 ± 0.43	4.8 ± 0.61	4.6 ± 0.41	4.3 ± 0.15	4.5 ± 0.15	4.9 ± 0.88

		Male				Female			
	wk	**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
ppm		**0**	**1000**	**5000**	**25000**	**0**	**1000**	**5000**	**25000**
mg/kg bw/d		**0**	**44**	**221**	**1121**	**0**	**46**	**225**	**1102**
	6	4.2 ± 0.30	4.4 ± 0.22	4.3 ± 0.37	4.4 ± 0.24	4.4 ± 0.14	4.3 ± 0.22	4.3 ± 0.22	4.2 ± 0.32
	13	4.3 ± 0.39	4.6 ± 0.21	4.3 ± 0.25	4.6 ± 0.28	4.7 ± 0.43	4.3 ± 0.17	4.4 ± 0.37	4.3 ± 0.36
Cl (mmol/L)	-1	108 ± 2.4	109 ± 0.5	108 ± 1.5	109 ± 1.3	108 ± 1.3	108 ± 2.2	107 ± 1.0	109 ± 0.5
	6	111 ± 1.4	112 ± 0.5	111 ± 1.0	112 ± 1.5	111 ± 0.6	112 ± 1.5	113 ± 1.7	112 ± 1.3
	13	112 ± 3.3	113 ± 1.0	112 ± 2.1	114 ± 1.7	113 ± 1.7	114 ± 1.3	113 ± 2.9	113 ± 1.3
Ca Total (mmol/L)	-1	2.99 ±0.152	2.96 ±0.108	2.95 ±0.057	3.03 ±0.079	3.03±0.043	3.06±0.118	2.99±0.056	2.96 ±0.139
	6	2.89±0.092	2.88 ±0.059	2.82 ±0.112	2.88 ±0.050	2.91 ±0.058	2.81 ±0.059	2.89±0.042	2.84 ± 0.058
	13	2.62 ± 0.123	2.88 ± 0.077	2.78 ± 0.097	2.78 ± 0.064	2.86 ± 0.086	2.72 ± 0.076	2.80 ± 0.043	2.76 ± 0.035
Phos (mmol/L)	-1	2.64 ± 0.295	2.72 ± 0.111	2.68 ± 0.328	2.97 ± 0.083	2.95 ± 0.175	2.75 ± 0.233	2.77 ± 0.180	**2.61* ± 0.228**
	6	2.66 ± 0.102	2.50 ± 0.108	2.40 ± 0.222 (↓10%)	2.54 ± 0.173 (↓5%)	2.50 ± 0.193	2.35±0.193 (↓6%)	2.34±0.175 (↓6%)	**2.15* ± 0.075 (↓14%)**
	13	2.24 ± 0.131	2.28 ± 0.153	2.36 ± 0.201	2.15 ± 0.071	2.37 ± 0.242	2.10±0.131 (↓11%)	2.10±0.166 (↓11%)	**2.02** ± 0.154 (↓15%)**
Total Prot (g/L)	-1	53 ± 5.0	51 ± 1.3	54 ± 3.1	54 ± 3.5	53 ± 1.7	52 ± 1.9	53 ± 2.2	51 ± 1.5
	6	53 ± 5.1	53 ± 1.3	54 ± 2.2	53 ± 2.4	55 ± 1.4	**53* ±1.9** (↓4%)	**53* ±1.0** (↓4%)	**53* ±1.0** (↓4%)
	13	56 ± 3.9	54 ± 3.0	57 ± 3.4	54 ± 2.4	56 ± 2.2	54 ± 2.6	56 ± 1.5	**52** ±1.1** (↓7%)
Alb (g/L)	-1	29 ± 2.4	28 ± 0.6	29 ± 0.5	27 ± 1.9	28 ± 1.0	29 ± 2.1	30 ± 1.5	27 ± 1.4
	6	29 ± 2.4	29 ± 1.0	29 ± 1.3	27 ± 1.3	30 ± 0.8	29 ± 1.8	29 ± 1.6	29 ± 1.0
	13	30 ± 2.4	30 ± 1.5	30 ± 2.1	27 ± 1.5	32 ± 1.3	30 ± 2.2	31 ± 1.7	29 ± 1.2
A/G Ratio	-1	1.18 ± 0.064	1.20 ± 0.089	1.14 ± 0.103	**1.02* ± 0.124**	1.17 ± 0.061	1.25 ± 0.143	1.27 ± 0.057	1.12 ± 0.113
	6	1.16 ± 0.140	1.16 ± 0.097	1.12 ± 0.105	1.02 ± 0.079	1.20 ± 0.037	1.22 ± 0.100	1.23 ± 0.113	1.18 ± 0.054
	13	1.14 ± 0.071	1.22 ± 0.057	1.11 ± 0.093	1.04 ± 0.057	1.30 ± 0.062	1.26 ± 0.194	1.21 ± 0.082	1.25 ± 0.021

N° of animals examined 4/dose/sex;↑↓:statistically significant modification: *p<0.05, **p<0.01;

Organ weight (**Table B.6.3.2.3.1-4**)
Adrenals weights were high at all doses (♂) but unremarkable in the ♀.
Mean liver weight was slightly increased in the top-dose ♂ in comparison with controls, the differences being dose-related (adjusted weights), though statistical significance was not attained.
Thyroid weights were higher for ♂ and ♀ at 5000 ppm and above, and as the individual values showed some degree of overlap with the control values and in the absence of corroborative macroscopic or microscopic finding, this was not considered to be of toxicological importance during the initial peer review.
Thymus weight was reduced at 5000 ppm (♂) and above (♂,♀) in comparison with controls, with a dose related effect at top dose (♀), though statistical significance was not achieved and some degree of overlap of individual values between treated dogs and controls was evident.
Regarding spleen weight, its decrease 5000 ppm (♀) and above (♂,♀) cannot be neglected (the dose-response in ♀ is poor, though).
Regarding testes and ovaries, the toxicological relevance of the weight changes are uncertain, in the light of observed weight variations and concomitant absence of dose-responsiveness.
Overall, the organ weights weight changes of >10% at 5000 ppm and above, should be taken into account for the determination of the NOAEL, at least for the doses at 5000 ppm and above. RMS considers that the changes at the lowest dose (adrenals ♂, spleen ♀) are of less concern, taking into account the absence of gross or histopathological findings at this dose (see below).

Table B.6.3.2.3.1-4 90-day dog study ([redacted] 2002): organ weight (g).

		Male				Female			
Dose groups		**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
ppm		**0**	**1000**	**5000**	**25000**	**0**	**1000**	**5000**	**25000**
mg/kg bw/d		**0**	**44**	**221**	**1121**	**0**	**46**	**225**	**1102**
B.w.	un	10600 ±1639.1	10425 ±1062.6	10300 ±1414.2	10400 ±1555.6	10775 ±492.5	9550 ± 793.7	9875 ± 991.2	10025 ±1260.6
Brain	un	81.7 ±5.2	84.1 ± 4.9	78.1 ± 5.6	80.1 ± 4.7	78.1 ± 5.5	80.8 ± 8.0	81.0 ± 7.4	79.2 ± 8.8
Adrenals	un	1.013±0.108	**1.143±0.174 (↑13%)**	**1.155±0.096 (↑14%)**	**1.227±0.201 (↑21%)**	0.953±0.132	1.018 ±0.070	1.062±0.090 (↑11%)	0.931 ±0.167
Epididymides	un	2.97 ±0.35	2.88 ± 0.49	2.99 ± 0.88	2.86 ± 0.72				
Heart	un	82.7 ±19.3	81.5 ± 11.4	75.8 ± 12.1	76.2 ± 5.9	81.7 ± 6.0	75.9 ± 5.4	78.8 ± 7.6	81.1 ± 9.8
	ad	81.4	81.6	76.8	76.5	78.2	78.3	79.7	81.2
Kidneys	un	49.7 ±6.2	47.5 ± 4.1	45.1 ± 5.6	47.6 ± 6.3	44.1 ± 2.3	45.1 ± 2.7	44.0 ± 3.0	40.9 ± 4.9
	ad	49.2	47.6	45.5	47.7	42.4	46.3	44.4	40.9
Liver	un	337 ±46	351 ± 30	352 ± 51	**381 ±63 (↑13%)**	309 ± 16	292 ± 31	325 ± 21	325 ±21
	ad	333	351	355	**382 (↑15%)**	n.d.	n.d.	n.d.	n.d.
Ovaries	un					0.81 ± 0.17	0.87 ± 0.10 (↑7%)	0.88 ± 0.24 (↑9%)	**0.90 ± 0.11 (↑11%)**
Spleen	un	59.7 ±2.9	70.1 ± 7.6	72.3 ± 23.5	**50.6 ±7.2 (↓15%)**	78.5 ± 17.1	**69.1 ± 17.6 (↓12%)**	**51.9 ± 20.3 (↓34%)**	**55.9 ± 18.8 (↓29%)**
	ad	58.9	70.1	72.9	**50.7 (↓14%)**				
Testes	un	16.2 ±4.2	15.2 ± 5.5	14.2 ± 3.4 (↓12%)	14.5 ±2.3 (↓10%)				
	ad	15.9	15.2	14.4 (↓9%)	14.5 (↓9%)				
Thymus	un	14.24 ±3.60	13.02 ±2.72 (↓9%)	**12.80±5.62 (↓10%)**	**8.57±5.61 (↓40%)**	15.70 ± 5.08	12.67 ±3.29 (↓19%)	14.45 ±5.55 (8%)	**12.32 ±2.38 (↓22%)**
	ad	13.84	13.04	13.11	**8.65 (↓38%)**				
Thyroids + Para	un	0.672 ±0.123	0.704±0.046	**0.748±0.192 (↑11%)**	**0.806±0.125 (↑20%)**	0.751±0.202	0.777±0.206	**0.836±0.212 (↑11%)**	**0.934±0.143 (↑24%)**
	ad	0.659	0.705 (↑7%)	**0.758 (↑15%)**	**0.809* (↑23%)**				
Uterus & cervix	un					3.5 ± 1.7	3.8 ± 0.9	3.5 ± 0.8	3.6 ± 0.7

Un: "unadjusted"; Ad: "adjusted" mean organ weights; N° of animals examined 4/dose/sex; ↑↓ statistically significant modification: Williams' test *p<0.05;

Necropsy and histopathological findings:
Gross pathological findings included mainly the observation of small thymus at the top-dose (♂), in line with the observed thymus weight reduction.

Treatment related microscopic changes were noted in the liver for both sexes at at top-dose (♂,♀) and at 5000 ppm and above (♂). The finding was characterised as centrilobular and midzonal hepatocyte hypertrophy (**Table B.6.3.2.2.1-6**).
Marginally increased incidences of involution/atrophy in the thymus were seen at all doses (♂), with a clear increase mainly at the top-dose, when compared with controls (**table B.6.3.2.2.1-6**). This finding was associated with lower thymus weight at 5000 and 25000ppm (**table B.6.3.2.2.1-5**). This finding was low in incidence and severity, and the toxicological importance was considered equivocal during the initial peer review. However, RMS raises the question whether the thymus, along with other lymphoid organs (spleen, lymph nodes) could be at target of lenacil action. We note that subtle inflammatory infiltrates are seen at top-dose in brain, mammary, skin and thyroid tissue. This could be indicative of a haematomodulatory role of lenacil and perhaps of a possible immunomodulatory action of the substance, even if such outcomes are common in dogs.

Notifier: *"Historical control data for thymic involutions have been requested from Envigo (formerly HLS). The inflammatory changes are very common in dogs and this does not look like a test substance-related finding. This is supported in the book by McInnes, EF and Mann, P, Editors (2012) Background lesions in laboratory animals: a color atlas; Chapter 3. Beagle dog, by Cheryl Scudamore, where it says, "The most common observations are minimal inflammatory cell foci, usually predominantly mononuclear cells, of unknown etiology. Inflammatory cell foci are seen in many organs but are particularly common in the liver, lung, salivary glands and tongue."*

Table B.6.3.2.2.1-5 90-day dog study ([redacted] 2002): Macropathology

		Male				Female			
Dose groups	**Effect**	**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
ppm		**0**	**1000**	**5000**	**25000**	**0**	**1000**	**5000**	**25000**
mg/kg bw/d		**0**	**44**	**221**	**1121**	**0**	**46**	**225**	**1102**
Kidneys	Unilaterally small	0	0	0	0	0	0	0	**1**
Spleen	Pale area(s)	0	0	0	**1**	0	0	0	0
Thymus	Small	0	0	**1**	**2**	0	0	0	0

N° of animals examined 4/dose/sex.

Table B.6.3.2.2.1-6 90-day dog study ([redacted] 2002): Histopathology.

		Male				Female			
Dose groups	**Effect**	**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
ppm		**0**	**1000**	**5000**	**25000**	**0**	**1000**	**5000**	**25000**
mg/kg bw/d		**0**	**44**	**221**	**1121**	**0**	**46**	**225**	**1102**
Brain	Meningeal inflammatory cell infiltrate	0	0	0	1	0	0	0	0
Epididymides	No spermatozoa	0	0	0	**2**	-	-	-	-
Liver	Centrilobular hepatocyte hypertrophy	0	0	**2**	**3**	0	0	0	**1**
	Midzonal hepatocyte hypertrophy	0	0	**2**	**3**	0	0	0	**1**
	Hepatocyte vacuolation - focal	0	0	0	0	0	0	0	**1**
Lungs - bronchi	Pneumonitis	1	2	1	0	0	0	0	**2**
Mammary	Dermal inflammatory cell infiltrate	0	0	0	1	0	0	0	0
Pancreas	Prominent acinar cell apoptosis	0	0	0	1	0	0	0	0
Parathyroid	Cyst(s)	0	1	2	0	1	1	1	0
Pituitary	Cyst(s)	0	1	1	**2**	2	2	0	0
Skin	Epidermal inflammatory cell infiltrate	0	0	0	**1**	0	0	0	0
	Folliculitis/perifolliculitis	1	1	1	1	0	1	**2**	**2**
Spleen	Capsular thickening	0	0	0	1	0	0	0	0
Stomach	lymphoid aggregations in mucosa-glandular region	0	1	1	1	2	1	0	1
Thymus	Involution/atrophy	0	1	1	**2**	1	1	1	0
	Epithelial hyperplasia	0	0	0	0	1	0	0	0
Thyroids	Lymphocytic infiltration	0	1	0	0	0	0	0	0
	Inflammatory cell infiltrate	0	0	0	0	0	0	1	0
	Prominent ultimobranchial cysts	0	0	0	1	0	0	1	0
	Ectopic thymic tissue	1	1	0	1	0	0	0	0
Urinary bladder	Epithelial/subepithelial haemorrhage	0	0	0	0	0	0	0	**1**

N° of animals examined 4/dose/sex.

Conclusion (90d dog)

NOAEL = 1000 ppm = **44 mg/kg bw/d**
LOAEL = 5000 ppm = 221 mg/kg bw/d, based upon:
↓reticulocytes (♂), ↑γGT (♂), ↑cholesterol (♂,♀), ↓phosphorus (♀), ↑adrenal weight (♂), ↓spleen weight (♀)↓thymus weight (♂), ↑relative liver weight (♀), ↑thyroid+parathyroid weight (♂,♀), small thymus, ↑hepatocyte hypertrophy (♂), ↑skin folliculitis (♀).

Top-dose findings included:
↓body weight (♂), ↑APTT (♂), ↑AP (♂,♀), ↑liver weight (♂), ↑thymus involution (♂), ↓ epididymides sperm, ↑pituitary cysts, ↑ pneumonitis(♀)

While occasionally, some modifications were observed at the lowest dose (adrenals, spleen weight, cholesterol levels) it seems reasonable to maintain an overall NOAEL at the mid-dose, as originally proposed. The findings lacked corroboration by histopathological examination, or occurred at interim sampling but not at termination.

The notifier opinion (first peer review):
"Taking the two studies (28 day dog study and 90 day dog study) together it is apparent that considerable background variation occurs in a number of parameters following low dose administration of lenacil, without adverse effect on the animals over 4 or 13 weeks. The liver, rather than the kidney, is the target organ and at high doses this organ responds adaptively to the challenge of metabolizing lenacil. The test material is extensively metabolized following oral administration and so the functional liver changes are not unexpected.
Hence the low dose levels can reasonably be assumed to reflect biological variation and the high dose findings indicate an adaptive liver response. Based on these findings, the notifier disagrees with the RMS conclusion and respectfully requests reconsideration of an NOAEL of 25000 ppm.
Based on the results above the No Effect Level (NOEL) on this study was considered to be 1000 ppm (corresponding to a daily intake of 44 mg/kg in the males and 46 mg/kg/day in the females) based on adaptive histopathological findings in the liver. The highest No Adverse Effect Level (NOAEL) was 25000 ppm (equivalent to 1121 mg/kg/day for males and 1102 mg/kg/day for the females.
With the exception of increased liver weight, the minor changes noted in various haematological, blood chemistry, urinalysis, organ weight and pathology parameters show no dose relationship, no trends for increasing effect over time or with increasing dose and show no consistency between the sexes. The response in the liver is clearly an adaptive response to increase metabolic workload. The effects on liver weight, alkaline phosphatase and hepatic histopathology are consistent with an adaptive response which does not indicate an adverse effect of treatment.
The findings in the 28 day dog study and 90 day dog study do not show good correlation indicating the minor disturbances are not real toxic changes. The RMS expressed concern about renal dysfunction following the 28 day study but the 90 day study provides no evidence to support the proposition of renal effects. Opposing effects occurred in haematology parameters in the two studies.
Taking the two studies together it is apparent that considerable background variation occurs in a number of parameters following low dose administration of lenacil, without adverse effect on the animals over 4 or 13 weeks. The liver, rather than the kidney, is the target organ and at high doses this organ responds adaptively to the challenge of metabolizing lenacil. The test material is extensively metabolized following oral administration and so the functional liver changes are not unexpected.
Hence the low dose levels can reasonably be assumed to reflect biological variation and the high dose findings indicate an adaptive liver response. Based on these findings, the notifier disagrees with the RMS conclusion and respectfully requests reconsideration of an NOAEL of 25000 ppm."

RMS maintains its conclusions. The effects on hepatic histopathology could indeed be an adaptive response but liver enzyme induction was not measured and therefore not demonstrated.
A comparison between the findings in the 28d and 90d dog studies is difficult, taking into account the low number of animals investigated in the 28d studies. In addition, many uncertainties exist on the relevance of haematomodulation observed across different studies and different species.

B.6.3.3 (CA 5.3.3) Other routes

No other studies are required.

B.6.3.4 (CA 5.3.4) Summary of short-term toxicity

Lenacil was administered for a 13-week period in the diet of rats, mice and dogs at doses of approximately 15 mg/kg bw/d up to 4400 mg/kg bw/d.

In rat and mice, at doses of 100-400mg/kg bw/d, WBC count was decreased, without evidence of inflammatory change in any tissue, or any effect in lymphoid tissues.

In rats, at dose levels ranging from 400 to 4000 mg/kg bw/d, some blood electrolytes were altered and proteins were increased in urine suggesting a loss of the kidney ability to filter adequately blood. However, there were no effects upon kidney weight and kidney microscopy appeared normal. At these dose levels, liver weight was increased and hepatocyte centrilobular hypertrophy was noted at top dose. Some other organ weights were altered at top dose in rats without histological findings to support an adverse effect in these organs excepting for thyroid where thyroid follicular epithelium staining indicative of lipofuscin was observed at 5000 ppm onwards without any evidence of organ atrophy. After a 4 week rest, animals showed some recovery. Additional histopathological examinations of the thyroid were performed.

In mice at top doses of 1600-2500 mg/kg bw/d, white blood cell toxicity was observed and extramedullary haematopoiesis was increased in liver and spleen.

In dogs, at dose of 220 mg/kg bw/d onwards, liver weight was increased and centrilobular / midzonal hepatocyte hypertrophy was observed. At top dose, some dogs had thymus involution/atrophy.

The lowest NOAEL was agreed to be in the 13-week rat/dog study, around 41-44 mg/kg b.w./d, taking into account findings at >200 mg/kg b.w./d. and disregarding non-dose dependent effects of the a.s. on the WBC compartment in the mouse at a lower dose.
Overall, non-dose responsive blood toxicity was observed, further supported by extramedullary haematopoiesis at high doses, and occasional signs of cellularity changes in lymph nodes or thymus involution. The MoA remained unexplained.

Based on the available data on short-term toxicity and taking into account the type of effects observed, a classification of lenacil for repeated dose toxicity is not required according to the criteria laid down in Regulation (EU) No. 1272/2008 (CLP).

Results of short-term toxicity studies with lenacil are summarised in table 6.3-1

Table B6.3-1 Summary of short-term toxicity studies with lenacil

Type of test, test species, doses *(ppm)* - mg/kg b.w./d	Batch n°, purity (%)	NOAEL (mg/kg b.w./d)	LOAEL, critical effect (mg/kg b.w./d)	Reference
(B.6.3.1.1) 28-day oral, diet, Wistar rat *(0, 5000, 10000 (wk 1-2)/ 30000 (wk 3-4), 20000 (wk 1-2)/ 50000 (wk 3-4) ppm)* ♂: 0, 571, 1269/2978, 2545/5029 mg/kg bw/d ♀: 0. 631, 1288/3576, 2643/5913 mg/kg bw/d	B.n°. 141712003, purity 98.6%	5000 ppm = **571 mg/kg bw/d**	10000/30000 ppm = 1269/2978 mg/kg bw/d: ↑uterine fluid distention. Top-dose (20000/50000 ppm =2545/5029 mg/kg bw/d): ↑liver w	[redacted] 2002a
(B.6.3.1.2) 28-day oral, diet, Beagle dogs *(0, 5000, 20000, 50000 ppm)* ♂: 0, 219, 807, 1941 mg/kg bw/d ♀: 0, 242, 967, 2331 mg/kg bw/d	B.n°. 141712003, purity 98.6%	5000 ppm = **219 mg/kg bw/d**	20000 ppm = 807 mg/kg bw/d : ↓b.w. change, ↑liver w, ↓kidneys w ↓thymus w, ↓WBC, ↓neutrophils	[redacted] 2001
(B.6.3.2.1.1) 90-day oral, diet, Wistar rat, *(0, 500, 5000, 50000 ppm)* ♂: 0, 41, 412, 4357 mg/kg bw/d ♀: 0, 45, 468, 4893 mg/kg bw/d	B.n°. 141712003, purity 98.6%	500 ppm = **41 mg/kg bw/d**	5000 ppm = 412 mg/kg bw/d : ↓b.w. change, clinical signs, leukopenia, ↑proteinuria (♂),↑liver w, ↑Schmorl's^{+} pigment in thyroid cells Top-dose: ↑SG, ↑liver w, ↑thyroid w, hepatocyte hypertrophy, lymph node hypercellularity	[redacted] 2002b [redacted] 2004
(B.6.3.2.2.1) 90-day, oral, diet, CD-1 mice, *(0, 100, 1000, 5000, 10000 ppm)* ♂: 0, 16, 157, 787, 1616 mg/kg bw/d ♀: 0, 20, 207, 1127, 2150 mg/kg bw/d	B.n°. 9038, purity 98.2%	1000 ppm = **157 mg/kg bw/d**	1000 ppm = 787 mg/kg bw/d : ↑liver w Disregarding blood toxicity at the next-lower dose of 16 mg/kg bw/d Top-dose: ↑proteinuria, ↑liver w, ↑spleen w, ↑LN hyperplasia, ↑liver + spleen extramedullary haematopoiesis	[redacted] 1991
(B.6.3.2.3.1) 90-day, oral, diet, Beagle dog, *(0, 1000, 5000, 25000 ppm)* ♂: 0, 44, 221, 1121 mg/kg bw/d ♀: 0, 46, 225, 1102 mg/kg bw/d	Batch No. 141712003, purity 98.6%	1000 ppm = **44 mg/kg bw/d**	5000 ppm = 221 mg/kg bw/d : ↓reticulocytes, ↑γGT, ↑cholesterol, ↓phosphorus, ↑adrenal w, ↓spleen w, ↓thymus w, ↑liver w, ↑thyroid w, small thymus, ↑hepatocyte hypertrophy, ↑skin folliculitis. Top-dose: ↓body w, ↑APTT, ↑AP, ↑thymus involution, ↓epididymides sperm, ↑pituitary cysts, ↑pneumonitis.	[redacted] 2002

Notifier's opinion:

"*It is the opinion of the notifier that based on the overall response to 13 weeks administration and evidence of recovery, the appropriate NOAEL derived from short term toxicity studies is 412 mg/kg/day (5000 ppm). This conclusion was based on the occurrence of adaptive liver changes at the highest dose of 50000 ppm, which constituted the LOAEL. The NOAEL was defined by reduced white blood cell numbers at 5000 ppm, considered of uncertain toxicological significance, in that the findings were not consistently seen in the long-term rat study. Additional histopathological examinations were completed for this study.*

Following observation of thyroid changes in the multi-generation reproductive toxicity study additional histopathological examinations of thyroid tissue preserved from a 13 week dietary study in rats were instigated. In the original study (Point 5.3.2.1) thyroids from the control and high dose (50000 ppm) groups were examined. The additional investigation extended the examination to the low and intermediate groups.

The study authors concluded that examination of sections stained with haematoxylin and eosin revealed no changes indicative of any accumulation of pigment in the follicular epithelium or any other change indicative of a response to treatment. Schmorl's staining of the thyroids, however, revealed a background level of Schmorl's positive staining in all groups, particularly in males. Schmorl's positive staining is indicative of lipofuscin in the follicular epithelium. There was a treatment-related increase in the incidence and severity of Schmorl's-positive staining in females given lenacil technical at 50000 ppm, and a slight increase in the severity of this finding in males given 50000 ppm. The slightly increased incidence of Schmorl's-positive staining in females given 5000 ppm was within the background incidence and was, therefore, not attributed to treatment. Following a recovery period of four weeks there were no significant differences in incidence of Schmorl's-positive staining between control and high dose group males or females.

Further thyroid function tests were also completed in female rats dosed for 20 weeks at 250 or 50000 ppm lenacil. Investigations included assessment of T3 and T4 levels, thyroid weights, ^{125}Iodide uptake and displacement. The study concluded that there was no evidence to suggest that lenacil technical at doses of up to 50000 ppm affected the ability of the thyroid to take-up and organify ^{125}Iodide. Measurements of T3 during the study also indicated that lenacil does not act as an inhibitor of the deiodinase which converts T4 to T3. Overall, the results of the study showed that lenacil technical was not directly toxic to the thyroid."

RMS considers that the liver effects observed in rats at 5000 ppm could not be disregarded as long as enzyme induction was not demonstrated. We agree with the notifier that some liver parameters suggest an adaptive effect but the investigation was incomplete.

B.6.4 (CA 5.4) Genotoxicity testing

B.6.4.1 (CA 5.4.1) *In vitro* studies

Bacterial assays for gene mutation

B.6.4.1.1

Lenacil technical: Bacterial mutation assay (May K., 2001) - DuPont Report No.: ACD 016/013217

Guidelines: EC Directive 2000/32/EC Method B.13/14, OECD 471.

GLP status: yes

Materials and Methods

Salmonella typhimurium, strains TA1535, TA1537, TA98 and TA100, and *Escherichia coli*, strain WP2uvrA/pKM101 (CM891), were exposed to Lenacil technical (Batch No. 141712003, purity 98.6%) diluted in DMSO. Two independent mutation tests were performed in the presence and absence of liver preparations from Aroclor 1254-treated rats (S9 mix). The first test was a standard plate incorporation assay (TEST 1); the second involved a 30 minute pre-incubation stage (TEST 2). Concentrations of Lenacil technical up to 5000 µg/plate were tested. Mixtures of the test dilution, positive control or negative control, S9 mix or phosphate buffer and bacterial culture were added to agar containing a trace of histidine and tryptophan and overlaid onto Petri dishes containing minimal agar. All plates were incubated at 37 C for ca 72 hours. After this period, the appearance of the background bacterial lawn was examined and revertant colonies counted. Positive controls were sodium azide, 9-aminoacridine, 2-nitrofluorene, AF-2, 2-aminoanthracene, benzopyrene and gave the expected results.

The study is accepted.

Findings

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to Lenacil technical at any concentration in either the presence or absence of S9 mix (**Tables B.6.4.1.1-1 to B.6.4.1.1-5**). No cytotoxicity was observed.

Conclusion:

Lenacil technical showed no evidence of mutagenic activity in this bacterial system under the test conditions employed.

Table B.6.4.1.1-1 Lenacil, bacterial mutation assay (May, 2001) : results for TA98.

TEST 1 (range-finding)											
Dose	S9 mix	Revertant colony counts				Dose	S9 mix	Revertant colony counts			
		A	B	C	m ± S.D.			A	B	C	m ± S.D.
5000 µg/plate	-	30	32	34	**32±2**	5000 µg/plate	+	42	36	28	**35±7**
1500 µg/plate	-	30	37	41	**36±6**	1500 µg/plate	+	48	50	34	**44±9**
500 µg/plate	-	28	55	39	**41±14**	500 µg/plate	+	43	44	36	**41±4**
150 µg/plate	-	30	36	36	**34±3**	150 µg/plate	+	49	42	46	**46±4**
50 µg/plate	-	27	44	39	**37±9**	50 µg/plate	+	30	35	26	**30±5**
15 µg/plate	-	31	32	52	**38±12**	15 µg/plate	+	43	44	37	**41±4**
5 µg/plate	-	39	39	34	**37±3**	5 µg/plate	+	50	35	28	**38±11**
DMSO (0.1 ml/plate)	-	45	38	44	**42±4**	DMSO (0.1 ml/plate)	+	44	42	44	**43±1**
2-nitrofluorene (1µg/plate)	-	383	313	389	**362±42**	Benzo[a]pyrene (5µg/plate)	+	746	684	673	**701±39**
TEST 2 (with pre-incubation)											
	S9 mix	Revertant colony counts					S9 mix	Revertant colony counts			
		A	B	C	m ± S.D.			A	B	C	m ± S.D.
5000 µg/plate	-	23	22	31	**25±5**	5000 µg/plate	+	32	31	27	**30±3**
1500 µg/plate	-	36	20	24	**27±8**	1500 µg/plate	+	38	31	41	**37±5**
500 µg/plate	-	24	22	26	**24±2**	500 µg/plate	+	43	38	30	**37±7**
150 µg/plate	-	28	19	35	**27±8**	150 µg/plate	+	31	26	38	**32±6**
50 µg/plate	-	27	22	23	**24±3**	50 µg/plate	+	41	49	42	**44±4**
DMSO (0.1 ml/plate)	-	38	35	41	**38±3**	DMSO (0.1 ml/plate)	+	31	42	44	**39±7**
2-nitrofluorene (1µg/plate)	-	232	195	182	**203±26**	Benzo[a]pyrene (5µg/plate)	+	391	363	383	**379±14**

Table B.6.4.1.1-2 Lenacil, bacterial mutation assay (May, 2001) : results for TA100.

TEST 1 (range-finding)											
	S9 mix	Revertant colony counts					S9 mix	Revertant colony counts			
		A	B	C	m ± S.D.			A	B	C	m ± S.D.
5000 µg/plate	-	92	117	92	**100±14**	5000 µg/plate	+	130	106	118	**118±12**
1500 µg/plate	-	102	102	96	**100±3**	1500 µg/plate	+	100	115	118	**111±10**
500 µg/plate	-	106	132	85	**108±24**	500 µg/plate	+	107	111	110	**109±2**
150 µg/plate	-	110	109	107	**109±2**	150 µg/plate	+	95	115	115	**108±12**
50 µg/plate	-	100	109	93	**101±8**	50 µg/plate	+	110	116	111	**112±3**
15 µg/plate	-	109	121	108	**113±7**	15 µg/plate	+	132	107	117	**119±13**
5 µg/plate	-	109	100	93	**101±8**	5 µg/plate	+	107	130	118	**118±12**
DMSO (0.1 ml/plate)	-	104	117	132	**118±14**	DMSO (0.1 ml/plate)	+	126	128	130	**128±2**
Sodium azide (0.5 µg/plate)	-	460	477	482	**473±12**	Benzo[a]pyrene (5 µg/plate)	+	593	561	721	**625±85**
TEST 2 (with pre-incubation)											
	S9 mix	Revertant colony counts					S9 mix	Revertant colony counts			
		A	B	C	m ± S.D.			A	B	C	m ± S.D.
5000 µg/plate	-	132	125	129	**129±4**	5000 µg/plate	+	165	132	136	**144±18**
1500 µg/plate	-	137	132	118	**129±10**	1500 µg/plate	+	150	146	155	**150±5**
500 µg/plate	-	144	155	119	**139±18**	500 µg/plate	+	159	161	140	**153±12**
150 µg/plate	-	117	132	124	**124±8**	150 µg/plate	+	159	136	164	**153±15**
50 µg/plate	-	144	107	119	**123±19**	50 µg/plate	+	144	158	158	**158±14**
DMSO (0.1 ml/plate)	-	132	141	146	**140±7**	DMSO (0.1 ml/plate)	+	158	138	143	**146±10**
Sodium azide (0.5 µg/plate)	-	709	654	655	**673±31**	Benzo[a]pyrene (5 µg/plate)	+	819	837	776	**811±31**

Table B.6.4.1.1-3 Lenacil, bacterial mutation assay (May, 2001) : results for TA1535

TEST 1 (range-finding)											
	S9 mix	Revertant colony counts					S9 mix	Revertant colony counts			
		A	B	C	m ± S.D.			A	B	C	m ± S.D.
5000 µg/plate	-	17	21	14	**17±14**		5000 µg/plate	+	14	16	14	**15±1**
1500 µg/plate	-	16	19	14	**16±3**		1500 µg/plate	+	17	14	15	**15±2**
500 µg/plate	-	13	17	19	**16±3**		500 µg/plate	+	20	19	23	**21±2**
150 µg/plate	-	14	21	20	**18±4**		150 µg/plate	+	22	14	19	**18±4**
50 µg/plate	-	26	21	20	**22±3**		50 µg/plate	+	22	14	21	**19±4**
15 µg/plate	-	12	19	13	**15±4**		15 µg/plate	+	14	14	26	**18±7**
5 µg/plate	-	13	22	13	**16±5**		5 µg/plate	+	22	14	24	**20±5**
DMSO (0.1 ml/plate)	-	19	16	21	**19±3**		DMSO (0.1 ml/plate)	+	22	16	23	**20±4**
Sodium azide (0.5 µg/plate)	-	282	288	254	**275±18**		2-Aminoanthracène (2µg/plate)	+	361	346	296	**334±34**
TEST 2 (with pre-incubation)											
	S9 mix	Revertant colony counts					S9 mix	Revertant colony counts			
		A	B	C	m ± S.D.			A	B	C	m ± S.D.
5000 µg/plate	-	16	12	15	**14±2**		5000 µg/plate	+	26	24	15	**22±6**
1500 µg/plate	-	22	12	12	**15±6**		1500 µg/plate	+	24	23	21	**23±2**
500 µg/plate	-	14	20	15	**16±3**		500 µg/plate	+	23	20	21	**21±2**
150 µg/plate	-	12	16	16	**15±2**		150 µg/plate	+	23	20	24	**22±2**
50 µg/plate	-	19	23	14	**19±5**		50 µg/plate	+	30	16	21	**22±7**
DMSO (0.1 ml/plate)	-	20	24	19	**21±3**		DMSO (0.1 ml/plate)	+	27	22	22	**24±3**
Sodium azide (0.5 µg/plate)	-	264	378	285	**309±61**		2-Aminoanthracène (2µg/plate)	+	210	271	233	**238±31**

Table B.6.4.1.1-4 Lenacil, bacterial mutation assay (May, 2001): results for TA1537

TEST 1 (range-finding)												
	S9 mix	Revertant colony counts						S9 mix	Revertant colony counts			
		A	B	C	m ± S.D.				A	B	C	m ± S.D.
5000 µg/plate	-	12	13	12	**12±1**		5000 µg/plate	+	16	15	15	**15±1**
1500 µg/plate	-	10	17	9	**12±4**		1500 µg/plate	+	14	20	17	**17±3**
500 µg/plate	-	16	15	13	**15±2**		500 µg/plate	+	17	17	16	**17±1**
150 µg/plate	-	13	16	13	**14±2**		150 µg/plate	+	22	17	21	**20±3**
50 µg/plate	-	7	14	16	**12±5**		50 µg/plate	+	20	13	20	**18±4**
15 µg/plate	-	12	16	7	**12±5**		15 µg/plate	+	14	23	13	**17±6**
5 µg/plate	-	12	15	17	**15±3**		5 µg/plate	+	23	16	16	**18±4**
DMSO (0.1 ml/plate)	-	15	14	15	**15±1**		DMSO (0.1 ml/plate)	+	24	16	22	**21±4**
9-Aminoacridine (30 µg/plate)	-	573	591	495	**553±51**		Benzo[a]pyrene (5 µg/plate)	+	351	325	334	**337±13**
TEST 2 (with pre-incubation)												
	S9 mix	Revertant colony counts						S9 mix	Revertant colony counts			
		A	B	C	m ± S.D.				A	B	C	m ± S.D.
5000 µg/plate	-	9	12	13	**11±2**		5000 µg/plate	+	21	19	16	**19±3**
1500 µg/plate	-	20	8	9	**12±7**		1500 µg/plate	+	20	16	19	**18±2**
500 µg/plate	-	12	13	16	**14±2**		500 µg/plate	+	20	22	22	**21±1**
150 µg/plate	-	10	12	9	**10±2**		150 µg/plate	+	24	19	22	**22±3**
50 µg/plate	-	9	9	8	**9±1**		50 µg/plate	+	19	16	23	**19±4**
DMSO (0.1 ml/plate)	-	9	15	12	**12±3**		DMSO (0.1 ml/plate)	+	20	19	23	**21±2**
9-Aminoacridine (30 µg/plate)	-	1090	905	1009	**1001±93**		Benzo[a]pyrene (5 µg/plate)	+	341	308	336	**328±18**

Table B.6.4.1.1-5 Lenacil, bacterial mutation assay (May, 2001) : results for *Escherischia coli* WP2uvrA/pKM101 (CM891)

TEST 1 (range-finding)												
	S9 mix	Revertant colony counts						S9 mix	Revertant colony counts			
		A	B	C	m ± S.D.				A	B	C	m ± S.D.
5000 µg/plate	-	129	131	110	**123±12**		5000 µg/plate	+	130	167	150	**149±19**
1500 µg/plate	-	148	144	107	**133±23**		1500 µg/plate	+	158	136	157	**150±12**
500 µg/plate	-	109	132	115	**119±12**		500 µg/plate	+	161	159	168	**163±5**
150 µg/plate	-	122	116	133	**124±9**		150 µg/plate	+	133	137	159	**143±14**
50 µg/plate	-	112	146	148	**135±20**		50 µg/plate	+	130	158	148	**145±14**
15 µg/plate	-	135	146	131	**137±8**		15 µg/plate	+	133	157	150	**147±12**
5 µg/plate	-	137	159	150	**149±11**		5 µg/plate	+	161	176	165	**167±8**
DMSO (0.1 ml/plate)	-	147	124	146	**139±13**		DMSO (0.1 ml/plate)	+	184	157	170	**170±14**
AF-2 (0.05 µg/plate)	-	949	895	903	**916±29**		2-Aminoanthracène (10 µg/plate)	+	750	756	738	**748±9**
TEST 2 (with pre-incubation)												
	S9 mix	Revertant colony counts						S9 mix	Revertant colony counts and means			
		A	B	C	m ± S.D.				A	B	C	m ± S.D.
5000 µg/plate	-	100	111	101	**104±6**		5000 µg/plate	+	162	135	141	**146±14**
1500 µg/plate	-	123	122	118	**121±3**		1500 µg/plate	+	144	132	153	**143±11**
500 µg/plate	-	112	118	125	**118±7**		500 µg/plate	+	169	128	161	**153±22**
150 µg/plate	-	155	143	110	**136±23**		150 µg/plate	+	183	143	161	**162±20**
50 µg/plate	-	112	131	121	**122±8**		50 µg/plate	+	160	154	139	**151±11**
DMSO (0.1 ml/plate)	-	116	125	124	**122±5**		DMSO (0.1 ml/plate)	+	137	152	138	**142±8**
AF-2 (0.05 µg/plate)	-	1044	909	921	**958±75**		2-Aminoanthracène (10 µg/plate)	+	977	881	988	**949±59**

AF-2 : 2-(2-Furyl)—3-(5-nitro-2-furyl)acrylamide

Table B.6.4.1.1-6 Lenacil, bacterial mutation assay (May, 2001) : Historical control data

A. Dimethyl sulfoxide (DMSO) solvent control

Strain	TA98		TA100		TA1535		TA1537		WP2uvrA/pKM 101 (CM891)	
S9 mix	-	+	-	+	-	+	-	+	-	+
Minimum	22	24	77	77	11	10	6	6	94	81
Maximum	53	59	156	156	35	38	23	33	227	216
Mean	37	39	105	107	19	19	12	12	124	134
No of values	393	407	391	405	391	405	395	409	243	257
Standard deviation	4	4	13	16	3	3	3	4	17	23
Upper 99% limit	54	60	158	158	35	38	23	33	230	220
Lower 99% limit	21	23	75	75	11	10	6	6	91	77

B. Positive controls

Strain	TA98		TA100			TA1535			TA1537		WP2uvrA/pKM101 (CM891)		
S9 mix	-	+	-	-	+	-	-	+	-	+	-	-	+
	(f)	(h)	(a)	(d)	(h)	(b)	(d)	(i)	(g)	(h)	(c)	(e)	(j)
Minimum	126	123	212	237	263	53	112	63	39	67	294	244	188
Maximum	869	1031	860	972	1109	781	1126	850	1671	543	2312	1285	1704
Mean	312	499	405	516	508	205	388	216	245	250	1331	650	630
No of values	617	630	346	272	631	341	268	622	398	631	112	259	385
Standard deviation	111	196	120	134	220	90	497	312	160	90	331	263	207

(a) ENNG 3 µg
(b) ENNG 5 µg
(c) ENNG 2µg
(d) Sodium azide 0.5 µg
(e) AF-2 0.05 µg
(f) 2-Nitrofluorene
(g) 9-Aminoacridine 30 µg
(h) Benzo[a]pyrene 5 µg
(i) 2-Aminoanthracene 2 µg
(j) 2-Aminoanthracene 10 µg

B.6.4.1.2

Mutagenic activity of uracil, 3-cyclohexyl-5,6,-trimethylene in the *Salmonella*/microsome assay (Russell J.F., 1977)

DuPont Report No.: HLR 601-77

Guidelines: study is not fully in compliance with Dir EEC 2000/32/EEC Annex 4D 92/69 or 84/449 or OECD test guideline n° 471 (1997-83).

Deviation from official protocol: strain TA 102 and E.coli WP2uvrA were not included. Strains were not tested for their quality criteria. Experiment was not repeated. Pre-test was not performed. Limited experimental information.

GLP status: no

Materials and Methods

Doses of Lenacil technical (Code: INB-634-50), purity not specified) were selected on the basis of the test article to tester strain S. typhymurium TA1535.

Therefore doses of up to 500µg/plate were chosen for the test with activation and also up to 500 mg/plate without activation. Tester strains chosen were TA1535, TA1537, TA1538T, TA98 and TA100. Positive control substance was 2-aminoanthracene.

The study is considered to provide complementory information.

Findings and conclusions

Lenacil technical did not produce a positive response in any of the tester strains with and without metabolic activation.

B.6.4.1.3

Mutagenicity testing of IN E1512-2 in the *Salmonella typhimurium* plate incorporation assay (Reynolds V.L., 1989) - DuPont Report No.: HLR 550-89

See confidential section (Volume 4).

B.6.4.1.4

Mutagenicity testing of DPX-B634-107 (lenacil) in the *Salmonella typhimurium* plate incorporation assay (D'Amico S.W., 1994)

DuPont Report No.: HLR 413-94

Guidelines: study is not fully in compliance with Dir EEC 2000/32/EEC Annex 4D, 92/69 or 84/449 or OECD test guideline n 471 (1997-83).

Deviation from official protocol: strain TA 102 and E.coli WP2uvrA were not included in the study. Experimental protocol not described.

GLP status: no

Materials and Methods

Lenacil technical (DPX-B634-107), purity: not specified. Doses of Lenacil technical were selected on the basis of the test article to tester strain *S. typhimurium* TA98. Dose levels of up to 5000 µg/plate were chosen for the test with activation and also up to 5000 mg/plate without activation. Tester strains chosen were TA1535, TA97, TA98, and TA100.

The study is accepted as additional information.

Findings

Lenacil technical did not produce a positive response in any of the tester strains with and without metabolic activation.

Conclusion

Lenacil technical was non-mutagenic in the reverse mutation assay with and without metabolic activation.

B.6.4.1.5

(Open literature study)

- Lack of genotoxic and cytotoxic effects of the herbicide lenacil on mouse tumor cells and on some Salmonella typhimurium strains (Grancharov K, Gorneva G, Mladenova J, Norpoth K, Golovinsky E (Arzneimittelforschung, 1986, 36(11), 1660-1663.)

A review of this publication indicates that it does not meet the current OECD Test Guideline 473 and has been superseded by the *in vivo* mouse micronucleus study (ACD 018/013472).

The study is considered to provide complementary information.

Findings and conclusions

Abstract:

"The effects of 3-cyclohexyl-6,7-dihydro-1H-cyclopentapyrimidine-2,4(3H,5H)-dione (lenacil) on macromolecular synthesis, thymidilate synthetase activity, and viability and cell cycle progression were studied using Friend leukemia (FL). P388 and Ehrlich ascites tumor cells in suspension, and its cytogenetic effects were studied in a Salmonella/mammalian microsome assay using both frameshift and base-substitution tester strains. At a concentration of 0.5mmol/l lenacil inhibited 45 to 70% thymidine incorporation into DNA fraction, while incorporations of uridine into RNA and leucine into protein were less affected. Thymidilate synthetase activity in P388 cells as assayed by the release of tritiated water from 5-^{3}H-deoxyuridine was inhibited by the compound to about 20%. Lenacil neither showed an in vivo inhibitory action on thymidine incorporation into acid-insoluble material in P388 cells, nor on thymidilate synthetase activity after a 24 or 48 h treatment. The compound did not change the melting temperature of isolated DNA. Studies of lenacil's effect on cell cycle kinetics of FL cells demonstrated that 48 h treatment increased the percentage of S-phase cells. Lenacil exerted a weak cytotoxic effect on FL cells. At concentrations above 0.1 mmol/l it inhibited cell growth, the effect being nonlethal.

Cytogenetic studies of lenacil revealed no indication of its mutagenicity against Salmonella typhimurium TA97, TA98, TA100 and TA102."

B.6.4.1.6

(Open literature study)

-Evaluation of genotoxic and cytotoxic properties of pesticides employed in Italian agricultural practices. De Marco A, De Salvia R, Polani S, Ricordy R, Sorrenti F, Perticone P, Cozzi R, D'Ambrosio C, De Simone C, Guidotti M, Albanesi T, Duranti G, Festa F, Gensabella G, Owczarek M. Environ Res. 2000 Jul;83(3):311-21.

The study is considered to provide complementary information.

Abstract

"In a program coordinated by the Italian Ministry of Works, we tested in vitro four pesticides widely employed in a developed agricultural region of central Italy. The four commercial agents were chosen on the basis of their diffusion in agricultural practice, knowledge of their active principle(s), and scant availability of data concerning their toxic and genotoxic activity. The agents were Cirtoxin, Decis, ***Tramat Combi (TC****), and Lasso Micromix (LM). All substances were tested in three in vitro systems: Chinese hamster ovary (CHO) cells, a metabolically competent hamster cell line (Chinese hamster epithelial liver; CHEL), and root tips of Vicia faba (VF). The cytotoxic and genotoxic end points challenged were micronuclei and root tip length (RTL) in VF and mitotic index (MI), proliferation index (PI), cell survival (CS), cell growth (CG), cell cycle length (CCL), sister chromatid exchanges, chromosomal aberrations, and single-cell gel electrophoresis, or comet assay, in CHEL and CHO cells. Tested doses ranged from the field dose up to 200× the field dose to take into account accumulation effects. On the whole, tested agents appear to induce genotoxic damage only at subtoxic or toxic doses, indicating a low clastogenic risk. MI, PI, CS, CG, RTL, and CCL appear to be the less sensitive end points, showing no effects in the presence of a clear positive response in some or all of the other tests. Using cytogenetic tests, we obtained positive results for TC and LM treatments in CHO but not in CHEL cells. These data could be accounted for by postulating a detoxifying activity exerted by this cell line. However, cytogenetic end points appear to be more sensitive than those referring to cytotoxicity."*

RMS:

In this publication (not reported by the notifier) De Marco *et al.* investigated the effect of several plant protection products on the genetic material using different test systems, including root tips of *Vicia faba* (VF), Chinese hamster ovary (CHO) cells, and a Chinese hamster epithelial liver cell line (CHEL).

The product "Tramat Combi" (Agrevo Italia SpA, No. 6032, purity unreported),containing approximately 12% **lenacil** and 30% ethofumesate). was chosen by the authors because of the *"few available data"respectively only 10 and 2 references for the active principles and in both cases no cell data). For ethofumesate the last reference were in 1986. The commercial product is used at field dose of 2 liters/ha (ap: respectively 240 and 600 g/ha)."*

The publication is of poor relevance for the a.s. lenacil under evaluation, since not the substance but a product was investigated. Since ethofumesate is not a clastogen and lenacil might be clastogenic *in-vitro* at excessive concentrations, the present study is not particularly helpul for the risk assessment, but the main results are reported for the sake of completeness.

In **table B.6.4.1.6 – 1**, the several outcomes of the micronucleus, chromosome aberration, SCE and SCGE test are represented.

A. Micronuclei and root tips length in *Vicia faba*[a]

Dose (g/kg soil)	MN % of MN	MN SD	RTL Length	RTL SD
Control	0.09	0.04	34.2	2.4
0.016 (fd)	0.10	0.05	30.3	2.4
0.16	0.12	0.08	28.1	2.8
0.32	0.10	0.06	20.9	2.0*
1.66	0.18	0.11	21.3	1.7*
3.32	0.12	0.09	19.8	1.2*

B. Cell survival and cell growth[b]

CS in CHO cells Dose (mg/ml)	Mean	CG in CHEL cells Dose (mg/ml)	0 h	24 h	48 h	72 h
Control	280	Control	500	1650	3730	3820
0.016 (fd)	267	0.016 (fd)	500	1390	3550	4450
0.064	246	0.064	500	1420	2800	3320
0.128	236	0.128	500	1240	2000	3240

C. Cell cycle length in CHO and CHEL cells[c]

	Phase	24 h	48 h	72 h	96 h
Control					
	G_1	35–33	50–47	81–41	88–48
	S	52–38	42–12	13–13	2–6
	G_2	13–28	8–40	2–45	5–45
TC					
	G_1	76–36	62–51	63–47	59–50
	S	9–32	16–12	8–11	4–2
	G_2	6–31	6–36	8–41	9–48

D. Single-cell gel electrophoresis in CHO and CHEL cells[d]

Dose (mg/ml)	CHO T. length	CHO T. moment	CHO T. inertia	CHEL T. length	CHEL T. moment	CHEL T. inertia
Control	13.06	8.23	551.6	4.08	1.15	122.48
H_2O_2	40.15**	35.55**	3457.1**	14.3**	7.3**	723.1**
0.016 (fd)	2.91*	0.8*	57.74*	6.8	4.19	429.11
0.16	5.39	1.74	113.23	5.28	2.02	236.51

E. Chromosomal aberrations in CHO and CHEL cells[e]

Dose (mg/ml)	Chromosomal aberrations % abnor.	Gaps	Breaks	Ctid exs	Cme exs	CA-gaps
2-h pulse						
Control	2–3	2–1	1–2	0–0	0–0	1–2
0.016 (fd)	16–14	2–0	10–21	6–4	6–0	22** 25**
0.07	10–22	0–9	7–12	6–10	0–2	13*–24**
0.23	12–10	0–0	16–9	3–2	0–2	19**–13*
0.70	12–8	0–0	13–0	3–8	2–0	18**–8
18-h pulse						
0.006	8–2	0–0	13–2	1–0	0–0	14**–2
0.016	22–10	2–0	33–8	0–4	0–0	33**–12*

F. Mitotic and proliferation indices and sister chromatid exchanges in CHO cells[f]

Dose (mg/ml)	SCE	SE	MI	PI
Control	9.33	1.04	4.6	1.0
0.016 (fd)	10.65	1.03	4.1	0.89
0.16	16.7	2.19*	4.7	0.94
1.60	12.8	1.03	4.4	0.89
3.20	11.0	0.55	2.7	0.87

Note. fd, Field dose.

[a] Controls were performed in tap water. $*P<0.01$, Duncan's multirange test. MN, % of micronucleated cells/20,000 cells. RTL, mean length of the primary root (mm)/25 roots.

[b] Each of four dishes was plated with 200 cells. Cells treated 24 h after plating and fixed 96 h later. All values are intended $\times 1000$. Treatments 3.5 h after seeding of cells. 0 h to 72 h, harvesting time after treatments.

[c] Values are the % of cells in that phase of the cell cycle. Underlined values = CHEL. Dose = 0.16 mg/ml.

[d] $H_2O_2 = 10^{-4}$ M as positive control. For a description of tail length, moment, and inertia, please refer to Materials and Methods. $*P<0.05$ and $**P<0.001$, t test.

[e] Chromosomal aberrations in 100 cells. Treatment pulses of 2 and 18 h $*P<0.05$ and $**P<0.01$, Fisher's exact test. % abnor., % abnormal cells. Ctid exs and Cme exs, respectively, chromatid and chromosome exchanges. Underlined values = CHEL cells.

[f] Mitotic index of 100 cells. $*P<0.01$, t test.

Data are presented in the table, containing six parts (A-F).
Part A addresses micronucleus (MN) frequency and root tip length (RTL) in *Vicia faba*. Control values were obtained by the use of H_2O. Doses range from the field dose up to a dose 200× higher.
Parts B and C contain data in in the mammalian cell survival (CS in CHO) and cell growth (CG in CHEL), and cell cycle length (CCL) in both CHO and in CHEL.
Part D of each table contains data on SCGE in both CHEL and CHO, including tail length, tail moment, and inertia.
Chromosome aberrations (CA) after both 12- and 18-h pulses and Sister Chromatid Exchange (SCE), mitotic index (MI), and proliferation index (PI) are listed in Parts E and F, respectively.

-The authors found the product able to affect root tip length (RTL) at a dose 20× the field dose (w/o statistical significance).
-Cell cycle analysis, performed at a single concentration of 0.16 mg/mL, were told to exhibit a decrease in G_1

cells *"at all times tested"* after the treatment in CHO cells alone, whereas in CHEL, cell cycle parameters did not differ from controls. **RMS** considers the relative amounts of CHO cells in G_1 slightly high at 24-48h, and slightly low at 72-96h in treated cells when compared with controls. It is unclear whether the modifications were significant, as it was not reported (probably not). It may indicate a slight arrest in the S-phase because of toxicity, although the effect is perhaps relevant at 24h, but less so at 48h, and unremarkable at 72-96h.

-Authors reported cell survival (cells incubated 1 week) slightly affected in CHO cells (at 0.128 mg/mL ~↓15%), and cell growth (cells incubated 24h and trypsinised 96h later) affected in a dose-dependent way in CHO (**RMS**: error, since CG not recorded in CHO) and more evidently so in CHEL cells.
RMS: Based upon the mean figures (no s.d., no statistics) , it may be anticipated that the doses applied in the CHO cells in the CA test (up to 0.23 and 0.7 mg/mL in 2h treatment) may be toxic. However, the numbers are not backed up by neither the MI nor the PI.

-Authors consider SCGE apparently "affected" only in CHO cells, but in fact it pertains a significant *decrease* when compared to control cells.

Authors highlight that both mitotic index and proliferation index MI and PI were decreased in CHO cells at the higest dose of 3.2 mg/mL in the SCE assay (**RMS**: data in the CA assay itself are absent, though).

-Chromosome aberration incidences were considered increased *"more clearly than SCE"* in CHO and CHEL, whereas both MI and PI (only available in CHO cells) were said affected in 24h treatments (not deducible from the tables, **RMS**). However, while statistically significant differences were observed in the category ("CA-gaps", unclear what is exactly meant), no statistical significance was noted in the other separate categories, and dose-dependence in the 2h-pulse seemed to be absent). The results should be interpreted cautionously in the light of all noted uncertainties. **RMS** also notes that the SCE assay showed no dose-dependent increases, either.
No increase is evident in the MN test (in root tips, **RMS**).

Authors interpreted their results as follows:
"Data presented here seem to indicate;for all agents tested;that a slight clastogenic risk is present, as indicated by genotoxicity tests (SCGE, MN, SCE, and CA). In fact, even in the case of positive cytogenetic results, the effects appear at toxic or subtoxic doses (as indicated by comparisons with cytotoxicity tests). Moreover, due to the different sensitivities of the tests used, an equivocal picture appears. The MN test seems to us the easiest to execute with clear indications. Data obtained with this test clearly indicate that all four agents tested appear to be slightly genotoxic (only Cirtoxin showed a signifcant increase in MN and only when tested at higher doses) even when tested at toxic or subtoxic doses. Tramat Combi and Cirtoxin appear to be toxic (RTL) starting at doses 20x the field dose. CA and SCE appear to be more sensitive than MN, as shown by the positive results obtained with all tested drugs. In the case of Tramat Combi, both tests indicate genotoxicity at doses very near the field dose: this effect appears in the presence of a similar toxic effect (CCL, CS, CG, MI in CHO cells), clear in the case of Tramat Combi, slight in the case of the other agents."

RMS considers that:
- in the absence of clear conclusions on MI and PI (the doses used for the SCE test do not match the CA test), results on CA (the most relevant effects) are difficult to interpret; at best, it could be assumed that doses tested in the CA were not cytotoxic and thus relevant for this reason, but the dose-response is not convincing.
- In general, the genotoxicological picture is unclear, in view of the uncertainty on the outcome, as well as the inconsistent response throughout all reported tests. The product is negative in the VF micronucleus test, equivocally positive in the SCGE, CA and SCE (no dose-responsive and/or with doubtful statistical significance).
- The data pertain to products, therefore the confounding effects of surfactants may obscur any relevant effect of the a.s. (which in addition are both ethofumesate and lenacil, making conclusions on lenacil impossible to draw).

In conclusion, for both methodological and reporting deficiencies, this study does not allow RMS to conclude on a potential clastogenicity of lenacil.

Photomutagenicity

Although no validated test guideline for photomutagenicity is available, the potential photomutagenicity of lenacil can be assessed considering the results of the phototoxicity study performed with lenacil and taking into account the recommendations of the "Committee on Mutagenicity of Chemicals in Food Consumer Products and the Environment" on photomutagenicity testing.

In the available *in vitro* 3T3 NRU phototoxicity test, lenacil was clearly demonstrated not to be phototoxic, which indicates that the substance is not transformed in a photoreaction. Since the substance is neither transformed nor activated by non-ionising radiation, it is unlikely that lenacil will be photomutagenic. The absence of a photomutagenicity potential is confirmed by the statement of the "Committee on Mutagenicity of Chemicals in Food Consumer Products and the Environment" on photogenotoxicity testing where it is concluded that

"…if an in vitro 3T3 NRU phototoxicity test was negative there would be no need for photogenotoxicity testing…".

A comparison of the results of phototoxicity and photomutagenicity testing showed a > 90% concordance for 3T3 NRU phototoxic positives for chemicals which resulted in one or more of the three photoreactive products following exposure to UVR. Furthermore, chemicals known to be strong photogenotoxins were predicted to be positive in photoreactivity assays. A number of chemicals were negative for photogenotoxicity despite being positive for photochemical reactivity and *in vitro* phototoxicity. Taking into account the negative result in the *in vitro* 3T3 NRU phototoxicity test with lenacil and considering the conclusions of the Committee on Mutagenicity of Chemicals in Food Consumer Products and the Environment on photomutagenicity testing, lenacil is not expected to be photomutagenic.

***In vitro* cytogenetic studies in mammalian cells**

B.6.4.1.6

Lenacil technical, *in vitro* mammalian chromosome aberration test in human lymphocytes (Allais L., 2001) - DuPont Report No.: ACD 017/013707

Guidelines: the study is in compliance with Directive 2000/32/EC Method B.10 (2000), equivalent to OECD 473 (1997).

GLP status: yes

Materials and Methods
Human blood was collected aseptically from two healthy non-smoking ♂ donors, pooled and diluted with RPMI tissue culture medium supplemented with foetal calf serum, heparin, glutamine and antibiotics.
Lenacil technical (Batch No. 141712003, purity 98.6%) was tested as a suspension in culture medium at the highest final concentration of 5000 µg/mL The study was performed on two separate occasions and on duplicate cultures.
A three hour exposure followed by a 17 hour recovery period was used in both tests and in both the absence and presence of S9 mix derived from rat liver.
In the first test, cultures were exposed to the test substance at final concentrations of 39.06, 78.13, 156.05, 312.5, 625, 1250, 2500 and 5000 µg/mL.
In the second test, cultures were exposed to the test substance at 312.5, 625, 1250, 2500 and 5000 µg/mL in the absence of S9 mix, and at 625, 1250, 2500 and 5000 µg/mLin the presence of S9 mix. Solvent and positive control cultures were also prepared.
Two hours before the cells were harvested; mitotic activity was arrested by addition of Colcemid. After two hours incubation, the cells were treated with a hypotonic solution and fixed. Slides were then prepared and stained with Giemsa.
One hundred metaphase figures were examined, where possible, from each culture. The incidence of polyploid metaphase cells, out of 500 metaphase cells, was determined quantitatively for negative control cultures and cultures treated with the highest dose level of the test substance used in the analysis for chromosomal aberrations. The number of aberrant metaphase cells in each treatment group was compared with the solvent control value using Fisher's test. Criteria for evaluation of the results are well defined.

RMS:
In the study, it is stated: "*Prior to commencing testing, the solubility of the test substance in solvents compatible with the test system was assessed. Lenacil Technical was found to be insoluble in purified water at 50 mg/mL and insoluble in dimethyl sulphoxide, ethanol and acetone at 250 mg/mL. However, a doseable suspension was formed in culture medium at 10 mg/mL. On dosing at 50% v/v into aqueous tissue culture medium, giving a final concentration of 5000 µg/mL, precipitate was observed. Precipitate was also observed at final concentrations of 2500, 1250 and 625 µg/mL*".
It is not clear from the data whether precipitation was systematically observed at the doses 625 µg/mL and 1250 µg/mL (3h ±S9) during the two mutagenicity tests. From the data, including the consideration of cytotoxicity (Mitotic index), it appears to the RMS that the tested doses are acceptable.

The study is accepted.

Findings

On the basis of the mitotic index data, the following concentrations were selected for metaphase analysis:

- First test, without S9 mix: 625, 1250, 2500 and 5000 µg/mL.
- First test with S9 mix: 1250, 2500 and 5000 µg/mL.
- Second test, without S9 mix: 625, 2500 and 5000 µg/mL.
- Second test with S9 mix: 1250, 2500 and 5000 µg/mL.

In the absence of S9 mix, Lenacil technical caused statistically significant increases in the proportion of metaphase figures containing chromosomal aberrations, at 5000 µg/mL in the first test ($P<0.001$), and at 2500 and 5000 µg/mL in the second test ($P<0.01$ and $P<0.001$, respectively), when compared with the solvent control.

In the presence of S9 mix, Lenacil technical caused no statistically significant increases in the proportion of metaphase figures containing chromosomal aberrations at any dose level, in either test.

No increases in the proportion of polyploid cells were seen in either test.

All positive control compounds caused large, statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the assay.

Table B.6.4.1.6-1: Summary of results of chromosomal aberrations in human lymphocytes (Test 1)

Exposure period /S9-mix	**Concentration of Lenacil technical**	**Cells with aberrations Excluding gaps**			**Cells with aberrations Including gaps**			**Mitotic Index (%)**
-S9-mix	**(µg/mL)**	**Individual values (%)**		**Mean (%)**	**Individual values (%)**		**Mean (%)**	
3hours	0 (Culture medium)	1	2	1.5	1	2	1.5	100
	625	1	1	1.0	1	1	1.0	82
	1250	1	1	1.0	1	1	1.0	82
	2500	2	4	**3.0**	2	4	**3.0**	68
	5000	7	16	**11.5****	7	16	**11.5****	54
	0.2 (Mitomycin C)	*17*	*12*	*14.5***	*17*	*12*	*14.5***	-
+ S9-mix								
3hours	0 (Culture medium)	1	0	0.5	1	0	0.5	100
	1250	0	0	0.0	1	0	0.5	90
	2500	2	0	1.0	2	0	1.0	84
	5000	1	0	0.5	1	0	0.5	75
	6 (Cyclophosphamide)	*12*	*13*	*12.5***	*12*	*13*	*12.5***	-

Statistically significant at **p<0.001; *: p<0.01

Table B.6.4.1.6-2: Summary of results of chromosomal aberrations in human lymphocytes (Test 2)

Exposure period/ S9-mix	**Concentration of Lenacil technical**	**Cells with aberrations Excluding gaps**			**Cells with aberrations Including gaps**			**Mitotic Index (%)**
- S9-mix	**(µg/mL)**	**Individual values (%)**		**Mean (%)**	**Individual values (%)**		**Mean (%)**	**Index (%)**
3hours	0 (Culture medium)	1	1	1.0	1	1	1.0	100
	625	0	1	0.5	0	1	0.5	124
	2500	5	6	**5.5***	5	6	**5.5***	61
	5000	16	11	**13.5****	16	11	**13.5****	39
	0.1 (Mitomycin C)	*13*	*11*	*12.0***	*13*	*11*	*12.0***	-
+ S9-mix								
3hours	0 (Culture medium)	0	1	0.5	0	1	0.5	100
	1250	1	0	0.5	1	0	0.5	79
	2500	2	2	2.0	2	2	2.0	58
	5000	1	1	1.0	1	1	1.0	56
	6 (Cyclophosphamide)	*11*	*11*	*11.0***	*11*	*11*	*11.0***	-

Statistically significant at **p<0.001; *: p<0.01

Conclusion

It is concluded that Lenacil technical has shown evidence of clastogenic activity, in this *in vitro* cytogenetic test system, in the absence of S9 mix only, under the experimental conditions described. No clastogenic activity was observed in the presence of S9 mix.

During the first peer review, more information was requested on the study of chromosomal aberration test, and additional information was provided below. From the figures, **RMS** concludes that the incidence of cells containing gaps and breaks are the same (excluding gaps gives the same incidence as including them) in the absence of S9, and that lenacil is an *in-vitro* clastogen in a dose-dependent way, in the absence of metabolic activation. The presence of S9 mitigates this incidence, lowering the level of concern.

It should in addition be highlighted that clastogenicity was apparent only at doses at the limit of solubility (2.5 mg/mL and 5 mg/mL), however with still an acceptable cytotoxicity.

The resultsof the testing of Lenacil, In the *in vitro* mammalian chromosomal aberration test in human lymphocytes (Allais, 2001) are further detailed in the **tables 6.4.1.6-1** and **-6.4.1.6-1 2** (see below).

Table 6.4.1.6-1 Lenacil, *in vitro* chromosome aberration test in human lymphocytes (Allais, 2001) : detailed results (Test 1)

Exposure period/ S9-mix	Chromatid type		Chromosome type		Concentration of Lenacil technical	Cells with aberrations Excluding gaps			Cells with aberrations Including gaps			Mitotic Index (%)
3 hours	ctb %	cte %	csb	cse	(µg/mL)	Individual values (%)		Mean (%)	Individual values (%)		Mean (%)	
-S9 mix	1 3	1			0 (Culture medium)	1	2	1.5	1	2	1.5	100
	1 1				**625**	1	1	1.0	1	1	1.0	82
	1 1				**1250** [P]	1	1	1.0	1	1	1.0	82
	2 6				**2500** [P]	2	4	**3.0**	2	4	3.0	68
	10 23				**5000** [P]	7	16	**11.5****	7	16	**11.5****	54
	12 *12*	*4* *2*	*1* *1*		*0.2 (Mitomycin C)*	*17*	*12*	*14.5***	*17*	*12*	*14.5***	-
+ S9 mix												
	1				0 (Culture medium)	1	0	0.5	1	0	0.5	100
					1250	0	0	0.0	1	0	0.5	90
	3				**2500** [P]	2	0	1.0	2	0	1.0	84
	1	1			**5000** [P]	1	0	0.5	1	0	0.5	75
	10 *11*	*1* *3*	*2* *2*		*6 (Cyclophosphamide)*	*12*	*13*	*12.5***	*12*	*13*	*12.5***	-

Statistically significant at **p<0.001; *: p<0.01
Ctb/csb= chromatid /chromosome break
Cte/cse= chromatid/chromosome exchange
[P] :precipitation observed on the slide.

Table 6.4.1.6-2 Lenacil, *in vitro* chromosome aberration test in human lymphocytes (Allais, 2001) : detailed results (Test 2)

Exposure period/ S9-mix	Chromatid type		Chromosome		Concentration of Lenacil technical	Cells with aberrations Excluding gaps			Cells with aberrations Including gaps			Mitotic Index (%)
- S9-mix	ctb %	cte %	csb%	cse%	(µg/mL)	Individual values (%)		Mean (%)	Individual values (%)		Mean (%)	
3 hours	1 1				0 (Culture medium)	1	1	1.0	1	1	1.0	100
	 1				**625**	0	1	0.5	0	1	0.5	124
	5 6				**2500 P**	5	6	**5.5***	5	6	**5.5***	61
	25 14		1		**5000 P**	16	11	**13.5****	16	11	**13.5****	39
	10 *11*	*4* *2*			*0.1 (Mitomycin C)*	*13*	*11*	*12.0***	*13*	*11*	*12.0***	-
+ S9-mix												
3 hours	 1				0 (Culture medium)	0	1	0.5	0	1	0.5	100
			1		**1250**	1	0	0.5	1	0	0.5	79
	1 2		1 3		**2500 P**	2	2	2.0	2	2	2.0	58
	1 2		 1		**5000 P**	1	1	1.0	1	1	1.0	56
	9 *8*	 *1*	*1* *3*	*Other1* *1*	*6 (Cyclophosphamide)*	*11*	*11*	*11.0***	*11*	*11*	*11.0***	-

Statistically significant at **p<0.001; *: p<0.01
Ctb/csb= chromatid /chromosome break
Cte/cse= chromatid/chromosome exchange
P :precipitation observed on the slide.

B.6.4.1.7

Lenacil (DPX-B0634) technical: *In vitro* mammalian chromosomal aberration test in human peripheral blood (Kellum S.N., 2017) - DuPont Report No.: 49348

Guidelines: the study is *partly* in compliance with Directive 440/2008/EC Method **B.10** (2017), equivalent to OECD 473 (2016).
GLP status: yes
Deviations from the current test method lenacil was diluted in DMSO, which seemed to be suboptimal in terms of solubility.Taking into account the solubility data of a former chromosome aberration assay (Allais, 2001) it is evident that doses up to and including 1250 µg/mL could have been tested to remain within an acceptable solubility and cytotoxicity concentration fork

Materials and Methods
Human peripheral blood lymphocytes (HPBL) were drawn from a healthy 34 year old ♀ without previous chemotherapy or radiotherapy; and without recent (within the last 3 months) viral disease or X-ray exposure. HPBL cultures were initiated in RPMI tissue culture medium supplemented with foetal bovine serum, L-glutamine and antibiotics, and with 1-2% of the mitogen phytohemagglutinin-M (PHA-M). Forty-eight hours later, the cell cultures were exposed to 9 concentrations (10, 50, 100, 250, 500, 750, 1000, 1500 and the limit dose, as per OECD 473 (2014), of 2000 µg/mL) of lenacil (DPX-B0634-150, lot number 047303003, purity 99.33 %) and the vehicle control substance for each test condition (one culture per concentration level). Positive control substances were mitomycin-C (MMC) for the non-activated system and cyclophosphamide (CP) for the S9-activated system. The concentrations for MMC were 0.4 and 0.6 µg/mL for the 4-hour condition and 0.2 and 0.4 µg/mL for the 22-hour condition. The concentrations for CP were 10 and 15 µg/mL. The cells were treated for approximately 4 and 22 hours in the absence of S9 metabolic activation, and ~4 hours in the presence of Aroclor induced rat liver S9 metabolic activation.
The cells were harvested at a single time point, approximately 22 hours from the initiation of treatment (~1.5 times the normal cell cycle). Approximately 19 hours after the initiation of exposure to the test substance, Colcemid® was added at a final concentration in the culture medium of 0.1 µg/mL to all cultures. Approximately 3 hours after the Colcemid® addition, the cells were collected by centrifugation and the medium removed. Two hours before the cells were harvested; mitotic activity was arrested by addition of Colcemid. After two hours incubation, the cells were treated with a hypotonic solution and fixed. Slides were then prepared and stained with Giemsa.
After selection of the slides for cytogenetic analyses, the slides were coded and scored blind to control for bias. Metaphase cells were selected for scoring based on good chromosome morphology and staining characteristics. Only metaphase cells with 46 centromeres were analysed for structural aberrations. At least 300 metaphases per concentration level (150 from each duplicate culture), when available, were analysed for structural aberrations. Numerical aberrations were recorded as well.

The study is considered to provide complementary information.

Findings

The test substance formed an opaque, beige suspension at 200 mg/mL, the highest stock concentration used in the study.

Test substance precipitation was observed at the beginning at 100 µg/mL in all test conditions.

Test substance precipitation was observed at the end of treatment at 100 µg/mL in both 4 hour conditions and at 50 µg/mL in the 22-hour non-activated test condition.

Cytotoxicity of 55 ± 5% mitotic reduction in relation to the vehicle control, or greater, was not observed in any test condition. Yet, based on these findings, the doses chosen for the chromosomal aberration assay were 5, 10, 25, 50, and 100 µg/mL for each test condition. In the chromosomal aberration assay, test substance precipitation was observed at the beginning and end of treatment at 100 µg/mL in all test conditions. HPBL were treated for 4 hours (activated and non-activated test system) and 22 hours (non-activated test system). Metaphase cells were harvested about 19 hours following the initiation of treatment (Colcemid® was added 3 hours prior to harvest to arrest cells in metaphase). Cytogenetic evaluations were conducted at 25, 50, and 100 µg/mL for all test conditions. The positive and solvent controls fulfilled the requirements for a valid test.

The percentage of cells with structural aberrations in the test substance-treated groups were not significantly increased above that of the vehicle control in any test condition ($p > 0.05$, Fisher's exact test). No dose responsive trend was observed ($p > 0.05$, Cochran-Armitage test). Positive controls induced the appropriate response (see results in **table B.6.4.1.7-1**).

Conclusion

Based on the findings of this study, lenacil was concluded to be negative for the induction of structural and numerical chromosomal aberrations in cultured human peripheral blood lymphocytes with and without an exogenous metabolic activation system.

However, RMS is uncertain that the current study overrules the earlier study of 2001, since RMS thinks that the tested concentratyions could have been higher, when compared to the concentrations tested in the first *in-vitro* CA test in human lymphocytes. Precipitation was observed at 100 µg/mL and above in the new test, while doses up to 1250 µg/mL without precipitation have been tested in the former CA test (Allais, 2001).

Table B.6.4.1.7-1 Lenacil: *In vitro* chromosomal aberration test in human peripheral blood (Kellum, 2017): assay summary

Treatment[a] (µg/mL)	S9 activation	Treatment time	Mitotic index (%)	Cells scored		Aberrations per cell		Cells with aberrations[b]	
				Numerical	Structural	Mean	SD	Numerical (%)	Structural (%)
Vehicle[c]	-S9	4	6.9	300	300	0.003	0.005	0.0	0.3
25	-S9	4	7.5	300	300	0.003	0.005	0.0	0.3
50	-S9	4	7.3	300	300	0.007	0.009	0.0	0.7
100[e]	-S9	4	6.1	300	300	0.003	0.005	0.0	0.3
MMC 0.4	*-S9*	*4*	*6.0*	*300*	*300*	*0.093*	*0.019*	*0.0*	*9.3[d]*
Vehicle	+S9	4	6.2	300	300	0.007	0.000	0.0	0.7
25	+S9	4	7.2	300	300	0.003	0.005	0.0	0.3
50	+S9	4	6.5	300	300	0.003	0.005	0.0	0.3
100[e]	+S9	4	7.7	300	300	0.003	0.005	0.0	0.3
CP 10	*+S9*	*4*	*4.3*	*300*	*300*	*0.120*	*0.000*	*0.0*	*11.7[d]*
Vehicle	-S9	22	6.8	300	300	0.007	0.000	0.0	0.7
25	-S9	22	6.1	300	300	0.013	0.009	0.3	1.3
50	-S9	22	4.3	300	300	0.007	0.009	0.0	0.7
100[e]	-S9	22	6.9	300	300	0.017	0.005	0.0	1.7
MMC 0.2	*-S9*	*22*	*4.9*	*200*	*200*	*0.150*	*0.160*	*0.0*	*12.5[d]*

a: human peripheral blood lymphocyte (HPBL) cells were treated at 37°C;
b: excluding cells with only gaps;
c: DMSO;
d: statistically significant difference from control at $p < 0.05$ by Fisher's test;
e: test substance precipitation observed at the beginning and end of treatment.
MMC: mitomycin-C;
CP: cyclophosphamide.

Discussion of the *in-vitro* chromosome aberration assays.

RMS:
The large discrepancy between the chromosome aberration assay conducted in 2001 *vs* 2017 is puzzling. In the 2001 assay, concentrations as high as 5000 µg/mL were tested, while the maximal dose in the 2017 study was 100 µg/mL. The study director in the 2017 study highlights the occurrence of precipitation at this dose. It is confusing that the first author did even not mention any form of precipitation at concentrations going up to 12× higher.

In the most recent study, mitotic index was unaltered, while it was decreased in the 2001 study by 40-50%, which would constitute an adequate degree of cytotoxicity as prescribed.
Notifier was invited to clarify this discrepancy in behaviour of the a.s. in the 2 studies. No data as regards the pH or osmolality of the medium after dissolution of the test article, giving some indication of the confounding factors explaining a potential false positivity.
Another reason for the difference in outcome between the first and the second study could be found in the degree of purity, the latter being somewhat higher in the more recent study. It is questioned whether the tested a.s. in the most recent version corresponds to the purity for which approval is sought. However, there are no indications that the impurities would be completely different in terms of genotoxicity, when compared to the a.s. itself.

Notifier:
"The 2001 study used a doseable suspension in culture medium (selection of solvent, p. 12) and precipitate was seen starting at 625 µg/mL. It also mentions precipitate on slides at the end of treatment. Page 20 (Table 2) shows the mitotic index data in the 1st test without S9, where precipitate was observed on slides at ≥1250 µg/mL. On p. 21 (Table 2 continued) the data for the with S9 treated cells shows precipitate starting at ≥2500 µg/mL. Page 24 (table 4) shows mitotic index data for the 2nd test without S9 with precipitate observed again at ≥1250 µg/mL. In the 2nd test with S9, precipitate was observed at ≥ 2500 µg/mL (p. 25, Table 4 continued). In all these cases, precipitate likely occurred at even doses lower, as the cells were washed before preparing slides.
Comparing the 1997 versus 2016 OECD Guideline 473, there is change in regard to testing when precipitate is present. In the 1997 update paragraph 17 says, "In some cases (e.g. when toxicity occurs only at higher than the lowest insoluble concentration) (**RMS**: which is the case here) *it is advisable to test at more than one concentration with visible precipitation. In the 2016 update paragraph 23 says, "For poorly soluble test chemicals that are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration analysed should produce turbidity or a precipitate visible by eye or with the aid of an inverted microscope at the end of the treatment with the test chemical. Even if cytotoxicity occurs above the lowest insoluble concentration, it is advisable to test at only one concentration producing turbidity or with a visible precipitate because artefactual effects may result from the precipitate."*
Applying the 2016 guideline to the 2001 study means that the without S9 treated groups would only have been tested up to 1250 µg/mL, based on precipitate. None of the without S9 metaphase results tested up to 1250 µg/mL showed an increase in chromosome aberrations.
Procedural differences between the 2 labs could have also contributed to differences in precipitate the 2001 test treated cells for 3 hours, the 2017 test treated for a minimum of 4 hours. The 2001 test used growth medium to prepare a workable suspension, the 2017 test used DMSO. The 2001 test had 10% serum and the 2017 test had 15%. The 2017 study complies with current guidelines, but the 2001 study does not, and thus, the 2001 study should not be relied on."

RMS agrees that there are differences between the 2001 and 2017 studies, but does not agree that the differences as regard the current guidelines would invalidate the former study from the scientific and formal point of view. In the 2001 assay, it was clearly stated that Lenacil was insoluble *[…]* in DMSO *[…]* at 250 mg/mL. Thus, the second test was conducted with a.s. in a solvent (DMSO) which would have been unsuitable, and consequently, caused precipitation at low concentration, limiting the highest tested dose at a level about 12× below that observed in the original test.

In addition, notifier does not mention an important study recommendation under §1.4.2.2 of EC TM B.10 (OECD TG 473), *i.e.*
"If no cytotoxicity or precipitate is observed, the highest test concentration should correspond to 10 mM, 2 mg/mL or 2 µl/mL, whichever is the lowest. This applies to test chemicals of defined composition. Justification for not testing individual components of the composition should be provided."
For lenacil, the lowest is 2000 µg/mL. The level of 0.01M would correspond with 2340 µg/mL, comparable to 2500 µg/mL in the first study for which chromosome aberrations were observed without S9. In addition, the increases were not proven associated with large changes in osmolality of the treatment medium or extreme toxicity.

For these reasons, it is rather the 2017 study which should not be relied upon, and the conclusion that lenacil may intrinsically be clastogenic *in-vitro* (in the absence of S9, not in the presence), is not refuted. However, it may be underlined that the clastogenicity occurs only at the first dose level producing some precipitation, which is however still acceptable when checked against TM B.10, but in the presence of an acceptable cytotoxicity, not <45% MI.

However, if the *in-vivo* study is found negative, it does not change the overall conclusion.

***In vitro* mammalian cell assay for gene mutation**

B.6.4.1.8 Lenacil technical: *in vitro* mammalian cell gene mutation test (Clare, G., 2003) - DuPont Report No.: ACD 053/023530

Guidelines:

study is not fully in compliance with Dir EEC 2000/32/EEC Annex 4E or 87/302 or OECD test guideline n 476 (1997-84).

Deviation from official protocol: diameter of colonies was not measured for control cells (OK for 87/302).

GLP status: yes

Materials and Methods

Cultures of mouse lymphoma L5178Y cells were exposed to Lenacil technical (Batch No. 141712003, purity 98.6%) suspended in culture medium at concentrations up to 5000 µg/mL for 3 hours in both the absence and presence of supplemented Aroclor-induced rat liver fraction (S9 mix) and for 24 hours in the absence of S9 mix. The cells were washed and resuspended.

Aliquots were diluted and plated for determination of Day 0 survival. Further aliquots were diluted to 2 x 10^5 cells/mL and incubated for 48 hours, with readjustment of cell density after 24 hours. Using 96-well plates, cloning efficiency was assessed by plating at 1.6 cells/well, incubating at 37 C in an atmosphere of 5% CO_2 in air for at least 7 days, and counting empty wells. Cells were also plated at 2 x 10^3 cells/well in selective medium containing trifluorothymidine (lethal to TK^{-}/- mutants) and incubated for 10-14 days.

Mutant frequency (forward mutation to the homozygous TK^{-}/- form) was calculated relative to survival. 3MC, and MMS were used as positive controls and induced significant increases in mutant frequency.

The study is accepted.

Findings:

There were no significant increases in mutant frequency in either the presence or absence of S9 mix.

The details of the results are reported in table **B.6.4.1.8-1**

Conclusion:

Lenacil technical did not demonstrate mutagenic potential in this *in vitro* cell mutation assay, under the experimental conditions described.

Table B.6.4.1.8-1 Lenacil: *in vitro* mammalian cell gene mutation test (Clare, 2003): test data

Lenacil		3 Hours					
		-S9			+S9		
(µg/mL)		Mean relative survival	Mean relative % CE	Mean MF	Mean relative survival	Mean relative % CE	Mean MF
0		100	100	340 10^{-6} (100%)	100	100	366 10^{-6} (100%)
39		107	128	275 10^{-6} (81%)	100	97	361 10^{-6} (99%)
78		97	127	250 10^{-6} (74%)	99	90	369 10^{-6} (101%)
152		97	116	338 10^{-6} (99%)	95	91	305 10^{-6} (83%)
313		74	92	321 10^{-6} (94%)	90	80	318 10^{-6} (87%)
625		79	114	252 10^{-6} (74%)	89	116	225 10^{-6} (61%)
1250		73	109	277 10^{-6} (81%)	84	110	311 10^{-6} (85%)
2500		71	79	343 10^{-6} (101%)	81	117	272 10^{-6} (74%)
5000		56	123	246 10^{-6} (72%)	72	113	298 10^{-6} (81%)
MMS (10 µg/mL)		*91*	*72*	*1631 10^{-6}** (479%)*			
MC (2.5 µg/mL)					*67*	*94*	*874 10^{-6}** (239%)*
Lenacil		**24 hours**					
		-S9			+S9		
(µg/mL)		Mean relative survival	Mean relative % CE	Mean MF	Mean relative survival	Mean relative % CE	Mean MF
0		100	100	303 10^{-6} (100%)	100	100	282 10^{-6} (100%)
39		109	86	267 10^{-6} (88%)	99	112	314 10^{-6} (113%)
78		87	94	271 10^{-6} (89%)	91	119	294 10^{-6} (104%)
152		85	82	300 10^{-6} (99%)	94	151	273 10^{-6} (97%)
313		92	104	194 10^{-6} (64%)	108	141	287 10^{-6} (102%)
625		93	85	223 10^{-6} (76%)	97	133	216 10^{-6} (77%)
1250		66	102	170 10^{-6} (56%)	81	121	272 10^{-6} (96%)
2500		64	115	151 10^{-6} (50%)	77	104	231 10^{-6} (82%)
5000		26	122	95 10^{-6} (31%)	93	98	209 10^{-6} (74%)
MMS (5 µg/mL)		*52*	*57*	*1947 10^{-6}** (643%)*			
MC (2.5 µg/mL)					*35*	*64*	*2014 10^{-6}** (714%)*

MC: 3-Methylcholanthrene; MMC: Methyl methanesulphonate; CE: cloning efficiency; MF: mutant frequency
**: p<0.01, significant outside the historical control range

Table B.6.4.1.8-2 Lenacil: *in vitro* mammalian cell gene mutation test (Clare, 2003): Historical control data:

		Mean Day_0 relative survival	Mean suspension growth	Mean day_2 cloning efficiency	Mean mutant frequency
Solvent controls	Mean	88.42	8.09	101.90	192 10^{-6}
	Maximum	141.36	14.18	188.17	459 10^{-6}
	S.D.	17.77	2.22	24.95	76 10^{-6}
Positive controls	Mean	67.72	6.52	75.95	1138 10^{-6}
	Minimum	22.23	3.02	30.46	346 10^{-6}
	S.D.	23.25	2.70	25.72	575 10^{-6}

Number of tests : 154; Data collection period: [01-Nov-99, 12-Jul-02]

DNA interaction or damage *in vitro (e.g., In vitro* unscheduled DNA synthesis)

B.6.4.1.9

Lenacil: assessment of genotoxicity in an unscheduled DNA synthesis assay using adult rat hepatocyte primary cultures (Riach & Mohammed 1990)

DuPont Report No.: IRI 6135

Guidelines:

study is in compliance with Dir EEC 87/302/EEC Annex VB.

Renewal assessment: The guidelines according to which the study was performed were not reported. A review of this study indicates it does not meet the current OECD Test Guideline 486 and has been superseded by the in vivo mouse micronucleus study (ACD 018/013472-[redacted] 2001)).

GLP status: yes (no attest of competent authority)

Materials and Methods

Lenacil (batch no. 8903, purity not stated in report) in DMSO was tested for its ability to induce unscheduled DNA synthesis (UDS) in primary cultures of adult rat hepatocytes as measured by silver grain counts in photographic emulsion formed by radiation from [6-^{3}H]-thymidine taken up by the cells. Cultures were established with cells derived from the collagenase-perfused liver of Fischer 344 rats. Eight one-half decreasing concentrations of Lenacil from 0.078 µg/mL^{-1} to 10 µg/mL^{-1} were tested. Two independent assays were performed. Vehicle controls were treated with DMSO only. Positive control substance 2AAF and Michler's ketone demonstrated the sensitivity of the test system. Criteria for a positive test were well defined.

In the study:

"*Several criteria have been established which, if met, provide a basis for classifying a test material as positive in the UDS assay. These criteria are formulated on the basis of published results and laboratory experience and are used in lieu of a statistical treatment to indicate a positive response. Wulie the criteria are arbitrary guidelines that may not be applicable to all assays and may need revision as the database alters, they represent a reasonable approach to the evaluation of the test material.*

The test material was considered to be positive in the UDS assay at concentrations that caused:

1. *an increase in the mean net nuclear grain count to at least 6 grains per nucleus in excess of the concurrent vehicle control value, **and***
2. *the % of nuclei with 6 or more net grains to increase above 20% of the examined population, in excess of the concurrent vehicle control value, **and/or***
3. *the % of nuclei with 20 or more net grains to reach or exceed 2% of the examined population.*"

The study is accepted.

Findings

Cell viability at the completion of the dosing period (Table B.6.4.1.9-1)

In assay 1, vehicle controls had a viability of 51% and 52%, respectively. There was no clear dose-related trend in terms of reduction of survival in Lenacil-treated cultures, although the 4 higher concentrations had generally lower survival than the 4 lower concentrations.

In assay 2, vehicle controls had a viability of 51% and 52%, respectively. There was no significant reduction in cell survival in any of the Lenacil-treated cultures.

UDS data (Table B.6.4.1.9-2)

In both experiments there were a number of cultures where one of the triplicate coverslips did not contain sufficient scorable cells for acceptance. It was considered that the results from the remaining coverslips were, on all occasions, sufficiently well-defined for both experiments to be deemed acceptable.

The vehicle controls were considered valid, having met the requirements as specified by the acceptance criteria

Positive controls: in both assays, primary cultures treated with Michler's ketone (direct acting control) and 2-AAF (indirect acting control) gave significant increases by all 3 criteria, at all concentrations.

Lenacil:

In assay 1, no increases over control values were obtained in any Lenacil-treated cultures, by any of the 3 criteria. In assay 2, small increases in both mean net grains (2.3 over a vehicle mean of -0.55) and in % nuclei with ≥6 net grains (22.67% over a vehicle mean of 5.67%) were obtained at a concentration of 5 µg/ml Lenacil.

Neither increase was significant by the stated criteria, and there was no indication of a dose-related trend at lower concentrations. Furthermore, toxicity was observed in the cultures at the 5 µg/mLconcentration, and, while only cells not visibly affected by physical signs of toxicity were scored, the possibility remains that small increases observed at toxic concentrations may be artifacts of the toxic effects. From these considerations, and the lack of any similar effect in assay 1, it was concluded that the increases obtained in assay 2 were not significant.

In conclusion, lenacil does not induce unscheduled DNA synthesis in primary cultures of adult rat hepatocytes when tested in DMSO at concentrations extending into the toxic range.

Conclusion

Lenacil technical did not induce unscheduled DNA synthesis in cultures of primary rat hepatocytes when tested at concentrations extending into the toxic range.

Table B.6.4.1.9-1. Lenacil: assessment of genotoxicity in an unscheduled DNA synthesis assay using adult rat hepatocyte primary cultures (Riach & Mohammed.1990): cell survival data

Assay 1			Assay 2		
Compound (µg/mL)	**% viable cells**	**% survival ***	**Compound (µg/mL)**	**% viable cells**	**% survival ***
DMSO (10 µL)			**DMSO (10 µL)**		
Solvent I	51.0	99	Solvent I	58.0	99
Solvent II	52.0	101	Solvent II	59.0	101
Michler's ketone			***Michler's ketone***		
2	*17.3*	*34*	*2*	*$*	
8	*14.5*	*28*	*8*	*$*	
2-Acetylaminofluorene			***2-Acetylaminofluorene***		
0.5	*38.2*	*74*	*0.5*	*48.5*	*83*
2	*36.5*	*71*	*2*	*$*	
Lenacil			**Lenacil**		
0.078	39.0	76	0.078	57.5	98
0.156	40.5	79	0.156	$	
0.312	40.0	78	0.312	40.0	68
0.625	31.0	60	0.625	43.0	74
1.25	24.0	**47**	1.25	45.0	77
2.5	20.0	**39**	2.5	19.0	84
5	18.0	**39**	5	44.5	76
10	15.0	**49**	10	41.0	70

*: Viability/vehicle mean

$:not possible to judge accurately the number of viable cells, due to the large number of overlapping cells present.

Table B.6.4.1.9-2. Lenacil: assessment of genotoxicity in an unscheduled DNA synthesis assay using adult rat hepatocyte primary cultures (Riach & Mohammed.1990): UDS data.

Assay 1					Assay 2				
Compound (µg/mL)	Cells scored	Mean net grains/nucleus	% nuclei with ≥6 net grains	% nuclei with ≥20 net grains	Compound (µg/mL)	Cells scored	Mean net grains/nucleus	% nuclei with ≥6 net grains	% nuclei with ≥20 net grains
DMSO (10 µL)					**DMSO (10 µL)**				
Solvent I	150	-0.86	10.67	0.00	Solvent I	150	-0.47	5.33	0.00
Solvent II	100	-1.51	2.67	0.00	Solvent II	150	-0.62	6.00	0.00
Michler's ketone					**Michler's ketone**				
2	150	9.59	71.33	4.00	2	100	10.13	70.00	10.00
8	150	10.69	88.67	2.67	8	100	17.41	80.00	34.00
2-Acetylaminofluorene					***2-Acetylaminofluorene***				
0.5	*150*	*17.46*	*92.00*	*36.67*	*0.5*	*150*	*12.60*	*62.67*	*20.67*
2	*150*	*11.91*	*78.00*	*19.33*	*2*	*100*	*14.14*	*86.00*	*15.00*
Lenacil					**Lenacil**				
0.078	100	0.04	5.33	0.00	0.078	100	-1.17	5.00	0.00
0.156	150	-5.23	3.33	0.67	0.156	150	-0.42	6.00	0.00
0.312	150	1.25	**18.00**	0.00	0.312	150	0.95	**10.67**	0.00
0.625	100	0.46	**15.00**	0.00	0.625	150	0.84	6.67	0.00
1.25	100	0.41	**12.67**	1.30	1.25	150	-0.32	6.67	0.00
2.5	150	-0.63	7.33	0.00	2.5	100	-0.16	6.00	0.00
5*	100	-0.40	6.67	0.00	5*	150	2.3	22.67	0.00
10	100	-1.22	4.67	0.00	10	NS	NS	NS	NS

NS: not scorable due to toxicity

*: indications of toxicity to cell cultures present on slides

No significant evidence of unscheduled DNA synthesis was obtained at any test concentration of Lenacil, in either of the 2 independent experiments. Direct and indirect acting positive control compounds demonstrated the sensitivity of the test system.

B.6.4.2 (CA 5.4.2) ***In vivo* studies in somatic cells**

Metaphase or micronucleus analysis rodent bone marrow

B.6.4.2.1 Lenacil technical - mouse micronucleus test ([redacted] 2001) - DuPont Report No.: ACD 018/013472

Guidelines: OECD 474, OPPTS 870.5395 Method B.12 (1998), JMAFF NohSan No. 4200

Deviations from official protocol:

- ♀ *were not included in the test;*
- *oral route was used although it was not demonstrated that lenacil reached bone marrow in this assay. However, from the ADME studies it appeared that marrow was reached, and since lymphoid tissue is at target in the studies with repeated a.s. administration, it may be assumed that bone marrow was also attained.*
- *Results are reported w/o standard deviation.*
- *Only 7 mice were tested.*

The deviations are nont considered to invalidate the study

Renewal assessment:
The in vivo mouse micronucleus assay (ACD 018/013472) was originally submitted under EU Rev8 Point IIA 5.4.2 and has been conducted with lenacil technical. The study was conducted according to OECD 474, OPPTS 870.5395 Method B.12 (1998), and JMAFF NohSan No. 4200. A review of this study indicates that it meets the current OECD Test Guideline 473 or OPPTS guideline 870.5395; a deviation includes that the results are reported without standard deviation. However, reconduct is unlikely to yield a significantly different result because of the fact that study was conducted according to current guidelines and under GLP conditions.

GLP status: yes

Materials and Methods

Mice were treated with a single oral administration of Lenacil technical in 0.5% methylcellulose (Batch No. 141712003, purity 98.6%) at dose levels of 500, 1000 and 2000 mg/kg bodyweight. A preliminary toxicity test had previously shown that a dose of 2000 mg/kg (the standard limit dose for the micronucleus test) was tolerated. This level was therefore selected as an appropriate maximum for use in the micronucleus test.

The test substance, negative and positive control groups were administered orally by intragastric gavage. The negative control group received the vehicle, 0.5% w/v methylcellulose and the positive control group received mitomycin C at 12 mg/kg bodyweight. Following the preliminary toxicity test, no substantial differences in toxicity were observed between the sexes, in line with current guidelines, the micronucleus test was performed using ♂animals only. Bone marrow smears were obtained from 7 ♂ animals in the negative control, each of the test substance groups and 5 ♂ animals in the positive control group 24 hours after dosing. In addition bone marrow smears were obtained from 7 ♂animals in the negative control and high level treatment groups 48 hours after dosing. One smear from each animal was examined for the presence of micronuclei in 2000 immature erythrocytes. The proportion of immature erythrocytes was assessed by examination of at least 1000 erythrocytes from each animal. A record of the incidence of micro-nucleated mature erythrocytes was also kept. Criteria for positive test are clearly reported and acceptable.

The study is accepted.

Findings

Following the preliminary toxicity test performed at the limit dose of 2000 mg/kg bw with ♂ and ♀, no substantial difference in toxicity were observed between sexes and the main test was performed using ♂ only.

No statistically significant increases in the frequency of micronucleated immature erythrocytes and no substantial decreases in the proportion of immature erythrocytes were observed in mice treated with Lenacil technical and killed 24 or 48 hours later, compared to vehicle control values (P>0.01 in each case).

The positive control compound, mitomycin C, produced significant increases in the frequency of micronucleated immature erythrocytes (P<0.01).

Table B.6.4.2.1-1 Lenacil technical - mouse micronucleus test (2001) : summary of results and statistical analysis.

Sampling time	Treatment	Dose (mg/kg b.w.)	% ie/(ie+me)$ (mean)	Incidence mie (mean)	Incidence mme (group mean)
24 hours	Vehicle control	-	43	0.1	0.0
	Lenacil	**500**	39	0.0	0.0
		1000	42	0.3	0.5
		2000	47	0.1	0.5
	Mitomycin C	*12*	*39*	*29.4***	*0.6*
48 hours	Vehicle control	-	37	0.1	0.4
	Lenacil	**2000**	37	0.3	0.0

Vehicle control: 0.5% w/v Methylcellulose
% ie/(ie+me): proportion of immature erythrocytes
mie: Number of micronucleated cells observed per 2000 immature erythrocytes examined
mme: Number of micronucleated cells calculated per 2000 mature erythrocytes
Results of statistical analysis using the appropriate nonparametric method of analysis based on permutation (one-sided probabilities):
** P < 0.01 (significant)
$: Occasional apparent errors of ± 1% may occur due to rounding of values for presentation in the table.

Conclusion

Lenacil technical did not show any evidence of causing chromosome damage or bone marrow cell toxicity when administered orally by intra-gastric gavage in this *in vivo* test procedure.

Co-RMS highlighted that (see EFSA opinion, 2017) "*Haematological changes observed in a repeated-dose toxicity study such as e.g. decreased erythrocyte count, haemoglobin concentration, haematocrit, leukocyte count, if evaluated as being test-substance related and toxicologically significant, are lines of evidence of systemic bioavailability, and consequently, lines of evidence of bone marrow exposure.*"

Haematologic perturbations (especially decreases in white blood cell count) are observed throughout this dossier in different species (also in the 90-day mouse study), and *might be* an evidence for systemic bioavailability and therefore bone marrow exposure.

In the ADME study, a difference is made in table between "bone" and "marrow". Therefore, also according to the ADME study, presence of Lenacil in the bone marrow could be expected. Also, radioactivity was detected in red blood cells (RBCs) as well as in plasma (0.5h up to 72 h post dosing) and in urine, which might also be seen as an evidence for systemic bioavailability.

However, as a positive result was obtained in the *in vitro* chromosome aberration test a detailed weight of evidence approach for bone marrow exposure (including also a rationale for considering data from the ADME study obtained from the rat as representative for the mouse used in the MN study) was be provided by the applicant.

RMS: since compared to vehicle control, the % PCE/NCE was unaltered, it is important to obtain a final demonstration of BM toxicity, and consequently that this target organ was reached.
Notifier cited some arguments indicating that the bone marrow of the mouse was adequately reached:

(i) "In rat ADME study ([redacted] 1996) separated bone from marrow and analysed them separately. Increased levels of residue were seen in the marrow of high dose group compared to the marrow in the low dose group (although the increase was not proportional to the increased dose, compare marrow from 10 mg/kg 1000 mg/kg. The high dose of 1000 mg/kg in the rat ADME study is noteworthy, as the mouse micronucleus test used doses of 500, 1000 and 2000 mg/kg.

(ii) The high level of residue in the urine of the rat (ca. ≥80%) suggests that there should be adequate systemic exposure in other species, including the mouse. This position is in line with the RMS's recommendation to assume 100% absorbance for derivation of the AOEL. The rat ADME study demonstrated the test substance in the urine of the rat as well as high levels of radioactive residue in the plasma. This is critical since the blood percolates through the bone marrow, ensuring adequate exposure of the marrow."

RMS agrees partly with the argumentation, and considers lenacil probably absorbed and targeting the lymphoid compartment in the rat, and thereby possibly the bone marrow as progenitor of it. This is not conclusively proven in the mouse. On the other hand, it would appear that in both in the 90d study ([redacted] 1991) and the long-term mouse study (Malek, 1994), there is *limited* evidence that the WBC compartment would also been affected (albeit not in a dose-responsive way, casting some doubt on the relation with substance administration).

On balance, it may be considered that the bone marrow has probably been targeted.

Cited reference:
EFSA opinion "Clarification of some aspects related to genotoxicity", EFSA Journal, 15(12):5113, 2017.

***In vivo* unscheduled DNA synthesis or a mouse spot test**

Neither the *in vitro* unscheduled DNA synthesis nor gene mutation tests were positive, and no clastogenicity was observed in the *in vivo* MNT in mice; therefore, no additional *in vivo* testing of unscheduled DNA synthesis or a mouse spot test is needed.

B.6.4.3 (CA 5.4.3) *In vivo* genotoxicity testing in germ cells

In vivo studies in germ cells are required when results from *in vivo* somatic cells studies are positive. Since lenacil technical was negative in most *in vitro* and all *in vivo* studies, *in vivo* genotoxicity testing in germ cells would not be needed. However, the BM *in-vivo* micronucleus assay provided no evidence that the target organ was reached, putting into doubt the validity of this assay.

B.6.4.4 (CA 5.4.4) Summary of genotoxicity testing

Lenacil technical showed no evidence of mutagenic activity *in vitro*, in the *Salmonella typhimurium* bacterial system.

No mutagenic potential was exhibited in the *in vitro* mouse lymphoma cell mutation assay and the substance did not induce unscheduled DNA synthesis in cultures of primary rat hepatocytes when tested at concentrations extending into the toxic range.

However, lenacil technical did show evidence of clastogenic activity in human lymphocytes in *in vitro* cytogenetic test system in the absence of S9 mix only, and in the presence of slight precipitation (2500-5000 µg/mL). The positive result was not replicated in a more recent study. However, although the top concentration in this recent assay (not higher than 100 µg/mL) exhibited some precipitation, no cytotoxicity was demonstrated. It was therefore questioned if the second assay was tested at sufficiently high concentration since chromosome aberrations were noted in the first assay, tested at >12× higher but at acceptable cytotoxicity (not <45% MI) compared with the more recent assay.

No clastogenic activity was observed in the presence of S9 mix in either test.

Lenacil technical did not show any evidence of causing chromosome damage or bone marrow cell toxicity *in vivo* when administered orally to mice. The results of the ADME study ([redacted] 1996) suggested that lenacil becomes quantitatively bioavailable in the bone marrow after oral dosing in the rat. Repeated dosing in the mouse also also indicates some haemotoxicity in the WBC, providing limited evidence that the test substance reached the bone marrow in the *in vivo* micronucleus assay.

Based on the battery of genetic toxicology studies that have been conducted with lenacil, it can be overall concluded that lenacil does not cause genetic damage and, therefore, does not pose a mutagenic risk, and classification of lenacil with respect to mutagenicity/genotoxicity is not required according to the criteria laid down in Regulation (EU) No. 1272/2008 (CLP).

A summary of the results of genotoxicity testing is given in table B.6.4-1:

Table B.6.4-1: Summary of *in vitro* and *in vivo* genotoxicity studies for lenacil

Type of study	Test system	Batch no, purity (%) Concentration range tested	Result	Reference
In vitro bacterial mutagenicity (Ames)	*S. typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* strain WP2uvrA/pKM101 (CM891)	B.n°: 141712003, purity 98.6% 5-5000 µg/plate (±S-9)	negative	**B.6.4.1.1** ACD 016/013217 (May, 2001)
In vitro bacterial mutagenicity (Ames)	*S. typhimurium* TA1535, TA1537, TA1538T, TA98, TA100	B.n°: INB-634-50, purity not specified up to 500 µg/plate	negative	**B.6.4.1.2** HLR 601-77 (Russell, 1977)
In vitro bacterial mutagenicity (Ames)	*S. typhimurium* TA1535, TA97, TA98, TA100	B.n°: DPX-B634-107, purity: not specified up to 5000 µg/plate	negative	**B.6.4.1.4** HLR 413-94 (D'Amico, 1994)
In vitro chromosome aberration (clastogenicity)	Cultured human peripheral blood lymphocytes	B.n°: 141712003, purity 98.6% 625-5000 µg/mL (±S-9)	positive without S9 mix/negative with S9 mix	**B.6.4.1.6** ACD 017/013707 (Allais, 2001)
In vitro mammalian chromosomal aberration	Cultured human peripheral blood lymphocytes	B.n°: 047303003, purity 99.33% 25-100 µg/mL (±S-9)	Negative	**B.6.4.1.7** 49348 (Kellum, 2017
In vitro mammalian cell mutagenicity (CHO/HGPRT)	Mouse lymphoma L5178Y cells	B.n°:.141712003, purity 98.6% 39-5000 µg/mL (±S-9)	negative	**B.6.4.1.8** ACD 053/023530 (Clare, 2003)
In vitro unscheduled DNA synthesis	Rat primary hepatocytes	B.n°: 8903, purity not stated in report 0.078-10 µg/mL^{-1}	negative	**B.6.4.1.9** IRI 6135 (Riach & Mohammed, 1990)
In vivo micronucleus	Mouse bone marrow	B.n°: 141712003, purity 98.6% 500, 1000, 2000 mg/kg bw	negative	**B.6.4.2.1** ACD 018/013472 ([redacted] 2001)

B.6.5 (CA 5.5) Long term toxicity and carcinogenicity

The chronic toxicity and/or carcinogenicity of lenacil were evaluated in rats and mice. The studies in rats and mice are summarised below.

B.6.5.1 (CA 5.5.1) Long-term oral toxicity and carcinogenicity in the rat

Combined chronic toxicity and carcinogenicity study by dietary administration to Han Wistar rats over 104 weeks ([redacted] 2003)

The **toxicity phase** of this study was initially reported after 52 weeks under DuPont Report No. ACD 045/024288 (reported here as **B.6.5.1.1**), while the **carcinogenicity phase** was reported after 104 weeks under DuPont Report No.ACD 045/042214 (reported here as **B.6.5.1.2**).

B.6.5.1.1

DuPont Report No.: ACD 045/024288 (Toxicity phase – 52 weeks)

Guidelines: study is in compliance with Dir EEC 87/302/EEC Annex V B or OECD test guideline n° 453 (1981).

GLP status: yes

Materials and Methods (DuPont Report No. ACD 045/042214, see below)

Lenacil technical (Batch No. 141712003, purity 98.6%) was administered via dietary admixture into the powdered diet. At specified intervals (weeks 1, 13, 26 and 52) during the toxicity phase, prepared dietary formulations were sampled and analysed for concentration. The homogeneity and stability of Lenacil, conducted as part of an earlier study, were confirmed at nominal concentrations of 50 ppm and 50000 ppm during ambient temperature storage for 22 days. The mean concentrations of Lenacil technical in test formulations during the toxicity phase of the study were between – 4.8 and + 2.0% of intended, which were within the acceptable limits of -15% to 10%, confirming the accuracy of formulation.

Three groups of 50 ♂ and 50 ♀ rats HsdBrl Han:Wist (Han Wistar) are receiving Lenacil technical orally, via the diet, at concentrations of 250, 2500 or 25000 ppm. Together with a similarly constituted control group receiving the vehicle, untreated diet, these animals comprise the carcinogenicity phase of the study. A further 20 ♂ and 20 ♀ rats were allocated to each group. These animals comprised the toxicity phase of the study and were sacrificed after the completion of 52 weeks of treatment. In this toxicity phase, 250, 2500 and 25000 ppm are equivalent to 14.3, 139.1 and 1446 mg/kg bw/d (♂) and to 18.8, 188.5 and 1894 mg/kg bw/d (♀). The achieved doses are tabulated as follows:

	0 ppm		250 ppm		2500 ppm		25000 ppm	
	M	F	M	F	M	F	M	F
Week 1-52	0	0	14.3	18.8	139.1	188.5	1446	1894
Week 1-104	0	0	12	15.9	118.4	160.2	1223.2	1699.2

Animals were observed daily for evidence of a reaction to treatment. During the study detailed physical and arena observations, sensory reactivity and grip strength, motor activity, bodyweight, food consumption, ophthalmic examination, haematology, blood chemistry, urinalysis, organ weight, macroscopic and microscopic pathology investigations were undertaken.

Statistics: were carried out separately for males and females using the individual rat as unit. For categorical data, including pathological findings, the proportion of rats were analyzed using Fisher exact test for each group compared to control. For continuous data, Bartlett test was applied to test homogeneity of variance. When statistically different a Behrens-Fisher test was used to perform pair wise comparisons otherwise a Dunnett test. Intergroup differences in mortality and tumour incidence were performed using the Peto approach.

The study is accepted.

Findings

Note: the findings were initially summarised in **table B.6.5.1.1-6**, and are presented at a higher level of detail during AIR-3 renewal

Mortality (**Table B.6.5.1.1-1**)*:*

Two rats assigned to the toxicity phase died or were killed during the 52 week treatment period. One ♂ had a large ventral mass and 1 ♀ had ocular damage. These deaths were considered unrelated to treatment.

During the 104-week treatment period a total of 43 ♂ and 50 ♀rats died or were killed prematurely. The distribution of deaths was considered unaffected by treatment.

Table B.6.5.1.1-1 Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted], 2003): mortality.

	Male				Female			
Dose (mg/kg bw/d)	**0**	**12.0**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
Weeks 1-52		1/20						1/20
Weeks 1-104	14/50	15/50	5/50	9/50	9/50	17/50	9/50	15/50

Weeks 1-52: toxicity phase; weeks 1-104: carcinogenicity phase

Clinical signs: in ♀ receiving 25000 ppm the incidence of exfoliation on the tail and yellow staining in the peri-genital region was higher than the control, but the number affected animals was small.

(see Table B.6.5.1.1-2) There was an increased incidence of yellow staining in the peri-genital region in top-dose females at interim kill (1 yr). Compared to control, the incidences were high at all doses at 2 yr termination.

There were no signs observed at the physical examinations and arena investigations that were clearly attributable to lenacil, nor was there any treatment-related effect upon the group distribution, multiplicity and mean time of onset of palpable swellings.

At top-dose at termination, there was an increased incidence of exfoliation on the tail. Firm and swollen area incidences in ventral abdomen were slightly higher at top-dose.

Table B.6.5.1.1-2a Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): selected clinical signs; toxicity phase (12 months)

	Males				Females			
Dose (mg/kg bw/d)	**0**	**12.0**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
Staining, yellow, perigenital								
	(N=0)	(N=0)	(N=0)	(N=1)	(N=0)	(N=0)	**(N=2)**	**(N=4)**
Individual data				#423 (7, 28-34, 42-44, 47-53)			545 (7, 42-46) 556 (7, 47-53)	502 (7, 46-47, 52) 506 (7, 44-46, 50) 510 (7, 25, 44-50) 520 (7, 44-46)
Build (deformity), firm area, ventral abdomen								
	(N=0)	(N=0)	(N=0)	(N=0)	(N=0)	(N=0)	(N=0)	**(N= 1)**
Individual data								504 (7, 46-53)

N= total number of animals with sign. In the individual data section, values indicate: animal number (phase: 7-terminal kill; K-killed in extremis; H-humane kill; F-found dead, week(s) when clinical signs were observed).

Table B.6.5.1.1-2b Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): selected clinical signs; carcinogenicity phase (24 months)

	Males				Females			
Dose (mg/kg bw/d)	**0**	**12.0**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
Staining, yellow, perigenital								
	(N=3)	(N=3)	(N=3)	(N=1)	(N=2)	(N=7)	(N=9)	(N=12)
Individual data	11 (K, 79-81) 31 (H, 27) 37 (K, 86)	51 (K, 91-92) 56 (7, 80-81) 65 (K, 86-89)	123 (7, 105) 128 (7, 104-105) 144 (K, 89)	196 (K, 26-27, 32-44, 51-104)	205 (7, 99-106) 231 (7, 48-53)	263 (K, 97-102) 267 (K, 102) 270 (H, 47-57) 275 (7, 102-105) 284 (7, 47-55) 288 (H, 92-93) 294 (K, 42, 47-52)	301 (7, 40-106) 302 (7, 40-53) 307 (7, 43-106) 317 (F, 53) 331 (K, 48-69) 332 (7, 23-60) 333 (H, 53) 335 (H, 42-86) 337 (7, 106)	351 (7, 95-106) 354 (K, 96) 357 (H, 96) 359 (K, 47-90) 363 (H, 44-53) 371 (7, 48-60) 390 (7, 62-100) 391 (K, 102) 394 (K, 44-90) 396 (7, 42-53) 399 (7, 44-91, 103-106) 400 (7, 44-53, 74-91)
Skin, exfoliation, tail								
	(N=5)	(N=3)	(N=2)	(N=2)	(N=3)	(N=3)	(N=2)	(N=12)
Individual data	1 (7, 28-29) 3 (F, 28-30) 4 (7, 28-29) 9 (7, 77) 16 (7, 96-105)	75 (7, 97-105) 99 (7, 9-20, 41-66) 100 (7, 22-24)	125 (7, 24) 138 (7, 4-24)	173 (7, 42-79) 195 (7, 23-29)	208 (7, 22-23) 240 (7, 33-35) 250 (7, 22-30)	266 (7, 4-10) 287 (7, 33-38) 300 (7, 97-106)	322 (7, 22, 70-74) 347 (7, 33-38)	352 (7, 1-12) 356 (7, 22-24, 33-38) 358 (7, 22-23) 364 (H, 65-85) 367 (7, 22-24) 368 (7, 33-38) 377 (F, 28-29) 380 (7, 33-38) 389 (7, 22, 33-35, 85-90) 394 (K, 22-24, 33-46) 396 (7, 22-31) 399 (7, 17-24)
Build (conformation), thin								
	(N=4)	(N=3)	(N=4)	(N=4)	(N=2)	(N=4)	(N=4)	(N=9)
Individual data	3 (F, 100) 11 (K, 79-81) 37 (K, 86) 42 (K, 101-102)	65 (K, 89) 76 (H, 95) 89 (K, 101)	108 (7, 2) 112 (K, 97-98) 120 (7, 2-4) 150 (K, 35, 79)	159 (K, 99-100) 161 (7, 105) 181 (K, 98-100) 199 (7, 105)	209 (K, 96-97) 236 (K, 85-87)	253 (K, 60-64) 262 (K, 10) 276 (K, 95-96) 294 (K, 50-52)	303 (7, 106) 306 (K, 64) 319 (7, 61) 344 (K, 95-96)	354 (K, 97-100) 357 (H, 96) 362 (H, 39) 375 (K, 98-100) 376 (7, 99-106) 377 (F, 92-94) 385 (F, 73-75) 390 (7, 104-106) 394 (K, 94)
Build (deformity), firm area, ventral abdomen								
	(N=0)	(N=1)	(N=2)	(N=0)	(N=2)	(N=2)	(N=0)	(N=4)
Individual data		81 (K, 87-88)	103 (7, 44-45) 138 (7, 44-45)		218 (H, 26) 244 (7, 68-72)	284 (7, 62-69) 296 (K, 89)		359 (K, 90) 373 (7, 103-105) 374 (7, 100-106) 391 (K, 101-102)
Build (deformity), swollen area, ventral abdomen								
	(N=0)	(N=0)	(N=1)	(N=0)	(N=1)	(N=0)	(N=0)	(N=4)
Individual data			147 (7, 38-39)		295 (K, 89)			359 (K, 90) 379 (7, 103-106) 385 (F, 74-75) 391 (K, 101-102)

N= total number of animals with sign. In the individual data section, values indicate: animal number (phase: 7-terminal kill; K-killed in extremis; H-humane kill; F-found dead, week(s) when clinical signs were observed)

Effect on motor activity (see Tables B.6.5.1.1-3a and –3b, bold values statistically and/or biologically relevant)

There was no evidence of neurotoxicity from arena observations or assessment of sensory reactivity or grip strength.

Notifier considered the sensory reactivity and grip strength were considered unaffected by treatment, on which RMS agrees.

Motor activity in week 50 in ♂ receiving 2500 or 25000 ppm was lower than controls at certain time points in the 60-minute assessment period, resulting in low total motor activity scores but in the absence of any other indications of reduced motor activity, these findings were not considered toxicologically significant by the notifier. This was critically re-assessed and further detailed during renewal.

Motor activity in treated ♂ was significantly lower than controls ($p<0.05$) at certain time points in the 60-minute assessment period and the overall total motor activity scores of these animals were similarly affected at 118 mg/kg b.w./d and above. Reduced "low beam" breaks (total) values were 85%, 72% and 70% of at 12 mg/kg bw/d and above, expressing some dose-response relationship. It was remarkable that the "low beam" breaks were more affected than "high beam" breaks. The reduced high beam activity did not attain statistical significance. The decreased incidence of low-beam interruptions indicate that the males at these dietary concentrations were moving less than the controls during the observation period.

The total count dropped from the lowest dose on, but the finding (i) was not statistically significant at the lowest dose, (ii) was weak taking into account the inherent variability of the endpoint, and (iii) a dose-dependent decrease across all doses, including the lowest dose was only observed at two occasions (12' and 24') and when summing up all interval results.

Females were not affected. RMS confirms during renewal that there were no significant differences seen in the activities of treated females when compared to controls.

Whereas the notifier considered this modification of no adversity, RMS considers that the average decrease may indicate some (mild) neurotoxic effect, which should not be ignored for the establishment of the NOAEL. The detailed analysis confirms the establishment of an overall motor activity NOAEL at the lowest dose.

Notifier: *"Given that the only times that this effect was seen was in the 2nd and 4th intervals, it is unlikely that this is a test substance-related finding. If it was test substance-related, one would also expect statistically significant reductions in high beam breaks. Additionally, one would expect to see related changes occurring in adjacent sampling intervals (1st and 5th). While there was a statistically significant decrease at the 3rd interval, it was not dose-dependent. Finally, no marked increase in motor activity was observed in the 90-day rat study which, while of shorter duration, used dietary concentrations double that of the chronic/cancer study (top dose in the 90-day study was 50000 ppm versus 25000 ppm in the chronic/carcinogenicity study). Lastly, there were no corroborating clinical signs or histopathological findings to suggest a neurotoxic effect."*

Table B.6.5.1.1-3a Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): motor activity – (beam breaks, beam level low) at wk 50 in the ♂ animals

Time (')	Dose (mg/kg b.w./d)				DR
	0	12.0	118.4	1223.2	
6	105.3 ± 31.8	104.6 (99%) ± 40.7	73.7 (70%) ± 33.7	85.1 (81%) ± 47.6	
12	80.4 ± 34.6	54.5 (68%) ± 36.7	**46.8[a] (58%) ± 22.8**	**47.3[a] (59%) ± 29.4**	+
18	53.5 ± 20.3	24.8 (46%) ± 14.3	42.6 (80%) ± 29.3	**25.5[b] (48%) ± 19.7**	
24	42.7 ± 24.1	38.4 (90%) ± 27.8	**36.1 (85%) ± 31.2**	**28.7 (67%) ± 13.1**	+
30	36.6 ± 26.5	36.7 (100%) ± 20.4	29.3 (80%) ± 32.2	33.7 (92%) ± 20.7	
36	31.3 ± 34.3	40.7 (130%) ± 26.9	35.7 (114%) ± 60.4	**21.0 (67%) ± 23.3**	
42	49.2 ± 36.0	29.3 (60%) ± 16.8	19.4 (39%) ± 20.0	31.3 (64%) ± 27.2	
48	54.6 ± 52.1	41.4 (76%) ± 21.5	23.4 (43%) ± 18.2	28.7 (53%) ± 30.2	
54	22.9 ± 31.0	21.4 (93%) ± 19.7	28.1 (123%) ± 20.1	**15.1 (66%) ± 10.8**	
60	16.1 ± 18.6	28.4 (176%) ± 30.1	18.4 (114%) ± 16.7	28.2 (175%) ± 29.4	
total	492.6 ± 172.2	420.2 (85%) ± 61.0	**353.5[a] (72%)±153.0**	**344.6[a] (70%)±103.1**	+

group mean scores ± s.d. (% of control). Statistically significant modification: [a]: p<0.05; [b]: p<0.01; N=10 ; DR: dose-response (taking into account all experimental doses)

Table B.6.5.1.1-3b Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): motor activity – (beam breaks, beam level high) at wk 50 in the male animals

Time (')	Dose (mg/kg b.w./d)				DR
	0	12.0	118.4	1223.2	
6	32.4 ± 17.1	31.6 (98%) ± 13.2	21.0 (65%) ± 13.0	23.6 (73%) ± 19.1	
12	12.9 ± 5.6	18.6 (144%) ± 16.4	15.1 (117%) ± 16.4	15.4 (119%) ± 12.5	
18	10.0 ± 8.8	5.9 (59%) ± 5.1	8.3 (83%) ± 6.9	7.2 (72%) ± 7.4	
24	7.9 ± 9.4	10.2 (129%) ± 11.4	**5.1 (65%) ± 5.3**	**3.6 (46%) ± 3.2**	
30	6.6 ± 8.9	4.9 (74%) ± 4.3	4.1 (62%) ± 5.5	6.6 (100%) ± 7.8	
36	5.5 ± 12.7	8.0 (145%) ± 9.7	**1.6 (29%) ± 2.4**	**1.5 (27%) ± 2.2**	
42	5.7 ± 5.8	4.1 (72%) ± 5.8	1.3 (23%) ± 2.5	6.1 (107%) ± 6.6	
48	6.5 ± 6.4	6.1 (94%) ± 10.3	2.6 (40%) ± 2.5	5.3 (82%) ± 7.9	
54	2.6 ± 6.9	4.8 (185%) ± 7.7	3.8 (146%) ± 3.3	3.0 (115%) ± 5.0	
60	2.5 ± 4.8	5.9 (236%) ± 8.8	4.3 (172%) ± 6.4	3.3 (132%) ± 3.8	
	92.6 ± 34.9	100.1 (108%) ± 26.7	67.4 (73%) ± 31.0	75.6 (82%) ± 40.7	

group mean scores ± s.d. (% of control) Statistically significant modification: [a]: p<0.05; [b]: p<0.01; N=10 ; DR: dose-response

Body weight: overall bodyweight during the 104-wk treatment period was low in comparison with the controls in top-dose ♀, reaching statistical significance. The overall weight gain of ♀at top dose was also slightly lower than control. Body weight gain was decreased (>10%) without reaching statistical significance (**Table B.6.5.1.1-4**).

Food consumption was not affected by treatment (**table B.6.5.1.1-4**). There was no effect on food conversion efficiency.

Table B.6.5.1.1-4 Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): bodyweight (gain) and food consumption.

		Male				Female			
	Mg/kg b.w./d	0	12	118.4	1223.2	0	15.9	160.2	1699.2
Body weight	Week 52		↓3%		↓2%		↓2%		↓3%
	Week 104		↓2%	↓4%	↓6%				**↓9%***
Body weight gain	Week 52		↓3%		↓2%		↓2%		↓5%
	Week 104		↓3%	↓6%	↓8%			↓2%	**↓13%**
Food consumption	Week 52		↓2%	↓3%	↓1%		↓4%		↓3%
	Week 104		↓2%	↓5%	↓2%			↓2%	↓1%

Statistically significant modification: *p<0.05

Haematology: according to the notifier, a number of differences were noted, some of which attained statistical significance when compared with controls. These differences were minor or lacking dose-relationship and were attributed to normal biological variation. These changes also included the small variations of prothrombin and activated partial thromboplastin times at week 78 and 104. The details of the the parameters are given under **table B.6.5.1.1-5**.

Table B.6.5.1.1-5a Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): details on coagulation parameters.

		Male				Female			
Mg/kg b.w./d	**week**	**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
Plt ($\times 10^9$/L)	**13**	984 ± 76.4	933 ± 107.8	960 ± 72.0	979 ± 96.1	972 ± 80.8	1012 ± 73.1	1029 ± 124.4	1017 ± 79.4
	26	928 ± 82.1	865 ± 104.5	887 ± 63.9	900 ± 108.9	889 ± 81.2	911 ± 72.0	948 ± 90.2 (↑7%)	**982[a] ± 103.2 (↑10%)**
	52	859 ± 97.1	761 ± 119.0	849 ± 82.2	804 ± 88.6	820 ± 90.4	833 ± 74.8	869 ± 103.3	**911 ± 82.5 (↑11%)**
	78	858 ± 98.9	824 ± 73.6	779 ± 262.6	841 ± 104.5	812 ± 143.3	811 ± 74.5	843 ± 73.8	**897 ± 58.6 (↑10%)**
	104	838 ± 113.6	836 ± 198.3	820 ± 68.4	768 ± 158.9	741 ± 129.9	777 ± 123.8 (↑5%)	**832 ± 79.1 (↑12%)**	**835 ± 103.7 (↑13%)**
PT (sec)	**13**	15.3 ± 0.60	15.4 ± 1.43	15.4 ± 0.98	14.9 ± 0.77	14.3 ± 1.11	14.3 ± 0.44	14.4 ± 0.71	14.1 ± 0.86
	26	15.2 ± 0.68	14.6 ± 1.29	14.3 ± 1.57	14.0 ± 1.84	14.2 ± 1.29	13.6 ± 0.74	14.5 ± 0.78	13.7 ± 1.00
	52	14.6 ± 1.34	15.0 ± 1.75	14.7 ± 1.91	14.5 ± 0.91	16.1 ± 0.58	16.8 ± 0.62	15.9 ± 0.78	**15.3[a] ± 0.41 (↓5%)**
	78	15.7 ± 2.31	14.1± 0.61 (↓10%)	**13.2[b] ± 2.27 (↓16%)**	**12.7[b] ± 1.04 (↓19%)**	13.7 ± 0.73	**15.0[b]±0.56 (↑9%)**	**15.3[b] ±0.72 (↑12%)**	**14.6[b] ± 0.77 (↑7%)**
	104	16.8 ± 0.90	16.4 ± 1.53	17.0 ± 1.21	16.4 ± 1.21	15.7 ± 0.89	15.7 ± 0.79	16.0 ± 0.75	14.5 ± 2.09
APTT (sec)	**13**	19.5 ± 1.44	19.5 ± 3.47	19.2 ± 2.20	19.9 ± 2.13	18.7 ± 2.72	17.7 ± 1.39	18.7 ± 1.15	17.7 ± 2.04
	26	18.9 ± 3.89	18.9 ± 2.65	17.9 ± 1.99	18.9 ± 2.88	17.4 ± 3.73	19.0 ± 2.64	18.8 ± 1.68	**15.6 ± 3.30 (↓10%)**
	52	18.8 ± 1.62	20.4 ± 2.82	18.4 ± 1.94	18.0 ± 2.27	18.2 ± 1.20	18.6 ± 1.62	16.9 ± 3.48	17.1 ± 2.53
	78	18.9 ± 4.05	**16.8±1.63 (↓11%)**	**14.7[b] ± 2.71 (↓22%)**	**16.8[b] ± 3.77 (↓11%)**	16.6 ± 1.40	16.7 ± 0.82	17.1 ± 2.28	16.9 ± 1.56
	104	17.5 ± 1.91	**15.4[a]±1.27 (↓12%)**	**14.8[b] ± 2.01 (↓15%)**	**14.7[b] ± 2.79 (↓16%)**	16.4 ± 2.09	16.7 ± 3.46	**14.5 ± 2.09 (↓12%)**	**14.5 ± 2.42 (↓12%)**

Statistically significant modification: [a]: $p<0.05$; [b]: $p<0.01$

During renewal evaluation, RMS considers that erythron parameters could be considered altered at 104 weeks at 118 mg/kg b.w./d and above, based upon mild thrombocytomia (♀), and slight drops of APTT (♂,♀) reaching high significance in the ♂. Although occasional excursions at the lowest dose are observed, the overall pattern is not very consistent, except a predominant effect at the top-dose, and possibly also at the mid-dose. RMS feels that an overall NOAEL for erythron may be set at the lowest dose.

*White blood cell compartment (see **tables B.6.5.1.1-6a-d**).*

RMS observes an intrinsic high variability in cell counts (notably as a function of time), as well as unclear/absent dose-response relationships However, it appears throughout this DRAR that, even taking into account the considerable cell number variations (WBC, L, N, M -by an unexplained mechanism), lenacil modulates WBC cell counts in an incoherent way. This is notably shown by the lymphocyte count data, reflected in the WBC count accordingly (lymphocyte generally represent 20%-40% of WBC). It would appear that the weak trend observed (at least in rodents), points to a decrease in lymphocyte counts in the presence of lenacil. It may be speculated that

the absence of simple dose-response relationship in some cases could reflect a "plateau", already reached at the lowest dose, or reflect a complex modulation in which lenacil could act on several factors themselves determining, in combination, the cell count. The inconsistent pattern (across both sexes, duration of exposure, and doses tested) obscures any meaningful interpretation of the data.

It could be useful to examine further relevant associations between related substances and potential (adverse) effects. From the open scientific literature (www.t3db.ca, see also Lim, 2010) , it is *e.g.* known that uracil herbicides like terbacil, bromacil and butafenacil may interact with amongst others CD38, which might in some way impact on immune responses and related metabolic pathways. While RMS clearly realises that such association with herbicides related to lenacil (the latter is not highlighted in this interaction) may be highly speculative as regards the observed effects (WBC counts), the effect of lenacil on immunologic functions is currently not entirely known, and further studies revealing the inocuity on immunogenic function is desired.

Notifier: *"As mentioned above, this data is highly variable in nature. Note, for example, the WBC changes in ♀ at the middle dose group at the week 26 sampling show a decrease as large as the increase at 52 weeks. The best way to interpret this type of data is to compare to the reference range, or to test it over time as done in this study. Without reproducibility, the findings are suspect.*

For WBCs only one sampling was statistically different from the control and that being in geriatric animals. (males at week 78). As mentioned for the mouse the transient nature of the hematology findings suggests that they are of borderline adversity.

Although the uracil herbicides terbacil, bromacil and butafenacil belong to the same herbicide class, they are structurally diverse when compared to lenacil. Tanimoto structural similarity scores calculated via the OECD Toolbox v3.4 shows that the lenacil is structurally disparate when compared to terbacil (19% similar), bromacil (24% similar) and butafenacil (4% similar). The immunomodulatory potential of these uracils has not been reported in in vivo mammalian studies. In addition, these uracils were not included in a recent analysis of immunotoxic effects of pesticide active substances reviewed by Dewhurst et al. (2015) for EFSA.

In addition, the Bioseek-BioMap system used in the EPA-ToxCast program assesses CD38 activity (positive or negative) that should be taken into consideration with 9 other assay components; that means the CD38 assay is to be used in conjunction with 9 other assays. Lenacil has not been tested in the EPA-ToxCast program. Mammalian studies with lenacil show some transient and inconsistent white blood cell changes suggesting weak, if any, immunomodulatory activity. Based on ToxCast data, only butafenacil and terbacil exhibited a decrease in CD38 activity; however, the AC_{50} values for butafenacil (10 µM) and terbacil (13 µM) were 609 and 775 times higher than colchicine (0.0169 µM), thereby showing very weak activity for the uracils against CD38 activity in vitro. Thus, comparing immunomodulatory potential via CD38 activity for these uracils and applying it to lenacil does not appear to provide useful information. More details can be provided upon request."

From the data, the **RMS** suggests that lenacil could possess limited haemato-modulating properties. From the apparent weak trend (decrease) observed on lymphocyte counts in several studies, a potential immune-modulating role of lenacil may be questioned. RMS recognises the structural differences between cited a.s., but considers also that, in the absence of a proper MoA for the immunomodulatory findings (at least for the a.s. under investigation), the effects cannot be ignored. On the other hand, it is likely that the established reference doses are protective for any such weak effect.

However, it is also suggested to conduct a guideline immunotoxicity study in order to exclude any functional adverse effect on immunocompetent tissue, for which there is very limited evidence in the existing regulatory studies at this stage.

Table B.6.5.1.1-5a Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): details on WBC counts.

		Male				Female			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
Time	**N**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Wk 13		9.34 ± 1.376	9.32 ± 1.602	10.15 ± 1.065 (↑9%)	9.26 ± 1.009	8.45 ± 1.362	7.43 ± 1.015 (↓12%)	6.79 ± 2.530 (↓20%)	7.20 ± 1.403 (↓15%)
	N	*10*	*8*	*10*	*8*	*10*	*9*	*10*	*10*
Wk 26		8.56 ± 1.074	6.79 ± 1.917 (↓21%)	7.67 ± 0.921 (↓10%)	7.53 ± 1.587 (↓12%)	5.97 ± 1.381	**4.29[a] ± 0618 (↓28%)**	**4.78[a] ± 1.539 (↓20%)**	**5.38[a] ± 0.953 (↓10%)**
	N	*8*	*10*	*10*	*10*	*9*	*10*	*10*	*8*
Wk 52		6.16 ± 1.959	5.99 ± 1.534	6.72 ± 0.940 (↑9%)	5.87 ± 1.156 (↓5%)	3.45 ± 0.928	3.81 ± 0.670 (↑10%)	4.21 ± 1.177 (↑22%)	3.84 ± 0.709 (↑11%)
	N	*10*	*9*	*10*	*10*	*10*	*10*	*10*	*10*
Wk 78		5.74 ± 1.112	6.11 ± 2.097 (↑6%)	5.21 ± 1.678 (↓9%)	**5.10 ± 0.872 (↓11%)**	3.92 ± 1.146	3.06 ± 0.609 (↓22%)	3.72 ± 1.551 (↓5%)	3.74 ± 0.620 (↓5%)
	N	*10*	*9*	*10*	*10*	*10*	*9*	*9*	*10*
Wk 104		5.82 ± 1.071	6.37 ± 4.035 (↑9%)	5.06 ± 1.003 (↓13%)	5.41 ± 1.477 (↓7%)	4.02 ± 1.542	3.89 ± 1.242	4.51 ± 1.359 (↑12%)	4.20 ±1.044 (↑4%)
Grand MEAN		7.12	6.92	6.96	6.63 (↓7%)	5.16	4.50 (↓13%)	4.80 (↓7%)	4.87 (↓6%)

Table B.6.5.1.1-5b Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): details on lymphocyte (L) counts.

		Male				Female			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
Wk 13	*N*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
		7.13 ± 1.201	7.13 ± 1.227	7.92 ± 1.037 (↑11%)	7.21 ± 0.989	7.02 ± 1.250	5.99 ± 0.828 (↓15%)	**5.37[a] ± 1.782 (↓24%)**	**5.74[a] ± 1.278 (↓18%)**
Wk 26	*N*	*10*	*8*	*10*	*8*	*10*	*9*	*10*	*10*
		6.79 ± 1.113	5.15 ± 1.026 (↓24%)	6.07 ± 0.744 (↓11%)	6.18 ± 1.280 (↓9%)	5.09 ± 1.267	**3.46[a] ± 0.594 (↓32%)**	**3.83[a] ± 1.218 (↓25%)**	**4.32[a] ± 0.796 (↓15%)**
Wk 52	*N*	*8*	*10*	*10*	*10*	*9*	*10*	*10*	*8*
		4.26 ± 1.300	4.55 ± 1.117 (↑7%)	4.97 ± 0.738 (↑17%)	4.36 1.064	2.69 ± 0.682	2.96 ± 0.634 (↑10%)	3.26 ± 1.010 (↑21%)	2.91 ± 0.592
Wk 78	*N*	*10*	*9*	*10*	*10*	*10*	*10*	*10*	*10*
		4.10 ± 0.915	4.25 ± 1.422 (↑4%)	**3.60 ± 1.246 (↓12%)**	**3.16 ± 0.718 (↓23%)**	2.80 ± 0.75	2.10 ± 0.445 (↓25%)	2.40 ± 0.614 (↓14%)	2.46 ± 0.498 (↓12%)
Wk 104	*N*	*10*	*9*	*10*	*10*	*10*	*9*	*9*	*10*
		4.26 ± 0.681	3.98 ± 1.253 (↓7%)	**3.38 ± 0.780 (↓21%)**	**3.40 ± 1.072 (↓20%)**	2.51 ± 0.753	2.35 ± 0.452 (↓6%)	2.85 ± 0.925 (↑14%)	2.60 ± 0.593
Grand MEAN		5.31	5.01 (↓6%)	5.19 (↓2%)	4.86 (↓8%)	4.02	3.37 (↓16%)	3.54 (↓12%)	3.61 (↓10%)

Statistically significant modification: [a]: $p<0.05$

Table B.6.5.1.1-5c Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): details on neutrophil (N) counts.

		Male				Female			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
Time	*N*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
Wk 13		1.69 ± 0.728	1.66 ± 0.411	1.64 ± 0.247	1.55 ± 0.351 (↓8%)	1.06 ± 0.231	1.06 ± 0.439	1.06 ± 0.725	1.10 ± 0.403
	N	*10*	*8*	*10*	*8*	*10*	*9*	*10*	*10*
Wk 26		1.34 ± 0.312	1.32 ± 0.890	**1.17 ± 0.286 (↓13%)**	**1.00 ± 0.324 (↓25%)**	0.62 ± 0.106	0.61 ± 0.286	**0.71 ± 0.363 (↑15%)**	**0.77 ± 0.224 (↑24%)**
	N	*8*	*10*	*10*	*10*	*9*	*10*	*10*	*8*
Wk 52		1.46 ± 0.623	1.06 ± 0.333 (↓26%)	**1.28 ± 0.280 (↓12%)**	**1.14 ± 0.330 (↓22%)**	0.55 ± 0.199	0.64 ± 0.227 (↑16%)	0.70 ± 0.319 (↑27%)	0.72 ± 0.252 (↑31%)
	N	*10*	*9*	*10*	*10*	*10*	*10*	*10*	*10*
Wk 78		1.16 ± 0.286	1.40 ± 0.682 (↑21%)	1.10 ± 0.525 (↓5%)	1.51 ± 0.358 (↑30%)	0.81 ± 0.411	0.72 ± 0.255 (↓10%)	1.03 ± 1.080 (↑27%)	0.95 ± 0.257 (↑17%)
	N	*10*	*9*	*10*	*10*	*10*	*9*	*9*	*10*
Wk 104		1.10 ± 0.418	1.91 ± 2.589 (↑74%)	1.33 ± 0.495 (↑21%)	1.58 ± 0.592 (↑44%)	1.24 ± 1.046	1.26 ± 0.880	1.33 ± 0.650 (↑7%)	1.28 ± 0.599 (↑3%)
Grand MEAN		1.35	1.47	1.30	1.36	0.86	0.86	0.97 (↑13%)	0.96 (↑12%)

Table B.6.5.1.1-5d Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): details on monocte (M) counts.

		Male				Female			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
time	*N*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
Week 13		0.31 ± 0.111	0.26 ± 0.056 (↓16%)	0.33 ± 0.063 (↑6%)	0.28 ± 0.080 (↓10%)	0.19 ± 0.039	0.20 ± 0.063 (↑5%)	0.18 ± 0.006 (↓5%)	0.20 ± 0.050 (↑5%)
	N	*10*	*8*	*10*	*8*	*10*	*9*	*10*	*10*
Week 26		0.24 ± 0.064	0.17 ± 0.065 (↓29%)	0.24 ± 0.073	0.18 ± 0.055 (↓25%)	0.13 ± 0.061	0.12 ± 0.033 (↓8%)	0.13 ± 0.060	0.17 ± 0.056 (↑31%)
	N	*8*	*10*	*10*	*10*	*9*	*10*	*10*	*8*
Week 52		0.22 ± 0.113	0.17 ± 0.075 (↓23%)	0.22 ± 0.057	0.19 ± 0.057 (↓14%)	0.10 ± 0.041	0.11 ± 0.048 (↑10%)	0.14 ± 0.047 (↑40%)	0.12 ± 0.047 (↑20%)
	N	*10*	*9*	*10*	*10*	*10*	*10*	*10*	*10*
Week 78		0.28 ± 0.087	0.27 ± 0.083 (↓4%)	0.20 ± 0.098 (↓29%)	0.25 ± 0.042 (↓11%)	0.18 ± 0.087	0.15 ± 0.055 (↓17%)	0.17 ± 0.049 (↓6%)	0.19 ± 0.043 (↑6%)
	N	*10*	*9*	*10*	*10*	*10*	*9*	*9*	*10*
Week 104		0.20 ± 0.072	0.26 ± 0.218 (↑30%)	0.18 ± 0.066 (↓10%)	0.20 ± 0.084 (=)	0.14 ± 0.081	0.15 ± 0.054 (↑7%)	0.17 ± 0.054 (↑21%)	0.17 ± 0.078 (↑21%)
Grand MEAN		0.25	0.23	0.23	0.22 (↓12%)	0.15	0.15	0.16	0.17 (↑13%)

According to the notifier, the blood smears did not indicate any differences attributed to treatment. Minor variations occurred, some of which attained statistical significance, but they were considered fortuitous.

RMS further detailed the findings, as described hereunder.

Table B.6.5.1.1-6 Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): details on blood smears.

		Males		Females	
Mg/kg b.w./d	**Time (week)**	**0**	**1223.2**	**0**	**1699.2**
Neutrophils (N)	Wk 52	26.1 ± 6.76	**29.6 ± 6.68 (↑13%*)**	30.7 ± 6.33	31.9 ± 8.40 (↑4%)
	Wk 78	27.8 ± 9.30	29.4 ± 8.29 (↑6%)	26.8 ± 7.08	29.3 ± 6.04 (↑9%)
	Wk 104	30.0 ± 7.43	**37.3± 11.15 (↑24%*)•**	41.3 ±10.52	39.5 ± 13.31 (↓4%)
Lymphocytes (L)	Wk 52	67.5 ± 6.60	**64.5 ± 6.88** (↓4%*)•	62.8 ± 6.40	61.6 ± 8.32 (↓2%)
	Wk 78	68.4 ± 9.78	68.1 ± 8.67 (↓4%)	71.3 ± 7.09	68.8 ± 6.66 (↓4%)
	Wk 104	65.0 ± 8.29	**59.5 ± 11.31 (↓8%*)•**	55.3 ± 10.36	56.8 ± 13.74 (↓3%)
Eosinophils (E)	Wk 52	1.6 ± 1.0	1.6 ± 1.25 (=)	1.6 ± 1.37	1.7 ± 1.34 (↑7%)
	Wk 78	1.1 ± 1.25	0.7 ± 0.75 (↓36%)	0.6 ± 0.68	0.5 ± 0.65 (↓17%)
	Wk 104	1.8 ± 1.57	2.4 ± 2.01 (↑33%)	2.1 ± 1.66	2.1 ± 2.01 (=)
Monocytes (M)	Wk 52	4.8 ± 1.91	4.2 ± 1.66 (↓13%)	4.9 ± 2.34	4.8 ± 2.20 (↓2%)
	Wk 78	2.7 ± 1.81	**1.8 ± 1.17 (↓33%*)**	1.3 ± 1.02	1.4 ± 1.24 (↑8%)
	Wk 104	3.2 ± 2.17	**0.8 ± 0.86 (↓75%*)**	1.3 ± 1.42	1.6 ± 1.66 (↑23%)

Values correspond to the relative incidence (%), •: indicates concordance with the absolute cell count; *: $p<0.05$

RMS considers that the effects in top-dose ♂, *i.e.* neutrophils (↑) and lymphocytes (↓), reported at week 52 and at week 104 (concordant with absolute cell count modification) could precautionously be considered related to treatment, as such effects were also reported in short term studies. However, the effect is unremarkable at week 78, rendering the toxicological relevance somewhat equivocal, in the absence of clear time-dependency.

Other blood smears findings did not indicate any differences attributed to treatment, also because no clear concordance could be found with cell counts in the peripheral blood.

It is of note that blood smear analysis was restricted to controls and top-dose groups. Since the toxicological relevance of the findings is discutable, and overall the modifications at the top-dose were small to moderate, it seems unlikely that the analysis at low and intermediate doses would be particularly informative, and it is considered that further investigation is not necessary.

*Blood chemistry (see table **B.6.5.1.1-7**)*

There were no changes in the blood plasma that were attributed to treatment according to the notifier. Changes such as transiently reduced plasma urea, creatinine and glucose in week 26 in ♀and minor differences in plasma protein and electrolytes were considered as normal biological variations. Exception could be made for a slight increase of triglyceride level in the ♂ at 118 mg/kg b.w./d and above at week 104.

In 5♂and 3♀at top dose TSH was increased without reaching statistical significance. Recorded individual values: ♂: 11.3; 11.5; 12.7; 13.4; 18.5, compared to 6.3 ± 1.12 (controls, n = 20); ♀: 9.2; 11.7; 18.8, compared to 5.4 ± 0.72 (controls, n = 20) . T_3 and T_4 levels remained unchanged (including in the above-mentioned animals). The notifier concluded that thyroid hormone levels were not affected by treatment; this conclusion is not completely agreed upon by the RMS. The results are obviously not consistent with a severely disrupting effect on the thyroid hormones, however, the thyroid weight is slightly elevated in the top-dose animals. These animals show in addition increased luminal concretions (histopathology), and thus an adverse effect is not excluded. Notifier further made a case to clarify the MoA of this effect.

According to the **notifier**, the increase in TSH noted by the RMS looks like a very weak, high dose effect. Supporting that this is a very weak finding is the fact that it occurred in the absence of changes in T_3 and T_4.

Notifier expressed its intention on trying to identify a mechanistic study. Preliminary data would show a mild increase in UDPGT activity, as well as an increase in CYP2B1 gene expression in the liver of rats. This work would include an *in vitro* thyroid peroxidase inhibition assay, where lenacil is expected to show no thyroid peroxidase inhibiting activity.

RMS: the existing study (perchlorate discharge test, see **B.6.8.2.1)** indeed indicates no organification disturbance. RMS proposes that the announced *in-vitro* study should be requested during a SoC.

Table B.6.5.1.1-7 Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): clinical chemistry.

	Week	Male				Female			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
ALP (U/L)	**26**	63 ± 9.9	64 ± 13.4	56 ± 10.1 (11%)	62 ± 11.6	26 ± 4.6	21 ± 2.9 (↓19%)	26 ± 8.1	24 ± 6.7
	52	57 ± 7.3	64 ± 11.3 (↑12%)	59 ± 9.9	59 ± 10.5	25 ± 6.8	21 ± 4.6 (↓16%)	26 ± 7.6	21 ± 8.2 (↓16%)
	78	64 ± 17.3	59 ± 6.8	51 ± 13.4 (↓20%)	62 ± 15.0	22 ± 6.7	26 ± 5.8 (↑18%)	22 ± 5.7	22 ± 7.2
	104	60 ± 10.5	59 ± 13.6	52 ± 22.1 (↓13%)	57 ± 12.7	27 ± 8.6	24 ± 5.7 (↓11%)	21 ± 7.2 (↓22%)	25 ±8.2
CPK Total (U/L)	**26**	262 ± 200.8	309 ± 244.7 (↑18%)	380 ± 304.3 (↑45%)	331 ± 254.1 (↑26%)	211 ± 197.3	188 ± 62.8 (↓11%)	160 ± 34.9 (↓24%)	**287[a] ±164.6 (↑36%)**
	52	416 ± 607.0	99 ± 17.1 (↓76%)	232 ± 201.3 (↓44%)	310 ± 388.1 (↓25%)	224 ± 194.9	212 ± 112.2 (↓5%)	216 ± 294.0 (↓4%)	**113[a] ± 13.6 (↓50%)**
	78	233 ± 160.1	198 ± 108.7 (↓15%)	151 ± 59.3 (↓5%)	255 ± 129.1 (↑9%)	137 ± 38.2	193 ± 94.4 (↑41%)	167 ± 69.8 (↑22%)	**266[a] ±156.1 (↑94%)**
	104	193 ± 108.2	364 ± 629.8 (↑90%)	359 ± 392.3 (↑86%)	284 ± 338.4 (↑47%)	185 ± 100.8	395 ± 714.0 (↑114%)	172 ± 91.2 (↓7%)	183 ± 97.1
Urea (mmol/L)	**26**	6.03 ± 0.890	4.42 ± 0.641 (↓27%)	6.01 ± 0.757	6.18 ± 0.543	10.14 ± 2.123	**7.71[a] ±0.652 (↓24%)**	**9.17[a] ±1.749 (9%)**	**7.42[b] ±0.996 (↓27%)**
	52	4.85 ± 0.706	5.44 ± 0.602 (↑12%)	4.77 ± 0.532	4.64 ± 0.565	6.74 ± 0.552	6.91 ± 1.062	7.11 ± 1.116	6.13 ± 0.927 (↓9%)
	78	4.64 ± 0.637	4.98 ± 0.645	4.84 ± 0.965	4.51 ± 0.494	6.56 ± 0.914	5.71 ± 1.080 (↓13%)	6.26 ± 0.763	5.80 ± 0.689 (↓12%)
	104	4.41 ± 0.788	4.79 ± 0.447	4.26 ± 0.699	4.14 ± 0.624	5.92 ± 0.828	5.25 ±0.911 (↓11%)	5.18 ±0.864 (↓13%)	5.18 ±0.926 (↓13%)
Creat (µmol/L)	**26**	52 ± 1.7	54 ± 2.7	53 ± 3.3	53 ± 3.1	64 ± 4.6	**59[a] ± 3.5 (↓8%)**	**60[a] ± 4.0 (↓6%)**	**60[a] ± 3.5 (↓6%)**
	52	48 ± 1.6	47 ± 1.9	48 ± 2.5	48 ± 2.0	56 ± 2.6	56 ± 4.1	59 ± 4.1	57 ± 2.2
	78	50 ± 1.0	51 ± 2.9	51 ± 2.4	50 ± 2.0	58 ± 4.7	56 ± 2.8	56 ± 2.9	56 ± 3.1
	104	50 ± 2.1	52 ± 1.9	53 ± 3.3	52 ± 3.6	56 ± 3.7	56 ± 3.9	54 ± 2.8	56 ± 2.5
Gluc (mmol/L)	**26**	7.41 ± 0.718	7.85 ± 0.796	7.87 ± 0.656	7.91 ± 1.303	7.05 ± 1.131	6.90 ± 0.705	6.72 ± 1.083	**5.99[a]±1.053 (↓15%)**
	52	7.62 ± 0.642	8.25 ± 0.650 (↑8%)	**8.69[b] ±0.465 (↑14%)**	**8.72[b] ±1.374 (↑14%)**	7.15 ± 0.832	6.80 ± 0.788	7.20 ± 0.870	7.78 ±0.997 (↑9%)
	78	6.95 ± 1.017	7.20 ± 0.873	7.23 ± 1.432	7.21 ± 1.683	6.73 ± 0.861	6.80 ± 0.617	7.24 ± 0.475	6.81 ± 0.893

	Week	Male				Female			
Mg/kg b.w./d		0	12	118.4	1223.2	0	15.9	160.2	1699.2
	104	7.01 ± 0.994	6.57 ± 1.376	7.86 ± 1.317 (↑11%)	8.11 ± 1.849 (↑16%)	6.67 ± 1.227	7.27 ± 0.805 (↑9%)	7.26 ± 0.412 (↑9%)	7.29 ± 1.039 (↑9%)
Chol (mmol/L)	26	1.57 ± 0.154	1.62 ± 0.422	1.86 ± 0.396 (↑18%)	1.74 ± 0.323 (↑11%)	1.62 ± 0.361	1.99 ± 0.430 (↑17%)	1.81 ± 0.424 (↑12%)	1.95 ± 0.380 (↑14%)
	52	1.86 ± 0.233	1.79 ± 0.491	2.12 ± 0.422 (↑14%)	1.87 ± 0.401	2.00 ± 0.361	2.21 ± 0.607 (↑11%)	2.17 ± 0.665 (↑9%)	2.22 ± 0.456 (↑11%)
	78	2.00 ± 0.473	2.11 ± 0.470	2.21 ± 0.646 (↑11%)	2.15 ± 0.643	1.96 ± 0.376	2.18 ± 0.617 (11%)	1.88 ± 0.729	2.29 ± 0.569 (↑17%)
	104	2.53 ± 0.710	2.60 ± 0.731	2.30 ± 0.661	2.66 ± 0.858	2.29 ± 0.312	2.37 ± 0.439	2.41 ± 0.879	2.71 ± 0.698 (↑18%)
Trig (mmol/L)	26	1.23 ± 0.426	1.27 ± 0.450	1.16 ± 0.408	1.16 ± 0.382	0.57 ± 0.181	0.65 ± 0.163 (↑14%)	0.84 ± 0.447 (↑47%)	0.66 ± 0.160 (↑16%)
	52	1.37 ± 0.503	1.14 ± 0.395 (↓17%)	**1.74 ± 0.478 (↑27%)**	**1.87[a] ±0.715 (↑36%)**	0.83 ± 0.616	0.60 ± 0.157 (↓28%)	1.18 ± 0.606 (↑42%)	0.73 ± 0.237 (↓12%)
	78	1.71 ± 0.458	1.75 ± 0.423	1.71 ± 0.828	**2.22 ± 1.053 (↑30%)**	1.08 ± 0.460	1.29 ± 0.707 (↑19%)	0.97 ± 0.263 (↓10%)	0.98 ± 0.640 (↓9%)
	104	1.58 ± 0.368	1.59 ± 0.387	**1.89 ±1.10 (↑20%)**	**2.37 ±1.28 (↑50%)**	1.26 ± 0.737	1.16 ± 0.432 ()	1.19 ± 0.835	**1.03 ±0.83 (↓18%)**
Phos (mmol/L)	26	1.85 ± 0.119	1.70 ± 0.136	1.75 ± 0.160	**1.71[a]±0.167 (↓8%)**	1.49 ± 0.112	1.40 ± 0.117	1.40 ± 0.229	1.47 ± 0.215
	52	1.51 ± 0.140	1.44 ± 0.108	1.42 ± 0.112	1.46 ± 0.197	1.33 ± 0.167	1.28 ± 0.099	1.30 ± 0.174	1.27 ± 0.160
	78	1.73 ± 0.931	1.38 ± 0.087 (↓20%)	1.35 ± 0.162 (↓22%)	1.51 ± 0.112 (↓13%)	1.37 ± 0.146	1.25 ± 0.161 (9%)	1.24 ± 0.120 (9%)	1.32 ± 0.134
	104	1.41 ± 0.126	1.29 ± 0.190 (↓9%)	1.25 ± 0.154 (↓11%)	1.42 ± 0.336	1.21 ± 0.191	1.08 ± 0.159 (↓11%)	1.22 ± 0.191	1.25 ± 0.102
Total Prot (g/L)	26	65 ± 2.0	64 ± 1.6	66 ± 2.4	67 ± 3.3	40 ± 2.5	41 ± 1.8	40 ± 1.8	42 ± 2.2
	52	65 ± 2.5	64 ± 1.2	66 ± 1.3	**67[b]±2.8 (↑3%)**	74 ± 2.3	72 ± 1.8	72 ± 3.3	75 ± 3.3
	78	69 ± 1.9	67 ±2.2	66 ± 3.4	69 ± 3.0	39 ± 2.7	40 ± 1.5	39 ± 1.6	39 ± 1.6
	104	70 ± 1.9	67 ± 2.4	68 ± 2.9	69 ± 3.3	74 ± 3.4	74 ± 3.6	74 ± 2.6	76 ±3.6
A/G ratio	26	1.24 ± 0.111	1.25 ± 0.086	1.23 ± 0.088	1.21 ± 0.097	1.42 ± 0.089	**1.33[a] ±0.087 (↓6%)**	**1.29[b] ±0.112 (↓9%)**	**1.27[b]±0.074 (↓11%)**
	52	1.22 ± 0.069	1.25 ± 0.049	1.24 ± 0.094	1.27 ± 0.087	1.33 ± 0.057	1.32 ± 0.043	1.33 ± 0.094	**1.25[a]±0.081 (↓6%)**
	78	1.02 ± 0.029	1.04 ± 0.082	1.05 ± 0.081	1.05 ± 0.046	1.15 ± 0.096	1.21 ± 0.057	1.19 ± 0.060	1.11 ± 0.060
	104	1.08 ± 0.066	1.10 ± 0.099	1.10 ± 0.084	1.10 ± 0.089	1.24 ± 0.131	1.22 ± 0.091	1.23 ± 0.049	**1.14[a]±0.095 (↓8%)**
Free T3 (pmol/L)	N	20			20	20			19
	52	1.1 ± 0.26			1.2 ± 0.22 (↑9%)	1.4 ± 0.42			1.5 ± 0.34 (↑7%)
Free T4 (pmol/L)	N	20			20	20			20
	52	12.4± 2.20			13.0± 2.50 (↑5%)	8.9 ± 2.94			**8.0 ± 2.29 (↓10%)**
	N	20			20	20			19

	Week	Male				Female			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
TSH (ng/mL)	**52**	6.3 ± 1.12			**8.4 ± 3.58 (↑33%)**	5.4 ± 0.72			**6.9 ± 3.37 (↑28%)**

N=9-10 animals/sex/dose, except where indicated otherwise

The details partly confirmed original conclusions. Creatinin phosphokinase activity was significantly altered in the top-dose♀ on weeks 26, 52, 78, but the modifications occurred in different directions, and any effect was unremarkable at termination. Triglyceride levels were slightly elevated in the top-dose ♂, attaining significance only on week 52. Reduced A/G ratio were observed in top-dose at all sampling times except week 78. As mentioned, thyroid stimulating hormone level was marginally high at the top-dose, however, in the absence of effective alterations of thyroid hormones themselves, the toxicological relevance in this LT rat study remains unclear.Other clinical chemical findings were irrelevant in the absence of any coherent pattern throughout treatment and across dose-levels.

Overall, the results of the present study does not put RMS in a position to attribute in an unequivocal way serious clinical chemistry modification to the treatment with lenacil.

However, taking into account the slightly increased triglyceride levels at 118 mg/kg b.w./d and above, the NOAEL is precautionously established at the lowest dose-level. The modified Trig level is considered potentially adverse keeping in mind the slight hepatic effects at the same doses, while top-dose animals also exhibited slightly elevated relative livcer weights (see organ weight and histopathology findings). Notifier requested the reference range for triglycerides requested from Envigo (HLS).

Urinalysis: slightly high protein concentration was noted in week 12 and 51 in ♂ at top dose.

It is of note that decreased urinary volume was also observed in this 2yr rat study ([redacted] 2003), but the effect lacked a dose-related response. There was no aggravation of urinary volume modification with time either, as terminal animals (both ♂ and ♀, wk 103) were unremarkable. It is of note that urine volume was slightly decreased, however without dose-responsiveness in the former 90d rat dietary study with lenacil too ([redacted] 2002).

Regarding proteinuria, in this 2-yr study, urinary proteins were observed in the top-dose ♂ on week 12 and 51, but not in the ♂ animals sampled at other times (wk 25, 77, 103). Proteinuria was observed in the ♀ at week 51, but not at the other sampling times (week 12, 25, 71, 103).

Thus, whereas some effects was observed on occasions, both the subchronic and chronic study failed to reveal a consistent pattern, both in terms of dose-response and time-relationship. In the abovementioned 90d-study, the findings were significant in the ♂ at 118 mg/kg b.w./d and above, and a slight dose-response was observed. The values were slightly high, but insignificant, in all treated ♀.

In conclusion, whereas some effect of the substance on the fluid homeostasis cannot entirely be excluded, the measured parameters show a too disparate response in order to consider this a true adverse finding. In addition, no kidney alteration was demonstrated by morphology and histopathology. Only a slight increase of relative kidney weight was observed at the top-dose in terminal animals. The significance of this proteinuria remains thus unclear, in absence of a consistent pattern (dose, duration), or a plausible mechanism of action for lenacil in rat.

As a possible explanation for this slight hypovolumia in association with proteinuria and increased SG, it appears that a slight exsiccation could be suspected. Notifier confirmed that dehydration could explain the low volume of urine, which would consequently increase the amount of protein in urine and since the urine is concentrated an increase in the SG would also occur. The increased kidney weights sometimes co-occurs with enzyme induction in the liver, as enzyme induction is often also detected in the tubules of the kidneys. There are generally no histopathologic findings for enzyme induction in the kidneys, only weight changes.

Table B.6.5.1.1-7 Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): urinalysis.

		Male				Female			
Mg/kg b.w./d	**Wk**	**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
Volume (mL)	**12**	5.2 ± 1.65	**2.9[b] ±1.28 (↓44%)**	**2.7[b] ±1.14 (↓48%)**	**3.0[b] ±1.36 (↓42%)**	2.3 ±1.14	1.9 ±0.78 (↓17%)	2.2 ±0.93	1.6 ±0.96 (↓30%)
	25	4.3 ± 1.83	3.7 ±1.35 (↓14%)	4.2 ±1.46	5.1 ±1.80 (↑19%)	2.1 ±1.01	2.2 ±1.06	1.7 ±0.90 (↓21%)	3.1 ±1.75 (↑48%)
	51	6.3 ± 2.30	4.2 ±1.04 (↓33%)	5.4 ±1.69 (↓14%)	**4.5[a] ±1.39 (↓29%)**	3.2 ±1.01	2.9 ±2.81	3.5 ±2.36	3.5 ±2.00
	77	5.4 ± 2.45	3.7 ±1.98 (↓31%)	3.7 ±1.22 (↓31%)	4.8 ± 1.92 (↓11%)	4.9 ± 1.91	5.5 ± 3.25 (↑12%)	4.2 ± 2.12 (↓14%)	4.8 ± 2.39
	103	7.1 ± 2.77	7.4 ± 3.55	6.2 ± 2.51 (↓13%)	7.6 ± 3.22	8.1 ± 2.84	7.8 ± 1.83	6.7 ± 2.35 (↓17%)	8.4 ±2.29
pH	**12**	6.8 ± 0.30	6.7 ± 0.36	6.6 ± 0.39	6.5 ± 0.28	5.8 ± 0.31	5.8 ± 0.35	5.6 ± 0.22	5.7 ± 0.28
	25	7.4 ± 0.46	**6.9[b] ± 0.24**	**6.9[b] ± 0.28**	**6.9[b] ± 0.30**	5.8 ± 0.27	5.7 ± 0.12	5.6 ± 0.23	5.7 ± 0.18
	51	7.3 ± 0.55	7.2 ± 0.27	7.1 ± 0.42	7.0 ± 0.51	5.8 ± 0.35	5.9 ± 0.31	5.8 ± 0.74	5.7 ± 0.50
	77	6.8 ± 0.49	6.6 ± 0.49	6.7 ± 0.49	6.7 ± 0.28	6.1 ± 0.33	6.1 ± 0.32	5.9 ± 0.29	6.1 ± 0.46
	103	6.7 ± 0.43	6.8 ± 0.52	6.8 ± 0.47	6.7 ± 0.60	5.9 ± 0.22	5.8 ± 0.33	5.8 ± 0.32	5.9 ± 0.31
SG (g/L)	**12**	1036 ± 5.9	1048 ± 21.0	**1048[a] ± 11.0**	**1048[a] ± 15.4**	1054 ± 25.1	1046 ± 7.6	1041 ± 5.3	1053 ± 12.9
	25	1040 ± 12.1	1042 ± 9.8	1043 ± 17.2	1036 ± 7.8	1052 ± 21.2	1048 ± 12.4	1054 ± 19.6	1043 ± 11.2
	51	1028 ± 6.1	1035 ± 8.0	1032 ± 4.0	1032 ± 6.3	1036 ± 7.2	1038 ± 10.8	1041 ± 9.6	1041 ± 14.4
	77	1039 ± 12.0	1047 ± 15.1	1041 ± 13.4	1040 ± 11.8	1032 ± 6.9	1032 ± 11.3	1034 ± 12.0	1035 ± 13.6
	103	1030 ± 6.0	1029 ± 8.3	1030 ± 6.5	1028 ± 6.0	1026 ± 5.8	1028 ± 6.4	1030 ± 5.4	1025 ± 4.8
Proteins (g/L)	**12**	1.17 ± 0.094	1.38 ± 0.228 (↑18%)	1.36 ± 0.295 (↑16%)	**1.55[b] ± 0.300 (↑32%)**	0.16 ± 0.031	0.17 ± 0.039	0.14 ± 0.047	0.14 ± 0.024
	25	1.02 ± 0.188	1.17 ± 0.118 (↑15%)	1.11 ± 0.373 (9%)	1.19 ± 0.297 (↑17%)	0.17 ± 0.037	0.16 ± 0.031	0.16 ± 0.034	0.16 ±0.023
	51	1.14 ± 0.309	1.45 ± 0.546 (↑27%)	1.25 ± 0.336 (↑10%)	**1.71[b] ± 0.467 (↑50%)**	0.22 ± 0.064	0.25 ± 0.149 (↑14%)	0.28 ± 0.100 (↑27%)	**0.37[a] ± 0.269 (↑68%)**
	77	1.35 ± 0.305	1.57 ± 0.713 (↑16%)	1.15 ± 0.579 (↓15%)	1.56 ± 0.540 (↓16%)	0.31 ± 0.202	0.27 ± 0.190 (↓13%)	0.26 ± 0.091 (↓16%)	0.27 ± 0.143 (↓13%)
	103	0.71 ± 0.344	0.61 ± 0.621 (↓14%)	0.56 ± 0.313 (↓21%)	0.77 ± 0.644 (↑8%)	0.26 ± 0.224	0.21 ± 0.069 (↓19%)	0.20 ± 0.060 (↓23%)	0.18 ± 0.058 (↓31%)

N=8-10 animals/sex/dose; Statistically significant modification: [a]p<0.05, [b]p<0.01

Organ weight:

Organ weights were further detailed in following tables, highlighting both absolute (Abs) and relative (Rel) organ weights.

RMS: after 104 weeks, adrenals absolute and relative weight was increased at top-dose in both the ♂ and the ♀ (**table B.6.5.1.1-6a**). Relative adrenal weights were high in the ♀ at 160 mg/kg bw/d and above.

Table B.6.5.1.1-6a Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): focus on adrenals weight

	Sex	Male				Female			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
52 weeks	Abs.(g)	0.048 ± 0.008	0.045 ± 0.008	0.046 ± 0.005	0.048 ± 0.005	0.057 ± 0.012	0.057 ± 0.009	0.061 ± 0.012 (↑7%)	0.060 ± 0.044 (↑5%)
	Rel.(%)	0.0103 ± 0.0013	0.0098 ± 0.0010	0.0101 ± 0.0014	0.0105 ± 0.0016 (↑2%)	0.0219 ± 0.0035	0.0223 ± 0.0034	0.0226 ± 0.0035 (↑3%)	0.0236 ± 0.0036 (↑8%)
104 weeks	Abs.(g)	0.062 ± 0.013	0.064 ± 0.013	0.062 ± 0.020	**0.153 ± 0.621 (↑147%)**	0.067 ± 0.011	0.073[a] ± 0.014 (↑9%)	0.072 ± 0.016 (↑7%)	**0.078 ± 0.062 (↑16%)**
	Rel.(%)	0.0111 ± 0.0023	0.0117 ± 0.0021	0.0116 ± 0.0033	**0.0240 ± 0.0851 (↑116%)**	0.0193 ± 0.0034	0.0196 ± 0.0039 (2%)	**0.0213[a] ± 0.0050 (↑10%)**	**0.0250 ± 0.0202 (↑30%)**

Statistically significant modification: [a]p<0.05, [b]p<0.01

Epidipymides weight was not altered by lenacil after 104 weeks **(table B.6.5.1.1-6b).**

Table B.6.5.1.1-6b Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): focus on epididymes weight.

	Sex	Male			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**
52 weeks	Abs.(g)	1.313 ± 0.128	1.245 ± 0.119	1.307 ± 0.135	1.328 ± 0.146
	Rel.(%)	0.2850 ±0.0367	0.2736 ±0.0300	0.2862 ± 0.0464	0.2929 ± 0.0332
104 weeks	Abs.(g)	1.373 ± 0.186	1.305 ± 0.167	1.314 ± 0.235	1.306 ± 0.208
	Rel.(%)	0.2472 ±0.0406	0.2441 ±0.0414	0.2466 ± 0.0459	0.2512 ± 0.0399

Relative kidney weight was slightly but significantly increased in ♂+♀ at top-dose after 104 weeks (**table B.6.5.1.1-6c**).

Table B.6.5.1.1-6c Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): focus on kidney weight.

	Sex	Male				Female			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
52 weeks	Abs.(g)	2.07 ± 0.30	2.08 ± 0.31	2.13 ± 0.23	2.08 ± 0.22	1.45 ± 0.16	1.48 ± 0.14	1.55 ± 0.17	1.53 ± 0.14
	Rel.(%)	0.443 ± 0.032	0.455 ± 0.041	0.463 ± 0.044	0.457 ± 0.035	0.560 ± 0.053	0.576 ± 0.053	0.579 ± 0.044	**0.605[a]±0.043 (↑8%)**
104 weeks	Abs.(g)	2.70 ± 0.28	2.76 ± 0.38	2.69 ± 0.35	2.74 ± 0.39	2.03 ± 0.24	2.08 ± 0.27	2.08 ± 0.34	2.07 ± 0.30
	Rel.(%)	0.485 ± 0.048	0.505 ± 0.051	0.504 ± 0.052	**0.529[a]±0.096 (↑9%)**	0.585 ± 0.058	0.558 ± 0.066	0.615 ± 0.120	**0.655[b]±0.099 (↑12%)**

Statistically significant modification: [a]p<0.05, [b]p<0.01

Relative liver weight was slightly but significantly increased in ♂ and ♀ at top-dose after 104 weeks (**table B.6.5.1.1-6d**).

Table B.6.5.1.1-6d Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): focus on liver weight.

	Sex	Male				Female			
Mg/kg b.w./d		0	12	118.4	1223.2	0	15.9	160.2	1699.2
52 weeks	Abs.(g)	12.49 ± 1.77	12.28 ± 1.92	12.90 ± 1.64	13.33 ± 1.72 (↑7%)	7.76 ± 1.00	7.44 ± 0.86	7.74 ± 0.97	8.00 ± 0.68
	Rel.(%)	2.682 ± 0.183	2.672 ± 0.195	2.790 ± 0.236	**2.921[b] ± 0.231 (↑9%)**	2.980 ± 0.239	2.891 ± 0.201	2.883 ± 0.256	3.164 ± 0.304 (↑6%)
104 weeks	Abs.(g)	15.35 ± 1.92	15.49 ± 2.68	15.15 ± 2.74	16.45 ± 2.31	10.55 ± 1.50	11.24 ± 1.63	10.59 ± 2.15	10.73 ± 1.83
	Rel.(%)	2.794 ± 0.317	2.826 ± 0.255	2.814 ± 0.343	**3.149[b] ± 0.311 (↑13%)**	3.035 ± 0.316	3.001 ± 0.312	3.110 ± 0.571	**3.365[b] ± 0.301 (↑11%)**

Statistically significant modification: [a]p<0.05, [b]p<0.01

RMS: non statistically significant, ovaries weight changes (>10%) were observed at all lenacil doses. The dose-response relationship was absent (**table B.6.5.1.1-6e**), and at both interim kill and termination the direction of the changes was inconsistent among the doses tested. Therefore, the toxicological relevance of the finding is considered equivocal.

Table B.6.5.1.1-6e Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): focus on ovaries weight.

		Female			
Mg/kg b.w./d		0	15.9	160.2	1699.2
52 weeks	Abs.(g)	0.113 ± 0.177	0.073 ± 0.019 (↓35%)	0.131 ± 0.232 (↑16%)	0.074 ± 0.028 (↓35%)
	Rel.(%)	0.043 ± 0.065	0.029 ± 0.007 (↓34%)	0.051 ± 0.097 (↑19%)	0.029 ± 0.011 (↓31%)
104 weeks	Abs.(g)	0.122 ± 0.205	0.087 ± 0.027 (↓29%)	0.317 ± 1.436 (↑160%)	0.082 ± 0.028 (↓33%)
	Rel.(%)	0.037 ± 0.072	0.024 ± 0.008 (↓37%)	0.109 ± 0.519 (↑194%)	0.026 ± 0.011 (↓30%)

RMS: although non statistically significant, spleen weight changes (>10%) were observed at top-dose ♀ (**table B.6.5.1.1-6e**).

Table B.6.5.1.1-6e Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): focus on spleen weight .

	Sex	Male				Female			
Mg/kg b.w./d		0	12	118.4	1223.2	0	15.9	160.2	1699.2
52 weeks	Abs.(g)	0.713 ± 0.097	0.712 ± 0.137	0.761 ± 0.116	0.725 ± 0.101	0.504 ± 0.075	0.503 ± 0.083	0.524 ± 0.103	0.508 ± 0.081
	Rel.(%)	0.1538 ± 0.0190	0.1545 ± 0.0163	0.1647 ± 0.0204	0.1595 ± 0.0211	0.1939 ± 0.0269	0.1958 ± 0.0313	0.1942 ± 0.0264	0.2003 ± 0.0299 (↑3%)
104 weeks	Abs.(g)	0.949 ± 0.135	1.038 ± 0.246	0.968 ± 0.281	0.902 ± 0.237	0.673 ± 0.142	0.683 ± 0.132	0.648 ± 0.142	**0.746 ± 0.362 (↑11%)**
	Rel.(%)	0.1705 ± 0.0258	0.1900[a] ± 0.0361 (↑11%)	0.1806 ± 0.0537 (↑6%)	0.1722 ± 0.0435 (↑1%)	0.1945 ± 0.0451	0.1831 ± 0.0373	0.1930 ± 0.0509	**0.2399 ± 0.1348 (↑23%)**

Statistically significant modification: [a]p<0.05, [b]p<0.01

RMS: as observed for epididymides, testes weight was not significantly altered by lenacil after 104 weeks (**table B.6.5.1.1-6f**).

Table B.6.5.1.1-6f Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): focus on testes weight.

		Male			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**
52 weeks	Abs.(g)	3.76 ± 0.31	3.75 ± 0.44	3.74 ± 0.27	3.96 ± 0.35
	Rel.(%)	0.814 ± 0.078	0.826 ± 0.112	0.815 ± 0.097	0.875 ± 0.101 (↑7%)
104 weeks	Abs.(g)	3.92 ± 0.52	3.82 ± 0.45	3.69 ± 0.65	3.74 ± 0.52
	Rel.(%)	0.703 ± 0.099	0.707 ± 0.111	0.693 ± 0.126	0.720 ± 0.104 (↑2%)

RMS: no substance-related effect was seen on thymus weight (**table B.6.5.1.1-6g**).The highly average weight increase in the ♀ mid-dose group is clearly caused by one outlier ♀ animal #334 having thymus weight of :7.782 g (absolute) and 2.54 (relative), and thus unlikely of relevance.

Table B.6.5.1.1-6g Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): focus on thymus weight.

	Sex	**Male**				**Female**			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
52 weeks	Abs.(g)	0.167 ± 0.055	0.156 ± 0.065 (↓8%)	0.170 ± 0.051 (↑2%)	0.152 ± 0.048 (↓9%)	0.165 ± 0.050	0.171 ± 0.058 (↑4%)	0.162 ± 0.043 (↓2%)	0.159 ± 0.070 (↓4%)
	Rel.(%)	0.0356 ± 0.0094	0.0332 ± 0.0102 (↓7%)	0.0367 ± 0.0108 (↑4%)	0.0335 ± 0.0104 (↓6%)	0.0626 ± 0.0156	0.0665 ± 0.0204 (↑6%)	0.0603 ± 0.0145 (↓4%)	0.0619 ± 0.0243 (↓1%)
104 weeks	Abs.(g)	0.090 ± 0.045	0.098 ± 0.036 (↑9%)	0.092 ± 0.048 (↑2%)	0.089 ± 0.037 (↓1%)	0.101 ± 0.063	0.100 ± 0.061 (↓1%)	0.286 ± 1.201 (↑183%)	0.093 ± 0.032 (↓7%)
	Rel.(%)	0.0160 ± 0.0079	0.0180 ± 0.0063 (↑13%)	0.0172 ± 0.0083 (↑8%)	0.0167 ± 0.0057 (↑4%)	0.0291 ± 0.0178	0.0274 ± 0.0194 (↓6%)	0.0904 ± 0.3928 (↑211%)	0.0298 ± 0.0109 (↓2%)

RMS: although not always statistically significant, thyroids + parathyroids weight changes (>10%) were observed at 118 mg/kg b.w./d (♂) and above (♂,♀) (**table B.6.5.1.1-6h**). It was of note that the distinction between thyroid and parathyroid was not made.

Table B.6.5.1.1-6h Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): focus on thyroids + parathyroids weight.

	Sex	**Male**				**Female**			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
52 weeks	Abs.(g)	0.020 ± 0.006	0.020 ± 0.005	0.021 ± 0.007	**0.024 ± 0.006 (↑20%)**	0.014 ± 0.003	0.014 ± 0.002	0.015 ± 0.004	**0.017 ± 0.004 (↑21%)**
	Rel.(%)	0.0043 ± 0.0009	0.0044 ± 0.0008	0.0045 ± 0.0015	**0.0053[a] ± 0.0011 (↑23%)**	0.0055 ± 0.0008	0.0054 ± 0.0009	0.0056 ± 0.0010	**0.0066[a] ± 0.0016 (↑20%)**
104 weeks	Abs.(g)	0.029 ± 0.010	0.029 ± 0.009	**0.033 ± 0.021 (↑14%)**	**0.038 ± 0.048 (↑31%)**	0.022 ± 0.009	0.022 ± 0.006	0.022 ± 0.010	**0.029 ± 0.039 (↑32%)**
	Rel.(%)	0.0052 ± 0.0015	0.0053 ± 0.0014	**0.0061 ± 0.0035 (↑17%)**	**0.0073 ± 0.0095 (↑40%)**	0.0061 ± 0.0021	0.0057 ± 0.0011	0.0066 ± 0.0035 (↑8%)	**0.0091 ± 0.0112 (↑49%)**

Statistically significant modification: [a]p<0.05, [b]p<0.01

RMS: non statistically significant, uterus+cervix weight decreases (>10%) were observed at all lenacil doses. The dose-response relationship was however absent. (**table B.6.5.1.1-6i**).

Table B.6.5.1.1-6i Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): focus on uterus + cervix weight.

		Female			
Mg/kg b.w./d		**0**	**15.9**	**160.2**	**1699.2**
52 weeks	Abs.(g)	0.850 ± 0.373	0.807 ± 0.281 (↓5%)	0.793 ± 0.177 (↓7%)	0.770 ± 0.217 (↓9%)
	Rel.(%)	0.3326 ±0.1648	0.3150 ± 0.1059 (↓5%)	0.2960± 0.0685 (↓11%)	0.3037 ± 0.0799 (↓9%)
104 weeks	Abs.(g)	0.846 ± 1.174	0.662 ± 0.232 (↓17%)	0.763 ± 0.464 (↓10%)	0.726 ± 0.307 (↓14%)
	Rel.(%)	0.2550 ±0.3888	0.1784 ±0.0641 (↓30%)	0.2299 ±0.1553 (↓10%)	0.2311 ±0.1028 (9%)

In summary, at termination, treatment-related increase of organ weights was observed at :

- 118 mg/kg b.w. (♀) and above (♂,♀) in adrenals,
- 118 mg/kg b.w. (♂) and above (♂,♀) in thyroids and parathyroids,
- 1232 mg/kg b.w. in spleen (♀), kidney (♂,♀) and liver (♂,♀).

Therefore, the lowest dose of 12 mg/kg b.w./d was without effect for abovementioned organ weights.

Other occasional organ weight changes were considered unrelated to lenacil treatment, in the absence of either time- or dose-dependent relationship or both.

Macroscopy:

Dose-dependent increased incidences of enlarged thyroids were observed at 118 mg/kg b.w./d (♂) and above (♂,♀). Fluid distended uterus was also observed at 118 mg/kg b.w./d and above.

At the top-dose, the incidence of following gross pathology findings was meaningfully increased: scabs in skin or subcutis (♂), enlarged or swollen spleen (♀), subcapsular fluid accumulation in testes or small testes, dark thyroid (or dark areas) (♂,♀). The latter was particularly remarkable in the ♀, which were affected during the whole experiment, including animals at interim kill (52 weeks), dying or killed during the study, and at termination, with an overall incidence of about 24%.
The incidence of dark areas in the thymus was high in treated ♀ attaining high statistical significance at mid-dose but decreased in the top-dose ♀ animals. While the observed incidences were elevated when compared to the study controls, the absence of dose-dependency leaves the observation to be of uncertain toxicological significance.

Both the nature of the darkening of thymus (also observed in the generational study) and thyroids remained unexplained.

According the notifier, only dark colouration of thyroid seen in ♂ and ♀ rats at top dose after 52 week, affecting ♀ solely after 104 week, was considered adverse and no other notable changes were observed. RMS concurs with the potential adversity of the thyroid findings, corroborated by clinical-chemical modifications (slight ↑TSH), elevated thyroid/parathyroid weights and concordant histopathological findings (see below).

However, abovementioned subtle changes in other organs should also be mentioned for the sake of completeness.

In conclusion, the target organ identified during gross pathological observations was the thyroid and confirmed the initial conclusion during renewal, and the lowest dose remains the NOAEL for necropsy findings.

Details were put in the extended table B.6.5.1.1-7

Table B.6.5.1.1-7 Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): macropathology.

		Males				Females			
Mg/kg b.w./d		0	12	118.4	1223.2	0	15.9	160.2	1699.2
Adrenals, dark	52 weeks	-	-	-	-	-	-	-	-
	DOT	0/14	0/15	1/5	0/9	1/9	4/17	1/9 (11%)	0/15
	104 weeks	0/36	0/35	1/45	0/41	0/41	0/33	1/41 (2%)	0/35
	∑	0/50	0/50	2/50	0/50	1/50	4/50	2/50	0/50
Adrenals, dark areas	52 weeks	0/20	0/19	0/20	0/20	0/20	0/20	1/20 (5%)	0/19
	DOT	2/14	0/15	0/5	1/9	0/9	1/17	1/9 (11%)	**5/15 (33%)**
	104 weeks	13/36	13/35	10/45	12/41	11/41	12/33	9/41	7/35
	∑	15/50	13/50	10/50	13/50	11/50	13/50	10/50	12/50
Mammary masses	52 weeks					1/20	0/20	0/20	0/20
	DOT					3/9	7/17	4/9	6/15
	104 weeks					4/41	11/33	10/41	8/35
	∑					7/50	**18/50***	**14/50**	**14/50**
Skin/ subcutis, scab(s)	52 weeks	2/20	1/19	0/20	1/20	0/20	0/20	0/20	0/19
	DOT	1/14	1/15	0/5	**3/19 (16%)**	0/9	0/17	2/9 (22%)	3/15 (20%)
	104 weeks	1/36	0/35	0/45	**4/41 (10%)**	3/41	0/33	2/41	1/35
	∑	2/50	1/50	0/50	**7/50 (14%)**	3/50	0/50	4/50 (8%)	4/50 (8%)
Spleen, enlarged	52 weeks	-	-	-	-	-	-	-	-
	DOT	0/14	3/15	1/5	1/9	3/9	2/17	2/9	2/15 (13%)
	104 weeks	0/36	0/35	1/45	0/41	0/41	0/33	0/41	**3/35 (9%)**
	∑	0/50	3/50	2/50	1/50	3/50	2/50	2/50	**5/50 (10%)**
Spleen, swollen	52 weeks	-	-	-	-	-	-	-	-
	DOT	1/14	4/15	0/5	1/9 (11%)	2/9	2/17 (12%)	1/9 (11%)	2/15 (13%)
	104 weeks	5/36	3/35	3/45	1/41	1/41	1/33	1/41	**5/35 (14%)**
	∑	6/50	7/50	3/50	2/50	3/50	3/50	2/50	**7/50 (14%)**
Testis, subcapsular fluid	52 weeks	-	-	-	-				
	DOT	0/14	0/15	0/5	1/9 (11%)				
	104 weeks	1/36	3/35	1/45	**4/41 (10%)**				
	∑	1/50	3/50	1/50	**5/50 (10%)**				
Testis, unilaterally small	52 weeks	-	-	-	-				
	DOT	-	-	-	-				
	104 weeks	1/36	2/35	1/45	**5/41 (12%)**				
	∑	1/50	2/50	1/50	**5/50 (10%)**				
Thymus, dark	52 weeks	-	-	-	-	-	-	-	-
	DOT	0/14	0/15	0/5	0/9	0/9	1/17	0/9	0/15
	104 weeks	0/36	0/35	0/42	0/41	0/41	0/33	1/41	0/35
	∑	0/50	0/50	0/50	0/50	0/50	1/50	1/50	0/50
Thymus, dark areas	52 weeks	-	-	-	-	-	-	-	-
	DOT	1/14	0/15	0/5	1/9	0/9	0/17	0/9	0/15
	104 weeks	5/36	2/35	6/45	6/41	1/41	3/33 (10%)	8/41**(20%)	4/35 (11%)
	∑	6/50	2/50	6/50	7/50 (14%)	1/50	3/50	8/50**(16%)	4/50 (8%)
Thyroid, dark	52 weeks	0/20	0/19	0/20	**5/20* (25%)**	0/20	0/20	0/20	**10/19** (53%)**
	DOT	0/14	0/15	0/5	**1/9 (11%)**	0/9	0/17	0/9	**2/15 (13%)**
	104 weeks	0/36	0/35	0/45	0/41	0/41	0/33	0/41	**10/35** (29%)**
	∑	0/50	0/50	0/50	**1/50 (2%)**	0/50	0/50	0/50	**12/50 (24%)**
Thyroid, dark areas	52 weeks	0/20	0/19	0/20	0/20	0/20	0/20	0/20	**3/19 (16%)**
	DOT	0/14	0/15	0/5	**1/9 (11%)**	0/9	0/17	0/9	**1/15 (7%)**
	104 weeks	0/36	0/35	0/45	**1/41 (2%)**	0/41	0/33	0/41	**2/35 (6%)**
	∑	0/50	0/50	0/50	**2/50 (4%)**	0/50	0/50	0/50	**3/50 (6%)**
Thyroid, enlarged	52 weeks	0/20	0/19	0/20	0/20	0/20	0/20	0/20	**2/19 (11%)**
	DOT	-	-	-	-	-	-	-	-
	104 weeks	0/36	0/35	**2/45 (4%)**	**5/41 (12%)**	1/41	0/33	0/41	1/35
	∑	0/50	0/50	**2/50 (4%)**	**5/50 (10%)**	1/50	0/20	0/50	1/50
Uterus, fluid distention	52 weeks					5/20	5/20	3/20	1/19
	DOT					0/9	0/17	**1/9 (11%)**	**3/15 (20%)**
	104 weeks					0/41	0/33	**2/41 (5%)**	**3/35 (9%)**
	∑					0/50	0/50	**3/50 (6%)**	**6/50* (12%)**

52 weeks: interim kill after 52 weeks; DOT: killed or dying on test; 104 weeks: terminal sacrifice after 104 weeks; ∑: all animals. Statistically significant modification: *p<0.05, **p<0.01

Histopathology:

Top-dose ♀ showed an increased incidence of adrenal alterations: ceroid inclusions and accessory tissue. The etiology of these findings remained unexplained, but it may indicate oxidative stress. Notifier considered accessory tissue a normal variation of adrenal tissue. This is a congenital (spontaneous) background lesion, which has not been associated with experimental procedures. Notifier further thinks that the finding of "ceroid inclusions' is actually "pigment" according to INHAND terminology. The pigment in this case could be due to a variety of things, including ceroid, but found it difficult to identify an underlying cause.

Changes were evident in liver of ♂rats at top dose where there was an increased incidence of centrilobular hepatocyte hypertrophy and increased vacuolation accumulation. Vacuolation is considered a toxic change and normally represents fat accumulation, suggesting that the compound influences the uptake, intracellular fat metabolism or fat release by the hepatocyte. During the initial peer review, it was considered that there was no evidence of any effect upon plasma cholesterol and triglycerides as a result of the fatty vacuolation in the liver. However, during renewal a more precautiounous approach was considered more appropriate, and the hepatic effects were not discarded, although it was acknowledged that ♀ were less affected.

In thyroids, an increased incidence of luminal concretions was seen in ♂and ♀at the top dose.

While these findings were initially considered to be a common background change which is exaggerated by treatment at top dose, it is now considered that the various effects of lenacil on the thyroids should be given more attention, and that further investigation to unravel the MoA should be conducted by the notifier. Notifier (cfr above): *"We are working on trying to identify a mechanistic study. Preliminary data has shown that we have a mild increase in UDPGT activity, as well as an increase in CYP2B1 gene expression in the liver of rats. This work includes an in vitro thyroid peroxidase inhibition assay. In that assay lenacil has shown no thyroid peroxidase inhibiting activity."*

A slight increase in the incidence of luminal dilatation was seen in uterus of top-dose rats, and to a lesser extent in the rats treated at the next-lower dose As this finding is commonly seen in animals of this age, this was considered to be an exaggeration over the background level and was not attributed to treatment during the first evaluation. Of note: the readings for both low-dose and mid-dose are lacking, in order to have a full assessment up to 50 animals/dose, like for control- and top-dose groups. Notifier agreed and has started the process to get the rest of the histopathology of uterus in lower dose groups examined.

Associated with previous observation **RMS** notes an increase of endometrial gland hyperplasia at the top-dose. Mammary ducts were characterised by acinar hyperplasia. Both mammary and uterus findings are considered treatment-related.

Notifier underlined that an incidence of 0/41 in controls seems very low, and that found it surprising that none were observed in controls. Notifier cited Blankenship and Skaggs (2013) who reported a control incidence of up to 2/60 cases of uterine dilation in Han Wistar rats in a 26-week study. RMS noted indeed such an incidence in only 1 experiment, where 60♀ were sacrificed after 4, 13 and 26 weeks, which is not really a sufficient database to be considered a HC.

Notifier pointed out that the incidence in older ♀ would likely be more variable. Further notifier stated: *"Uterine dilation is not necessarily associated with endometrial gland hyperplasia. Uterine dilatation is normal during certain oestrus cycle periods and this incidence may reflect differences in oestrus cycle. Since these are reproductively senescent animals the oestrus cycles are not uniform, and drawing conclusions about uterine effects in these animals is not informative. Thickening is not informative in this context and a more detailed description of the thickening (location) might have been useful"*

Table B.6.5.1.1-8a Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): histopathology - non neoplastic findings.

		Males				Females			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
Adrenals, ceroid accumulation	DOT	0/14	0/15	0/5	0/9	0/9	1/17	0/9	0/15
	104 weeks	0/36	0/20	1/24	3/41	1/41	2/28	2/36	**7/35 (20%)**
	∑	0/50	0/25	1/29	3/50	1/50	3/45	2/45	**7/50 (14%)**
Adrenals, prominent accessory adrenocortical tissue	DOT	2/14	4/15	0/5	1/9	2/9	2/17	0/9	**5/15 (33%)**
	104 weeks	3/36	2/20	4/24	2/41	4/41	3/28	5/36	**7/35 (20%)**
	∑	5/50	6/35	4/29	3/50	7/50 (14%)	5/45 (11%)	5/45 (11%)	**12/50 (24%)**
Liver, centrilobular vacuolation hepatocytes	DOT	3/14	6/15	1/5	**7/9* (78%)**	2/9	2/17	1/9	2/15
	104 weeks	13/36	15/35	17/45	**21/41 (51%)**	0/41	2/33	1/41	2/35
	∑	16/50	21/50	18/50	**28*/50 (56%)**	2/50	4/50	2/50	2/50
Liver, centrilobular hypertrophy hepatocytes	DOT	1/14	1/15	1/5	1/9	0/9	0/17	0/9	**2/15 (13%)**
	104 weeks	10/36	10/35	**14/45**	**25/41**(61%)**	1/41	0/33	1/41	**2/35 (6%)**
	∑	11/50	11/50	**15/50**	**26*/50 (52%)**	1/50	0/50	1/50	**4/50 (8%)**
Mammary acinar hyperplasia	52 weeks					5/9	7/17	3/9	7/15
	DOT					0/20	0/20	0/20	0/19
	104 weeks					17/41	18/33	23/41	21/35
	∑					22/50	25/50	**26/50 (52%)**	**28/50 (56%)**
Thyroid, increased luminal concretions	DOT	2/14	8/15	1/5	**6/9 (67%)**	1/9	3/17	2/9	**8/14* (57%)**
	104 weeks	9/36	8/35	16/44	**27/41**(66%)**	4/41	3/33	8/41	**24/35**(69%)**
	∑	11/50 (22%)	16/50 (32%)	17/49 (35%)	**33*/50 (66%)**	5/50 (10%)	6/50 (12%)	10/50 (20%)	**32*/49 (65%)**
Uterus, endometrial gland hyperplasia	DOT					0/9	0/17	0/9	**1/15 (7%)**
	104 weeks					2/41	1/20	2/25	**5/35 (14%)**
	∑					2/50 (4%)	1/37 (3%)	2/34 (6%)	**6/50 (12%)**
Uterus, luminal dilatation	DOT					0/9 (0%)	4/17 (24%)	2/9 (22%)	**7/15* (50%)**
	104 weeks					17/41 (41%)	16/20 (80%)	17/25 (68%)	**20/35 (57%)**
	∑					17/50 (34%)	20/37 (54%)	19/34 (56%)	**27/50 (54%)**

DOT: killed or dying on test; 104 weeks: terminal sacrifice after 104 weeks; ∑: all animals.
Statistically significant modification: *p<0.05, **p<0.01.

In the context of a more thorough investigation of potential endocrine effects, also the uterus luminal dilatation findings (in line with the observed endometrial gland hyperplasia) was further investigated. Both additional MoA studies and a sound rationale were necessary to further discard a potential endocrine effect of the substance. The presence of mammary acinary hyperplasia may be related to the neoplastic findings.

Therefore, **RMS** requested further tests to discard any potential hazard of the a.s. to interact by any means with ER, taking into account abovementioned findings. For example, the MCF-7 cell proliferation assay (testing for ER ant/agonism) considered by OECD as a possible level 2 *in vitro* assay could provide data about selected endocrine mechanism(s) / pathways(s). Notifier therefore evaluated oestrogenic agonism and antagonism in the stably transfected human oestrogen receptor-α transactivation assay, and has evaluated oestrogen receptor binding using rat uterine cytosol. In addition notifier looked at androgen receptor binding and evaluated androgenic agonism and antagonism in an androgen receptor transactivation assay. In contrast, notifier did not conduct the MCF-7 cell proliferation assay, as there is no OECD guideline. The receptor and transactivation assays were negative. In the meanwhile, notifier also conducted an uterotrophic assay for detecting oestrogenic activity in ovariectomised rats, which was also negative - please refer to **B.6.8.3**).

Also the neoplastic findings were further detailed during the renewal evaluation (see **table B.6.5.1.1-8b**). Increased incidences of of neoplasia were only observed in the ♀.

Table B.6.5.1.1-8b Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): histopathology - neoplastic findings.

		Males				Females			
Mg/kg b.w./d		**0**	**12**	**118.4**	**1223.2**	**0**	**15.9**	**160.2**	**1699.2**
Adrenals, cortical adenoma	DOT	0/14	0/15	0/5	1/9 (11%)	0/9	0/17	0/9	1/15 (7%)
	104 weeks	2/36	0/20	2/24	1/41	2/41	1/28	1/36	3/35
	∑	2/50	0/35	2/29	2/50	2/50	1/45	1/45	**4/50 (8%)**
Adrenals, cortical carcinoma	DOT								
	104 weeks	0/36	0/20	0/24	1/41	0/41	0/28	0/36	1/35
	∑	0/50	0/35	0/29	1/50	0/50	0/45	0/45	1/50
Mammary tissues, adenoma	DOT					0/9	1/17	0/9	1/15
	104 weeks					0/41	0/33	0/41	2/35
	∑					0/50	1/50	0/50	**3/50**
Mammary tissues, fibroadenoma	DOT					3/9	3/17	0/9	3/15
	104 weeks					4/41	9/33	8/41	5/32
	∑					7/50	12/50	8/50	8/50
Mammary tissues, adenocarcinoma	DOT					0/9	2/17	3/9	3/15
	104 weeks					0/41	0/33	3/41	2/35
	∑					0/50	2/50 (4%)	**6/50 (12%)**	**5/50 (10%)**
Thyroid, C-cell adenoma	DOT	1/14	2/15	0/5	0/9	0/9	1/17	1/9	4/14
	104 weeks	3/36	1/35	5/44	5/41	2/41	1/33	7/41	3/35
	∑	4/50	3/50	5/49	5/50	2/50 (4%)	2/50 (4%)	**8/50 (16%)**	**7/49 (14%)**
Uterus, endometrial adenocarcinoma	DOT					1/9	0/17	1/9	2/15
	104 weeks					1/41	2/20	3/25	3/35
	∑					2/50 (4%)	2/37 (5%)	4/34 (12%)	5/50 (10%)

DOT: killed or dying on test; 104 weeks: terminal sacrifice after 104 weeks; ∑: all animals. Statistically significant modification: *p<0.05, **p<0.01

The incidence of adrenal adenoma was increased in the top-dose ♀; however, no increase of malignant adrenal tumours were observed. Mammary adenocarcinoma were slightly increased at 160 mg/kg b.w./d and above. Adenoma were slightly high at the top-dose, but the incidence of mammary fibroadenoma were unaltered when compared to the study controls. Thyroid C-cell adenoma were also increased at 160 mg/kg b.w./d and above. An in-depth discussion of the cancer incidences is provided under

All other changes observed in this study were of the types normally encountered in Han Wistar rats at these laboratories.

An in-depth discussion on the carcinogenicity part of the study is found in next section B.6.5.1.2.

RMS: Table B.6.5.1.1-8b The assessment of uterus, endometrial adenocarcinoma was not performed on all animals. Taking into account suspected findings regarding uterus, endometrial gland hyperplasia, notifier should provide an assessment of the uterus tumour histopathology for all doses tested, including the mid-doses (and for all animals, up to N=50). As mentioned, the uterus will be examined in all dose groups.

Conclusion from the RMS (2yr chronic rat toxicity study):

The NOAEL's, established during the previous peer-review (initial inclusion of lenacil) were confirmed.
Toxicity NOAEL= 250 ppm = **12 mg/kg b.w./d**
Toxicity LOAEL = 2500 ppm = 118 mg/kg b.w./d, based upon:
Clinical signs (perigenital staining, eye discolouration), ↓motility, ↓lymphocyte count (♂), ↑triglyceride level, ↑organ weight (adrenal and thyroids), enlarged thyroid, fluid distended and hyperplastic uterus.

Top-dose (1223 mg/kg b.w./d) findings included:
clinical signs (skin/subcutis exfoliation/scabs, ventral swollen/firm areas, exophthalmos), thin appearance, ↓body weight (gain), proteinuria, haematology (↓platelet count, ↓APTT), ↓A/G ratio (♀)), ↑TSH, ↑organ weight (spleen, kidney, liver), thyroids black and with luminal concretions, enlarged or swollen spleen, subcapsular fluid

accumulation in testes or small testes, adrenal ceroid accumulation and accessory tissue, liver CL vacuolation/hypertrophy.

Carcinogenicity NOAEL= 250 ppm = **12 mg/kg b.w./d**
Carcinogenicity LOAEL = 2500 ppm = 118 mg/kg b.w./d,
based upon the increased incidence of mammary adenocarcinoma and thyroid C-cell adenoma.
Top-dose (1223 mg/kg b.w./d) findings included adrenal cortical adenoma and mammary adenoma.

Notifier concluded that the NOAEL for rats dosed in a one/two year long term toxicity study is 2500 ppm, based upon non-specific toxicity in ♀ at 25000ppm, and adaptive and toxic change in the liver in ♂at 25000ppm, equivalent to 139 mg/kg b.w./d :"*The long-term NOAEL for systemic toxicity in rats was determined to be 139.1 mg/kgbw/day (♂rats), based on increased thyroid and liver weights, increased hepatocellular hypertrophy/vacuolaton in both sexes, increased urinary protein excretion and kidney weight, some effects on eyes in ♂ and ♀and abnormal blood smear in males at the top dose level of 1446mg/kg bw/day*."

Taking into account the abovementioned rationale, the proposal of the notifier was not agreed upon.

Cited references:

- *Blankenship B, Skaggs H, Findings in Historical Control Harlan RCCHan™: WIST Rats from 4-, 13-, 26-Week Studies, Toxicol Pathol.,41(3):537-547, 2013.*
- *Lim E, Pon A, Djoumbou Y, Knox C, Shrivastava S, Guo AC, Neveu V, Wishart DS, T3DB: a comprehensively annotated database of common toxins and their targets, Nucleic Acids Res.,38, D781-786, 2010.*

Annex: for the sake of transparency, the original table, summarising all critical endpoints for general toxicity, haematology, clinical chemistry, necropsy, and histopathology are reproduced hereunder.

Table B.6.5.1.1-9 Combined chronic toxicity and carcinogenicity study by dietary administration to Han Wistar rats over 104 weeks ([redacted] P.M., 2003): summary of data.

	0 ppm		250 ppm		2500 ppm		25000 ppm	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Dose mg/kg/d Wk 1-52	0	0	14.3	18.8	139.1	188.5	1446	1894
Wk 1-104	0	0	12	15.9	118.4	160.2	1223.2	1699.2
Mortality week 1-52			1/20					1/20
Mortality weeks 1-104	14/50	9/50	15/50	17/50	5/50	9/50	9/50	15/50
Body weight:								
Week 52			↓3%	↓2%			↓2%	↓3%
Week 104			↓2%		↓4%		↓6%	↓9%*
Bw gain								
week 52			↓3%	↓2%			↓2%	↓5%
week 104			↓3%		↓6%	↓2%	↓8%	↓13%
Food consumption								
Week 52			↓2%	↓4%	↓3%		↓1%	↓3%
week 104			↓2%		↓5%	↓2%	↓2%	↓1%
Haematology:								
Week 13								
Large Unstained Cells					↓37%*		↓12%*	
Lymphocytes						↓23%*		↓19%*
Week 26								
Lymphocytes				↓32%*		↓25%*		↓16%*
WBCs				↓28%*		↓20%*		↓10%*
PT wk 52								↓5%*
PT wk 78				↑9%*	↓16%*	↑11%*	↓20%*	↑6%*
APTT wk 78					↓23%*		↓12%*	
Hct wk 78								↓4%*
APTT wk 104			↓12%*		↓16%*		↓16%*	

	0 ppm		250 ppm		2500 ppm		25000 ppm	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Dose mg/kg/d Wk 1-52	0	0	14.3	18.8	139.1	188.5	1446	1894
Wk 1-104	0	0	12	15.9	118.4	160.2	1223.2	1699.2
Blood smears:								
Neutrophils wk 52							↑13%*	
Neutrophils wk 104							↑24%*	
Lymphocytes wk 52							↓4%*	
Lymphocytes wk 104							↓8.5%*	
Monocytes wk 52							↓33%*	
Monocytes wk 104							↓75%*	
Blood chemistry								
Week 26								
Ca^{2+}							↓1%*	
Phosphate							↓8%*	
Na^{+}				↓8%*		↓1.4%*		↓0.7%*
Urea				↓24%*		↓10%*		↓27%*
Creatinine				↓8%*		↓6%*		↓6%*
A/G ratio				↓6%*		↓9%*		↓11%*
Week 52								
glucose					↑14%*		↑14%*	
triglycerides					↑27%*		↑36%*	
Total proteins							↑3%*	
Albumin							↑5.5%*	
CPK								↓50%*
A/G ratio								↓6%*
Free T3, T4	No compound related effect							
TSH							↑33%	↑27%
Week 78								
CPK						↑22%		↑94%*
Week 104								
Ca^{2+}			↓3%*	↑2.9%*	↓4%*	↑3.6%*	↓1.5%*	↑0.35%*
A/G ratio								↓8%*
Urinalysis								
Week 12								
Volume			↓45%*		↓48%*		↓42%*	↓30%
SG					↑1.1%*		↑1.1%*	
Proteins							↑32%*	
Week 25								
pH	7.4		6.9*		6.9*		6.9*	
Week 51								
volume							↓29%*	
Proteins							↑50%*	↑68%*
Organ weight								
Week 52								
Kidney relative								↑8%*
Liver							↑9%*	↑6%
Thyroid +para							↑23%*	↑20%*
Week 104								
Kidney relative							↑9%*	↑12%*
Liver							↑14%*	↑10%*
Thyroid +para							↑40%	↑49%
Brain							↑7%*	↑8%
Heart							↑8%*	↑9%*

*significant when compared with control group at a < 0.05% level

Table B.6.5.1.1-10 Combined chronic toxicity and carcinogenicity study by dietary administration to Han Wistar rats over 104 weeks ([redacted] P.M., 2003): summary of data.

	0 ppm		250 ppm		2500 ppm		25000 ppm	
	M	F	M	F	M	F	M	F
	20	20	19	20	20	20	20	19
Week 52								
Macroscopy:								
Thyroid dark							5*	10*
Carcinogenicity phase:								
Macroscopy:								
Rats killed/dying during study								
Liver: pale area							3/9	1/15
Lung: pale area	4/14	6/9	6/15	6/17	2/5	4/9	6/9	10/15
Thyroid dark area	0/14	0/9	0/15	0/17	0/5	0/9	1/9	1/15
Thyroid dark	0/14	0/9	0/15	0/17	0/5	0/9	1/9	2/15
Uterus:								
Fluid distension		0/9		0/17		1/9		3/15
cysts		0		5		1		4
thickened		0		1		1		2
Skin scabs	1/14	0/9	1/15	0/17	0/5	2/9	3/9	3/15
Rats killed after 104 weeks								
Kidneys depression		2/41		2/33		0/41		4/35
Liver dark depression	1/36	2/41	0/35	2/33	3/45	4/41	3/41	4/35
Lung dark area	9/36	7/41	8/35	8/33	14/45	10/41	13/41	10/35
Spleen swollen	5/36	1/41	3/45	1/33	1/41	1/41	1/41	5/35
Testes subcapsular fluid	1/36		3/35		1/45		4/41	
Thymus dark area	5/36	1/41	2/35	3/33	6/45	8*/41	6/41	4/35
Thyroid dark	0/36	0/41	0/35	0/33	0/45	0/41	0/41	10*/35
Enlarged	0/36	1/41	0/35	0/33	2/45	0/41	5/41	1/35
All animals:								
Spleen swollen	6/50	3/50	7/50	3/50	3/50	2/50	2/50	7/50
Testes subcapsular fluid	1/50		3/50		1/50		5/50	
Unilaterally small	1/50		2/50		1/50		5/50	
Thymus dark area	6/50	1/50	2/50	3/50	6/50	8*/50	7/50	4/50
Thyroid dark	0/50	0/50	0/50	0/50	0/50	0/50	1/50	12*/50
Enlarged	0/50	1/50	0/50	0/50	2/50	0/50	5/50	1/50
Uterus fluid distension		0/50		0/50		3/50		6*/50
Mammary area masses		7/50		18*/50		14/50		14/50

B.6.5.1.2

DuPont Report No.: ACD 045/042214

Guidelines: study is in compliance with EC TM B.30 of Dir EEC 87/302/EEC Annex V B or OECD test guideline n 453 (1981).

GLP status: yes

Materials and Methods
Lenacil technical (Batch No. 141712003, purity 98.6%) was administered via dietary admixture into the powdered diet. At specified intervals, (weeks 1, 13, 26 and 52) during the toxicity phase, prepared dietary formulations were sampled and analysed for concentration. The homogeneity and stability of Lenacil, conducted as part of an earlier study, were confirmed at nominal concentrations of 50 ppm and 50000 ppm during ambient temperature storage for 22 days. The mean concentrations of Lenacil technical in test formulations during the Toxicity phase of the study were between – 4.8 and + 2.0% of intended, which were within the acceptable limits of -15% to 10%, confirming the accuracy of formulation.
Three groups of 50 ♂ and 50 ♀ rats HsdBrl Han:Wist (Han Wistar) are receiving Lenacil technical orally, via the diet, at concentrations of 250, 2500 or 25000 ppm. Together with a similarly constituted control group receiving the vehicle, untreated diet, these animals comprise the carcinogenicity phase of the study. A further 20 ♂ and 20 ♀ rats were allocated to each group. These animals comprised the toxicity phase of the study and were sacrificed after the completion of 52 weeks of treatment. In this oncogenicity phase, 250, 2500 and 25000 ppm are equivalent to **12**, **118.4** and **1223.2** mg/kg bw/d (♂) and to **15.9**, **160.2** and **1699.2** mg/kg bw/d (♀).
Animals were observed daily for evidence of a reaction to treatment. During the study, detailed physical and arena observations, sensory reactivity and grip strength, motor activity, bodyweight, food consumption, ophthalmic examination, haematology, blood chemistry, urinalysis, organ weight, macroscopic and microscopic pathology investigations were undertaken.
Statistics were carried out separately for ♂ and ♀ using the individual rat as unit. For categorical data, including pathological findings, the proportion of rats were analysed using Fisher exact test for each group compared to control. For continuous data, Bartlett test was applied to test homogeneity of variance. When statistically different a Behrens-Fisher test was used to perform pair wise comparisons otherwise a Dunnett test. Intergroup differences in mortality and tumour incidence were performed using the Peto approach.
The study is accepted.

Findings

The results reported here are limited to the carcinogenicity findings; they are complementary to study B.6.5.1.1.

Neoplastic findings:

In ♂, no statistically significant results were found.

In ♀:

Thyroids: for benign follicular cell adenoma the trend test was found to be statistically significant when taking the top dose into account. Pair wise comparison of control and top dose was statistically significant. When follicular cell adenoma and malignant follicular cell carcinoma were combined the trend test was statistically significant if the top dose was included.

Notifier highlighted that the thyroid follicular cell adenomas and carcinomas occurred to some extent in all groups. The percentage incidence of follicular cell adenomas in treated groups was well within the background range for both sexes. In addition, the group distribution, and lack of clear dosage relationship indicates that these particular tumours are not related to the administration of Lenacil and are not considered to be toxicologically significant. The incidence of follicular cell adenomas was not associated with follicular cell carcinomas. The group incidence of other non-neoplastic proliferative lesions such as follicular cell hyperplasia did not show any effects of treatment.

RMS considered those thyroid follicular cell adenomas are within historical control data of the laboratory. The laboratory background incidence of follicular cell carcinoma is not reported.

An increased incidence of C-cell adenoma was seen in ♀ at 160 mg/kg b.w./d and above. The incidences observed however, were either within background range or marginally outside. There was, in addition, no dose-relation in

the occurrence of these tumors which were considered unrelated to treatment during the first evaluation. Although the plateau-type of response, showing approximately the same incidence at 2500 ppm as in 25000 ppm, is not an *a priori* condition to be considered unrelated to treatment for this reason alone (a saturation process could be expected at high dose-levels), it should be acknowledged that C-cells, unlike other thyroid cells, are not particularly targeted. The latter are involved in Ca-metabolism, and no adverse findings are observed at that level with lenacil. In conclusion, taking into account a relatively high background incidence of C-cell tumours, RMS is of the opinion that the contribution of the C-cell lesions in the carcinogenicity classification in the rat remains equivocal.

It was concluded (ECHA): The incidence of follicular cell adenoma was significantly increased in high-dose ♀ but remained within the historical control data (HCD) for the laboratory. The incidence of carcinomas was not elevated at any dose when compared to the controls. The incidence of combined adenomas and carcinomas was within the HCD for adenomas only and there was no evidence that Lenacil induced follicular cell tumours.

The finding C-cell carcinoma was seen in top-dose ♀. Although the study (4%) incidence of these carcinoma was higher than the ♀ background range (1.8%), the incidence was within the ♂ background range (0-5.1%). The pairwise comparison between the control and the top dose treated group was found to be statistically significant, but it is of note that there is a poor dose-response, with 0, 2, 0, 2* incidences /50 at 0, 16, 160 and 1699 mg/kg b.w./d, respectively. The notifier considers that C-cell carcinomas of the thyroid in the 2 top-dose ♀ have arisen incidentally and the etiology probably related to age.

A position paper was provided by the notifier (Gopinath, 2004) in which it was concluded that C-cell tumours are spontaneous age-related lesions with a widely variable incidence in laboratory rats. The carcinogenicity study in Wistar rats reported an increased incidence of C cell adenomas in females receiving 2500 or 25000 ppm lenacil technical. The incidences reported were only marginally greater than the historical control rats from Huntingdon Life Sciences laboratories. The incidence of C-cell carcinoma was well within the control range. Male rats did not reveal similar changes. C-cell lesions including C-cell tumors are seldom observed as treatment related end points. There was no treatment related C-cell hyperplasia in the study. The overall proliferative lesions of C-cells did not show any intergroup differences from controls. The examination of clinical biochemical parameters did not reveal any evidence of disturbance of calcium homeostasis to suggest any C-cell involvement.

The review of two other short term studies using higher dosages up to 50000 ppm did not show any treatment related changes in C-cells or any indications for disturbances in calcium/phosphorus levels. The two studies reviewed revealed a few minor changes in follicular epithelium of thyroid, such as increased Schmorl's positive pigment and or follicular cell hypertrophy at high dosages. These changes have no connection or impact on C-cell lesions. In view of the above mentioned facts, the minor increased incidence reported of C-cell adenoma in the female rats receiving 2500 or 25000ppm in this study is considered incidental and of no toxicological importance.

According to the open literature, in many rat strains, C-cell hyperplasia occurs in an age-dependent manner and is often associated with multifocal C-cell carcinoma. The incidence of C-cell hyperplasia shows a significant increase with age ($p<0.001$) and is much higher in ♀ rats than in ♂ rats ($p<0.05$). From 3 to 24 months of life, 27.5% of female rats showed a normal C-cell pattern, 55.0% showed C-cell hyperplasia, and 17.5% showed C-cell tumors; while 57.5% of ♀ rats showed a normal C-cell pattern, 32.5% showed C-cell hyperplasia, and 10% showed C-cell tumours. Although the overall frequency of C-cell neoplasms in ♀was nearly double that in ♂, these data are not statistically significant. However, the number of C-cell tumours showed a significant increase with age ($p<0.05$) (Martín-Lacave *et al.,* 1999). **Notifier**: "*The position by Gopinath seems to be a reasonable assessment for this is an age related tumour."*

Therefore, RMS accepts that the significant differences in the incidence of the total spectrum of C-cell proliferative abnormalities in the thyroid gland of Wistar rats are both age- and gender-dependent. Overall, RMS considers the treatment-relationship of C-cell carcinoma induction unclear.

It was concluded (ECHA): An increased incidence of C-cell adenomas was observed in females, which was not (although borderline) statistically significant at mid-dose (p=0.051). The incidence exceeds the laboratory HCD at the two highest doses but without clear dose-response relationship. Two females in the high dose group had C-cell carcinomas. This incidence is above available HCD. A dose-response was observed for the incidence of combined C-cell tumours. Overall, considering that the incidence of C-cell tumours in female rats was marginally above HCD, there is equivoqual evidence of carcinogenicity of Lenacil on the thyroid in the rat.

Mammary tissue

For benign mammary adenoma the trend test was found to be statistically significant. Upon exclusion of the top dose the trend test was no longer statistically significant. For malignant mammary adenocarcinoma, the pairwise comparison between the control and the treated groups (160 and 1699 mg/kg b.w./d) were both found to be statistically significant. For benign mammary adenoma, benign mammary fibroadenoma and malignant mammary adenocarcinoma combined the pairwise comparison between control and the 12 mg/kg b.w./d-treated group was found to be statistically significant.

According to the notifier, the incidence of mammary fibroadenoma was well within background range in all ♀ groups. RMS observes that in any case, no increase is seen across treated groups (7/12/8/8), indicating no relationships with the treatment in the first place; however, the incidences are also within HCD range (6.7%-32%)

RMS observes that mammary adenocarcinomas were seen in all treated ♀; the incidences seen in ♀ at 160 mg/kg b.w./d and above (respectively 12% and 10%) were higher than the background historical data of the first HCD (1996-2001) set (up to 6.7%), but below the background historical data of the (more appropriate) second HCD (2001-2006) set (up to 22%). However, it could be argued that the range of the 'updated' laboratory HCD (19 studies) was *varying* from 0-22%, but that 18/19 studies exhibit an incidence rate up to maximally 8%. There was only *one* study, exhibiting 22% of adenocarcinoma. It was questioned what weight should be attributed to one outlier in the HCD, and notifier was of the opinion that the incidence of 22% is not inconsistent with other sources of data from other suppliers and from published references, and that the incidence in this individual study should not be excluded from the historical control dataset. RMS considers the second HCD set as more appropriate, taking into account the 5 years window 2001-2006 as compared to the reference study (2003), and the overall HCD range indeed points to a spontaneous incidence of 6.7-22%), although there could be s some reservation to compare such wide range, knowing that the average HCD value (4.8%) is about half the reference study incidence (10-12%).

Although the control incidence of mammary adenocarcinoma in this study was the same as the lowest recorded HCD incidence (0.0%), it is considered atypical as out of 10 compatible background studies examined, only two had a mammary adenocarcinoma incidence of 0.0%. An increase in mammary adenocarcinomas would normally be associated with an increase in mammary fibroadenomas and acinar hyperplasia (Boorman *et al,* 1990). Although there is an increased incidence of the mammary adenocarcinomas over background range in the intermediate and high dose ♀in this study, in the absence of a similar increase in mammary fibroadenomas ~~and acinar hyperplasia~~, and in the absence of dosage relationship, the increase in adenocarcinomas is not considered to be associated with the administration of Lenacil. RMS observed during renewal that, contrarily to the opinion during first inclusion, some evidence of weakly increased acinar hyperplasia incidence in the ♀ of 44, 50, **52**, **56**% at 0, 16, 160 and 1699 mg/kg b.w./d. Although the increase was not reported as being statistically significant, there was a weak trend, which cannot be completely ignored.

Although RMS considers that the incidence of malignant mammary adenocarcinoma in ♀ at top dose (10%) and at intermediate dose (12%) were slightly outside the historical controls of the laboratory (6.7%) but within the data of [redacted] (13.33%) and of the updated in-house time-matched HCD range (0-22%), and consequently the incidence represents an equivocal finding, it collegially accepts the RAC decision, also keeping in mind the subtle signs of mammary duct hyperplasia observed.

However, it could have been helpful that the notifier would have initiated some mechanistic study in order to further elucidate a possible MoA, confirming or infirming the causal relationship between treatment of the Wistar rat and apparent mammary carcinoma incidence at doses >160 mg/kg b.w./d. **Notifier** highlighted that several *in*

vitro assays were conducted, along with the uterotrophic assay (see B.6.8.3), and that all were negative for oestrogenic activity. RMS agrees with this argument, indicating that lenacil is aunlikely an endocrine disrupting substance, but this does still not explain the slightly higher incidence of mammary adenoma, which, in the absence of genotoxicity, could be driven by another epigenetic MoA.

Table B.6.5.1.2-1 Combined chronic toxicity and carcinogenicity of lenacil in rats over 104 weeks ([redacted] 2003): histopathology - .tumour incidence / laboratory/published HCD

	0 ppm		250 ppm		2500 ppm		25000 ppm	
	♂	♀	♂	♀	♂	♀	♂	♀
Mg/kg b.w./d	**0**	**0**	**12**	**16**	**118**	**160**	**1223**	**1699**
Total animals investigated	50	50	50	50	50	50	50	50
Adrenals								
cortical adenoma + carcinoma	2	2					3	5
pheochromocytoma	1						2	
Liver Hepatocellular adenoma	3	2	0	0	1	2	3	0
Hepatocellular carcinoma	0	0	0	0	1	0	1	0
Pancreas islet cell adenoma	3						5	
Pituitary: adenoma *pars distalis*	10	32					8	25
Leydig cell adenoma	0						**2**	
Thyroid:								
follicular cell adenoma	3	1	2	0	2	1	**5** (10%)	**4***,** (8%)
Laboratory HCD (years)							*0.0-16%*	*0.0 -11.7%*
follicular cell carcinoma	0	2	0	0	1	2	1 (2%)	**4** (8%)▪
HCD Poteracki and Walsh, 1998§							*0.0-1.7%*	*0.0 -3.3%*
HCD Crl Wistar Han rats, 2003							*1.67-3.64%*	*1.82-3.64%*
Thyroid								
C-cell adenoma	4	2	3	2	5	**8*** (16%)▪	5	7 (14%)(▪)
HCD Laboratory adenoma (years)						*0-13.6%*		*0-13.6%*
HCD Crl Wistar Han rats, 2003								*3.64-21.82%*
C-cell carcinoma	0	0	0	**2**	0	0	0	**2****,* (4%)
HCD Laboratory carcinoma (years)								*0-1.7%*
HCD Crl Wistar Han rats, 2003								*0-1.82%*
Uterus								
polyps		5						5
adenocarcinoma		2						5
Mammary gland								
Mammary gland adenoma		0		1		0		**3**** (6%)(▪)
1: HCD Laboratory (1996-2001, N=10)								*0.7% (0.0-2.0%)*
2: HCD Laboratory (2001-2006, N=19)								***1.96% (0.0-5.5%)***
3: HCD RCC Wistar Han rats, <1999								*1.4% (0.0-14.0%)*
4: HCD Crl Wistar Han rats, 2003								*1.4% (1.8-3.6%)*
5: HCD Poteracki and Walsh, 1998§								*3.9% (2.0-6.7%)*
fibroadenoma		7		12		8		8

	0 ppm		250 ppm		2500 ppm		25000 ppm	
	♂	♀	♂	♀	♂	♀	♂	♀
Mg/kg b.w./d	**0**	**0**	**12**	**16**	**118**	**160**	**1223**	**1699**
Mammary gland adenocarcinoma		0		2		**6****(12%)		**5****(10%)
1: HCD Laboratory (1996-2001, N=10)						*3.6% (0.0-6.7%)*		*3.6% (0.0-6.7%)*
2: HCD Laboratory (2001-2006, N=19)						***4.8% (0.0-22.0%)***		***4.8% (0.0-22.0%)***
3: HCD RCC Wistar Han rats, <1999						*5.6% (0.0-18.0%)*		*5.6% (0.0-18.0%)*
4: HCD Crl Wistar Han rats, 2003						*5.5% (1.8-13.3%)*		*5.5% (1.8-13.3%)*
5: HCD Poteracki and Walsh, 1998§						*6.7% (1.7-12.4%)*		*6.7% (1.7-12.4%)*
Total mammary tumours		7 14%		15* 30%		13 26%		10 20%

Statistically significant in *pair wise comparison ** trend test; HCD (N=number of HCD studies) given as average (minimum, maximum)
▪: above (approximately at if between brackets) HCD range; (i.e. years mentioned in the table)
The cited HCD for the mammary adenocarcinoma comprise 5 sources:

1. 10 studies initiated at the test laboratory during 1996-2001 (i.e. immediately prior to the [redacted] study), referred to in the original DAR
2. **19 studies initiated at the test laboratory during 2001-2006 (an updated database)**
3. Published data for HsdRCCHan (Wistar Hannover) rats from 50 carcinogenicity studies performed at RCC (Switzerland) 1981-2006.
4. Published data for Wistar Han Rats from Charles River Laboratories (10 studies terminated in 1999 or earlier).
5. Published data compiled from reviews of tumour incidence in Wistar rats (Poteracki & Walsh, 1998).

Note: RMS considers the **HCD n°2** the most relevant, as it comprises 5 years around the reference study;

§:Cited references:
Poteracki J, Walsh KM, Spontaneous neoplasms in control Wistar rats: a comparison of reviews, Toxicol Sci. 45(1):1-8, 1998.

Conclusion:

According to the RMS,

The NOAEL for oncogenicity should be set at 250 ppm (12 mg/kg bw/d) taking into account the increased incidence of for mammary gland malignant adenocarcinoma and thyroid C-cell adenomata at 2500 ppm (118 mg/kg bw/d).

The notifier concluded that
"the administration of Lenacil technical to Han Wistar rats, via the diet, at concentrations up to 25000 ppm for 104 weeks caused non-specific toxicity in ♀ at 25000 ppm and adaptive and toxic change in the liver in males at 25000ppm. Lenacil technical was not associated with the occurrence of any of the tumours observed in the study. The no-observed-effect level (NOEL) in this study was 250ppm (equivalent to 12.0 mg/kg/day in males and 15.9 mg/kg/day in females) due to slightly reduced motor activity in males at 2500 ppm.
The no-observed-adverse –effect Level (NOAEL) is considered to be 2500ppm, (equivalent to 118 mg/kg/day for males and 160 mg/kg/day for females).
Further comment from notifier:
The notifier suggests that the data support the proposition that the administration of lenacil is not associated with mammary tumour incidence, since the incidence at high dose levels is less than that in background data. The notifier proposes that the same information is used to set a NOAEL for oncogenicity, where, if lenacil is not associated with induction of any of the tumours observed, as concluded by Notifier and supported by RMS in text above, then 2500 ppm is the appropriate NOAEL

According to the notifier, no statistically significant differences for neoplastic lesion were found in ♂. The carcinogenic NOAEL in rats was established at 15.9 mg/kg bw/day. This was based on the incidence of malignant mammary adenocarcinomas observed at the next higher dose level of 160.2mg/ bw/day in ♀, which was above the historical background range of the laboratory but well within the historical control range of the [redacted] database. For this reason, the RMS considered the increased incidence of mammary adenocarcinoma as being an equivocal finding. Since an increase in mammary adenocarcinoma is usually associated with increases in mammary fibroadenomas and acinar hyperplasia and as these associated increases were not observed, the increased incidences in mammary adenocarcinoma were regarded to be unrelated to lenacil treatment by the applicant. In the framework of the assessment of the CLH report by the RAC Committee of ECHA, the increased incidences of mammary adenocarcinoma were considered relevant for humans by the RAC Committee. As a consequence, lenacil was classified as a category 2 carcinogen (Carc. 2; H351). This harmonized classification proposal has meanwhile been adopted (see Regulation (EC) No. 2015/1221) and the substance is included into Annex VI of the CLP regulation (Regulation (EC) No. 1272/2008).

RMS:
It was concluded (ECHA):
Overall, RAC considered that the classification of Lenacil in category 2 for carcinogenicity under CLP (Carc 2 – H351) and carcinogenicity 3 under DSD (Carc. cat. 3; R40) was warranted, **based on some evidence of induction of mammary gland tumours in female rats.**

B.6.5.2 (CA 5.5.2) Carcinogenicity study in the mouse

Oncogenicity study with DPX-B634-91 (lenacil) eighteen-month feeding study in mice ([redacted] 1994)

DuPont Report No.: HLR 336-93

Guidelines: study is not fully in compliance with Dir EEC 87/302/EEC Annex V B or OECD test guideline n° 451 (1981). A review of this study indicates that it partially meets the current OECD Test Guideline 453; deviations include:

- *Differential blood counts were obtained from only ten mice in the control and high dose groups*
- *The ovaries were not weighed*

These deviations are not considered to have had a severe impact on the reliability and the intpretation of the study results. The study is considered valid.

Materials and Methods

Four groups of each 80 ♂and 80 ♀ CRL-CD®-1(ICR)BR were fed diets containing 0, 100, 2500 or 7000 ppm of Lenacil technical (synonyms DPX-B634-91 (B634-91) DPX-B634; IN B634-91(Batch No. 9038, purity 98.2% (reanalysis 98.5%) administered via dietary admixture into the powdered diet during 18 months. The technical material was analysed for stability at the beginning, in the middle and at the end of the study. On test day –1, samples were collected from each dietary concentration to verify concentration, homogeneity and stability. At approximately three-month intervals throughout the study, feed samples were collected for concentration analyses. Measured concentrations ranged from 86.8 to 104% of nominal and appeared to be stable in the diet. The homogeneity was confirmed.

Body weight and food consumption were measured and clinical signs conducted weekly (first three months) or bi-weekly during the remainder of the study. Ophthalmoscopic examinations were performed during pre-test and at study end. 332Haematology and clinical chemistry analyses were conducted after 3, 6, 12 and 18 months. After 18 months, all survivors were sacrificed, selected organs were weighed and tissues examined for the presence of gross or microscopic lesions.

Statistical analyses: bw, bw gain, organ weight, clinical pathology were analysed by analysis of variance. Pairwise comparison between test and control were made with the Dunnett's test. Clinical observations were evaluated by the Fisher exact test with a Bonferroni correction and if significant followed by the Cochran Armitage test for trend. The incidence of all primary neoplastic hyperplastic and compound related non neoplastic lesions and survival among groups observed microscopically were evaluated by the Cochran Armitage test for trend and or the Fisher exact test. The Barletts test for homogeneity of variances was performed on the organ weight and clinical laboratory data. Compound intake was calculated and tabulated as follows:

Dose (ppm)	0		100 ppm		2500 ppm		7000 ppm	
sex	♂	♀	♂	♀	♂	♀	♂	♀
Compound intake (mg/kg bw/d)	0	0	13.8	19.6	332	482	977	1358

The study is accepted.

Findings

(the original overview table was divided in different parts and expanded where deemed necessary)

Mortality: no compound-related mortality was observed.

Table B.6.5.2-1 Carcinogenicity of lenacil in CD mice over 18 months ([redacted] 1994): mortality

Endpoints/dose (mg/kg bw/d)	0		13.8		332		977	
	♂	♀	♂	♀	♂	♀	♂	♀
Mortality (/80)	25	24	23	9	15	18	23	15

Clinical signs: no signs were attributed to the dietary administration of lenacil.

Body weight: mean bw and bw gains of male and female mice were comparable to controls at all dose levels.

Food consumption and efficiency were comparable with controls at all dose levels.

Ophthalmoscopy: at the end of the study the most common ocular findings were unilateral or bilateral central corneal opacities which were not considered to be compound-related.

Table B.6.5.2-2 Carcinogenicity of lenacil in CD mice over 18 months ([redacted],1994): ophthalmoscopy

Endpoints/dose (mg/kg bw/d)	0		100		2500		7000 ppm	
	♂	♀	♂	♀	♂	♀	♂	♀
Ocular opacity %	21	31	14	21	21	22	19	29

Haematology:

Occasional statistically significant findings such as decreases in platelet, total leukocyte, neutrophil, or lymphocyte counts in ♂ and/or ♀ mice were not dose- or time related, and were not considered toxicologically important during the first peer-review. However, in the perspective of the newly triggered discussion, the relevance of haematological parameters was further detailed. The non-dose-dependent modification and the absence of temporality in the effects indicates that the association with treatment remains of uncertain toxicological significance.

Table B.6.5.2-3 Carcinogenicity of lenacil in CD mice over 18 months ([redacted] 1994): haematology

Endpoints	time	Male				Female			
dose (ppm)		**0**	**100**	**2500**	**7000**	**0**	**100**	**2500**	**7000**
dose (mg/kg bw/d)		**0**	**13.8**	**332**	**977**	**0**	**19.6**	**482**	**1358**
RBC (× 10^6/µL)	**3**	9.12 ± 0.43	9.31 ± 0.87	9.77 ± 0.73	9.14 ± 0.77	8.84 ± 0.52	9.19 ± 0.57	9.38 ± 0.73	9.23 ± 0.71
	6	9.29 ± 0.81	9.32 ± 1.88	9.68 ± 0.70	9.89 ± 0.85	9.67 ± 0.63	9.88 ± 0.75	10.08 ± 0.56	10.14 ±0.61
	12	8.74 ± 0.83	8.81 ± 1.21	9.02 ± 0.50	8.94 ± 1.43	8.75 ± 1.81	8.49 ± 0.96	8.81 ± 0.59	9.03 ± 0.52
	18	8.45 ± 2.07	8.94 ± 1.12	8.02 ± 1.47	8.23 ± 1.90	8.09 ± 1.20	8.35 ± 1.02	8.64 ± 0.66	8.31 ± 0.79
Hb (g/dL)		No variation > 10%							
Ht (%)		No variation > 10%							
MCV (fL)		No variation > 10%							
MCH (pg)		No variation > 10%							
MCHC (g/dL)		No variation > 10%							
PLAT (×10^3/mL)	**3**	1054 ± 135	1000 ± 221 (↓5%)	**772[a] ± 238 (↓27%)**	1030 ± 191 (↓2%)	1036 ± 254	782[a] ± 135 (↓25%)	819 ± 185 (↓21%)	796[a] ± 249 (↓23%)
	6	1325 ± 305	1114 ± 264 (↓16%)	1097 ± 133 (↓17%)	1071 ± 257 (↓19%)	973 ± 263	811 ± 110 (↓27%)	882 ± 211 (↓9%)	866 ± 136 (↓12%)
	12	1378 ± 377	1344 ± 572 (↓2%)	1229 ± 187 (↓11%)	1174 ± 303 (↓15%)	914 ± 325	903 ± 222 (↓1%)	944 ± 201 (↓3%)	929 ± 233 (↓2%)
	18	1615 ± 295	**1358[b] ± 196 (↓16%)**	1492 ± 379 (↓8%)	1736 ± 656 (↑7%)	1177 ± 387	1174 ± 270 (=)	1095 ± 175 (↓7%)	1063 ± 368 (↓10%)
WBC (×10^3/µL)	**3**	8.8 ± 1.9	7.0 ± 1.6 (↓20%)	6.3 ± 2.2 (↓28%)	7.8 ± 2.3 (↓11%)	6.6 ± 2.2	5.6 ± 1.4 (↓15%)	5.5 ± 2.8 (↓17%)	4.4 ± 1.7 (↓33%)
	6	8.9 ± 3.2	8.4 ± 4.3 (↓6%)	7.8 ± 2.8 (↓12%)	8.5 ± 5.2 (↓4%)	7.9 ± 2.3	**5.1[a] ± 1.8 (↓35%)**	**5.7[a] ± 1.3 (↓28%)**	6.2 ± 1.7 (↓22%)
	12	8.0 ± 2.6	10.2±7.1 (↑28%)	6.4 ± 1.4 (↓20%)	7.8 ± 2.4 (↓3%)	**5.5* ± 3.4**	4.7 ± 2.6 (↓15%)	5.5 ± 1.8 (=)	4.6 ± 1.1 (↓16%)
	18	10.9 ± 7.1	7.3 ± 2.7 (↓33%)	10.3 ± 5.0 (↓6%)	10.1 ± 4.8 (↓7%)	6.4 ± 3.4	7.5 ± 3.9 (↑17%)	4.4 ± 1.8 (↓31%)	5.8 ±2.8 (↓9%)
NEUT (WBC×%)	**3**	1101 ± 543	967 ± 400 (↓12%)	1036 ± 958 (↓6%)	1199 ± 603 (↑9%)	635 ± 235	692 ± 390 (↑9%)	**437 ± 214 (↓31%)**	**376 ± 237 (↓41%)**

Endpoints	time	Male				Female			
dose (ppm)		0	100	2500	7000	0	100	2500	7000
dose (mg/kg bw/d)		0	13.8	332	977	0	19.6	482	1358
	6	1845 ± 1325	1520 ± 1523 (↓18%)	**845[b] ± 210 (↓54%)**	2335 ± 4477 (↑26%)	891 ± 702	443 ± 160 (↓50%)	514 ± 193 (↓42%)	**404 ± 206 (↓55%)**
	12	1910 ± 904	3064 ± 3432 (↑60%)	1554 ± 837 (↓19%)	1582 ± 960 (↓17%)	1205* ± 1288	779 ± 395 (↓35%)	1031 ± 654 (↓14%)	775 ± 285 (↓36%)
	18	3079 ± 2511	2205 ± 1626 (↓28%)	3602 ± 4587 (↑17%)	3344 ± 2598 (↑9%)	1866 ± 1677	2294 ± 2095 (↑23%)	1044 ± 673 (↓44%)	1267 ± 812 (↓32%)
Band (WBC×%)	3	28 ± 47	6 ± 18	0 ± 0	5 ± 15	0 ± 0	0 ± 0	0 ± 0	4 ± 14
	6	8 ± 25	15 ± 32	0 ± 0	17 ± 36	20 ± 42	0 ± 0	0 ± 0	0 ± 0
	12	15 ± 33	0 ± 0	6 ± 20	0 ± 0	0* ± 0	6 ± 19	44 ± 94	20 ± 27
	18	105 ± 221	4 ± 12	69 ± 140	94 ± 200	27 ± 26	7 ± 21	7 ± 23	22 ± 53
Lymph (WBC×%)	3	6992 ± 1536	5497 ± 6535 (↓21%)	**4868[a] ± 1514 (↓30%)**	6087 ± 1642 (↓13%)	5727 ± 2034	4625 ± 1116 (↓19%)	4868 ± 2557 (↓15%)	3795 ± 1458 (↓34%)
	6	6787 ± 2031	6535 ± 2774 (↓4%)	6432 ± 2451 (↓5%)	5649 ± 1184 (↓17%)	6424 ± 1904	**4293[a] ± 1765 (↓33%)**	**4647[a] ± 1000 (↓28%)**	5281 ± 1566 (↓18%)
	12	5736 ± 2162	6569 ± 3378 (↑15%)	4528 ± 1388 (↓21%)	6023 ± 2031 (↑5%)	3943* ± 1577	3681 ± 2185 (↓7%)	4252 ± 1513 (↑8%)	3581 ± 820 (↓9%)
	18	7330 ± 4270	4242 ± 1286 (↓42%)	5832 ± 2112 (↓20%)	5584 ± 2231 (↓24%)	3900± 1721	4393 ± 2661 (↑13%)	3102 ± 1398 (↓20%)	4083 ± 2645 (↑5%)
	MEAN	*6711*	*5711 (↓15%)*	*5415 (↓19%)*	*5836 (↓13%)*	*4998*	*4248 (↓15%)*	*4217 (↓16%)*	*4185 (↓16%)*
Lymph (WBC×%)	3	28 ± 47	16 ± 35	12 ± 24	28 ± 72	0 ± 0	0[b] ± 0	6 ± 19	14 ± 25
	6	8 ± 25	15 ± 31	15 ± 46	11 ± 24	22 ± 38	8 ± 25	14 ± 30	11 ± 23
	12	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4* ± 12	0 ± 0	0 ± 0	0 ± 0
	18	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Mono (WBC×%)	3	595 ± 356	473 ± 223 (↓21%)	378 ± 176 (↓36%)	431 ± 320 (↓28%)	213 ± 166	252[b] ± 179 (↑18%)	188 ± 143 (↓12%)	144 ± 95 (↓32%)
	6	252 ± 196	321 ± 185 (↑27%)	484 ± 490 (↑92%)	403 ± 237 (↑60%)	454 ± 229	355 ± 199 (↓22%)	469 ± 291 (↑3%)	424 ± 196 (↓7%)
	12	309 ± 300	530 ± 731 (↑72%)	294 ± 217 (↓5%)	220 ± 231 (↓29%)	361* ± 763	190 ± 303 (↓47%)	161 ± 160 (↓55%)	179 ± 183 (↓50%)
	18	1315 ± 1134	792 ± 435 (↓40%)	734 ± 514 (↓44%)	1011 ± 972 (↓23%)	569 ± 362	766 ± 526 (↑35%)	273 ± 274 (↓52%)	406 ± 300 (↓29%)
Eosin (WBC×%)	3	16 ± 33	31 ± 43 (↑94%)	36 ± 41 (↑125%)	41 ± 48 (↑156%)	47 ± 49	22[b] ± 29 (↓53%)	41 ± 51 (↓13%)	47 ± 56 (=)
	6	31 ± 40	25 ± 44 (↓19%)	14 ± 31 (↓55%)	42 ± 43 (↑35%)	60 ± 55	32 ± 50 (↓47%)	16 ± 50 (↓73%)	40 ± 47 (↓33%)
	12	50 ± 38	37 ± 49 (↓26%)	27 ± 49 (↓46%)	25 ± 42 (↓50%)	20* ± 47	14 ± 32 (↓30%)	12 ± 26 (↓40%)	6 ± 18 (↓70%)
	18	85 ± 59	37 ± 60 (↓56%)	33 ± 47 (↓61%)	36 ± 47 (↓57%)	46 ± 63	41 ± 66 (↓11%)	14 ± 36 (↓70%)	61 ±77 (↓33%)

a: $p<0.05$ (Dunnett) b: $p<0.05$ (Mann-Whitney U) *: elimination of leukocytic data from 1 animal (#78741), an outlier.

Organ weight:

Of note, thyroid absolute/relative weights were not measured.

Relative liver weight was increased at top dose. This could be a normal physiological response of the liver to xenobiotic administration, but there is insufficient experimental evidence to assert this position.

However, also spleen weights were low at 332 mg/kg bw/d and above, although even at the lowest dose, spleen weights were >10% lower than average control weights (overall, a dose-dependent trend is observed, further confirming the relationship with treatment). No explanation was provided by the notifier concerning this weight drop. Taking into account the non-dose-dependent modification of WBC counts, but in the absence of any immunotoxicity study, the relevance of this finding should be discussed. The study NOAEL could have been considered <13.8 mg/kg bw/d on the basis of the spleen weight. Therefore, it was discussed with co-RMS which NOAEL was most appropriate. Further inspection of the individual data revealed that there was a higher incidence of the finding "large spleen" in the control groups (both, ♂ and ♀) than in the treated groups. The incidences are 20, 18, 10 and 15 in ♂, and 25, 17, 21 and 13 in ♀, at 0, 100, 2500 and 7000 ppm, respectively. Strikingly, in ♀ control mice there is a very high incidence of multicentric malignant lymphoma. The incidence of this finding is 16, 10, 6, and 4 at 0, 100, 2500 and 7000 ppm (in the two mid-doses not all animals were examined, though). When individual animal data in ♀ are examined, where the effect observed on spleen weights is more pronounced, there is a high inter-individual variability in the control group (2 animals with the highest spleen weights were diagnosed with multicentric malignant lymphoma, animals #78729 and #78744, with relative spleen weights of 4.99% and 3.08%). The high variability inside the control group is also reflected by the high standard deviation (0.63 ± 0.80 % b.w.). Considering this, the relevance of the obvious decrease in spleen weight in ♀ could be considered questionable, especially at the low dose level. In ♂, there is no dose-relationship.

Notifier further explained that there is no trend in ♂, but there does appear to be a trend with the ♀. The ♀ control data is very scattered (absolute spleen weight 0.197 g ± 0.264).

"Historical control data provided by the lab performing the study reported a mean absolute spleen weight in 18-month-old female CD-1 mice of 0.1693 g (based on 381 control mice from 1983 – 1996, reference study is 1994) with a 95% confidence interval of 0.0613 to 0.6984 g. In the lenacil study, 3 control ♀ are outside the 95% confidence interval (#78715, 78729, and 78744 weighing 0.776, 1.673 and 0.979 g, respectively). These weights may have skewed the mean control value higher than normal, making the treated groups look more impacted. If these 3 spleens are excluded, the mean for the control becomes 0.143 g, compared to means of 0.159, 0.133 and 0.126 g for the 100, 2500 and 7000 ppm groups, respectively. Thus, the spleen weight appears lower in the higher dose groups, but the data is influenced, in part, by outliers in the control group."

Since there were no histological changes in the spleen to correlate with the weight changes, hence these changes appear inconsequential (*i.e.* non-adverse) for the notifier.

Therefore, **RMS** did the exercise to exclude three outliers (#78715, #78729, and #78744) from the control group and this exclusion decreased the corresponding mean from 0.197 to 0.143. However, outliers should then also be removed in the other dose-groups. For instance, excluding #78784, #78803 and #78832 (spleen weights 0.527, 1.257 and 0.497) from the 100 ppm group would also decrease the corresponding group mean (from 0.159 ± 0.160 to 0.132 ± 0.066). Other examples of outliers are #78863 (spleen weight 0.619) in the 2500 ppm group,as well as #78954 (spleen weight 0.317) and #78958, (spleen weight 0.292) in the 7000 ppm group. It is of note that removing all outliers in the top-dose group for example (*i.e.* in addition: #78922, #78963, #78982, #78984) taking into account the 95th C.I. as notifier suggests (*i.e.* ≥2× s.d.) would of course have decreased further the average value of the remaining top-dose population), but RMS refrained from doing so, considering that exclusion ≥2×s.d. on a general basis, would be excessive.

The table with average values ± s.d. was extended, and a graphical representation was produced to visualise the decreasing trends accordingly. It was concluded that, when excluding the outlying values in each dose-group, the difference of both absolute spleen weight between the control and the lowest dose-group was not meaningful any more, and that the lowest dose could indeed be considered a NOAEL for the splenic weights, while the mid- and

high dose still exhibited a possible biologically relevant trend towards lower spleen weights, although the difference was mitigated because of the removal of the extreme control values.

Kidney weight was decreased in ♀at all dose levels but did not correlate with any microscopic lesions and was considered by the notifier to be unrelated to lenacil, on which RMS agrees. At top-dose (♂) the incidence of tubular kidney cysts was slightly increased, but the latter was not observed in the ♀.

Table B.6.5.2-3 Carcinogenicity of lenacil in CD mice over 18 months ([redacted],1994): organ weights

		Male				Female			
Dose	**(ppm)**	**0**	**100**	**2500**	**7000**	**0**	**100**	**2500**	**7000**
Dose	**(mg/kg bw/d)**	**0**	**13.8**	**332**	**977**	**0**	**19.6**	**482**	**1358**
Liver	Abs. (g)	1.859 ± 0.468	2.013 ± 0.784	2098 ± 0.824 (↑11%)	2.138 ± 0.705 (↑15%)	1.549 ± 0.326	1.536 ± 0.298	1.564 ± 0.279	1.638 ± 0.218 (↑6%)
	Rel (%)	4.97 ± 1.16	5.33 ± 2.06	5.29 ± 1.71 (↑6%)	**5.77 ±1.60 (↑16%)**	5.04 ± 0.95	4.99 ± 0.75	5.06 ± 0.70	**5.38* ±0.63 (↑7%)**
Kidneys	Abs. (g)	0.817 ± 0.098	0.822 ± 0.098	0.804 ± 0.116	0.782 ± 0.118 (↓4%)	0.602 ± 0.493	**0.526 ±0.067 (↓13%)**	**0.521 ±0.069 (↓14%)**	**0.498 ±0.049 (↓17%)**
	Rel (%)	2.19 ± 0.26	2.18 ± 0.27	2.06 ± 0.37	2.13 ± 0.30	1.96 ± 1.54	**1.72 ±0.26 (↓12%)**	**1.70 ±0.26 (↓13%)**	**1.64 ±0.19 (↓16%)**
Spleen	Abs. (g)	0.133 ± 0.141	0.109 ± 0.043	0.144 ± 0.094	**0.115 ±0.074 (↓14%)**	0.197▲ ± 0.264	**0.159▼ ±0.160 (↓19%)**	**0.133▼ ±0.086 (↓32%)**	**0.126▼ ±0.062 (↓36%)**
	Rel (%)	0.36 ±0.39	0.29 ± 0.12	0.37 ± 0.25	0.32 ± 0.24	0.63 ± 0.80	**0.52 ±0.49 (↓17%)**	**0.43 ±0.28 (↓32%)**	**0.41 ±0.20 (↓35%)**
	Abs. (g) [$]					0.143▼ ±0.103	0.138▼ ±0.08 (↓3.5%)	**0.125▼ ±0.075 (↓13%)**	**0.123▼ ±0.056 (↓14%)**
	Rel (%) [$]					0.462 ±0.312	0.454 ±0.280	**0.405 ±0.196 (↓12%)**	**0.406 ±0.180 (↓12%)**
Adrenals	Abs. (g)	0.008 ± 0.003	0.008 ± 0.003	0.009 ± 0.005	0.009 ± 0.005	0.012 ± 0.003	0.011 ± 0.003	0.012 ± 0.003	0.012 ± 0.003
	Rel (%)	0.0231 ± 0.0092	0.0222 ± 0.0084	0.0224 ± 0.0112	0.0241 ± 0.0077	0.0386 ± 0.0100	0.0377 ± 0.0096	0.0406 ± 0.0106	0.0394 ± 0.0090
Testes	Abs. (g)	0.228 ± 0.040	0.236 ± 0.044	0.227 ± 0.050	0.233 ± 0.039	-	-	-	-
	Rel (%)	0.61 ± 0.12	0.63 ± 0.13	0.58 ± 0.14	0.63 ± 0.11	-	-	-	-

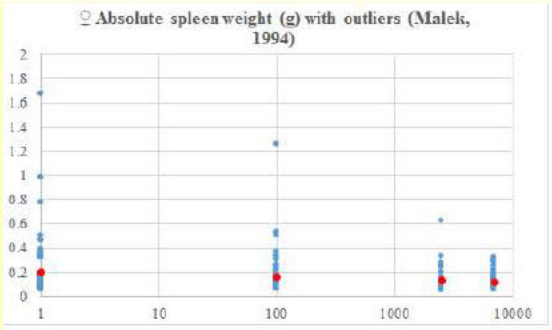
a: $p<0.05$ (Dunnett) b: $p<0.05$ (Mann-Whitney U) ; [$]: excluding outliers as explained in the text above; the excluded outlier values in the 1994 study (as proposed above):

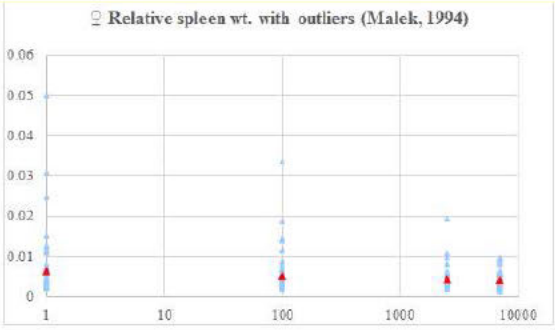
- Control 0.776 g, 1.673 g, 0.979 g;
- 100 ppm: 0.527 g, 1.257 g;
- 2500 ppm: 0.619 g,
- 7000 ppm: 0.317 g, 0.292 g

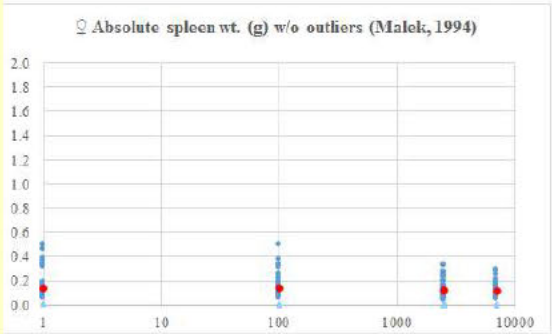
HCD (in-house, 1983-1996 (reference study 1994), N=381)
absolute spleen weight (g) = average 0.163g (95% CI= 0.0613g – 0.6984g), range: min-max=not cited);
study average above (▲) or below (▼) the HCD average, or below the 95th C.I. (▪)

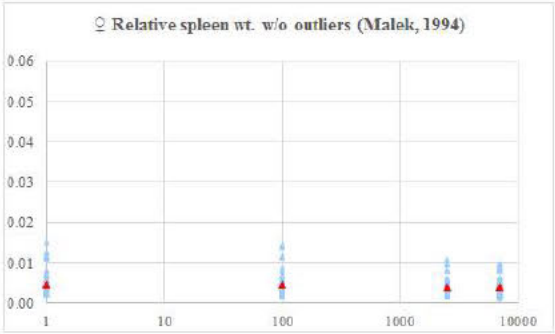
Figure B.6.5.2-1 Carcinogenicity of lenacil in CD mice over 18 months ([redacted],1994): spleen weights

In the scatterplots, values are depicted in the presence (upper panel) and in the absence (lower panels) of cited outlier values. Individual absolute (•) or relative (▲) values are plotted. Average values are in red.









Macroscopic findings:

In ♂mice at top dose, there was an increased incidence of lung masses which was not considered compound related, taking into account the WoE approach considering the lung tumours in the top-dose ♂ not relevant. Liver masses were considered attributable to a toxicologically significant increase in hepatocellular adenomas.

Further, RMS highlights the slightly increased eyes discolouration (332 mg/kg bw/d and above) and exophthalmos (top-dose) in the ♂. Top-dose ♂ also showed slightly elevated incidences of Harderian gland masses (table B.6.5.2-4).

Table B.6.5.2-4 Carcinogenicity of lenacil in CD mice over 18 months ([redacted],1994): gross pathology

Endpoints/dose	**0**		**100**		**2500**		**7000 ppm**	
Dose (mg/kg bw/d)	**0**	**0**	**13.8**	**19.6**	**332**	**482**	**977**	**1358**
	♂	♀	♂	♀	♂	♀	♂	♀
N° examined animals	***80***	***78***	***79***	***79***	***80***	***79***	***80***	***80***
Lung masses	6	3	3	3	6	1	**13**	2
Kidney cyst	8	3	12	4	9	5	13	2
Kidney discolouration	3	7	6	9	8	8	4	4
Eyes discoloration	1	3	2	3	**3**	3	**5**	1
Exophthalmos	0	0	0	0	0	0	**3**	0
Harderian gland masses	0	0	0	0	0	3	**4**	0

Harderian glands exhibited an increased incidence of hyperplasia (possibly explaining the exophthalmos), when compared to the concurrent controls, and RMS concluded that, in the absence of HCD of the latter finding, this top-dose effects could be considered treatment-related.

Histopathology:

Liver:

Centrilobular hypertrophy was observed in ♂ livers and the incidence was low. This effect was considered by the notifier to be the result of the induction of smooth endoplasmic reticulum and an increase in SER-associated enzymes but this was neither demonstrated, nor measured. The centrilobular hypertrophy observed in male mice was not considered as adverse by the notifier. In the absence of data to underpin this position, RMS considers this endpoint adverse.

***Notifier's** opinion: "The conclusion of an expert group of toxicologic pathologists on this matter said that, "hepatomegaly as a consequence of hepatocellular hypertrophy without histologic or clinical pathology alterations indicative of liver toxicity was considered an adaptive and a non-adverse reaction" (Hall et al.,2012). Non-adversity of liver hypertrophy. The lenacil study did not report corroborative evidence of a marked increase in liver toxicity (i.e. necrosis), hence it appears difficult to conclude that this is an adverse effect. Additionally, we now have evidence that lenacil induced liver enzymes in rats, which might bear on findings in other species."*

RMS: karyomegaly is not by definition linked to enzyme induction. Hepatocellular hypertrophy (enzyme induction)—increase in cytoplasmic volume is not typically associated with increased nuclear size or number. Karyomagaly could in this case be associated with the observed liver adenoma in the ♂ and reflects an increase of ploidy (often encountered in ageing mice) reflecting polyploidy not followed by cellular division, and should not be confounded with hypertrophy.

Cited reference:

Hall AP, Elcombe CR, Foster JR, Harada T, Kaufmann W, Knippel A, Küttler K, Malarkey DE, Maronpot RR, Nishikawa A, Nolte T, Schulte A, Strauss V, York MJ. Liver hypertrophy: a review of adaptive (adverse and non-adverse) changes-conclusions from the 3rd International ESTP Expert Workshop. Toxicol Pathol, 40(7):971-994, 2012.

Lung:

There was no significant statistical increase in the incidence of pulmonary alveolar adenomas or adenocarcinoma. However, there was a borderline increase in the combined incidence of alveolar adenomas and adenocarcinoma observed in ♂mice at top dose. Although this increase was significant by Cochran-Armitage trend test, the increase was not significant using the Fisher exact test. The incidence of various alveolar tumours observed in the concurrent control males was similar to those of historical controls in this laboratory, except at top dose. However, it was not considered compound related based on the following reasons:

1. Incidences of adenoma and adenocarcinoma, taken separately, were not statistically increased.
2. There was no statistical significance with the Fisher exact test at p=0.05 for any dose group.
3. There was no decrease in alveolar tumor latency; most tumours were observed in mice killed at terminal sacrifice.
4. There was no increase in focal hyperplasia of type II alveolar cells.
5. There was no shift in tumour cell anaplasia.

Tumours or hyperplasia were identified in testes and harderian gland in the top-dose ♂.
A slight but statistically non-significant increase was observed in the incidence of Leydig cell hyperplasia, which could be considered a putative precursor of benign Leydig cell tumours (no tumours were reported).
The incidence of Harderian gland adenoma was also slightly high, however without attaining statistical significance. Notifier is invited to submit a time-matched HCD set (within 5 years of the reference study of 1994) in order to verify the spontaneous background range of both Leydig cell hyperplasia and of Harderian gland adenoma. Notifier: *"Historical control data for the Harderian gland and Leydig cell hyperplasia in mice has been requested from [redacted]"*

Top-dose ♂ showed a slightly increased incidence of kidney tubular cysts. In this dose-group, lung findings included slightly elevated focal pleural fibrosis, alveolar histiocytosis and focal alveolitis.

Pituitary cysts were also elevated in the top-dose males. Notifier is invited to submit a time-matched HCD set (within 5 years of the reference study of 1994) in order to verify the spontaneous background range of pituitary cysts.
RMS: in top-dose ♂mice there were 6 cases of pituitary cysts *vs.* 2 cases in controls. Notifier stated that pituitary cysts in rodents are non-proliferative lesions and typically represent either the remnants of the craniophyrangeal (Rathke's) pouch (Morton and Tekeli, 1997) or cysts or pseudocysts (Isobe *et al.* 2017). Given the low numbers in this study and their lack of statistical significance, notifier considered this finding incidental (as supported by the interpretation of the study pathologist).
RMS retained the pituitary cyst conservatively as part of the top-dose-effects, but noted also the absence of other pituitary findings, and highlighted the absence of concerning endocrine effects in the thyroids, adrenals, ovaries, and testes, all primarily regulated by the pituitary, whose dysfunction would have caused pleiotropic effects none of which were observed, indicating an uncertain endocrine impact. The cited references are for now insufficient to ignore the effects, and in-house HCD are requested.

Notifier: *"Historical control data for this lesion has been requested from* [redacted] *."*
RMS thus would await the submission of the announced HCD.

Other organs or tissues were not considered having increased adverse histopathological findings when compared to the control animals. In order to verify a possible correlate between spleen findings and the observed decreased spleen weight, histopathological findings of this organs were tabulated (table B.6.5.2-6), but RMS considered that no treatment-related effects could be identified. Therefore, the weight loss is without morphological correlate, and the spleen weight decrease may have another origin.

Table B.6.5.2-5 Carcinogenicity of lenacil in CD mice over 18 months ([redacted],1994): histopathology, non-neoplastic findings

Endpoints/dose	0		100		2500		7000 ppm	
Dose (mg/kg bw/d)	**0**	**0**	**13.8**	**19.6**	**332**	**482**	**977**	**1358**
	♂	♀	♂	♀	♂	♀	♂	♀
N° examined animals	***80***	***78***	***79***	***79***	***80***	***79***	***80***	***80***
Kidney cysts tubular	15 18.8%	7	21 26.6%	10	22 27.5%	14	**25** 31.3%	8
Pleural fibrosis focal	2	1	6	0	5	1	**8**	1
Lung alveolar histiocytosis	6	6	7	8	**12**	5	**12**	5
Lung alveolitis focal	1	2	4	2	9	6	5	4
Pituitary cysts	2	1	0	-	0	-	**6**	1
LIVER: Hepatocellular								
Centrilobular hypertrophy	-	-	-	-	-	-	**7***	-
Karyomegaly	2	-	2	-	**4**	-	**5**	-

Cited references:

- *Morton, D and Tekeli S. 'Have You Seen This' Pituitary Cysts in a Mouse. Toxicologic Pathology 25:333, 1997.*
- *Isobe K, Baily J, Mukaratirwa S, Petterino C, and Bradley A, Historical control background incidence of spontaneous pituitary gland lesions of Han-Wistar and Sprague-Dawley rats and CD-1 mice used in 104-week carcinogenicity studies. J. Toxicol. Pathol., 30:339–344, 2017.*

Table B.6.5.2-6 Carcinogenicity of lenacil in CD mice over 18 months ([redacted],1994): histopathology, neoplastic findings

Endpoints/dose	0		100		2500		7000 ppm	
Dose (mg/kg bw/d)	**0**	**0**	**13.8**	**19.6**	**332**	**482**	**977**	**1358**
	♂	♀	♂	♀	♂	♀	♂	♀
N° examined animals	*80*	*78*	*79*	*79*	*80*	*79*	*80*	*80*
TESTES Leydig cell hyperplasia	7 (8.8%)	-	0	-	3	-	**12 (15%)**	-
Harderian gland adenoma	6	-	2	-	2	3	**9**	1
Urinary bladder								
Lymphoid hyperplasia		11		0		4		17
LIVER: Hepatocellular								
• Adenoma single (s)	11	2	10	0	10	0	11	1
• Adenoma multiple (m)	0	0	**5**	0	**4**	0	**13*,**(16.0%)**	0
Published HCD for adenoma							*0-19%*	*0.0-2%*
Combined adenoma (s or m)	11	2	15	0	14	0	**24*,****	1
• Carcinoma	5	0	3	0	3	0	2	0
Liver tumour, any type	14 (17.5%)	2	16	0	15	0	**25*,** (31.3%)**	1
LUNG alveolar								
• **Adenoma** single (s)	14 (17.5%)	5	9	5	15	4	17 (21.3%)	6
Laboratory HCD (2 studies)							*11.9-16.7%*	
Laboratory HCD (16 studies)							*5.0-17.5%*	
• **Adenoma** multiple (m)	1	1	2	0	0	2	3 (3.8%)	0
Laboratory HCD (2 studies)							*1.6-5.0%*	
Laboratory HCD (16 studies)							*0-6.7%*	
Combined adenoma (s or m)	15 (18.8%)	6	11	5	15	6	**20** (25.0%)	6
Laboratory HCD (2 studies)							*13.6-21.7%*	
Laboratory HCD (16 studies)							*1.8-21.7%*	
Published HCD (Crl 1995)							*0.0-26.0%*	
• **Carcinoma** single (s)	3 (3.8%)	3	4	4	4	2	**8 (10.0%)**	2
Laboratory HCD (2 studies)							*0.0-5.1%*	
Laboratory HCD (16 studies)							*2.5-11.3%*	
• **Carcinoma** multiple (m)	1	1	0	0	2	0	0	0
Laboratory HCD (2 studies)							*0.0*	
Laboratory HCD (16 studies)							*0.0-2.5%*	
Combined carcinoma (s or m)	4 (5%)	4	4	4	6 (7.5%)	2	**8 (10%)**	2
Laboratory HCD (2 studies)							*0.0-5.1%*	
Laboratory HCD (16 studies)							*0.0-12.5%*	
Published HCD (Crl 1995)							*0.0-23.2%*	
Lung tumour, any type	18 (22.5%)	10	15	8	18	7	**26*** (32.0%)	8
Laboratory HCD (2 studies)							*18.6-21.7%*	
Laboratory HCD (16 studies)							*3.8-25.0%*	
Published HCD (Crl 1995)							*n.a.%*	

*p<0.05 for Cochran Armitage trend test and for ** Fisher exact test; n.a.: not available

Historical control data from laboratory (initially, only 2 HC control groups were available due to a shift of breeding colony in this animal supplier, but notifier extended its HCD -see below) and Published historical control data from Charles River laboratories, 1995, Crl: CD-1 BR mouse

Lung tumours are known to occur in CD-1 mice (and particularly in ♂ CD-1 mice) with a high spontaneous incidence. The relevance of the alveolar tumours seen in the Lenacil study was therefore compared against 3 sources of historical control data:

- Data from 2 studies performed by the test laboratory and presented in the original study report.
- More extensive background data for the test laboratory (16 studies initiated between 1983-2000)
- Published data for CD-1 mice from Charles River Laboratories (25 studies performed from 1988-1995).

Table B.6.5.2-6 Carcinogenicity of lenacil in CD mice over 18 months ([redacted],1994): details on spleen histopathology

Endpoints/dose	0		100		2500		7000 ppm	
Dose (mg/kg bw/d)	**0**	**0**	**13.8**	**19.6**	**332**	**482**	**977**	**1358**
	♂	♀	♂	♀	♂	♀	♂	♀
N° examined animals	***80***	***78***	***32***	***24***	***24***	***31***	***79***	***78***
NON-NEOPLASTIC findings								
Amyloid	0		3		1		0	
Atrophy (lymphoid depletion)	3	2	0	1	0	2	1	2
↑Extramedullary haematopoiesis	22	11	19	11	13	17	19	7
↑Haemosiderin	0	4	0	1	1	3	0	5
Lymphoid hyperplasia	3	8	2	2	4	4	5	**12**
Plasmocytosis	4	1	1	1	1	0	2	0
Splenitis	0	0	1	0	2	0	0	1
NEOPLASTIC findings								
Haemangiosarcoma^{M}	0	1	2	0	0	0	0	1
Histiocytic sarcoma^{M} (multicentric)	0	0	0	0	1	1	0	0
Lymphoma^{M} (multicentric)	4	16	5	10	2	6	3	4

Apart from a marginal higher lymphoid hyperplasia in the top-dose ♀, which relevance is equivoval in the view of the poor dose-response, no histopathologically relevant finding could be detected in the spleen, neither in the ♂ the nor in the ♀. However, although of probably another origin, it is considered that the slightly decreased spleen weight at the mid- and top-dose onwards should not be disregarded.

Comment from RMS on the liver and lung histopathology:

LIVER:

The notifier did not provide the laboratory historical control data for liver tumours and RMS used historical control data published by Charles River laboratories for Crl:CD-1 BR mice, 1995. The incidence of liver cell adenoma multiple reported in males at top dose (16%) is within the maximum range of historical control data at [redacted] (19%).

Both RMS and Co-RMS noted that, ccording to RAC, there is equivoqual evidence of carcinogenicity of Lenacil in the mouse liver. Laboratory historical control data were not provided. It would be helpful if the notifier would also submit a time-matched HCD set for this endpoint.

LUNG:

The incidence of 17/80 (21%) lung alveolar adenomas for ♂at top-dose is slightly above the maximum range of historical control data at the testing laboratory (16%) and at [redacted] (12%). The incidence of 8/80 (10%) alveolar carcinomas in the same dose-group is above the maximum range of historical control data at the testing facility (0%) but inside [redacted] (21%) and not statistically significant.

The number of any type lung alveolar neoplasms in ♂at top-dose is also slightly increased (26/80, 32%) compared to the concurrent untreated control (18/80, 22.5%), it is statistically significant ($p<0.05$) and is outside the range of the historical controls at the testing facility (18-21%). However, because this increase is small, and did not

demonstrate decreased latency compared to controls, it is considered to represent only equivocal toxicologic significance.

The discussion of the mouse lung tumours was concluded as follows in the RAC (ECHA C&L experts)

- The incidences of single adenomas (11.3-21.3%) are comparable to the laboratory's very limited historical control data of 11.9-16.7%. Although it is noted that the tumour incidences in males at 2500 ppm (18.8%) and 7000 ppm (21.3%) lie outside the historical range, the fact that the laboratory's background incidence is derived from only two studies and that the range is only slightly exceeded in the Lenacil study does not provide a strong indication that the tumours are treatment-related. It is also notable that the concurrent control incidence of 17.5% exceeds the historical range. More extensive historical control data from the performing laboratory provided a background range of 5.0-17.5%. The marginal increase in the incidence of tumours seen in the Lenacil study at dose levels of 2500 ppm (18.8%) and 7000 ppm (21.3%) compared to that in the concurrent control group (17.5%) cannot be considered to be treatment-related in the absence of statistical significance and considering that the incidence in controls is also at the upper limit of the background incidence of this tumour type. The incidence of multiple alveolar adenomas was not significantly increased and was below the laboratory HCD.
- The incidences of total *(i.e.* single or multiple) adenocarcinomas in the Lenacil study are 3.8-10.0%. Although it is noted the tumour incidence at 7000 ppm (10.0%) lies outside the laboratory's original historical range (0-5.1%) reported in the study report, the incidence is clearly within the range (0.0-12.5%) based on the more extensive laboratory data.
- Overall, a significantly increased incidence of alveolar tumours is observed in male mice at the highest dose. The incidence is above laboratory historical control data. However, several studies in the literature provide evidence of the high incidence of bronchoalvealoar tumours in CD-1 male mice, up to 61.1% (Manenti, 2003), 43% (Fox, 2007) and 33.4% (Maita, 1988).
- Besides, it is noted that lung is not a target organ of Lenacil toxicity and that the observed increase was restricted to males.
- The link between the induction of bronchoalveolar tumours and Lenacil is therefore uncertain.

In conclusion, this confirms that the contribution of the mouse lung tumours do not contribute to the C&L of lenacil as a Cat.2 carcinogen.

Conclusion from the RMS (18 month mouse carcinogenicity study):

The NOAEL's, established during the previous peer-review (initial inclusion of lenacil) were confirmed.

Toxicity NOAEL = 100 ppm = **13.8 mg/kg b.w./d**

Toxicity LOAEL = 2500 ppm = 332 mg/kg b.w./d, based upon eye discolouration, ↓kidney and ↓spleen w, ↑hepatocellular karyomegaly

At top-dose (7000 ppm, equivalent to 977 mg/kg b.w./d) findings included:

Exophthalmos, ↑liver w, ↑alveolar histiocytosis, ↑hepatocellular CL hypertrophy, ↑pituitary cysts, ↑kidney tubular cysts.

Carcinogenicity NOAEL= 2500 ppm = **332 mg/kg b.w./d**

Carcinogenicity LOAEL = 7000 ppm = 977 mg/kg b.w./d,

based upon the increased incidence of lung alveolar carcinoma (♂) and liver adenoma (♂).

B.6.5.3 Summary of long-term toxicity and carcinogenicity

Results of long-term toxicity and carcinogenicity studies with lenacil are summarised below.

Table B.6.5.3 – 1: Summary of chronic toxicity studies for lenacil

Type of test, test species, doses *(ppm)* - mg/kg b.w./d	**Batch n°, purity (%)**	**NOAEL (mg/kg b.w./d)**	**LOAEL, critical effect (mg/kg b.w./d)**	**Reference**
(B.6.5.1.1) 2 yr- oral, diet, Wistar rat *(0, 250, 2500, 25000 ppm)* ♂: 0, 12, 118, 1223 mg/kg bw/d ♀: 0, 16, 160, 1699 mg/kg bw/d	Batch No. 141712003, purity 98.6%	250 ppm = **12 mg/kg bw/d**	**Chronic toxicity:** 2500 ppm = 118 mg/kg bw/d: Clinical signs (perigenital staining, eye discolouration°), ↓motility°, ↓lymphocyte count, ↑triglyceride level, ↑adrenal w, ↑thyroid weight°, enlarged thyroid, fluid distended/ hyperplastic uterus. Top-dose (25000 ppm =1223 mg/kg bw/d): clinical signs (skin/subcutis exfoliation/scabs, ventral swollen/firm areas, exophthalmos°), thin appearance, ↓body w (gain) °, proteinuria°, haematology (↓platelet count, ↓APTT,) ↓A/G ratio, ↑TSH, ↑organ weight° (spleen, kidney, liver), thyroids black°/luminal concretions°, enlarged/swollen spleen, small testes, adrenal ceroid accumulation and accessory tissue, liver CL hypertrophy°	[redacted] 2003
(B.6.5.1.2) 2 yr- oral, diet, Wistar rat *(0, 250, 2500, 25000 ppm)* ♂: 0, 12, 118, 1223 mg/kg bw/d ♀: 0, 16, 160, 1699 mg/kg bw/d	Batch No. 141712003, purity 98.6%	250 ppm = **12 mg/kg bw/d**	**Carcinogenicity:** 2500 ppm = 118 mg/kg bw/d: ↑mammary adenocarcinoma° and thyroid C-cell adenoma° Top-dose (25000 ppm =1223 mg/kg bw/d): ↑adrenal cortical adenoma and mammary adenoma	[redacted] 2003
(B.6.5.2.1) 18 month- oral, diet, CD-1 mouse *(0, 100, 2500, 7000 ppm)* ♂: 0, 14, 332, 977 mg/kg bw/d ♀: 0, 20, 482, 1358 mg/kg bw/d	Batch No. No. 9038, purity 98.2%-98.5%	**Chronic toxicity:** 100 ppm **=14 mg/kg bw/d** **Carcinogenicity:** 2500 ppm **=332 mg/kg bw/d**	**Chronic toxicity:** 2500 ppm = 332 mg/kg bw/d : eye discolouration, ↓kidney w, ↓spleen w ↑hepatocellular karyomegaly Top-dose (7000 ppm =977 mg/kg bw/d): Exophthalmos, ↑liver w, ↑alveolar histiocytosis, ↑hepatocellular CL hypertrophy, ↑pituitary cysts, ↑kidney tubular cysts **Carcinogenicity:** 7000 ppm = 977 mg/kg bw/d: ↑lung alveolar carcinoma° (♂), liver adenoma° (♂). Limited ↑Leydig cell hyperplasia and Harderian gland adenoma	[redacted] 1994

°: findings reported during the first peer review of lenacil

-In rats, the systemic NOAEL was determined to be 12 mg/kg b.w.d, based on clinical signs, decreased motility, decreased WBC counts, increased adrenal and thyroid weights (confimed by macroscopically enlarged thyroid) and fluid distended and hyperplastic uterus at 118 mg/kg b.w./d. Clinical chemistry findings at LOAEL were limited to increased cholesterol levels.

At top dose, further clinical signs (skin, eye) were detected. Top-dose animals exhibited haematological modifications (decreased platelet count, APTT), decreased A/G ratio and increased TSH level (however without effect on T_3-T_4). Increased weights were observed in spleen, kidney and liver.

The relevance of the liver, thyroid and spleen findings was corroborated by hypertrophy, discolouration of thyroids, and enlarged spleens respectively. Other notorious top-dose findings included increased incidences of alveolar histiocytosis, pituitary and kidney tubular cysts.

-In mice, the systemic NOAEL was determined to be 14 mg/kg b.w./d, based on spleen weight (exhibiting a dose-responsive decrease at the two highest doses). Major findings at the higher doses (mid and/or top-dose) included ocular effects, and increased liver weight associated with centrilobular hypertrophy. Other top-dose findings included increased incidences of alveolar histiocytosis, pituitary and kidney tubular cysts.

In concordance with observations in subchronic studies in rodents and dogs, the relevance of effects on the white blood cell compartment was questioned. Treated animals showed consistently alterations of WBC counts, however, often without a proper dose- or time-response. In the absence of any study investigating the immunotoxic potential of lenacil, the findings remain without explanation.

Thyroid and mammary gland tumours were observed in ♀ rats. Thyroid follicular cell adenomas were borderline within laboratory historical control data and C-cell tumours were considered as age and gender-dependent.

Therefore, thyroid tumours were not considered toxicologically relevant in terms of human exposure. Based on the incidences of mammary adenocarcinomas, the carcinogenic NOAEL in rats was established at 12 mg/kg b.w./d.

In the oncogenicity study in mice, liver and lung tumours were observed in ♂treated at the top-dose. The incidence of multiple liver adenomas was outside the laboratory historical control range but was covered by historical control data of [redacted] for Crl:CD-1 BR mice. The incidence of lung single alveolar adenoma was above the laboratory historical control range, but the incidence of lung single alveolar carcinoma was within the laboratory historical control data. When taken together, the combined lung adenoma and carcinoma incidence was outside the laboratory historical control data but it is presumably because of the adenoma incidence. However, because the increase was small and did not demonstrate a decreased latency period. Lung and liver tumours were considered of equivocal relevance for humans. The carcinogenic NOAEL in mice was established at 332 mg/kg bw/day.

The incidence of malignant mammary adenocarcinoma was above the historical background range of the laboratory but well within the historical control range of the [redacted] database. For this reason, the RMS considered the increased incidence of mammary adenocarcinoma as being an equivocal finding. Since an increase in mammary adenocarcinoma is usually associated with increases in mammary fibroadenomas and acinar hyperplasia and as these associated increases were not observed, the increased incidences in mammary adenocarcinoma were regarded to be unrelated to lenacil treatment by the notifier.

Summary and discussion of neoplastic findings in the rat carcinogenicity study

The data indicate a weak relationship to treatment with lenacil for the mammary gland findings in ♀ rats observed in the carcinogenicity study. The incidence of macroscopically observed masses was significantly higher in the low dose group only. Incidences of all tumours (adenoma, fibroadenoma and adenocarcinoma) lie within the range of published historical control data. Statistically significant increases in the incidence of adenocarcinoma are additionally not considered to be treatment-related due to their association with an

unusually low concurrent control value, the absence of correlative findings and in the absence of a dose-response relationship.

Summary and discussion of neoplastic findings in the mouse carcinogenicity study

Incidences of all tumours (single or multiple adenoma or adenocarcinoma) lie within the range of published historical control data. Published historical control data for male CD-1 mice from [redacted] report adenoma and adenocarcinoma incidences of 0.0-26.0% and 0.0-23.2%, respectively. The adenoma and adenocarcinoma incidence in all groups of male mice in the study lies within the background range. It can therefore be concluded that the adenoma and adenocarcinoma incidences in the mouse study is not related to treatment. Furthermore, there is insufficient evidence to indicate that lenacil induces bronchoalveolar tumours in CD-1 mice. The very high spontaneous occurrence of this tumour type in CD-1 mice is well known and means that they should not be used as a basis for classification of carcinogenicity. This conclusion is supported by the fact there were no statistically significant increases in the individual tumour types (i.e., single or multiple, adenoma or adenocarcinoma) or when total alveolar tumours were evaluated alone by the Fisher's exact test. Further, there was no decrease in tumour latency as most tumours were observed in animals at the end of the eighteen-month exposure period. There was no increase in focal hyperplasia of type II alveolar cells and no shift in tumour cell anaplasia. Finally, there was no treatment-related tumour response in females.

In the meanwhile, lenacil has been evaluated at ECHA onder Reg. (EU) no 1272/2008. The RAC evaluated the rat and mouse tumours, and concluded as follows:

Summary of the Dossier submitter's (BE) proposal

The DS reported the EFSA Conclusion on the peer review of Lenacil (2009) in which an increased incidence of malignant mammary adenocarcinoma in the rat carcinogenicity study was considered of relevance for humans. In the mouse carcinogenicity study, increased incidences of single, alveolar lung tumours (adenoma and carcinoma) and multiple liver adenomas were observed and were considered to be of equivocal relevance for humans. Based on the findings of mammary gland tumours in female rats and lung tumours in male mice, EFSA proposed classification as a Category 3 carcinogen under DSD (R40; 'Limited evidence of a carcinogenic effect') for Lenacil.

The significance of these findings was considered in the CLH report by the DS in the light of more extensive historical control data. According to the DS, evidence of the carcinogenic potential of Lenacil is equivocal and no mechanism of oncogenicity was established. Data from carcinogenicity studies in rats and mice, together with background incidence rates derived from various historical databases, supported the conclusion that Lenacil administration was not associated with a toxicologically significant increase in mammary tumour incidence. Similarly, pulmonary tumours in male mice were also shown to fall within historical ranges and no clear evidence of a treatment-association with Lenacil was established.

Overall, the DS concluded that Lenacil was not carcinogenic and no classification was proposed for carcinogenicity.

Comments received during public consultation

One MSCA and one company indicated their general support for the no classification proposed by the DS. One MSCA specifically mentioned its support for no carcinogenicity classification in contrast to the EFSA conclusion when considering the additional information provided on historical control values.

Assessment and comparison with the classification criteria

Various tumour types are induced by Lenacil in both rats (females and males) and male mice. They are discussed separately below:

Induction of thyroid tumours in female rats:

- The incidence of follicular cell adenoma was significantly increased in high-dose females but remained within the historical control data (HCD) for the laboratory. The incidence of carcinomas was not elevated at any

dose when compared to the controls. The incidence of combined adenomas and carcinomas was within the HCD for adenomas only and there was no evidence that Lenacil induced follicular cell tumours.

- An increased incidence of C-cell adenomas was observed in females, which was not (although borderline) statistically significant at mid-dose (p=0.051). The incidence exceeds the laboratory HCD at the two highest doses but without clear dose-response relationship. Two females in the high dose group had C-cell carcinomas. This incidence is above available HCD. A dose-response was observed for the incidence of combined C-cell tumours.

- Thyroid is a target organ of Lenacil in rats. The effects consist mainly of dark appearance of the thyroid. Microscopically, lipofuscin staining of the follicular epithelium indicates membrane degradation and in follicular cell hypertrophy. However, no microscopic treatment-related effects were reported in C-cells. The primary function of C-cells is to secrete calcitonin that reduces the blood calcium level. No effect was reported on calcium homeostasis in the 90-day study in the rat studies. Calcium levels were significantly decreased in males but significantly increased in females at the end of the carcinogenicity study at all doses but without a dose-response so, a link to treatment was unclear.

- Contrary to humans in which there is no great change in C cells with age, laboratory rats show an age-related increase in the number of C-cells and this may correlate with the fact that tumours of the C cells are relatively common findings in aged rats (Thomas & Williams, 1999), in particular in females as stated in the CLH report.

- Overall, considering that the incidence of C-cell tumours in female rats was marginally above HCD, there is equivoqual evidence of carcinogenicity of Lenacil on the thyroid in the rat.

Induction of mammary gland tumours in female rats

- The incidence of mammary adenoma was elevated in high-dose females (6%); this value was not statistically significant using a pair-wise comparison but attained statistical significance (p =0.028) using a trend test. The incidence of this benign tumour very marginally exceeds the laboratory's historical control range (0-5.5%).
- The incidence of benign fibroadenoma was not increased significantly or above HCD.
- The incidences of mammary adenocarcinoma in the mid- and high-dose groups of 6/50 (12%) and 5/50 (10%) respectively are significantly increased when compared to the concurrent control incidence of 0/50 (0%), but without a clear relationship to dose level. However, the absence of findings in the concurrent control is unusual and was seen only in one of the 19 studies constituting the updated laboratory historical data (mean HCD incidence 4.81%). The statistical significance of the findings at 2500 and 25000 ppm is therefore attributable to an unusually low concurrent control incidence. The incidences of this tumour type in the 2500 ppm and 25000 ppm dose groups lie within the laboratory's overall, updated historical control range (0-22%). However, detailed analysis of the distribution of HCD shows that the upper incidence of 22% was observed in a single study out of 19 and the maximum value in the 18 other studies was 8%. After exclusion of this outlier, the incidences of mammary adenocarcinomas were slightly above HCD at the mid- and high doses.
- The incidence of combined mammary adenomas and adenocarcinomas was not provided but a dose-response is likely for combined tumours (although this calculation may overestimate cumulative incidences as some animals may bear both adenomas and adenocarcinomas, addition of adenomas and adenocarcinomas incidences result in incidences of 0, 6, 12 and 16% in females exposed to 0, 250, 2500 or 25000 ppm).
- Combined incidences for all mammary tumour types (fibroadenomas, adenomas and adenocarcinomas revealed no dose-response relationship, with a statistical significant increase of tumours only at the low dose that is mainly due to fibroadenomas, but within HCD (and below mean HC incidence) for this tumour type alone.
- Overall, the incidence of adenocarcinomas in the mammary gland is significantly increased and elevated compared to expected incidence based on the analysis of HCD at the mid- and high-dose. With the support

of an elevated incidence of adenomas at the highest dose and an apparent dose-response when adenomas and adenocarcinomas are added, there is some evidence of carcinogenicity of Lenacil on the mammary gland in the rat.

Induction of liver adenomas in male mice

- No increase of liver single adenomas was observed. The incidence was similar in controls and high dose males.
- A statistically significant increase of multiple adenomas was observed in high dose males.
- Laboratory historical control data were not provided. Although of lower relevance, historical control data at [redacted] were considered but the incidence of liver cell multiple adenoma reported in males at the highest dose (16%) is within the maximum range of historical control data at [redacted] (28%, single or multiple type not specified). Cumulative incidence of single and multiple adenomas at the high dose (30%) is slightly above this HCD.
- No increase of liver carcinomas was observed.
- Incidence and historical control data for combined hepatocellular adenomas and carcinomas were not provided and no conclusion is possible on a combined analysis of tumours.
- Considering the lack of effect observed on hepatic single adenomas and carcinomas and that only benign tumours were increased, the significance of the isolated increase of multiple adenomas is unclear. There is equivoqual evidence of carcinogenicity of Lenacil in the mouse liver.

Induction of lung alveolar tumours in male mice

- The incidences of single adenomas (11.3-21.3%) are comparable to the laboratory's very limited historical control data of 11.9-16.7%. Although it is noted that the tumour incidences in males at 2500 ppm (18.8%) and 7000 ppm (21.3%) lie outside the historical range, the fact that the laboratory's background incidence is derived from only two studies and that the range is only slightly exceeded in the Lenacil study does not provide a strong indication that the tumours are treatment-related. It is also notable that the concurrent control incidence of 17.5% exceeds the historical range. More extensive historical control data from the performing laboratory provided a background range of 5.0-17.5%. The marginal increase in the incidence of tumours seen in the Lenacil study at dose levels of 2500 ppm (18.8%) and 7000 ppm (21.3%) compared to that in the concurrent control group (17.5%) cannot be considered to be treatment-related in the absence of statistical significance and considering that the incidence in controls is also at the upper limit of the background incidence of this tumour type. The incidence of multiple alveolar adenomas was not significantly increased and was below the laboratory HCD.
- The incidences of total (i.e. single or multiple) adenocarcinomas in the Lenacil study are 3.8-10.0%. Although it is noted the tumour incidence at 7000 ppm (10.0%) lies outside the laboratory's original historical range (0-5.1%) reported in the study report, the incidence is clearly within the range (0.0-12.5%) based on the more extensive laboratory data.
- Overall, a significantly increased incidence of alveolar tumours is observed in male mice at the highest dose. The incidence is above laboratory historical control data. However, several studies in the literature provide evidence of the high incidence of bronchoalvealoar tumours in CD-1 male mice, up to 61.1% (Manenti, 2003), 43% (Fox, 2007) and 33.4% (Maita, 1988).
- Besides, it is noted that lung is not a target organ of Lenacil toxicity and that the observed increase was restricted to males.
- The link between the induction of bronchoalveolar tumours and Lenacil is therefore uncertain

- Overall, RAC considered that the classification of Lenacil in category 2 for carcinogenicity under CLP (Carc 2 – H351) and carcinogenicity 3 under DSD (Carc. cat. 3; R40) was warranted, based on some evidence of **induction of mammary gland tumours** in female rats.

B.6.5.4 Mechanism of action for carcinogenicity and supporting data

In the expert statement on the assessment of the toxicological relevance of the groundwater metabolites of lenacil, the potential mode of action (MoA) of carcinogenicity of lenacil and its groundwater metabolites was also addressed in order to build a scientific basis for the performance of a health-based risk assessment on both lenacil and its groundwater metabolites (Kurubaran, S., 2016 (Vol.3 **B.6.8.1**). As the MOA for the induction of mammary tumours in ♀rats as observed in the carcinogenicity study with lenacil is unknown and as the absence of a genotoxic potential of lenacil does not suggest that mammary gland tumours are caused by a genotoxic MoA.

An epigenetic or an endocrine-mediated MoA could be the cause for the induction of these tumours. In order to prove or disprove an endocrine-mediated MoA of carcinogenicity, the potential endocrine disrupting (ED) properties of lenacil including its groundwater metabolites have been investigated in a first step using the OECD toolbox, considering the profilers oestrogen receptor (ER) binding and rtER Expert System ver. 1 – USEPA.

Lenacil revealed no structural alerts for ED properties in the rtER Expert System ver. 1 – USEPA profiler and was found to be a non-binder, without OH or NH_2 group in the ER binding profiler.

Based on the outcome of the OECD Toolbox screen, it could be anticipated in a first step that lenacil is not expected to display ED properties. An endocrine-mediated MOA for the induction of mammary tumours observed in the carcinogenicity study with lenacil was therefore not expected on the base of limited evidence from QSAR estimations, Notifier therefore considered that an endocrine MoA would have been *"very unlikely"* (Kurubaran, S., 2016).

Whereas **RMS** acknowledged the absence of ED MoA on the base of *in-silico* considerations, the renewal dossier could not be based solely on such considerations, and notifier was invited by RMS to make the evidence more robust.

Notifier planned and conducted the necessary Level 1 OECD EDS *in-vitro* screening assays on lenacil, as well as a level 3 study (uterotrophic assay). It would have been useful to conduct the same set of assays with the lenacil groundwater metabolites as well, but it appeared that it seems not possible to synthetise these metabolites at sufficient quantities to conduct such assays with the metabolites.

The endocrine evaluation is further detailed in section **B.6.8**. New studies were submitted by the notifier and an ED evaluation is performed.

Overall, **RMS** concluded that the possible emergence of mammary tumours, on which the C&L was based, was unlikely of endocrine origin, in the absence of evidence on oestrogenic effects of the a.s. Since lenacil is also considered devoid of genotoxic potential, no plausible explanation could be proposed for the lenacil tumourogenic potential.

B.6.6 (CA 5.6) Reproductive toxicity

A two-generation reproduction study was conducted in rats with lenacil and is summarised under CA 5.6.1 in this document. Developmental toxicity/teratogenicity studies in the rat and rabbit were also conducted and are summarised under CA 5.6.2 of this document.

B.6.6.1 (CA 5.6.1) Two-Generation study in the rat

B.6.6.1.1 Preliminary study

Lenacil technical: preliminary study of effects on reproductive performance in Han Wistar rats by dietary administration ([redacted] 2002) - DuPont Report No.: ACD 019/010186

Guidelines: Not stated in the report.

The preliminary reproductive toxicity study in ♂ and ♀ Han Wistar rats (ACD 019/010186) was originally submitted under EU Rev8 Point IIA 5.6.1, 5.8.2 and has been conducted with lenacil technical. The guidelines used were not reported. A review of this publication indicates that it does not meet the current guideline OECD Test Guideline 416 and has been superseded with ACD 020/023865.

The study is considered to provide complementary information.

Materials and Methods

See also materials and Methods in B.6.6.1.2 (DuPont Report No.: ACD 020/023865)

In the preliminary study, Lenacil technical (Batch No. 141712003, purity 98.6%) was administered continuously, at 0, 10000, 25000 and 50000 ppm, via the diet, to rats (Hsd Brl Han Wistar strain), through two successive generations. The F_0 generation comprised 8 ♂and 8 ♀per group, which were treated for 14 days prior to pairing, throughout pairing, during gestation and lactation and up to termination. Selected F1 animals, 12 ♂and 12 ♀in each group, received the treated diet from weaning up to completion of physical sexual maturation. The mean concentrations of lenacil technical in formulations prepared for dosing during weeks 1 and 12 of the study ranged from 95.2 to 103% of nominal concentrations.

Preliminary study: conversions

Dose (ppm)	10000		25000		50000	
Compound intake mg/kg bw/d	♂	♀	♂	♀	♂	♀
Prior pairing	**749**	**755**	**1952**	**2003**	**3840**	**4014**
Gestation		998		2406		4965
Lactation		1714		4555		9350
Mean achieved dose F_1 prior pairing	1314	1345	3173	3307	6814	6964

Findings

First generation (F_0):

Mortality: No F_0 animals died or were killed prematurely during the study.

Clinical signs: there were few signs at routine weekly physical examination that could be attributed to treatment.

RMS: at top-dose, an increase in occurrences of dorsal alopecia (F_0, F_1) and of brown staining (F_0) is observed, as also seen in the main study B.6.6.1.2.

Body weight:

Overall, body weight gain for the F_0 ♂was marginally affected by treatment. For ♀ at top dose, overall body weight gain prior to pairing was slightly low when compared to control (86% of the controls).

During lactation, the body weight gain from day 7-14 was low with an apparent dose response resulting in the overall low weight gain during lactation. Bodyweights in treated ♀during the second week of lactation were also

lower **(table B.6.6.1.1-2)**. Overall, body weight modifications were weak and/or poorly dose-dependent, except prior pairing in F_0 ♀generation.

Food consumption:

Prior pairing, and during gestation and lactation food consumption was unaffected by treatment (**table B.6.6.1.1-2).**

Second generation (F_1):

Clinical signs: there were no signs at routine weekly physical examination that could be attributed to treatment. No F_1 animals died or were killed prematurely during the study (see table B.6.6.1.1-1).

Body weight: the absolute body weight of treated rats on day 0 and the subsequent body weight gain up to termination during week 5 of the F1 generation did not show any adverse effect of treatment.

Food consumption was not affected and food conversion efficiency was slightly low at top dose.

Table B.6.6.1.1-1 Lenacil: preliminary 1-generation study ([redacted] 2002): clinical signs.

Endpoints/Dose		Males				Females			
Dose (ppm)		0	10000	25000	50000	0	10000	25000	50000
Coat, hairloss, dorsal body surface	F0	-	-	**1**	**1**	-	-	-	**1**
	F1	-	-	-	**1**	-	**1**	**1**	**2**
Coat, hairloss, head	F0	2	-	-	-				
	F1	-	-	**1**	-	**1**	-	-	-
Coat, hairloss, right forlimb	F0	-	-	-	-	-	-	2	-
	F1	-	-	-	-	-	-	-	-
Staining, brown, head	F0						**1**		
	F1	-	-	-	-	-	-	-	-
Staining, brown, upper dorsal thorax	F0	-	-	-	-	**1**	**1**	-	**2**
	F1	-	-	-	-	-	-	-	-
Staining, brown, dorsal body surface	F0	-	-	-	-	-	**1**		
	F1	-	-	-	-	-	-	-	-
Staining, brown, ventral body surface	F0							**1**	
	F1	-	-	-	-	-	-	-	-
Staining, yellow, perigenital	F0							**2**	**1**
	F1	-	-	-	-	-	-	-	-

Table B.6.6.1.1-2 Lenacil: preliminary 1-generation study ([redacted] 2002): compound intake, body weight and food consumption.

Endpoints/dose	10000 ppm		25000 ppm		50000 ppm	
	M	**F**	**M**	**F**	**M**	**F**
Compound intake mg/kg bw/d						
Prior pairing	**749**	**755**	**1952**	**2003**	**3840**	**4014**
Gestation		998		2406		4965
Lactation		1714		4555		9350
Mean achieved dose F1 prior pairing	1314	1345	3173	3307	6814	6964
Body weight:						
Prior pairing F0					**↓7%**	**↓14%**
Prior pairing F1					**↓8%**	**↓3%**
During gestation F0						**↓4%**
During lactation F0		**↓38%**		**↓22%**		**↓33%**
Food consumption:						
Before pairing F0		**↓5%**				**↓1%**
During lactation F0		**↓8%**				**↓5%**

Bold indication: reference for article intake calculation

Mating performance and fertility:

was unaffected by treatment. One ♀ at 10000 ppm was not pregnant and one ♀at 50000 ppm showed single implantation site post staining. These effects are not considered to be treatment related. Gestation length was not affected (**Table B.6.6.1.1-3**).

Table B.6.6.1.1-3 Lenacil: preliminary 1-generation study ([redacted] 2002): mating, fertility and gestation parameters.

Endpoint/Dose			0 ppm		10000 ppm		25000 ppm		50000 ppm	
Oestrous cycle – prior to mating (F_0)	Regular 4 or 5 days cycles		8/8		8/8		5/8		7/8	
	Irregular cycle [a]		0/8		0/8		3/8 (38%)		1/8 (13%)	
	Acyclic [b]		0/8		0/8		0/8		0/8	
Pre-coital interval (F_0-F_1)	1-4		8/8 (100%)		7/8 (88%)		8/8 (100%)		8/8 (100%)	
	5-8		0/8		1/8 (13%)		0/8		0/8	
	9-12		0/8		0/8		0/8		0/8	
	13-16		0/8		0/8		0/8		0/8	
	17-21		0/8		0/8		0/8		0/8	
			M	F	M	F	M	F	M	F
Mating performance and fertility (F_0-F_1)	# paired		8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8
	#mating		8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8
	#achieving pregnancy		8/8	8/8	7/8	7/8	8/8	8/8	8/8	8/8
	% mating		100%	100%	100%	100%	100%	100%	100%	100%
	Conception rate (%)		100	100	88	88	100	100	100	100
	Fertility index (%)		100	100	88	88	100	100	100	100
Gestation length and gestation index (F_0-F_1)	# pregnant animals			8/8		7/8		8/8 [c]		8/8
	Gestation length (days)	22		0		2 (29%)		0		2 (25%)
		22.5		3 (38%)		4 (57%)		2 (29%)		5 (63%)
		23		5 (63%)		1 (14%)		4 (57%)		1 (13%)
		23.5		0		0		1 (13%)		0
	#of live litters born			8		7		7		8
	Gestation index			100		100		88		100

[a]: at least one cycle of two, three, or six to ten days; [b]: at least 10 days without oestrus; [c]percentage distribution of gestation lengths calculated from 7 animals – one pregnant female (one implantation site visible post staining) failed to litter.

Macroscopy and microscopy:

Both F_0 and F_1 animals did not reveal any findings that could be attributed to treatment.

Table B.6.6.1.1-4 Lenacil: preliminary 1-generation study ([redacted] 2002): macropathology F_0

		Males				Females			
Endpoints/Dose (ppm)		**0**	**10000**	**25000**	**50000**	**0**	**10000**	**25000**	**50000**
Hairloss, ventral body surface	F_0	-	-			-	1	-	
	F_1	-	-	-	-	-	-	-	-
Hairloss, forlimbs	F_0	-	-	-	-	-	-	1	-
	F_1	-	-	-	-	-	-	-	-
Staining, brown (slight), dorsal body surface	F_0	-	-	-	-	-	1	-	-
	F_1	-	-	-	-	-	-	-	-
Staining, yellow, perigenital	F_0							2	-
	F_1	-	-	-	-	-	-	-	-
Staining, yellow, urogenital	F_0	-	-	-	-	-	-	-	1
	F_1	-	-	-	-	-	-	-	-

Litter response from F_0:

Litter size was normal (**table B.6.6.1.1-5**) and offspring survival was unaffected (**table B.6.6.1.1-6**). Sex ratio was unaffected by treatment (**table B.6.6.1.1-7**).

Body weight of offspring on day 1 of age and subsequent weight gain up to weaning on day 21 of age showed no clear effect of treatment.

Table B.6.6.1.1-5 Lenacil: preliminary 1-generation study ([redacted] 2002): litter size - group mean values (F_0-F_1).

Endpoints/Dose (ppm)			0	10000	25000	50000
Implantation sites		Mean ± SD	13.1 ± 1.7	12.0 ± 4.1	12.9 ± 1.8	12.6 ± 3.4
		N (litters)	8	7	7	8
Total on/before Day 1 (d)		Mean ± SD	11.6 ± 2.1	11.4 ± 4.0	12.0 ± 2.4	11.6 ± 3.1
		N (litters)	8	7	7	8
Number alive on day of age	Before cull (day 1)	Mean ± SD	11.5 ± 2.2	11.1 ± 4.2	12.0 ± 2.4	11.5 ± 3.1
		N (litters)	8	7	7	8
	Before cull (day 4)	Mean ± SD	11.4 ± 2.3	11.0 ± 4.2	12.0 ± 2.4	11.5 ± 3.1
		N (litters)	8	7	7	8
	After cull (day 4)	Mean ± SD	8.0 ± 0.0	7.3 ± 1.9	7.9 ± 0.4	7.6 ± 1.1
		N (litters)	8	7	7	8
	After cull (day 7)	Mean ± SD	8.0 ± 0.0	7.3 ± 1.9	7.9 ± 0.4	7.6 ± 1.1
		N (litters)	8	7	7	8
	After cull (day 14)	Mean ± SD	8.0 ± 0.0	7.3 ± 1.9	7.9 ± 0.4	7.6 ± 1.1
		N (litters)	8	7	7	8
	After cull (day 21)	Mean ± SD	8.0 ± 0.0	7.3 ± 1.9	7.9 ± 0.4	7.6 ± 1.1
		N (litters)	8	7	7	8

(d): includes offsprings that died prior to designated

Table B.6.6.1.1-6 Lenacil: preliminary study of effects on reproductive performance in Han Wistar rats by dietary administration ([redacted] 2002): offspring survival indices - group mean values (F_0-F_1).

Endpoints/Dose (ppm)			0	10000	25000	50000
Post-implantation		Mean ± SD	88.6 ± 10.7	95.6 ± 6.4	92.8 ± 11.3	92.5 ± 6.0
		N (litters)	8	7	7	8
Live birth index, Day 1 of age		Mean ± SD	98.9 ± 3.2	97.1 ± 7.6	100.0 ± 0.0	99.0 ± 2.9
		N (litters)	8	7	7	8
Viability index, Day 4 of age		Mean ± SD	98.8 ± 3.5	98.9 ± 2.9	100.0 ± 0.0	100.0 ± 0.0
		N (litters)	8	7	7	8
Lactation index, on Day of age	7	Mean ± SD	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
		N (litters)	8	7	7	8
	14	Mean ± SD	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
		N (litters)	8	7	7	8
	21	Mean ± SD	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
		N (litters)	8	7	7	8

Table B.6.6.1.1-7 Lenacil: preliminary 1-generation study ([redacted] 2002): sex ratio - group mean values (F0-F1).

Endpoints/Dose		0	10000	25000	50000
		%♂(Mean ± SD)	%♂ (Mean ± SD)	%♂ (Mean ± SD)	%♂ (Mean ± SD)
Total on Day 1 of age		46.0 ± 14.6	51.7 ± 11.3	41.3 ± 15.2	55.9 ± 15.7
Live (before cull)	D1	46.0 ± 14.6	51.7 ± 11.3	41.3 ± 15.2	55.9 ± 15.7
	D4	45.3 ± 14.5	52.3 ± 11.1	41.3 ± 15.2	55.4 ± 15.7
Live after cull	D21	50.0 ± 6.7	54.2 ± 7.2	44.9 ± 13.5	56.9 ± 12.8

Conclusion (preliminary 1G rat study)

Reproductive or offspring parameters were unaffected at any of the doses tested. Slight parental toxicity occurred at the top-dose (slight body weight decrease, clinical signs).

Maternal
NOAEL *25000 ppm* = **1952 mg/kg bw/d**
LOAEL *50000 ppm* = 3840 mg/kg bw/d based on ↓body w, ↑clinical signs (alopecia)

Offspring
NOAEL *50000 ppm* = 3840 mg/kg bw/d

Reproductive
NOAEL *50000 ppm* = 3840 mg/kg bw/d

Therefore, dietary doses of 1000, 10000 and 50000 ppm (> 3800 mg/kg bw/day) were considered suitable for the main two-generation study ([redacted] 2003) with this strain of rats.

B.6.6.1.2 Main study

Study of reproductive performance in Han Wistar rats treated continuously through two successive generations by dietary administration ([redacted] 2003) - DuPont Report No.: ACD 020/023865

Guidelines: EC test method B.35 (1999) equivalent to OECD 416 (1999), OPPTS 870.3800 (1998), JMAFF 12 Nohsan No. 8147 (2000).
The multigeneration study in ♂ and ♀ Han Wistar rats (ACD 020/023865) was originally submitted under EU Rev8 Point IIA 5.6.1, 5.8.2 and has been conducted with lenacil technical.
A review of this study indicates that it fully meets the current OECD Test Guideline 416 or OPPTS guideline 870.3800.
RMS: It was of note that the new 2G test protocol was **partially** respected:

- oestrous cyclicity data : performed
- sperm evaluation (performed F_0/F_1 adult animals: count, morphology, homogenisation-resistant spermatid count)
- sexual maturation data (F_1 selected offspring):
- F_1: vaginal opening d38 or preputial separation d28 (performed);
- F_1: measurement of anogenital distance (**not** performed) if clarified triggers (PND in F_2 if triggered by alterations in F_1 sex ratio or timing of sexual maturation; long-term effects in tha rat (carcinogenicity study) indicated a possible of an ED MoA which could not entirely be discarded.
- *functional tests* in F_1 offspring recommended (performed, however not NT; *functional developmental checks* included: surface/air righting and auditory/visual function);
- organ weights and histopathology:

	Selected organ weights:	Histopathology :
B.35:	• for organs of all P and F_1 parental animals *i.e.:* uterus, ovaries, testes, epididymides (total and cauda), prostate, seminal vesicles + coagulating glands and their fluids (as one unit), brain, liver, kidneys, spleen, pituitary, thyroid and adrenal glands and known target organs. • F_1 and F_2 pups selected* for necropsy: brain, spleen, thymus.	(i) P and F_1 (control + top-dose): vagina, uterus + cervix, ovaries, 1 testis, 1 epididymis, seminal vesicles, prostate, coagulating gland; (ii) target organ(s) from all P and F_1 animals selected for mating (iii) target organs (especially reproductive) from all pups (if external abnormalities or clinical signs), and from ≥ 1 pup/sex/ F_1 and F_2 litter not selected for mating
Study:	- Adrenals (paired) - Brain* - Epididymides (left and right) - Kidneys (paired) - Liver - Ovaries with oviduct (left and right) - Pituitary - Prostate -ventral lobe - Seminal vesicles with coagulation gland - Spleen* - Testes (left and right) - Thymus* - Thyroid with parathyroids, after fixation - Uterus	Preserved and examined microscopally: - Adrenals (paired) - Epididymides (right) - Liver - Ovaries with oviduct (left and right) - Pituitary - Prostate -ventral lobe - Seminal vesicles with coagulation gland - Spleen* - Testes (right) - Thymus - Thyroid with parathyroids, after fixation - Uterus + cervix - Vagina

GLP status: yes
Materials and Methods
In the main study, the F0 generation comprised 28 ♂ and 28 ♀ rats, received the diet (0, 1000, 10000, 50000 ppm) for 10 weeks before pairing, throughout pairing, gestation and lactation, until termination; F0 ♂ were terminated after 17 weeks of treatment and the F0 ♀ were terminated on day 28 post partum and the unselected F1 offspring were terminated at day 30 of age. Selected F1 rats, comprising 24 ♂ and 24 ♂ were exposed to diet from weaning until they were paired for mating at approximately 14 weeks of age.

Main study: conversions

	0 ppm		1000 ppm		10000 ppm		50000 ppm	
Compound intake mg/kg bw/d	♂	♀	♂	♀	♂	♀	♂	♀
Prior pairing F_0			**82**	**92.5**	**817**	**935**	**4279**	**4787**
F_1			99.5	107	1013	1115	5312	5762
Gestation F_0				92		919		4839
F_1				90		965.6		5060
Lactation F_0				166		1727		8659
F_1				164		1733		8839

Bold indication intake: reference for article intake calculation

Batches of the test diets were prepared and issued each week. The stability and homogeneity of the dietary formulations had been assessed and confirmed by a trial preparation prior to the study start. The stability was confirmed over 21 days. Concentration analyses were performed throughout the study at weeks 1, 11, 18, 28 and 32 and satisfactory levels were obtained (average –0.5%: range 5.0 to –4.4%)

The study is accepted.

Findings

RMS: it has to be noted that no haematological data were provided in this study. In the other repeated dose rat studies, haematological alterations were seen at doses including those lower than 20 mg/kg bw/d. It is therefore unnown whether blood changes would also occur here.

Parental data:

Mortality was not considered to be treatment related.

Clinical signs: During the initial assessment, RMS considered that F_0 rats did not show signs attributed to treatment.

However, at 817 mg/kg bw/d (♂) and above (♂,♀), rats showed an increased incidence of alopecia on head and back in both generations (table B.6.6.1.2-1). In F_1, the hairloss occurred from week 3 in the ♂, with ♀ being similarly affected up to week 8. Further, skin encrustation (dorsal body surface and tail), and staining (brown/yellow) were seen, mainly in the top-dose ♂. At least for hairloss and depigmentation, implication of the immune system, thyroid or more general stress-related etiology could be considered. **Notifier** provided some explanation on the etiology of hairloss: *"Alopecia and hair loss can be due to a variety of factors including environmental, hormonal, photosensitivity, inflammation, etc. The incidence in the study looks like normal variability, as it was observed to some extent in controls. Increased severity would be more indicative of a test substance effect. However, this would not be related to thyroid hormone changes, since we observed normal T_3 and T_4 levels."*. **RMS** takes note of the explanation, but stil considers the clinical signs treatment-related, although it is recognised that the etiology is uncertain.

Table B.6.6.1.2-1 Two generation study with lenacil in Wistar rats ([redacted] 2003):clinical signs

		Males				Females			
Dose (ppm)		0	1000	10000	50000	0	1000	10000	50000
		0	82	817	4279	0	92.5	935	4787
Behaviour, vocalisation	F0	0	0	0	0	2	2	4	3
	F1	0	0	0	0	2	4	2	1
Coat, hairloss, dorsal body surface	F0	2	1	**3**	**5**	7	3	**6**	**10**
	F1	1	1	**5**	**12**	6	4	5	**10**
Coat, hairloss, head	F0	1	0	**2**	**2**	3	2	2	**4**
	F1	1	1	0	**4**	0	1	0	0
Skin, encrustation(s), dorsal body surface	F0	0	0	**1**	**1**	1	1	0	0
	F1	1	0	**0**	**3**	0	0	0	0
Skin, encrustation(s), tail	F0	0	0	0	**1**	0	0	0	0
	F1	0	0	0	**2**	0	0	0	0
Staining, brown, head	F0	1	1	1	**4**	1	2	0	0
	F1	0	0	0	**2**	0	0	1	0
Staining, yellow, perigenital	F0	-	-	-	-	-	-	-	-
	F1	0	0	0	**2**	1	0	0	**4**
Staining, yellow, scrotum	F0	0	0	0	**1**	-	-	-	-
	F1	-	-	-	-	-	-	-	-

(-) not mentioned; number of animals: F0: n=28; F1: n=24

Body weight:

- Before mating F_0 rats were unaffected by treatment. At the start of the F_1 generation (week 0) weight was not affected. The overall bw for F_1♂ was unaffected by treatment. The top-dose♀ showed slightly lower weight gain for the 10-week period prior to pairing.

- During gestation, F_0♀ at 817 mg/kg bw/d and above, and top-dose F_1♀lost slightly weight.

- At top-dose, during the lactation period, maternal body weight gain tended to be superior to the controls and did not show the weight loss that is generally seen when the offspring become more independent and the lactation demand is reduced. This suggests that the lactation demand at this dietary concentration was not as high as in the controls and, consequently, there was no major impact on maternal weight gain as the offspring started to consume the diet.

- The initial birth weight of the F_1 and F_2 offspring was unaffected by maternal treatment but there was a reduction of weight gain at top-dose that occurred from d7 for the F_1 offspring and from d4 for the F_2 offspring. This effect occurred before that offspring began to consume solid food, suggesting an effect via lactation. Whether treatment caused a reduction in milk production or quality, or whether the offspring were exposed to lenacil via the milk cannot be ascertained in this study. **RMS** considered initially that that this effect needs a labelling of lenacil with Lact. H362 ("*May cause harm to breast-fed children*" - equivalent to R64). However, this was not agreed upon during the peer review, including the review pertaining classification and labelling (ECHA) of lenacil, considering that a weight effect was insufficient to classify, and that the effect was observed at doses around or largely exceeding the limit dose of 1000 mg/kg b.w./d. Whether treatment caused a reduction in milk production or quality, or whether the offspring was exposed to lenacil via milk could not be ascertained in this study, and was not further investigated during renewal.

Table B.6.6.1.2-1bis Two generation study with lenacil in Wistar rats ([redacted] 2003): body weight (change) and food conversion efficiency

		Males				Females			
Dose (ppm)		**0**	**1000**	**10000**	**50000**	**0**	**1000**	**10000**	**50000**
		0	**82**	**817**	**4279**	**0**	**92.5**	**935**	**4787**
Body weight (gain):									
Prior pairing	F_0				↓5%			↓4%	↓4%
	F_1		↓5%	↓2%	↓4%		↓1%	↓6%	↓9%
During gestation	F_0 d0-20							↓10%*	↓7%*
	F_1 d0-20								↓9%*
Bw change offspring	F_1 d1-21							↓6%*	↓6%*
	F_2 d1-21							↓11%*	↓11%*
Food conversion efficiency:	F_0						↓5%	↓7%	↓7%
	F_1							↓8%	↓11%

Notifier commented as follows (comment added for the sake of completeness):

The Notifier disagrees with the conclusion of the RMS to classify the active substance lenacil with R64.

The relevant legislation is Council Directive 67/548/EEC, as amended by Commission Directive 2001/59/EC, Annex 6 (Annex VI) Section3 2.8 and 4.2.3.3.

- Section 3.2.8 states the criteria for R64 as: For substances and preparations which are absorbed by women and may interfere with lactation or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child.

In rat metabolism studies, lenacil is primarily excreted via urine as water-soluble hydroxyl metabolites. It is generally considered that the high fat content of milk may lead to fat-soluble substances and metabolites being present in the milk. Residues in the target crop, sugar beet, are also hydroxyls and ketones, and it is predicted that in humans, these will be further hydroxylated and excreted via urine. There is no evidence that lenacil or its metabolites accumulate in the body, such that there is no implication that mobilisation of maternal fat reserves could lead to the presence of lenacil or its metabolites in milk. The ADI for Lenacil is 0,014 mg/kg bw/day. The NOAEL proposed by the RMS is 10,000 ppm or 1,727 mg/kg bw/day. This gives a margin of safety in excess of 120,000. The criterion for R64 includes the words 'in amounts sufficient to cause concern'.

-Furthermore, Section 4.1.3.3 states that 'For the purpose of classification, toxic effects on offspring resulting only from exposure via the breast milk, or toxic effects resulting from direct exposure of children will not be regarded as Toxic to reproduction, unless such effects result in impaired development of the offspring'.

It is accepted that offspring bodyweights were slightly lower than controls in the F0F1 (by 6%) and F1F2 (by 11%) during the lactation period, but offspring survival was not adversely affected, and the bodyweights of the F0F1 pups selected for the F1 generation were not different from controls at the start of the pre-mating maturation period. Also, the behavioural and developmental landmarks assessed prior to and after weaning were not adversely affected by either maternal treatment or by direct intake of the test material. Any marginal bodyweight effects on offspring prior to weaning are considered transient, and insufficient evidence for adverse effects via maternal milk.

The legislation states: 'This R-phrase may also be appropriate for substances which affect the quantity or quality of the milk'. Where there is an effect on quantity of the milk, there is usually evidence from the immediate post-partum period. The body wall of the newborn rat is translucent, and the technicians can see the presence of milk in the pups' stomach as a whitish crescent in the abdomen. Absence of this crescent is recorded in the data for the study as an indication that the dam is not nursing the pups. It is frequently accompanied by high post natal mortality in pups. Neither finding was made in this study.

The legislation gives further guidance:

R64 would normally be assigned on the basis of:

(a) toxicokinetic studies that would indicate the likelihood that the substance would be present in potentially toxic levels in breast milk; and/or

(b) on the basis of results of one or two generation studies in animals which indicate the presence of adverse effects on the offspring due to transfer in the milk; and/or

(c) on the basis of evidence in humans indicating a risk to babies during the lactational period.

The evidence from metabolism studies is that lenacil or its' metabolites would not be preferentially excreted in the milk, and if present at all, would be at a minute fraction of levels considered NOAEL in the rat. The effects on the offspring are minor, transient and there is no indication of impaired development or reduced survival. Finally, there is no evidence in humans.

In conclusion, lenacil should not be classified R64.

In view of the conclusion drawn here in respect of litter data and maturation of the F1 rats, the notifier requests re-evaluation of the proposal for classification with R64. The litter development shows no clear effects leading to impaired growth of maturation in these litters.

Hence, this proposal was discussed during the first peer review. The experts discussed the data presented in the DAR considering the very high dose level applied in the study (50000 ppm = 4279 mg/kg bw/d which exceeds the 1000 mg/kg bw/d limit dose for reproductive toxicity studies). The decrease in offspring weight gain was deemed insufficient to justify this classification at this very high dose level. Therefore, it was considered not appropriate to propose this classification, and the findings did not trigger this classification at the occasion of the CLH evaluation too. The conclusion is confirmed by the RMS during the renewal. However, it is of note that the finding qualifies for the establishment of the offspring toxicity NOAEL.

Food consumption and food conversion efficiency of F_0 animals was unaffected during the first 10 weeks of treatment.

The overall food efficiency of F_1 rats was slightly low during the 10-week period prior to pairing for mating and for top-dose animals.

Reproduction performance: oestrus cycle, mating performance, fertility, gestation index and length (**table B.6.6.1.2-2**), litter size, sex ratio and offspring survival were unaffected.

Lenacil did not delay the return to normal oestrus cycle of the F_0 and F_1 ♀, with all ♀ showing oestrus before termination on d28 post partum (PP). Sperm motility, morphology and concentration were unaffected by treatment.

Parents (F_0) reproductive performance tables: table B.6.6.1.2- 2a : mating, fertility and gestation parameters
2b : oestrous cycles – prior to termination, and -2c : sperm assessment and morphology

Table B.6.6.1.2-2a Two generation study with lenacil in Wistar rats ([redacted] 2003): mating, fertility and gestation parameters (F_0-F_1).

	ppm		0		1000		10000		50000	
	mg/kg bw/d		0		82		817		4279	
Oestrous cycle – prior to mating (F_0)	Regular 4 or 5d cycles		26/28 (93%)		25/28 (89%)		27/28 (96%)		26/27 (96%)	
	Irregular cycle [a]		0/28		1/28 (4%)		1/28 (4%)		0/27	
	Acyclic [b]		1/28 (4%)		2/28 (7%)		0/28		1/27 (4%)	
Pre-coital interval (F_0-F_1)	1-4		27/28 (96%)		28/28 (100%)		28/28 (100%)		27/27 (100%)	
	5-8		1/28 (4%)		0/28		0/28		0/27	
	9-12		0/28		0/28		0/28		0/27	
	13-16		0/28		0/28		0/28		0/27	
	17-21		0/28		0/28		0/28		0/27	
			M	F	M	F	M	F	M	F
Mating performance and fertility (F_0-F_1)	# paired		28	28	28)	28	28	28	27	27
	# mating		28	28	28)	28	28	28	27	27
	# achieving pregnancy		25	25	28)	28	28	28	27	27
	% mating		100%	100%	100%	100%	100%	100%	100%	100%
	Conception rate (%)		89	89	100	100	100	100	100	100
	Fertility index (%)		89	89	100	100	100	100	100	100
Gestation length and gestation index (F_0-F_1)	# pregnant			25		28		28		27
	Gestation length (days)	22		6 (24%)		5 (18%)		4 (14%)		5 (19%)
		22.5		11 (44%)		11 (39%)		16 (57%)		18 (67%)
		23		8 (32%)		12 (43%)		7 (25%)		4 (15%)
		23.5		0		0		1 (4%)		0
	# live litters born			25		28		28		27
	Gestation index			100		100		100		100

(a): at least one cycle of two, three, or six to ten days; (b): at least ten days without oestrus;.

Table B.6.6.1.2-2b Two generation study with lenacil in Wistar rats ([redacted] 2003): oestrous cycles – prior to termination (F_0).

	ppm	0	1000	10000	50000
	Mg/kg bw/d	0	82	817	4279
# animals		*28*	*28*	*27*	*27*
# showing oestrus by d28		28	28	27	27
first oestrus smear (LD)	25	13 (46%)	10 (36%)	7 (26%)	10 (37%)
	26	5 (18%)	4 (14%)	4 (15%)	5 (19%)
	27	1 (4%)	4 (14%)	3 (11%)	3 (11%)
	28	9 (32%)	10 (36%)	13 (48%)	9 (33%)
Cycle stage at kill (d28)	M	1 (4%)	4 (14%)	3 (11%)	3 (11%)
	D	7 (25%)	5 (18%)	4 (15%)	7 (26%)
	P	11 (39%)	9 (32%)	7 (26%)	8 (30%)
	E	9 (32%)	10 (36%)	13 (48%)	9 (33%)

LD: lactation day; M: metoestrus; D: dioestrus; P: pro-oestrus; E: oestrus

Table B.6.6.1.2-2c Two generation study with lenacil in Wistar rats ([redacted] 2003): sperm assessment and morphology – group mean values (F_0).

	ppm	0		1000	10000	50000	
	Mg/kg bw/d	0		82	817	4279	
# animals		*28*		*28*	*28*	*27*	
Motile sperm (%)		93 ± 4		92 ± 5	94 ± 4	92 ± 6	
Progressively motile sperm (%)		41 ± 10		38 ± 8	40 ± 7	39 ± 10	
Cauda epididymis	Weight (g)	0.214 ± 0.036				0.213 ± 0.027	
	Sperm count (10^6/g)	811 ± 237				868 ± 186	
	Total (10^6)	175 ± 60				186 ± 52	
Testis	Weight (g)	1.79 ± 0.21				1.76 ± 0.22	
	Sperm count (10^6/g)	99 ± 21				98 ± 21	
	Total (million)	178 ± 47				176 ± 45	
Total sperm examined		5603				5400	
Sperm morphology		#	%			#	%
	Normal	194 ± 8	97.1 ± 4.4			196 ± 2	98.0 ± 1.1
	Decapitate	3 8	1.4 ±3.9			1± 1	0.7± 0.7
	Abnormal	3 2	1.5 ±1.1			3± 2	1.3± 0.8

Parents (F1) reproductive performance tables:

Table B.6.6.1.2-3a : mating, fertility and gestation parameters; 3b : oestrous cycles – prior to termination;- 3c : sperm assessment and morphology

Table B.6.6.1.2-3a Two generation study with lenacil in Wistar rats ([redacted] 2003): mating, fertility and gestation parameters (F1-F2).

	ppm		0		1000		10000		50000	
	Mg/kg bw/d		0		82		817		4279	
Oestrous cycle–prior to mating (F1)	Regular 4 or 5 d cycles		22/24 (92%)		23/24 (96%)		23/24 (96%)		24/24 (100%)	
	Irregular cycle [(a)]		0/24		1/24 (4%)		0/24		0/24	
	Acyclic [(b)]		2/24 (8%)		0/24		1/24 (4%)		0/24	
Pre-coital interval (F0-F1-F2)	1-4		23/24 (96%)		22/24 (92%)		24/24 (100%)		22/24 (92%)	
	5-8		1/24 (4%)		2/24 (8%)		0/24		2/24 (8%)	
	9-12		0/24		0/24		0/24		0/24	
	13-16		0/24		0/24		0/24		0/24	
	17-21		0/24		0/24		0/24		0/24	
			M	F	M	F	M	F	M	F
Mating performance and fertility (F0-F1)	# paired		24	24	24	24	24	24	24	24
	#mating		24	24	24	24	24	24	24	24
	# achieving pregnancy		22	22	24	24	22	22	24	24
	% mating		100%	100%	100%	100%	100%	100%	100%	100%
	Conception rate (%)		92	92	100	100	92	92	100	100
	Fertility index (%)		92	92	100	100	92	92	100	100
Gestation length and gestation index (F0-F1)	# pregnant			22		24[(c)]		22		24
	Gestation length (days)	22		2 (9%)		2 (9%)		4 (18%)		4 (17%)
		22.5		12 (55%)		9 (39%)		7 (32%)		10 (42%)
		23		8 (36%)		11 (48%)		10 (45%)		8 (33%)
		23.5		0		1		1		2 (8%)
	# live litters born			22		23		22		24
	Gestation index			100		96		100		100

(a): at least one cycle of two, three, or six to ten days; (b): at least ten days without oestrus; (c): percentage distribution of gestation lengths calculated from 23 animals – one pregnant female failed to litter.

Table B.6.6.1.2-3b Two generation study with lenacil in Wistar rats ([redacted] 2003): oestrous cycles – prior to termination (F_1).

	ppm	0	1000	10000	50000
	Mg/kg bw/d	**0**	**82**	**817**	**4279**
# animals		*24*	*24*	*24*	*24*
# showing oestrus by d28		23 (96%)	24 (100%)	24 (100%)	23 (96%)
first oestrus smear (LD)	25	8 (35%)	10 (42%)	7 (29%)	3 (13%)
	26	1 (4%)	1 (4%)	8 (33%)	2 (9%)
	27	4 (17%)	8 (33%)	1 (4%)	7 (30%)
	28	10 (43%)	5 (21%)	8 (33%)	11 (48%)
Cycle stage at kill (d 28)	M	4 (17%)	8 (33%)	1 (4%)	6 (25%)
	D	2 (8%)	2 (8%)	9 (38%)	3 (13%)
	P	8 (33%)	9 (38%)	6 (25%)	3 (13%)
	E	10 (42%)	5 (21%)	8 (33%)	12 (50%)

LD: lactation day; M: metoestrus; D: dioestrus; P: pro-oestrus; E: oestrus

Table B.6.6.1.2-3c Two generation study with lenacil in Wistar rats ([redacted] 2003): sperm assessment and morphology – group mean values (F_0).

	ppm	0		1000	10000	50000	
	Mg/kg bw/d	**0**		**82**	**817**	**4279**	
# animals		*24*		*24*	*24*	*24*	
Motile sperm (%)		93 ± 4		94 ± 4	93 ± 6	93 ± 6	
Progressively motile sperm (%)		53 ± 9		56 ± 9	51 ± 10	53 ± 8	
Cauda epididymis	Weight (g)	0.211 ± 0.038				0.221 ± 0.019	
	Sperm count (10^6/g)	550 ± 113				541 ± 106	
	Total (10^6)	115 ± 32				120 ± 26	
Testis	Weight (g)	1.84 ± 0.23				1.84 ± 0.15	
	Sperm count (10^6/g)	105 ± 17				116 ± 17	
	Total (10^6)	192 ± 35				215 ± 38	
Total sperm examined		4801				4801	
Sperm morphology		#	%			#	%
	Normal	196 ± 2	98.0 ± 1.1			196 ± 3	98.2 ± 1.3
	Decapitate	2 ± 2	1.0 ± 0.8			1 ± 1	0.7 ± 0.6
	Abnormal	2 ± 1	1.0 ± 0.7			2 ± 2	1.1 ± 1.1

Organ weight:

Terminal body weights were very slightly decreased in the top-dose animals, and the difference with control was only significant in the top-dose ♀ at F_1, which was in line with the body weight observations throughout the study.

Liver weight was significantly increased in F_0 and F_1 parental rats at 817 mg/kg b.w./d (<10%) and above (8-16%).

Thyroid weight was high in the top-dose animals, and dose-dependent increases were also noted at the next-lower dose group (F_0, ♂,♀) and F_1 (♀).

Notifier considered the pituitary weights elevations after 17 weeks of treatment most likely due to *"excessively lower pituitary weights in several animals in the F_1 generation control group"*. In this regard, 4♂ from the F_1 generation controls had very low pituitary gland weights, as compared to the F_0 generation. The lowest pituitary weight measured for the F_0 generation control group ♂was a single animal weighing 0.05 grams. In contrast, for the F_1 generation control group ♂, the lowest weight measured was 0.03 grams (animal# 2010) and 3 other ♂weighed 0.04 grams (animal # 2018, 2023 and 2024); in addition, 4 animals weighed 0.05 grams. Therefore, the statistically significant increases in absolute pituitary weight in the F_1 generation ♂ at top-dose and relative

weights at 25000 and 50000 ppm may, in part, be due to an untypically low mean control value. The fact that this finding was not consistent across the generations suggests it may not be related to treatment with lenacil and may merely be a spurious finding.
RMS agreed that the pituitary weight was unaltered in F_0 but showed an increase in the F_1♂ at 817 mg/kg b.w./d and above. It was still questioned whether there could be an association with the increased thyroid weight in this cohort at the two highest doses.

The F_1 ♀at top dose had low uterine weight on day 28 *post partum*. A comparison of the individual uterine weights with the oestrus cycle classification at termination suggested a correlation between stage of the oestrus cycle on the morning of termination and the uterine weight at termination. Rats at *pro-oestrus* tended to have the highest uterine weights, whilst those at metoestrus tended to have the lowest uterine weights. According to the notifier, the apparent decrease in uterus weight at top dose may therefore be related to the stage of *oestrus* rather than a result of treatment because a high proportion of control ♀were at *pro-oestrus* prior to termination, whilst a high proportion of top-dose ♀ were at *metoestrus*. RMS considers that this explanation is in a limited extend supported by the data (see table B.6.6.1.2-2b, F_0 and -3b, F_1). Uterus changes are observed in several studies in the present DRAR and RMS considers them potentially treatment-related, although it is acknowledged that the significance of uterine weight *decrease* (of poor dose-response) in this 2G study is unclear.

Spleen and thymus weight were slightly decreased in ♀ in F_1 at the top-dose, and in both ♂ and ♀ in F_2 at 817 mg/kg b.w./d and above. It was of note that F_0 animals showed no change in these lymphoid organs, and that the weight effects became more prominent in the next generations. Although this exacerbation was not reflected by neither gross pathology nor histopathological findings, however, taking into account the recurrent (albeit poorly dose-dependent) finding on WBC and lymphoid organs, the association with lenacil-treatment cannot be ignored. The occasional effects on the weight the other organs was of uncertain toxicological relevance, in the absence of consistency among generations, absence of effects in other dietary studies or histopathological findings, and their weak magnitude. Sexual organ weights were unaffected by the treatment with lenacil.

Table B.6.6.1.2-4a Two generation study with lenacil in Wistar rats ([redacted] 2003): organ weight – F_0

		Males				Females			
Dose (ppm)		**0**	**10000**	**25000**	**50000**	**0**	**10000**	**25000**	**50000**
Mg/kg bw/d		**0**	**82**	**817**	**4279**	**0**	**82**	**817**	**4279**
Terminal body weight	A	434.4 ± 53.4	431.4 ± 43.1	433.9 ± 38.2	418.3 ± 43.7 (↓4%)	250.0 ± 12.1	249.6 ± 15.8	242.3 ± 15.2	244.3 ± 17.4 (↓2%)
Brain	A	2.02 ± 0.11	2.01 ± 0.08	2.03 ± 0.07	1.99 ± 0.10	1.88 ± 0.07	1.88 ± 0.08	1.88 ± 0.08	1.88 ± 0.08
	R	0.471 ± 0.052	0.470 ± 0.047	0.472 ± 0.039	0.481 ± 0.054	0.755 ± 0.031	0.756 ± 0.046	0.777 ± 0.049	0.773 ± 0.053
Pituitary	A	0.008 ± 0.002	0.008 ± 0.002	0.009 ± 0.002	0.008 ± 0.002	0.013 ± 0.002	0.013 ± 0.003	0.012 ± 0.003	0.012 ± 0.002
	R	0.0019 ± 0.0004	0.0019 ± 0.0005	0.0021 ± 0.0004	0.0019 ± 0.0004	0.0053 ± 0.0007	0.0053 ± 0.0009	0.0051 ± 0.0012	0.0050 ± 0.0007
Adrenals	A	0.058 ± 0.007	0.057 ± 0.009	0.057 ± 0.008	0.056 ± 0.009	0.078 ± 0.010	0.082 ± 0.010	0.080 ± 0.010	0.082 ± 0.011
	R	0.0134 ± 0.0020	0.0133 ± 0.0018	0.0131 ± 0.0022	0.0134 ± 0.0019	0.0311 ± 0.0036	0.0327 ± 0.0034	0.0329 ± 0.0045	0.0335 ± 0.0040
Epididymides L+R	A	1.193 ± 0.131	1.189 ± 0.140	1.186 ± 0.106	1.173 ± 0.118				
	R	0.2767 ± 0.0302	0.2774 ± 0.0369	0.2742 ± 0.0217	0.2829 ± 0.0370				
Kidneys	A	2.59 ± 0.26	2.64 ± 0.29	2.61 ± 0.21	2.61 ± 0.29	2.01 ± 0.14	2.00 ± 0.18	1.94 ± 0.15	1.98 ± 0.15
	R	0.599 ± 0.051	0.612 ± 0.042	0.603 ± 0.038	0.626 ± 0.047	0.803 ± 0.046	0.800 ± 0.056	0.800 ± 0.051	0.811 ± 0.046
Liver	A	15.18 ± 2.04	15.47 ± 1.83	15.90 ± 1.76	**16.46* ± 2.06 (↑8%)**	11.31 ± 0.96	11.65 ± 1.05	11.57 ± 1.10	**12.79** ± 1.20 (↑13%)**
	R	3.502 ± 0.293	3.583 ± 0.187	**3.663* ±0.226 (↑4%)**	**3.931** ±0.218 (↑12%)**	4.518 ± 0.260	4.670 ± 0.337	**4.774* ±0.352 (↑6%)**	**5.238** ±0.326 (↑16%)**
Ovaries + Ovid. L+R	A					0.150 ± 0.019	0.152 ± 0.022	0.154 ± 0.021	0.142 ± 0.016
	R					0.0601 ± 0.0068	0.0609 ± 0.0071	0.0636 ± 0.0095	0.0585 ± 0.0077
Prostate	A	0.440 ± 0.084	0.444 ± 0.089	0.465 ± 0.098	0.431 ± 0.063				
	R	0.1015 ± 0.0149	0.1040 ± 0.0229	0.1079 ± 0.0246	0.1040 ± 0.0177				
Seminal vesicles	A	1.873 ± 0.334	1.925 ± 0.256	1.884 ± 0.294	1.905 ± 0.268				
	R	0.4321 ± 0.0617	0.4495 ± 0.0684	0.4357 ± 0.0680	0.4582 ± 0.0649				
Spleen	A	0.671 ± 0.109	0.668 ± 0.081	0.673 ± 0.118	0.650 ± 0.087	0.512 ± 0.057	0.529 ± 0.061	0.497 ± 0.054	0.506 ± 0.045
	R	0.1547 ± 0.0176	0.1554 ± 0.0160	0.1551 ± 0.0228	0.1559 ± 0.0184	0.2048 ± 0.0224	0.2117 ± 0.0173	0.2054 ± 0.0203	0.2074 ± 0.0160
Testes L+R	A	3.600 ± 0.423	3.531 ± 0.366	3.635 ± 0.338	3.558 ± 0.337				
	R	0.8358 ± 0.1067	0.8232 ± 0.0945	0.8406 ± 0.0742	0.8561 ± 0.0879				

		Males				Females			
Dose (ppm)		**0**	**10000**	**25000**	**50000**	**0**	**10000**	**25000**	**50000**
Mg/kg bw/d		**0**	**82**	**817**	**4279**	**0**	**82**	**817**	**4279**
Thymus	A	0.348 ± 0.091	0.330 ± 0.084	0.321 ± 0.061	0.335 ± 0.073	0.284 ± 0.056 ()	0.315 ± 0.064	0.282 ± 0.050	0.279 ± 0.060
	R	0.0808 ± 0.0222	0.0765 ± 0.0170	0.0740 ± 0.0125	0.0800 ± 0.0142	0.1135 ± 0.0206	0.1265 ± 0.0263	0.1164 ± 0.0199	0.1141 ± 0.0227
Thyroids+parathyroids	A	0.018 ± 0.004	0.018 ± 0.003	**0.020 ±0.004 (↑11%)**	**0.021* ±0.005 (↑17%)**	0.015 ± 0.003	0.014 ± 0.002	0.015 ± 0.003	0.016 ± 0.003
	R	0.0042 ± 0.0009	0.0041 ± 0.0006	**0.0045 ±0.0009 (↑7%)**	**0.0050** ±0.0011 (↑19%)**	0.0060 ± 0.0012	0.0056 ± 0.0008	0.0063 ± 0.0011 (↑5%)	**0.0067* ±0.0011 (↑12%)**
Uterus + Cervix	A					0.751 ± 0.217	0.692 ± 0.258 (↓8%)	0.653 ± 0.228 (↓13%)	0.687 ± 0.294 (↓9%)
	R					0.3015 ± 0.0878	0.2787 ± 0.1089 (↓8%)	0.2700 ± 0.0937 (↓10%)	0.2782 ± 0.1066 (↓8%)

A: absolute (g); R: relative to b.w. (%), Statistically significant modification : *: p<0.05; **: p<0.01

Table B.6.6.1.2-4b Two generation study with lenacil in Wistar rats ([redacted] 2003): organ weight – F_1

		Males				Females			
Dose (ppm)		**0**	**10000**	**25000**	**50000**	**0**	**10000**	**25000**	**50000**
Mg/kg bw/d		**0**	**82**	**817**	**4279**	**0**	**82**	**817**	**4279**
Terminal body weight	A	442.2 ± 49.5	425.3 ± 30.5	435.9 ± 32.9	427.9 ± 41.7	254.0 ± 14.7	257.8 ± 22.1	243.5 ± 12.9	**241.4* ± 17.4** (↓5%)
Brain	A	2.00 ± 0.07	2.00 ± 0.09	2.01 ± 0.10	2.01 ± 0.06	1.89 ± 0.06	1.88 ± 0.10	1.86 ± 0.08	1.86 ± 0.07
	R	0.457 ± 0.046	0.472 ± 0.037	0.463 ± 0.034	0.474 ± 0.040	0.746 ± 0.051	0.734 ± 0.053	0.766 ± 0.039	0.774 ± 0.054
Pituitary	A	0.007 ± 0.002	0.007 ± 0.002	0.008 ± 0.003	**0.009* ±0.004 (↑29%)**	0.013 ± 0.002	0.013 ± 0.003	0.012 ± 0.004	0.011 ± 0.003
	R	0.0015 ± 0.0005	0.0016 ± 0.0006	**0.0019* ±0.0006 (↑27%)**	**0.0021* ±0.0009 (↑40%)**	0.0052 ± 0.0008	0.0049 ± 0.0009	0.0051 ± 0.0014	0.0047 ± 0.0010
Adrenals	A	0.058 ± 0.010	0.056 ± 0.009	0.056 ± 0.007	0.058 ± 0.009	0.090 ± 0.012	0.091 ± 0.013	0.089 ± 0.014	0.084 ± 0.010
	R	0.0131 ±0.0018	0.0132 ± 0.0018	0.0128 ± 0.0016	0.0136 ± 0.0020	0.0354 ±0.0048	0.0355 ± 0.0048	0.0365 ± 0.0049	0.0351 ± 0.0041
Epididymides L+R	A	1.148 ± 0.156	1.156 ± 0.108	1.192 ± 0.104	1.188 ± 0.083				
	R	0.2606 ± 0.0323	0.2728 ± 0.0272	0.2744 ± 0.0254	0.2793 ± 0.0257				
Kidneys	A	2.55 ± 0.33	2.41 ± 0.18	2.47 ± 0.20	2.50 ± 0.25	2.00 ± 0.016	2.11 ± 0.19	1.97 ± 0.13	1.98 ± 0.13
	R	0.581 ± 0.074	0.568 ± 0.031	0.568 ± 0.031	0.585 ± 0.045	0.791 ± 0.063	0.818 ± 0.051	0.808 ± 0.044	0.820 ± 0.0052

		Males				Females			
Dose (ppm)		**0**	**10000**	**25000**	**50000**	**0**	**10000**	**25000**	**50000**
Mg/kg bw/d		**0**	**82**	**817**	**4279**	**0**	**82**	**817**	**4279**
Liver	A	15.10 ± 2.43	15.24 ± 1.77	15.86 ± 1.43 (↑5%)	**16.48* ±2.06 (↑9%)**	12.06 ± 1.30	12.72 ± 1.85	12.23 ± 1.26	**13.29* ±1.46 (↑10%)**
	R	3.410 ± 0.367	3.575 ± 0.230 (↑5%)	**3.641* ±0.235 (↑7%)**	**3.847** ±0.244 (↑13%)**	4.741 ± 0.319	4.924 ± 0.491	5.022 ± 0.415 (↑6%)	**5.515** ±0.561 (↑16%)**
Ovaries + Ovid. L+R	A					0.160 ± 0.025	0.150 ± 0.018 (↓6%)	0.148 ± 0.019 (↓8%)	0.146 ± 0.024 (↓9%)
	R					0.0629 ± 0.0091	0.0584 ± 0.0068 (↓7%)	0.0607 ± 0.0075 (↓3%)	0.0604 ± 0.0099 (↓4%)
Prostate	A	0.437 ± 0.078	0.432 ± 0.093	0.448 ± 0.112 (↑3%)	0.462 ± 0.090 (↑6%)				
	R	0.0993 ± 0.0174	0.1018 ± 0.0210	0.1029 ± 0.0253 (↑4%)	0.1087 ± 0.0227 (↑9%)				
Seminal vesicles	A	1.665 ± 0.302	1.602 ± 0.257	1.616 ± 0.216	1.594 ± 0.211				
	R	0.3791 ± 0.0752	0.3786 ± 0.0654	0.3717 ± 0.0504	0.3730 ± 0.0196				
Spleen	A	0.691 ± 0.121	0.677 ± 0.083	0.692 ± 0.069	0.672 ± 0.107	0.559 ± 0.083	0.529 ± 0.060 (↓5%)	**0.507* ± 0.067 (↓9%)**	**0.510* ± 0.062 (↓9%)**
	R	0.1566 ± 0.0239	0.1593 ± 0.0176	0.1592 ± 0.0173	0.1570 ± 0.0196	0.2194 ± 0.0252	0.2058 ± 0.0228 (↓6%)	0.2084 ± 0.0268 (↓5%)	0.2125 ± 0.0324 (↓3%)
Testes L+R	A	3.65 ± 0.45	3.62 ± 0.36	3.68 ± 0.32	3.69 ± 0.31				
	R	0.83 ± 0.17	0.85 ± 0.09	0.85 ± 0.08	0.87 ± 0.09				
Thymus	A	0.371 ± 0.095	0.359 ± 0.058	0.349 ± 0.059	0.385 ± 0.087	0.352 ± 0.060	0.335 ± 0.052 (↓5%)	**0.315 ± 0.060 (↓11%)**	**0.277** ± 0.059 (↓21%)**
	R	0.0847 ± 0.0224	0.0846 ± 0.0131	0.0802 ± 0.0118	0.0900 ± 0.0185	0.1389 0.0255	0.1296 0.0146 (↓6%)	0.1294 0.0257 (↓7%)	**0.1148** 0.0240 (↓17%)**
Thyroids + parathyroids	A	0.019 ± 0.004	0.017 ± 0.004	0.019 ± 0.004	**0.022 ± 0.005 (↑16%)**	0.016 ± 0.002	0.016 ± 0.003	0.017 ± 0.003	0.017 ± 0.003
	R	0.0044 ± 0.0010	0.0039 ± 0.0010	0.044 ± 0.0008	**0.0051* ± 0.0009 (↑16%)**	0.0062 ± 0.0010	0.0062 ± 0.0012	**0.0070 ± 0.0014 (↑13%)**	**0.0071* ± 0.0010 (↑15%)**
Uterus + Cervix	A					0.731 ± 0.234	0.965 ± 0.240	0.670 ± 0.176 (↓8%)	**0.568* ± 0.162 (↓22%)**
	R					0.2882 0.0938	0.2697 0.0925	0.2768 0.0787 (↓4%)	**0.2362 0.0705 (↓18%)**

A: absolute (g); R: relative to b.w. (%), Statistically significant modification : *: $p<0.05$; **: $p<0.01$

Table B.6.6.1.2-4c Two generation study with lenacil in Wistar rats ([redacted] 2003): organ weight – F1 offspring

		Males				Females			
Dose (ppm)		**0**	**10000**	**25000**	**50000**	**0**	**10000**	**25000**	**50000**
Mg/kg bw/d		**0**	**82**	**817**	**4279**	**0**	**82**	**817**	**4279**
Terminal body weight	A	95 ± 6.1	90 ± 7.8	90 ± 9.4	**85**±9.9 (↓11%)**	86 ± 5.7	83 ± 7.5	**80* ± 6.1 (↓7%)**	**78** ± 8.5 (↓9%)**
Brain	A	1.61 ± 0.07	1.59 ± 0.09	1.61 ± 0.07	1.57 ± 0.07	1.54 ± 0.06	1.54 ± 0.08	1.53 ± 0.06	1.50 ± 0.07
	R	1.70 ± 0.10	1.77 ± 0.15	1.80* ± 0.16 (↑6%)	1.86** ± 0.19 (↑9%)	1.80 ± 0.12	4.87 ± 0.15	1.92** ± 0.13 (↑7%)	1.94** ± 0.16 (↑8%)
Spleen	A	0.36 ± 0.06	0.32* ± 0.06 (↓11%)	**0.33* ± 0.05 (↓8%)**	**0.31* ± 0.05 (↓14%)**	0.30 ± 0.05	0.28 ± 0.05	**0.27 ± 0.05 (↓10%)**	**0.26** ± 0.04 (↓13%)**
	R	0.38 ± 0.06	0.35 ± 0.06	0.37 ± 0.05	0.36 ± 0.04	0.35 ± 0.04	0.34 ± 0.05	0.34 ± 0.05	0.33 ± 0.05
Thymus	A	0.4 ± 0.04	0.38 ± 0.05 (↓5%)	**0.37* ± 0.06 (↓8%)**	**0.33** ± 0.05 (↓18%)**	0.38 ± 0.05	0.35 ± 0.05 (↓8%)	**0.34* ± 0.05 (↓11%)**	**0.33** ± 0.05 (↓13%)**
	R	0.42 ± 0.04	0.42 ± 0.05	0.41 ± 0.05	**0.38* ± 0.04 (↓10%)**	0.44 ± 0.05	0.42 ± 0.04 (↓5%)	0.42 ± 0.06 (↓5%)	0.42 ± 0.06 (↓5%)

A: absolute (g); R: relative to b.w. (%), Statistically significant modification : *: $p<0.05$; **: $p<0.01$

Taking into account the demonstrated effects of lenacil on liver, thyroid, spleen and thymus weight, corroborated by macro- or histopathological findings, the NOAEL for organ weight findings was conceivably 82 mg/kg b.w./d.
Organ weights in F_0 and F_1 ♂(after 17 weeks of treatment) and ♀(on d28 post-partum); in F_2 group mean values for unselected offspring on Day 30 of age (F2); tables B.6.6.1.2-4a: **F_0**; -4b: **F_1**; -4c: **F_2**

Macroscopic findings:

On d28 *post-partum*, both F_0 and F_1♀, and F_1♂ showed dark thyroids at 817 mg/kg b.w./d and above, in a dose-dependent manner. A marginal increased incidence of enlarged thyroids was also observed in few top-dose F_1 animals. It is also remarkable that F_1 offspring is more heavily affected than the F_0 parental animals.

Discoloration of the thyroid gland has been reported in the other studies as a treatment-related effect of administration of a variety of compounds and can be attributed either to an accumulation of the chemical/metabolite, or to increased cellular lipid oxidation. The findings were corroborated in histopathology examination, with thyroids exhibiting Schmorl's positivity, indicating staining of reducing substances including melanin, for which it is a useful method, enterochromaffin and lipofuscin. In the case of lenacil, the MoA for the thyroid blackening remains obscure.

Occasional effects in other organs observed in singularity were considered of no relevance by the RMS.

Macropathology in ♂ (after 17 weeks of treatment) and ♀ (on d28 post-partum): table B.6.6.1.2-5a: F_0, and table B.6.6.1.2-5b: F_1

Table B.6.6.1.2-5a Two generation study with lenacil in Wistar rats (2003): macropathology F_0

		Males				Females			
Dose	**(ppm)**	**0**	**10000**	**25000**	**50000**	**0**	**10000**	**25000**	**50000**
	mg/kg bw/d	**0**	**82**	**817**	**4279**	**0**	**82**	**817**	**4279**
# animals		*28*	*28*	*28*	*28*	*25*	*28*	*27*	*27*
Kidneys	pelvic dilatation	-	-	-	-	0	0	0	1
Seminal vesicles	pale area	0	0	0	1				
	small	0	0	0	1				
	dark	0	0	0	1				
Skin/ subcutis	scabs	0	0	1	1	0	0	0	1
Testes L+R	flaccid	0	0	0	1				
	blue	0	0	0	1				
Thyroids	dark	0	0	0	1	0	0	**1**	**25*** ** (93%)

Statistically significant modification : *: p<0.05; **: p<0.01;***: p<0.001

Table B.6.6.1.2-5b Two generation study with lenacil in Wistar rats (2003): macropathology F_1

		Males				Females			
Dose	**(ppm)**	**0**	**10000**	**25000**	**50000**	**0**	**10000**	**25000**	**50000**
	mg/kg bw/d	**0**	**82**	**817**	**4279**	**0**	**82**	**817**	**4279**
# animals		*24*	*24*	*24*	*24*	*22*	*23*	*22*	*24*
LN axillary	regional to mass					0	0	0	1
Mammary caud. (LN)	mass					0	0	0	1
Mammary protocol	thickened					0	0	0	1
	pale					0	0	0	1
Thymus	dark area(s)	1	0	1	**2**	-	-	-	-
Thyroids	dark	0	0	**5*** (21%)	**23**** (96%)	0	0	**8**** (36%)	**22***** (92%)
	enlarged	0	0	0	**2** (8%)	0	0	0	**1**
	not apparent	0	0	0	1	-	-	-	-

Statistically significant modification : *: p<0.05; **: p<0.01;***: p<0.001

Histopathology:

Examination of the thyroid sections stained with hematoxylin and eosin revealed a minimal or slight accumulation of pigment in the follicular epithelium of some animals at top dose.

In the F_0 and F_1 ♀ at 82 mg/kg b.w./d and above, there was an increased incidence and severity of Schmorl's positive pigment whilst in the top-dose F_0 and F_1 ♂, there was an increased severity of this change (taking into account the already high background incidence in the control animals). A slight increased incidence of follicular cell hypertrophy was observed in some animals, which may indicate hyperactivity of the thyroid.

A follicular cell adenoma was observed in a F_1 ♂given the top dose and, in view of this treatment related changes observed in the thyroids, involvement of treatment in this finding cannot be excluded. Follicular cell debris was present in the colloid of rats 817 mg/kg b.w./d and above, and was generally associated with the Schmorl's positive pigment. **RMS** notes that the marginal increase of Schmorl's $^+$ pigment at the lowest dose of the F_1♀ (incidence: 0, 2, **13**, **20**, at respectively 0, 1, 2.5 and 5% dietary a.s. level) in the absence of any other finding at that dose, in neither the thyroid nor any other organ, was unlikely to be considered an adverse effect. However, this position could be disagreed upon, knowing that the low-dose finding is part of a clear dose-dependent response.

The presence of cellular debris in the follicles of a few animals is indicative of increased follicular cell turnover, and according to the notifier, as a consequence of an *"increase in metabolic activity"*. Although it remains possible that the thyroid findings could perhaps be explained by enzymatic induction (in line with hepatic effects like liver weight increase, CL hypertrophy), thyroid stimulation with a possible feed-back on the pituitary gland (weight increase), the observed thyroid blackening can be suspected to be the result of another MoA, for which the relevance for the human cannot be excluded. Therefore, this should be further discussed.

Additional investigations (see B.6.8) were performed on thyroids to clarify the toxicological significance of the thyroid findings. These further thyroid studies provided evidence to suggest that lenacil did not affect the ability of the thyroid to take up and organify iodide and lenacil dose not act as an inhibitor of the deiodinase which converts T_4 toT_3. However, these studies provided no explanation on the possible MoA of the adverse effect on the thyroid.

Further, acute epithelium inflammatory infiltration in the uterine cervix, uterus myometrial hyalinisation, (F_0) pigmented macrophage (F_0, F_1) and glandular dilatation (F_1) and vaginal acute inflammatory infiltration of epithelium (F_0, F_1) was observed in the top-dose animals.

- **The incidence of the *uterine/vaginal* histopathological findings was not highly increased, but there was concordance between generations, and a further explanation should be provided for these observations, since these findings are consistent with possible endocrine effects of unknown MoA.**
- **The *thyroid effects* (also observed in several repeated toxicity studies) should be further discussed.**
- **RMS notes also that *inflammatory events* are observed. In association with the *altered haematology* (notably ↓lymphocyte/WBC counts) observed in other studies, possibly supported by effects in the thymus (weight decreases, macropathology and histopathological findings) and the clinical signs (coat hairloss, staining) observed in the present study (table B.6.6.1.2-1), a potential link to altered immunity could be suspected.**

Therefore, the notifier is kindly invited to discuss these issues.

Notifier: There was no effect on uterine luminal dilation in the multigeneration study:
F0 control:14/25, high dose group: 10/27
F1 control: 7/22, high dose group: 4/24

Other findings have been commented on in response to other questions.

Histopathology in ♂ (after 17 weeks of treatment) and ♀(on Day 28 post-partum), tables B.6.6.1.2-6a: F_0 and table B.6.6.1.2-6b: F_1.

The RMS concluded from this histopathological evaluation of all relevant tissues and organs in this 2G study that a NOAEL could be proposed at 82 mg/kg b.w./d, thereby disregarding marginal thyroid pigmentation at the lowest dose.

Table B.6.6.1.2-6a Two generation study with lenacil in Wistar rats ([redacted] 2003): Histopathology – F_0

		Males				Females			
Dose	**ppm**	**0**	**10000**	**25000**	**50000**	**0**	**10000**	**25000**	**50000**
	Mg/kg bw/d	**0**	**82**	**817**	**4279**	**0**	**82**	**817**	**4279**
Kidneys	hydronephrosis					0/0	0/0	0/0	1/1
Liver	hepatocyte hypertrophy, centrilobular	0/28	0/1	0/0	1/28	-	-	-	-
Pituitary	vacuolated cells –pars distalis, focal	0/28	0/1	0/0	1/28	-	-	-	-
Skin /subcutis	epidermal ulceration	0/0	0/0	1/1	0/1	0/0	0/0	0/0	1/1
	epidermal hyperplasia	0/0	0/0	1/1	0/1	0/0	0/0	0/0	1/1
	dermal inflammation	0/0	0/0	1/1	0/1	0/0	0/0	0/0	1/1
Testes L+R	seminiferous tubular atrophy	1/28	1/1	0/1	**3/28** (11%)				
Thymus	haemorrhage	2/28 (7%)	1/1	0/0	**5/28** (18%)	-	-	-	-
	cyst(s)	1/28	0/1	0/0	**2/28 (7%)**	5/25	0/0	0/0	6/27
	involution/ atrophy	0/28	1/28	0/28	**1/28**	-	-	-	-
Thyroids	Schmorl's^{+} pigment	14/20 (70%)	14/20 (70%)	**16/20** (80%)	**19/20** (95%)	0/25	0/28	**20/27***** (74%)	**24/27***** **(89%)**
	follicular cellular debris	0/20	0/20	**6/20*** (30%)	**15/20***** (75%)	0/25	0/28	**5/27** (19%)	**25/27***** (93%)
	follicular cell hypertrophy	1/20	0/20	**3/20** (15%)	**4/20** (20%)	0/25	0/28	0/27	**9/27**** **(33%)**
	follicular haemorrhage	0/20	0/20	0/20	**2/20** (10%)	-	-	-	-
	pigmented follicular epithelium	-	-	-	-	0/25	0/28	0/27	**2/27 (7%)**
Uterus	pigmented macrophage					14/25 (56%)	0/0	0/0	**18/27 (67%)**
	endometrial polyploid hyperplasia					0/25	0/0	0/0	**1/27** (4%)
Vagina	acute inflammatory infiltration of epithelium					0/25	0/0	0/0	**2/27** (7%)

Statistically significant modification : *: $p<0.05$; **: $p<0.01$;***: $p<0.001$

Table B.6.6.1.2-6b Two generation study with lenacil in Wistar rats ([redacted] 2003): Histopathology – F_1

		Males				Females			
Dose	**ppm**	**0**	**10000**	**25000**	**50000**	**0**	**10000**	**25000**	**50000**
	Mg/kg bw/d	**0**	**82**	**817**	**4279**	**0**	**82**	**817**	**4279**
Adrenals	cortical vacuolation	0/24	0/1	0/0	1/24	-	-	-	-
Liver	hepatocyte hypertrophy, centrilobular	0/24	0/24	0/24	**3/24** (13%)	-	-	-	-
	pigmented macrophages	-	-	-	-	0/22	0/0	0/0	**1/24**
	capsular fibrosis	-	-	-	-	0/22	0/0	0/0	1/24
Mammary	activated mammary tissue[$]	-	-	-	-	0/0	0/0	0/0	1/1
	fibrosis/inflammation	-	-	-	-	0/0	0/0	0/0	1/1
	necrosis	-	-	-	-	0/0	0/0	0/0	1/1
Skin/subcutis	abcessation	-	-	-	-	0/0	0/0	0/0	1/1
Thymus	haemorrhage	5/24 (7%)	0/0	1/1	**9/24 (38%)**	1/22	0/0	0/0	0/24
	cyst(s)	-	-	-	-	0/22	0/0	0/0	**2/24**
	involution/atrophy	-	-	-	-	0/22	0/0	0/0	**1/24**
Thyroids	B-follicular cell adenoma	0/20	0/20	0/20	**1/23**	-	-	-	-
	Schmorl's[+] pigment	14/20	15/20	**18/20 (90%)**	**23/23** (100%)**	0/20	2/20 (10%)	**13/20*** (65%)**	**20/22*** (90%)**
	Follicular cellular debris	0/20	0/20	1/20 (5%)	**5/23 (22%)**	1/20	0/20	2/20 (10%)	**15/22*** (68%)**
	Follicular cell hypertrophy	0/20	0/20	0/20	**2/23** (9%)	0/20	0/20	0/20	**2/22** (9%)
	Follicular dilatation	0/20	0/20	0/20	**1/23**	-	-	-	-
	Pigment in follicular epithelium	1/20	0/20	0/20	**4/23** (17%)	0/20	0/20	0/20	**2/22** (9%)
	Pigmented interstitial macrophage	0/20	0/20	0/20	**1/23** (4%)	-	-	-	
	Ectopic thymic tissue	1/20	1/20	0/20	0/23	2/20	0/20	0.20	0/22
Uterine cervix	Acute inflammatory infiltration of epithelium	-	-	-	-	2/22 (9%)	0/0	0/0	**4/23** (17%)
Uterus	Myometrial hyalinisation	-	-	-	-	12/22 (54%)	0/0	0/0	**19/24** (79%)
	Pigmented macrophage	-	-	-	-	10/22 (45%)	0/0	0/0	**16/24** (67%)
	Glandular dilatation	-	-	-	-	0/22	0/0	0/0	**1/24**
Vagina	Acute inflammatory infiltration of epithelium	-	-	-	-	1/21	0/1	0/0	**6/24** (25%)

Statistically significant modification : *: $p<0.05$; **: $p<0.01$;***: $p<0.001$

RMS: In table B.6.6.1.2-6b, 'activated mammary tissue'[$] was clarified as follows by the **notifier**: the laboratory (Envigo, who took over Huntington) confirmed that this means the mammary tissue was thickened at macroscopic examination and at microscopic examination had hypertrophic changes and hyperplastic changes which were normal for a lactating ♀rat.

In **table B.6.6.1.2-6c**, it appears that the increase observed in various parameters in gynecological organs (uterus/cervix/vagina) is somewhat stronger in F_1 than in F_0 animals. It is unclear if this represents a trend in terms of worsening of effects in F_1 when compared with F_0. However, the effect remains notable and it remains uncertain whether lenacil affects the immune system in some way, and the conduct of an immunotoxicity study is requested. It is of note that the effect was limited to the top-dose, and therefore, this top-dose should be investigated.

Table B.6.6.1.2-6c Two generation study with lenacil in Wistar rats ([redacted] 2003): Comparison of histological changes in the uterus, uterus/cervix and vagina between F_0 and F_1

Finding		0 ppm	50.000 ppm (4279 mg/kg bw/d)
Acute inflammatory infiltration of the uterine/cervical epithelium	F_0	0/25	0/27
	F_1	2/22	4/23 (↑**91%**)
Uterine myometrial hyalinisation	F_0	17/25	21/27 (↑14%)
	F_1	12/22	19/24 (↑**45%**)
Uterine pigmented macrophage accumulation	F_0	14/25	18/27 (↑19%)
	F_1	10/22	16/24 (↑**47%**)
Acute inflammatory infiltration of vaginal epithelium	F_0	0/25	2/27 (↑7%)
	F_1	1/21	**6**/24 (↑**432%**)

Litter data:

Pre-weaning surface and air righting reflex were unaffected and all offspring displayed normal auditory and visual responses. Physical sexual maturation of the selected rats, as assessed by the age and bw at completion of balano-preputial separation and vaginal opening, was unaffected by treatment. **RMS** detailed the physical exams performed pre-weaning. From these figures (table B.6.6.1.2-6d) it would appear that no abnormalities were observed, but is id noted that no full developmental neurotoxicity investigation was performed. Since lenacil has some effects on thyroid functionality, this consititutes a potential data gap.

Table B.6.6.1.2-6d Two generation study with lenacil in Wistar rats ([redacted] 2003): pre-weaning examinations- F_1

Dose	ppm	0	1000	10000	50000
	mg/kg bw/d	0	82	817	4279
#litters examined		*25*	*28*	*28*	*27*
#pups examined		*192*	*214*	*212*	*208*
Surface righting (d of age)		3.9 ± 1.0	3.8 ± 0.7	3.8 ± 0.8	3.9 ± 0.8
Air righting (d of age)		16.4 ± 1.0	16.2 ± 0.9	16.3 ± 0.8	16.5 ± 0.8
Pupil reflex (% passed)		100	100	100	100
Startle response (% passed)		100	100	100	100

Values expressed in group mean ± s.d.

Litter data F_1 (from F_0-F_1) : litter size, offspring survival indices, sex ratio, sexual maturation are tabulated in tables below B.6.6.1.2-7a: litter size; -7b: offspring survival indices; -7c: sex ratio, and -7d : sexual maturation

Table B.6.6.1.2-7a Two generation study with lenacil in Wistar rats ([redacted] 2003): litter size - F_0-F_1

Dose	ppm	0	1000	10000	50000
	mg/kg bw/d	0	82	817	4279
#litters examined		*25*	*28*	*28*	*27*
# Implantation sites		13.1 ± 2.5	12.8 ± 2.5	12.8 ± 3.1	12.8 ± 2.0
	Age (d)				
total	1§	12.5 ± 2.3	11.9 ± 2.6	11.9 ± 3.3	12.3 ± 2.3
# alive	1*	12.0 ± 2.5	11.9 ± 2.5	11.8 ± 3.4	12.0 ± 2.4
	4*	12.0 ± 2.5	11.7 ± 2.6	11.7 ± 3.3	12.0 ± 2.5
	4**	7.9 ± 0.4	7.8 ± 0.9	7.6 ± 1.1	7.9 ± 0.5
	7**	7.8 ± 0.5	7.7 ± 1.1	7.6 ± 1.1	7.8 ± 0.5
	14**	7.7 ± 0.6	7.6 ± 1.1	7.6 ± 1.2	7.7 ± 0.6
	21**	7.7 ± 0.6	7.6 ± 1.1	7.6 ± 1.2	7.7 ± 0.6

Values expressed in group mean ± SD; §: includes offsprings that died prior to designated; *before cull, **after cull

Table B.6.6.1.2-7b Two generation study with lenacil in Wistar rats ([redacted] 2003): survival indices - F_0-F_1

Dose	ppm	0	1000	10000	50000
	Mg/kg bw/d	0	82	817	4279
#litters examined		*25*	*28*	*28*	*27*
	Age (day)				
Post-implantation		95.3	92.7	92.1	95.5
Live birth index	1	96.2	99.7	99.0	97.5
Viability index	4	99.6	98.2	99.8	99.7
Lactation index	7	99.0	98.2	100.0	99.5
	14	97.0	97.8	98.8	98.1
	21	97.0	97.8	98.8	98.1

Values expressed in group mean (%)

Table B.6.6.1.2-7c Two generation study with lenacil in Wistar rats ([redacted] 2003): sex ratio - F_0-F_1

Dose	ppm	0	1000	10000	50000
	Mg/kg bw/d	0	82	817	4279
	Age (day)				
total§	1	45.1 ± 14.6	47.4 ± 18.5	52.7 ± 15.8	48.5 ± 17.0
live	1*	43.6 ± 16.4	47.2 ± 18.6	52.5 ± 16.1	47.9 ± 17.2
	4*	44.2 ± 16.7	47.4 ± 18.9	52.4 ± 16.1	47.7 ± 17.5
	4**	47.2 ± 13.7	50.4 ± 15.0	50.8 ± 12.8	48.8 ± 12.7
	21**	46.7 ± 13.7	50.6 ± 15.3	51.1 ± 13.3	48.8 ± 12.9

Values expressed in group mean ± SD; §: includes offsprings that died prior to designated; *before cull, **after cull

Table B.6.6.1.2-7d Two generation dietary study with lenacil in Han Wistar rats ([redacted] 2003): sexual maturation - group mean age and bodyweight for selected offspring at completion (F_0 - F_1).

Dose	ppm	0	1000	10000	50000
	Mg/kg bw/d	0	82	817	4279
Vaginal opening	Day of age	34 ± 2.5	34 ± 1.9	34 ± 2.1	**35 ± 2.5**
	Bodyweight (g)	102 ± 12.5	105 ± 11.8	103 ± 9.7	103 ± 13.9
Balano-preputial separation	Day of age	46 ± 2.5	46 ± 2.3	46 ± 2.9	46 ± 2.7
	Bodyweight (g)	184 ± 19.4	184 ± 16.5	181 ± 18.4	186 ± 26.9

RMS notes that VO is slightly delayed, in the absence of body weight changes.

Litter data F_2 (from F_1-F_2) : litter size, offspring survival indices, sex ratio, sexual maturation are tabulated in tables below B.6.6.1.2-8a: litter size; -8b: offspring survival indices; -8c: sex ratio, and -8d : sexual maturation

Table B.6.6.1.2-8a Two generation study with lenacil in Wistar rats ([redacted] 2003): litter size – F_1-F_2

Dose	ppm	0	1000	10000	50000
	Mg/kg bw/d	0	82	817	4279
#litters examined		22	23	22	24
# Implantation sites		12.2 ± 1.7	11.6 ± 2.6	12.3 ± 1.7	11.5 ± 2.2
	Age (d)				
total	1§	11.9 ± 1.7	11.3 ± 2.7	11.5 ± 2.2	11.0 ± 2.3
# alive	1*	11.8 ± 1.7	11.2 ± 2.8	11.4 ± 2.3	10.7 ± 2.4
	4*	11.7 ± 1.6	11.1 ± 2.8	11.3 ± 2.4	10.7 ± 2.4
	4**	8.0 ± 0.2	7.7 ± 1.3	7.9 ± 0.5	7.8 ± 1.2
	7**	8.0 ± 0.2	7.7 ± 1.3	7.8 ± 0.5	7.8 ± 1.2
	14**	7.9 ± 0.5	7.6 ± 1.3	7.7 ± 0.8	7.8 ± 1.2
	21**	7.9 ± 0.5	7.6 ± 1.3	7.7 ± 0.8	7.8 ± 1.2

Values expressed in group mean ± SD; §: includes offsprings that died prior to designated; *before cull, **after cull

Table B.6.6.1.2-8b Two generation study with lenacil in Wistar rats ([redacted] 2003): survival indices – F_1-F_2

Dose	ppm	0	1000	10000	50000
	Mg/kg bw/d	0	82	817	4279
#litters examined		*22*	*23*	*22*	*24*
Post-implantation	Age (day)	97.1	95.4	93.1	94.7
Live birth index	1	98.9	99.1	98.7	96.9
Viability index	4	99.3	99.6	99.2	100.0
Lactation index	7	100.0	100.0	99.4	100.0
	14	98.9	98.4	98.3	100.0
	21	98.9	98.4	98.3	100.0

Values expressed in group mean (%)

Table B.6.6.1.2-8c Two generation study with lenacil in Wistar rats ([redacted] 2003): sex ratio – F_1-F_2

Dose	ppm	0	1000	10000	50000
	Mg/kg bw/d	0	82	817	4279
	Age (day)				
total[§]	1	52.1 ± 12.3	49.5 ± 13.3	49.9 ± 12.0	45.5 ± 17.5
live	1*	51.8 ± 12.8	49.4 ± 13.2	49.4 ± 12.0	45.1 ± 17.7
	4*	52.2 ± 13.0	49.7 ± 13.3	49.0 ± 12.1	45.1 ± 17.7
	4**	51.4 ± 7.2	49.2 ± 6.7	49.0 ± 7.2	47.4 ± 13.8
	21**	50.6 ± 8.2	48.2 ± 7.5	49.5 ± 7.6	47.4 ±13.8

Values expressed in group mean ± SD; [§]: includes offsprings that died prior to designated; *before cull, **after cull

No meaningful effect on implantation number, fertility parameters, sex ratio, litter size. A slight delayed VO was observed but PPS was unaffected. The effect was without statistical significance and the isolated finding on developmental landmarks was deemed toxicologically of uncertain relevance.

Conclusion (2G study rats)

At 10000 ppm (817 mg/kg b.w./d) and 50000 ppm (4279 mg/kg b.w./d), maternal body weight was slightly altered, exhibited alopecia, and there was evidence of thyroid toxicity: increased weight, altered metabolism and histopathology. At these doses, dose-dependent decreases of spleen and thymus weight , and increased liver and pituitary weights were observed.

Reproductive organs and reproductive preformance and offspring survival were unaffected by treatment. Physical and sexual development of the offsprings were not altered.

At 817 mg/kg b.w./d and above, body weight of offsprings were reduced during lactation.

In summary:

Parental toxicity **NOAEL** = 1000 ppm = **82 mg/kg b.w./d**

Parental toxicity LOAEL= 10000 ppm = 817 mg/kg b.w./d, based upon:

↓body weight, ↑clinical signs (alopecia F_0,F_1), ↑liver w (F_0,F_1), ↑thyroid w (F_0), ↑pituitary w (F_1), ↓spleen w (F_1), ↓thymus w (F_1), ↑dark/Schmorl's$^+$ thyroids (F_0,F_1), ↑thyroid cell debris/hypertrophy (F_0,F_1).

Offspring toxicity **NOAEL** = 1000 ppm = **82 mg/kg b.w./d**

Offspring toxicity LOAEL= 10000 ppm = 817 mg/kg b.w./d, based upon:

↓b.w. gain lactation d1-21 (F_1,F_2), ↓terminal b.w. (F_1), ↓spleen w (F_1), ↓thymus w (F_1)

Reproductive **NOAEL** = 50000 ppm = **4279 mg/kg b.w./d**

It is of note that no full developmental neurotoxicity assessment was performed in the generational studies. Taking into account the possible impact of the treatment on thyroid, it was questioned whether a neurodevelopmental study would be necessary.

Notifier comment:

"The notifier proposes to set a NOAEL reproduction toxicity = 50000ppm (4278-5312mg/kg bw/d for males and 4787-8839mg/kg bw/d for females).

The notifier disagrees with the RMS proposal for a systemic parental NOAEL of 1000 ppm since this does not appear to take account of the additional thyroid investigations with the conclusion that lenacil is not directly toxic to thyroid function. The notifier has also submitted argumentation (see previous notifier comment) relating to the effects on offspring weight gain, which if accepted as non-adverse in the context of this study, will affect the derived NOAEL.

It is accepted that offspring bodyweights were slightly lower than controls in the F0F1 (by 6%) and F1F2 (by 11%) during the lactation period, but offspring survival was not adversely affected, and the bodyweights of the F0F1 pups selected for the F1 generation were not different from controls at the start of the pre-mating maturation period. Also, the behavioural and developmental landmarks assessed prior to and after weaning were not adversely affected by either maternal treatment or by direct intake of the test material. Any marginal bodyweight effects on offspring prior to weaning are considered transient, and insufficient evidence for adverse effects via maternal milk.

RMS reported that the initial birth weight of the F_1 and F_2 offspring was unaffected by maternal treatment but there was a reduction of weight gain at 50000ppm that occurred from day 7 of age for the F_1 offspring and from day 4 of age for the F_2 offspring. This effect occurred before that offspring begin to consume solid food suggesting an effect via lactation. Whether treatment caused a reduction in milk production or quality or whether the offspring were exposed to lenacil via the milk cannot be ascertained in this study.

The parental NOAEL in rats was determined to be 81.9 mg/kg bw/day, based on evidence of altered maternal body weigts, thyroid and liver metabolism at 1727 and 4300 mg/kg bw/day, respectively. The offspring and reproductive NOAELs were determined to be 1727 and 4300 mg/kg bw/day, respectively. The offspring NOAEL of 1727 mg/kg bw/day was based on the fact that body weight gain of offsprings during lactation was reduced at the dose level of 4300 mg/kg bw/day. During the peer review it was concluded that due to the very high dose level applied in the study (4300 mg/kg bw/d which exceeds the 1000 mg/kg bw/d limit dose for reproductive toxicity studies) the decrease in offspring weight gain during lactation was considered to reflect offspring rather than reproductive toxicity and that a classification for lactational effects is therefore not required.

Separate male and female studies

The two-generation reproductive study in the rat reported above provided sufficient information to fully interpret the effects of lenacil on reproduction and no further studies were deemed necessary.

Three segment designs

The two-generation reproduction study in the rat reported above provided sufficient information to fully interpret the effects of lenacil on reproduction and no further studies were deemed necessary.

Dominant lethal assay for male fertility

The two-generation reproduction study in the rat reported above provided sufficient information to fully interpret the effects of lenacil on reproduction and no further studies were deemed necessary.

Cross matings of treated males with untreated females and vice versa

The two-generation reproduction study in the rat reported above provided sufficient information to fully interpret the effects of lenacil on reproduction and no further studies were deemed necessary.

Effects on spermatogenesis

Sufficient information to fully interpret the effects of lenacil on reproduction has been provided and no further studies were deemed necessary.

Effects on oogenesis

Sufficient information to fully interpret the effects of lenacil on reproduction has been provided and no further studies were deemed necessary.

Sperm motility, mobility, and morphology

Sufficient information to fully interpret the effects of lenacil on reproduction has been provided and no further studies were deemed necessary.

Investigation of hormonal activity

The two-generation reproduction study in the rat reported above provided sufficient information to fully interpret the effects of lenacil on reproduction. No impairment of reproductive performance was evident in the rat multigeneration study even at doses levels far exceeding the limit dose of 1000 mg/kg bw/day and there were no effects in the study that could be potentially attributed to effects on hormone activity. Furthermore, a QSAR analysis using the OECD Toolbox (v3.3.5) was performed in order to screen lenacil for structural alerts for Endocrine Disrupting (ED) properties (Kurubaran, S. 2016). Lenacil was evaluated using the two profiling schemes, which are a) estrogen receptor (ER) binding and b) rtER Expert System ver. 1 – USEPA. Lenacil revealed no structural alerts for ED properties in the rtER Expert System ver. 1 – USEPA profiler and was found to be a non-binder, without OH or NH2 group in the ER binding profiler. Therefore, it can be concluded that lenacil is not an endocrine active substance. Please see Section CA 5.8.1/03 for further details."

B.6.6.2 (CA 5.6.2) Developmental toxicity studies

Developmental toxicity/teratogenicity study by the oral route in the rat

B.6.6.2.1

Embryotoxic and teratogenic study in rats with lenacil (INB-634) ([redacted] 1978) - DuPont Report No.: HLR 405-78

Guidelines: study is not fully in compliance with Dir EEC 87/302/EEC Annex V B or OECD test guideline n°414 (2001-1981).

At the time the study was performed, no specific Guidelines were compulsory. The developmental toxicity/teratogenicity study in [redacted] CD rats (HLR 405-78) was originally submitted under EU Rev8 Point IIA 5.6.2 and has been conducted with lenacil technical. The guidelines according to which the study was performed were not reported. A review of this study indicates that it does not meet the current OECD Test Guideline 414;

Deviations include:

- The ♀ were not acclimated and were *primigravida* when arriving;
- One group had a higher initial body weight; and
- Lenacil was given by diet and not by gavage and the doses were probably too low as no maternal toxicity was produced at the top dose level

GLP status: no

Materials and Methods

25 [redacted] CD rats/group were allocated to 4 experimental groups. Lenacil technical (Batch No.INB-634-61 ([redacted] No. 11848, MR-2949), purity approx. 100%) was administered continuously via the ground diet from Day 6 to 15 of pregnancy at levels of 500, 2500 or 5000 ppm. A fourth group received the basal diet without the test material and served as the Control. Rats were observed daily for clinical signs. Body weights were recorded on arrival and on days 6, 10, 16 and 21 of gestation. Food consumption was monitored and recorded throughout the test. Foetuses were developed by Caesarean section on Day 21 of pregnancy and corpora lutea, implantation sites, live and dead foetuses, resorptions, foetal weights, crown-rump length and gross abnormality recorded. Half of the foetuses were used for skeletal evaluation after Alizarin red staining. The other half was subjected to visceral examination according to the razor blade Wilson technique.

The study is considered to provide complementary information.

Findings

Clinical signs/mortality:

There were no clinical signs attributable to treatment. All animals survived the test period.

Bodyweights, food consumption, food efficiency:

Body weights and food consumption were unaffected by treatment.

Autopsy of dams:

There were no gross findings attributable to treatment.

Foetal parameters:

Treatment did not influence pregnancy and foetal parameters such as implantations, resorptions, body weight or crown-rump length (**table B.6.6.2.1-1**).

Skeletal and visceral examinations did not reveal findings which were attributed to treatment (**table B.6.6.2.1-2**).

The incidence of litters with early/partial resorptions was increased at the mid-sose, but not at the top-dose. Although a saturation could have occurred in the absorption at ≥2500 ppm, this isolated and non-dose-dependent increase is considered of doubtful toxicological significance.

Table B.6.6.2.1-1 Developmental study in rats with lenacil ([redacted] 1978): pregnancy and foetal parameters.

Endpoints/dose (ppm)	**0**	**500**	**2500**	**5000**
mg/kg bw/d	**0**	**48.4**	**241.9**	**485.7**
♀pregnant/♀bred (%)	21/25 (84%)	21/25 (84%)	24/25 (96%)	21/26 (81%)
Corpora lutea/pregnant ♀, mean ± S.D	12.0 ± 2.2	11.9 ± 2.0	12.7 ± 2.9	11.9 ± 2.4
Implantations/litter, mean ± S.D.	9.2 ± 3.6	8.9 ± 3.0	10.5 ± 1.7	9.4 ± 2.9
Live fetuses/litter, mean ± S.D.	8.2 ± 3.3	7.9 ± 3.2	9.5 ± 2.0	8.7 ± 2.7
Litters with early resorptions (%)	13 (61.9%)	12 (57.1%)	17 **(70.8%)**	7 (33.3%)
Litters with late resorptions (%)	0	0	0	0
Litters with dead foetuses (%)	0	0	0	0
Litters with partial resorptions (%)	13 (61.9%)	12 (57.1%)	17 **(70.8%)**	7 (33.3%)
Litters totally resorbed	0	0	0	0
Resorptions/litter with resorption	1.7 ± 0.9	1.8 ± 2.3	1.4 ± 1.0	2.0 ± 1.0
Initial body weight of pregnant ♀(g)	170.7 ± 7.0	177.1* ±12.9	169.9 ± 9.5	167.9 ± 10.4
Final body weight of pregnant ♀(g)	359.6 ± 25.4	362 ± 43.5	367 ± 20.6	358 ± 27.7
Fetal crown-rump length (cm)	3.0 ± 0.7	3.1 ± 0.5	2.8 ± 0.4	3.0 ± 0.6
Foetal weight (g)	3.5 ± 0.7	3.8 ± 0.7	3.3 ± 0.6	3.8 ± 1.0

Statistically significant modification: *: $p<0.05$

Table B.6.6.2.1-2 Developmental study in rats with lenacil ([redacted] 1978): incidence of abnormalities.

Endpoints/dose	**0**	**500**	**2500**	**5000 ppm**
Achieved dose (mg/kg bw/d)	**0**	**48.4**	**241.9**	**485.7**
External anomalies				
n° litters/foetuses examined	*21/172*	*21/165*	*24/229*	*21/183*
Subcutaneous haematomas	5 (24%) /6 (3%)	1 (5%) /1 (1%)	6 (25%) /7 (3%)	5 (24%) **/11 (6%)**
Petaechial haemorrhages	5 (24%) /7 (4%)	3 (14%) /3 (2%)	7 (29%) /9 (4%)	4 (19%) /5 (3%)
Visceral anomalies				
n° litters/foetuses examined	*21/83*	*21/77*	*24/108*	*21/88*
Hydronephrosis	-	-	-	1/1
Anophthalmia/microphthalmia	1/1	-	1/1	**3 (14%)/ 3 (3%)**
Umbilical hernia	1/1	-	1/1	-
Exencephalocele	1/1	-	-	-
Heart and vessel defect	1/1	-	1/1	-
Syndrome – multiple defects*	-	-	-	1/6 (7%)
Skeletal anomalies				
n° litters/foetuses examined	*21/89*	*21/88*	*24/121*	*21/95*
Delayed ossification of ≥1 sternebrae and/or ribs	11 (52%) /18 (20%)	7 (33%) /11 (13%)	17 (71%) /27 (22%)	12 (57%) /27 (28%)
14th rib(s)	-	-	1/1	2 (10%) /3 (3%)
14th rudimentary rib(s)	18 (86%) /51 (57%)	15 (71%)/ 41 (47%)	23 (96%) /60 (50%)	19 (90%) /48 (51%)
Wavy ribs	2 (10%) /3 (3%)	2 (10%) /4 (5%)	1 (4%) /2 (2%)	**3 (14%) /5 (10%)**
Centra bipartite	-	1/2	1/1	2/2
Cleft and/or misaligned sternebrae	-	-	1/1	**4 (19%) /4 (4%)**

*: generalised oedema, hydronephrosis, hydroureter, thickened bladder. Unilateral microphthalmia in 2 foetuses.

Conclusion and comment from RMS:

This study is performed at dose levels which are probably too low to demonstrate an effect of lenacil. At top dose, maternal toxicity was not demonstrated. The ♀were *primi gravida* when arriving and there is a slight increase in litters with visceral or skeletal anomalies at top dose when compared to the control. No statistical analysis was performed and the study director concluded that Lenacil technical was not considered to be embryotoxic or teratogenic under the experimental conditions.

It is of note that a slight increase of litter/foetus incidence of in *anophthalmia/microphthalmia* was observed at the top-dose. However, given the limitation of this early non-GLP study, the finding is not considered to outweigh the negative findings in the more recent developmental rat studies. However, in order to further assess this severe malformation, it could be of use to determine the likelihood that the finding is spontaneous, by indicating the HCD for this strain in preferably this lab. **Notifier**: *'[redacted] requested HCD on anophthalmia/microphthalmia from the 1978 study."*

Notifier's opinion:

"Oral administration of lenacil technical to ChR CD rats did not affect maternal or foetal parameters at any of the doses tested. The NOEL was therefore detemermined to be the top dose of 5000 ppm. This dietary concentration was equivalent to a mean daily intake of 485.7 mg/kg bw/day. Lenacil technical was not considered to be embryotoxic or teratogenic under the experimental conditions."

B.6.6.2.2

DPX-B634-91 (lenacil): Pilot developmental toxicity study in rats ([redacted] 1996) - DuPont Report No.: HLR 996-96

Guidelines: Not stated in the study report

The pilot developmental toxicity/teratogenicity study (HLR 996-96) was originally submitted under EU Rev8 Point IIA 5.6.2 and has been conducted with lenacil technical. The guidelines according to which the study was performed were not reported. A review of this publication indicates that it does not meet the current OECD Test Guidline 414 and has been superceded by the study on effects on embryo-fetal development in CD rats treated by oral gavage administration (ACD 058/032316).

Materials and Methods

A pilot study was performed using lenacil (batch n° DPX-B634091, 98.5%) in 0.5% methylcellulose which was administered to groups of 11 mated Crl:CD BR rats over day 7-16 of gestation at dose levels of 0, 500, 1000, 4000 mg/kg bw/d. On day 22, all rats were euthanised and gross necropsy was performed. The foetuses were removed from the uterus and were weighed, sexed and examined for external alterations.

The study is considered to provide complementary information.

Findings

Pretest study:

According to the authors of the study, there was no evidence of either maternal or developmental toxicity at any level tested.

Thus, under these experimental conditions, the maternal and developmental NOAEL was originally established at 4000 mg/kg bw/d. However, since b.w. were affected at different study periods, and alopecia was observed at the top-dose, the maternal toxicity NOAEL is more likely to be established at 1000 mg/kg b.w./d.

Table B.6.6.2.2-1 Pretest developmental rat study with lenacil ([redacted] 1996): Clinical signs, body weight and food consumption.

Endpoints/dose (mg/kg bw/d)	0	500	1000	4000
Clinical signs:				
Alopecia:				
Day 17-22	1/11	2/11	2/11	**4/11**
Maternal bw				
Day 7-9				**↓15%**
Day 9-11				**↓14%**
Day 11-13				↑14%
Day 17-22				↑11%
Food consumption				
Day 7-9				↑5%
Day 9-11				↑10%
Day 17-22				↑7.7%

Table B.6.6.2.2-2 Pretest developmental rat study with lenacil ([redacted] 1996): reproductive outcome.

Endpoints/dose (mg/kg bw/d)	0	500	1000	4000
♀ pregnant/ ♀ mated (%)	10/11	10/11	11/11	10/11
No. delivered early	0	0	0	0
No. deaths	0	0	0	0
No. with total resorptions	0	0	0	0
No. of litters	*10*	*10*	*11*	*10*
Corpora lutea (mean)	16.4	16.2	15.4	16.4
Nidations (mean)	15.6	14.4	14.5	15.2
Resorptions, total (mean)	0.6	0.7	0.9	0.0
Resorptions, early (mean)	0.6	0.7	0.9	0.0
Resorptions, late (mean)	0.0	0.0	0.0	0.0
Dead foetuses (mean)	0.0	0.0	0.0	0.0
Live foetuses[(a)], total (mean)	15.0	13.7	13.6	15.2
Live foetuses, ♂(mean)	6.6	6.4	6.2	7.3
Live foetuses, ♀(mean)	8.4	7.3	7.5	7.9
Mean foetal weight (grams)	4.98	5.19	5.19	5.11
Sex ratio[(b)]	0.44	0.47	0.46	0.48

[(a)]: statistical analyses were only conducted on the mean total number of live foetuses per litter.
[(b)]: Number ♂foetuses/total number foetuses per litter.

Table B.6.6.2.2-3 Pretest developmental rat study with lenacil ([redacted] 1996): incidence of abnormalities.

Endpoints/dose (mg/kg bw/d)	0	500	1000	4000
External				
n° litters/foetuses examined	*10/150*	*10/137*	*11/150*	*10/152*
n° litters/foetuses affected	0/0	0/0	0/0	**1/1**
Abdomen - Gastroschisis	0	0	0	**1/1**
Visceral anomalies				
n° litters/foetuses examined	0/0	0/0	0/0	0/0
n° litters/foetuses affected	0/0	0/0	0/0	0/0
Head anomalies				
n° litters/foetuses examined	0/0	0/0	0/0	0/0
n° litters/foetuses affected	0/0	0/0	0/0	0/0
Skeletal anomalies				
n° litters/foetuses examined	0/0	0/0	0/0	0/0
n° litters/foetuses affected	0/0	0/0	0/0	0/0

Conclusion:

RMS considers that at top dose, although food consumption was increased during gestation days 7-11, maternal body weight was decreased. Some ♀ had alopecia at the top dose. The NOAEL could be set at the 1000 mg/kg bw/d. Since apparently, no visceral nor skeletal examinations were performed (confirmed by the notifier), it is not possible to draw conclusion regarding the developmental effects of lenacil under these experimental conditios.

Notifier comment:
"Since maternal bodyweights were only reduced between days 7 and 11 and for the remainder of the dosing period, day 12 to 16 and after removal of treatment, bodyweight gains were recorded, it is our opinion that there are no indications of adverse effects or maternal toxicity at 4000 mg/kg bw/day. The notifier is therefore in agreement with the conclusions of the study authors in respect of this preliminary study and respectfully requests RMS to evaluate the endpoint of this study in respect of non-adverse changes."

B.6.6.2.3

Lenacil technical: preliminary study of effects on embryo-fetal development in CD rats treated by oral gavage preliminary study - ([redacted] 2003b) - DuPont Report No.: ACD 057/030001

Guidelines: Not stated in the study report
The developmental toxicity/teratogenicity study in CD rats (ACD 057/030001) was originally submitted under EU Rev8 Point IIA 5.6.2 and has been conducted with lenacil technical (**RMS**: Lot/Batch #: 141712003, purity 98.6%). The guidelines according to which the study was performed were not reported. A review of this publication indicates that it does not meet the current OECD Test Guideline 414 and has been superseded by the study on effects on embryo-fetal development in CD rats treated by oral gavage administration (ACD 058/032316) (RMS: see **B.6.6.2.4** below).

GLP status: yes

Materials and Methods:
Preliminary study (ACD 057/030001, **B.6.6.2.3)**: Lenacil technical (Batch No. 141712003, purity 98.6%) was administered once daily by oral gavage at dosages of 100, 300 or 1000 mg/kg/day to groups of 6 pregnant ♀ CD rats from day 1 to 19, inclusive. Control animals received the vehicle, 0.5% w/v methylcellulose. The ♀ were killed on day 20 of gestation for examination of their uterine contents and foetuses were examined externally for abnormalities.
The study is considered to provide complementary information.

Findings:

Clinical signs/mortality:

The outcome of the initial evaluation was that here were no clinical signs attributable to treatment. All animals survived the test period. RMS considered during renewal the increased incidence of clinical sings in top-dose maternal animals (brown staining on pinnae / head) adverse.

Bodyweights and food consumption: Body weights and food consumption were unaffected by treatment.

Autopsy of dams: There were no gross findings attributable to treatment.

Foetal parameters:

The treatment did not influence litter data such as *corpora lutea*, implantations, resorptions and live young, and the degree of pre-and post-implantation loss showed no evidence of an adverse effect of treatment.

Foetal and placental weights were unaffected by the treatment.

Further details of the preliminary study of effects on embryo-fetal development in CD rats treated by oral gavage ([redacted] 2003b) are given in table B.6.6.2.3-1 below.

Conclusion

According to RMS, oral administration of Lenacil technical to rats, weakly affected maternal parameters (increased incidence of clinical signs), and did not affect foetal parameters.

Maternal
NOAEL = 1000 mg/kg bw/d
LOAEL = 4000 mg/kg bw/d, based on ↓body w, ↑clinical signs (alopecia)

Developmental (n.a.)
(n.a.) investigation not available

Therefore, the same dosages as used in this preliminary study, *i.e.* dosages of 100, 300 and 1000 mg/kg b.w./d, were considered appropriate for use in the main embryo-foetal toxicity study in the rat.

(ACD 058/032316, see **B.6.6.2.4**).

Table B.6.6.2.3-1 Lenacil technical: preliminary study of effects on embryo-fetal development in CD rats treated by oral gavage ([redacted] 2003b) - developmental findings

Endpoints/dose (mg/kg b.w./d)	0	100	300	1000
Clinical signs:				
Brown staining (# affected/# examined)				
Pinnae	1/6 (17%)	2/6 (33%)	1/6 (17%)	**3/6 (50%)**
Head	2/6 (33%)	1/6 (17%)	1/6 (17%)	**5/6 (83%)**
Muzzle	0/6	1/6 (17%)	0/6	1/6 (17%)
Upper dorsal thorax	2/6 (33%)	2/6 (33%)	0/6	1/6 (17%)
Hairloss (# affected/# examined)				
Forelimbs	0/6	1/6 (17%)	0/6	1/6 (17%)
Ventral surface	0/6	0/6	1/6 (17%)	0/6
Body weight:				
Day 12-18		↓3-4%		
Adjusted BW day 20		↓5%		
Bw changes		↓10%	↑6%	↑3%
Food consumption		↓4%	↑3%	↑1%
***Corpora lutea*, mean ± S.D.**	14.8 ± 0.8	14.5 ± 2.1	17.0 ± 2.5	15.8 ± 0.8
Implantations, mean ± S.D.	14.7 ± 0.8	14.0 ± 1.7	16.7 ± 2.3	15.7 ± 1.0
Resorptions:				
total	0.2	0.8	0.8	0.8
early	0.2	0.8	0.8	0.8
late	0.0	0.0	0.0	0.0
Implantation loss %:				
Pre-	3.2	4.2	1.8	3.1
Post-	1.2	5.7	5.5	5.0
Live young:				
♂, mean ± S.D.	7.8 ± 1.5	6.8 ± 1.7	8.8 ± 2.6	6.3 ± 1.0
♀, mean ± S.D.	6.7 ± 1.0	6.3 ± 3.0	7.0 ± 1.1	8.5 ± 1.4
Total, mean ± S.D.	14.5 ± b1.0	13.2 ± 1.7	15.8 ± 2.9	14.8 ± 1.0
Sex ratio % ♂ (n=6/dose)	53.8	53.5	54.7	42.8
Placental weight, mean ± S.D.	0.54 ± 0.03	0.63 ± 0.18	0.50 ± 0.03	0.65 ± 0.25
Litter weight, mean ± S.D.	53.26 ± 8.03	49.77 ± 5.72	60.19 ± 10.08	54.09 ± 3.80
Live litter size, mean ± S.D.	14.5 ± 1.0	13.2 ± 1.7	15.8 ± 2.9	14.8 ±1.0
Foetal weight				
♂, mean ± S.D.	3.76 ± 0.38	3.92 ± 0.25	3.91 ± 0.21	3.77 ± 0.25
♀, mean ± S.D.	3.55 ± 0.38	3.66 ± 0.35	3.71 ± 0.19	3.57 ± 0.25
Overall, mean ± S.D.	3.66 ± 0.39	3.80 ± 0.31	3.81 ± 0.17	3.65 ± 0.25

Statistical analysis not performed

B.6.6.2.4

Lenacil technical: study on effects on embryo-fetal development in CD rats treated by oral gavage –main study ([redacted] 2003c) - DuPont Report No.: ACD 058/032316

Guidelines: study in compliance with EU Guideline B.31, equivalent to OECD 414.

GLP status: yes

Materials and Methods

Main test (ACD 058/032316, **B.6.6.2.4):** Adult virgin ♀rats of the CD (Sprague-Dawley) strain were allocated to one control and three experimental dose groups (22 animals per group). The animals received Lenacil technical (Batch No. 141712003, purity 98.6%) by gavage from Day 1 to 19 of gestation. Dose levels used were 100, 300 or 1000 mg/kg body weight per day. The control animals received the vehicle, a 0.5% w/v methylcellulose suspension.

Prior to commencement of treatment, the homogeneity and stability of Lenacil technical in 0.5% methylcellulose was confirmed. During the course of the study, samples from dosing suspensions were taken at all concentrations during the first and last week of treatment to assess achieved concentrations. All values were within the expected limits from nominal values.

All maternal parameters were taken regularly throughout the study. Females were killed on Day 20 of gestation for examination of their uterine contents and foetuses were examined externally for abnormalities.

The study is accepted.

Findings:

Maternal findings:
Clinical signs/mortality:
During intitial assessment, it was considered that there were no clinical signs or pre-terminal deaths attributable to treatment. However, in the absence of further justification, RMS proposes not to disregard forepaw alopecia and yellow ventral staining, indicating some adversity at 300 mg/kg b.w./d and above. As commented on above, the alopecia and ventral staining appears to be a random finding in the opinion of the notifier

Bodyweights and food consumption: Body weights and food consumption were unaffected by treatment.

Autopsy of dams: There were no gross findings attributable to treatment.

Table B.6.6.2.4-1: Lenacil technical: study on effects on embryo-fetal development in CD rats treated by oral gavage ([redacted] 2003c): main study - maternal findings

Endpoints/dose : mg/kg bw/d	**0**	**100**	**300**	**1000**
Clinical signs:				
Brown staining (number affected/number examined)				
Pinnae	7/21	4/21	4/22	7/22
Head	12/21	13/21	12/22	13/22
Muzzle	0/21	0/21	1/22	0/22
Forelimbs	3/21	1/21	1/22	**5/22**
Dorsal body surface	0/21	2/21	3/22	1/22
Ventral body surface	0/21	0/21	0/22	**1/22**
Upper dorsal thorax	3/21	4/21	4/22	4/22
Yellow staining (number affected/number examined)				
Ventral body surface	0/21	0/21	**1/22**	**1/22**
Sacral region	0/21	1/21	0/21	**2/22**
Staining resulting from **salivation** after dosing	0	0	0	**1/22**
Hairloss (forelimbs)	1/21	1/21	**2/22**	**5/22**
Body weight d1-20			↑3%	↑5%
Body weight gain d1-20				↑6%
Uterine weight gravid			↑8.8%	↑6.3%

Litter findings:

Foetal parameters:

Treatment did not influence litter data such as *corpora lutea*, implantations, resorptions and live young, and the extent of pre-and post-implantation loss showed no evidence of an adverse effect of treatment. Foetal and placental weights were unaffected by treatment. RMS confirms this evaluation.

Table B.6.6.2.4-2: Lenacil technical: study on effects on embryo-fetal development in CD rats treated by oral gavage (2003c): main study - litter findings

Endpoints/dose : mg/kg bw/d	0	100	300	1000
Corpora lutea, mean ± S.D.	15.2 ± 1.2	15.8 ± 2.1	15.8 ± 2.9	15.0 ± 1.9
Implantations, mean ± S.D.	14.2 ± 1.9	14.7 ± 2.1	14.7 ± 3.3	14.8 ± 1.8
Resorptions:				
total	1.0	1.0	0.6	0.7
early	1.0	1.0	0.6	0.7
late	0.0	0.0	0.0	0.0
Implantation loss %:				
Pre-	8.2	7.7	10.2	3.5
Post-	7.0	6.6	3.9	5.1
Live young:				
♂, mean ± S.D.	6.0 ± 2.2	6.6 ± 1.4	6.6 ± 2.9	7.5 ± 2.0
♀, mean ± S.D.	7.1 ± 2.4	7.1 ± 2.1	7.5 ± 2.8	6.6 ± 2.1
Total, mean ± S.D.	13.2 ± 2.0	13.7 ± 1.9	14.1 ± 3.2	14.1 ± 2.2
Sex ratio % M	46	48.7	44.8	53.1
Implantation loss %:				
Pre-	8.2	7.7	10.2	3.5
Post-	7.0	6.6	3.9	5.1
Placental weight	0.56 ± 0.05	0.55 ± 0.06	0.56 ± 0.12	0.56 ± 0.06
Litter weight				↑7.4%
Foetal weight				
♂, mean ± S.D.	3.89 ± 0.27	3.89 ± 0.28	3.83 ± 0.23	3.92 ± 0.24
♀, mean ± S.D.	3.73 ± 0.26	3.70 ± 0.17	3.67 ± 0.32	3.73 ± 0.21
Overall, mean ± S.D.	3.80 ± 0.26	3.79 ± 0.20	3.75 ± 0.30	3.83 ± 0.22

Developmental findings **(table B.6.6.2.4-3)**

During the first evaluation, the incidence of major and minor abnormalities and skeletal variants were considered having no relationship to treatment with lenacil technical. However, during renewal, it was considered that the observed slightly elevated incidences minor skeletal variants (thickened rib, incomplete ossification of cervical and sacrocaudal vertebrae) at 300 mg/kg b.w./d and above constituted a basis for the establishment of the developmental NOAEL at the lowest dose. It is however acknowledged that these minor findings do not qualify for classification and labelling for developmental effects.

Table B.6.6.2.4-3: Lenacil technical: study on effects on embryo-fetal development in CD rats treated by oral gavage (2003c): main study - maternal findings

Endpoints/dose : mg/kg bw/d	**0**	**100**	**300**	**1000**
Clinical signs:				
Brown staining (number affected/number examined)				
Pinnae	7/21	4/21	4/22	7/22
Head	12/21	13/21	12/22	13/22
Muzzle	0/21	0/21	1/22	0/22
Forelimbs	3/21	1/21	1/22	**5/22**
Dorsal body surface	0/21	2/21	3/22	1/22
Ventral body surface	0/21	0/21	0/22	**1/22**
Upper dorsal thorax	3/21	4/21	4/22	4/22
Yellow staining (number affected/number examined)				
Ventral body surface	0/21	0/21	**1/22**	**1/22**
Sacral region	0/21	1/21	0/21	**2/22**
Staining resulting from **salivation** after dosing	0	0	0	**1/22**
Hairloss (forelimbs)	1/21	1/21	**2/22**	**5/22**
Body weight d1-20			↑3%	↑5%
Body weight gain d1-20				↑6%
Uterine weight gravid			↑8.8%	↑6.3%

Table B.6.6.2.4-3: Lenacil technical: study on effects on embryo-fetal development in CD rats treated by oral gavage (, 2003c): main study - developmental findings

Endpoints/dose : mg/kg bw/d	**0**	**100**	**300**	**1000**
Major abnormalities				
N° foetus/n° litter examined	*277/21*	*287/21*	*310/22*	*310/22*
N° affected	2/2	1/1	1/1	-/-
Aortic pulmonary fistula	1/1	-/-	-/-	-/-
Diaphragmatic hernia	-/-	1/1	-/-	-/-
Duplicated inferior vena cava	1/1	-/-	-/-	-/-
Multiple malformations	-/-	-/-	1/1	-/-
Visceral abnormalities: minor				
N° foetus/n° litter examined	*137/21*	*142/21*	*152/22*	*154/22*
N° affected	32 (23.4%) /17 (81.0%)	24 (16.9%) /16 (66.7%)	39 (25.7%) /18 (81.8%)	30 (19.5%) /14 (63.6%)
Skeletal abnormalities/variants:minor				
N° foetus/n° litter examined	*138/21*	*144/21*	*157/22*	*156/22*
Cranial sutural bone	-/-	1/1	1/1	-/-
Ribs: thickened/kinked	1/1 (5%)	2/1 (5%)	**3/3 (14%)**	**4/3 (14%)**
Ribs: irregular ossification	-/-	-/-	1/1	1/1
Sternebrae: offset alignment	-/-	-/-	1/1	-/-
Appendicular: misshapen scapula	-/-	1/1	-/-	1/1
Total affected by ≥1 of the above	1 (0.7%) /1 (4.8%)	4 (2.8%) /3 (14.3%)	**5 (3.3%) /5 (22.7%)**	**6 (3.9%) /5 (22.7%)**

Endpoints/dose : mg/kg bw/d	**0**	**100**	**300**	**1000**
Rib and vertebral configuration				
Cervical rib	1/1	2/2	2/2	-/-
Short/absent 13th rib	-/-	-/-	-/-	7/2
Number with 13/14 or 14/14 ribs	24/9	15/8	20/10	31/**14**
Complete 14th rib(s)	1/1	-/-	1/1	1/1
18 thoracolumbar vertebrae	-/-	-/-	-/-	2/1
20 thoracolumbar vertebrae	2/1	-/-	2/1	-/-
Offset alignment pelvic girdle	-/-	1/1	-/-	-/-
Total rib and vertebral configuration	28 (20%) /12 (57%)	18 (13%) /11 (52%)	25 (16%) /14 **(64%)**	41 **(26%)** /18 **(82%)**
Incomplete ossification				
Cranial centres	19/11	21/8	24/11	29/13
Hyoid	6/3	7/5	9/8	9/5
Vertebrae, cervical	1/1 (5%)	1/1 (5%)	4/4 **(18%)**	5/4 **(18%)**
Vertebrae, thoracic	9/9	7/6	4/3	7/5
Vertebrae, lumbar	-/-	-/-	-/-	1/1
Vertebrae, sacrocaudal	12 (20%) /8 (38%)	10 (7%) /7 (33%)	19 (12%) /11 **(50%)**	16 (10%) /10 **(45%)**
Sternebrae, 5th and/or 6th	69/18	73/20	100/21	88/**22**
Sternebrae, other	3/3	2/2	4/4	4/2
Sternebrae, total	69/18	73/20	100/21	91/**22**
Pelvic bones	9/5	9/4	11/6	10/**9**
Metacarpals/metatarsal	1/1	1/1	2/1	2/**2**
Precocious ossification				
Cerebral cervical centra (>3 ossified)	4/2	-/-	5/4	6/4
Additional observations at necropsy				
N° foetus/n° litter examined	*138/21*	*144/21*	*157/22*	*156/22*
Renal cavitation	-/-	4/2	6/5	4/3
Hydroureter	5/3	6/5	5/4	1/1
Left umbilical artery	-/-	-/-	-/-	1/1
Subcutaneous haemorrhage	-/-	1/1	1/1	-/-

Conclusion (main rat developmental study)

During the first peer review, it was considered that oral administration of Lenacil technical to rats did not affect maternal or foetal parameters at any of the doses tested, and therefore, both the maternal and foetal NOAEL was at 1000 mg/kg body weight/day.

At renewal, NOAEL's were revised downwards on the basis of a more comprehensive assessment of earlier reported findings:

Maternal **NOAEL = 100 mg/kg b.w./d**
Maternal LOAEL = 300 mg/kg b.w./d, based on: ↑forepaw alopecia and yellow ventral staining.

Developmental **NOAEL = 100 mg/kg b.w./d**
Developmental LOAEL = 300 mg/kg b.w./d, based on:
↑skeletal variants (thickened rib, incomplete ossification of cervical and sacrocaudal vertebrae).

RMS sees no reason to disregard the adverse maternal and developmental findings without further justification.

Developmental toxicity/teratogenicity study by the oral route in the rabbit

B.6.6.2.5

Teratogenicity study of DPX-B634-91 in rabbits ([redacted] 1991) - DuPont Report No.: HLR 626-91

Guidelines: OECD 414, NohSan 59 No.4200, US EPA 83-3

The developmental toxicity/teratogenicity study in pregnant Hra:NZW SPF rabbits (HLR 626-91) was originally submitted under EU Rev8 Point IIA 5.6.2 and has been conducted with Lenacil technical. The study was conducted according to OECD 414, NohSan 59 No.4200, and US EPA 83-3. A review of this study indicates that it fully meets the current OECD Test Guideline 414 or US EPA guideline 83-3. The study is considered valid.

Guideline: study is in compliance with Dir EEC 87/302/EEC or OECD test guideline n° 417 (1984).

GLP status: yes (no attest of competent authority)

Materials and Methods

Lenacil technical (Code DPX-B634-91, Batch No. 9038, purity 98.5%) was administered once daily by oral gavage as an aqueous solution of methyl cellulose at daily dose levels of 0, 50, 200, 1000, or 4000 mg/kg of bodyweight to pregnant rabbits (Hra:NZW) SPF on days 7 to 19 of gestation. Control animals received the vehicle, 0.5% w/v methylcellulose. The dosing volume was 10 mL/kg of body weight. Samples of the dosing solution were taken on days 3, 10 and 17 for concentration analysis to verify concentration, uniformity and stability. Concentration analyses resulted in a recovery range from 87 to 109% of nominal values. Stability was confirmed over at least 5 hours. The homogeneity was confirmed. Clinical signs were recorded daily. Body weights and food consumption were measured daily throughout treatment and on days 24 and 29. Dams were sacrificed on day 29 and their intrauterine content examined. Foetuses were removed, foetal parameters taken and all of them were subjected to external, skull, visceral and skeletal examination for abnormalities.

The litter was considered to be the experimental unit for statistical evaluation.

The study is accepted.

RMS: of note, lenacil technical characteristics were mentioned as "Code DPX-B634-91, Batch No. 9038, purity 98.5%" in the original assessment. However, it appears that the Lot/Batch # is B634-91 (purity is still 98.5%). Notifier: confirmed that Batch No. 9038, purity 98.5% is the same as DPX-B634-91. The laboratory synthesising the sample used one numbering system, and the organiation conducting regulatory studies used another numbering system.

Findings

In a **pilot study** (data not available), doses of 0, 500, 1000, 2500 and 4000 mg/kg bw/d were administered daily to groups of 8 pregnant rabbits on day 7-19 of gestation.

RMS: Could notifier provide the lacking rabbit developmental pilot study? **Notifier**:*"The data that was generated from the pilot study was never put into a report. Hence, we have asked the laboratory to generate a report".*

A non-significant decrease in body weight gain for the dosing period was observed for all experimental groups compared to control.

A significant upward trend was observed in the mean number of resorptions/litter; however, the 4000 mg/kg bw/d value was not significantly different from control. No other significant compound-related effects were observed.

Based on these results and the fact that 4000 mg/kg bw/d was the maximum dose that could be mechanically administered, the dose levels tabulated here were selected for this study.

Main study

Mortality:

There were no deaths attributable to treatment.

Clinical signs:

High dosed dams showed a stained tail during the post-dosing period.

Bodyweights and food consumption:

A significant trend towards reduction in maternal weight gain was seen over day 13 to 16 as well as the overall dosing period (days 7-20). This trend attained statistical significance at the highest dose level. An upward trend was seen during the post-dosing period (days 20-29).

There was no change during pre-and dosing period in mean maternal food consumption, but a significant upward trend during post-dosing, attaining statistical significance at the highest dose level.

Autopsy of dams:

There were no gross findings attributable to treatment.

Foetal parameters:

Treatment did not influence litter data such as no. of live/dead foetuses, resorptions, corpora lutea, and implantations and mean foetal weights. The marginal variations of resorption rate or litter size at 1000 or 4000 mg/kg b.w./d were considered of no toxicological relevance.

Table B.6.6.2.2-1: Teratogenicity study of lenacil in rabbits ([redacted],1991) – maternal and litter findings

Endpoints/dose : mg/kg bw/d	**0**	**50**	**200**	**1000**	**4000**
N° inseminated	20	20	20	20	20
N° pregnant/aborted	16/0	16/0	15/0	15/1	16/0
N° with total resorptions	1	0	1	1	**2**
Mortality :		1			1
Clinical signs: Stained tail					
Day 0-6	4	4	3	3	6 **(↑50%)**
Day 7-19	6	7	7	6	11 **(↑83%)**
Day 20-29	5	5	8	6	12* **(↑140%)**
Body weight changes					
~~Day 13-16~~					~~↓75%*~~
~~Day 7-20~~					~~↓68%*~~
Day 10-13: grams	98.9	44.8 (↓55%)	53.5 (↓46%)	60.0 (↓39%)	47.0 (↓52%)
Day 13-16: grams	101.4	63.0 (↓38%)	93 (↓8%)	90.9 (↓10.3)	26.2** (↓**74%**)
Day 7-20: grams	162.6	82.2 (↓49%)	140.9 (↓13%)	136.7 (↓16%)	53.5** (↓**67%**)
Day 16-20: grams	-34.0	-21.9 (↑36%)	-20.2 (↑41%)	-20.6 (↑39%)	5.0 (↑115%)
Day 20-29: grams	77.1	123.7 (↑60%)	114.5 (↑49%)	129.8 (↑68%)	150.8** (↑96%)
Food consumption:					
Day 13-16	150.6	149.7	150.3	150.0	128.1 (↓**15%**)
Day 20-29	147.2	146.4	149.1 (↑1%)	149.8 (↑2%)	150.2* **(↑2%)**
# resorptions/# pregnant	1/16 (6%)	0/16 (0%)	1/15 (7%)	1/15 (7%)	2/16 (13%)
# litters/# inseminated	15/20 (75%)	16/20 (80%)	14/20 (70%)	13/20 (65%)	13/20 (65%)
Live/death foetuses mean	5.3/0.7	5.3/0.0	6.3/0.0	6.8/0.0	5.8/0.0
Resorptions late/total	0.1/0.5	0.1/0.3	0.1/0.7	0.2/0.7	0.2/0.4
Mean fœtal weight	44.8	47.2	45.9	45.7	47.9

Incidences expressed as #foetuses / #litters; *: Significantly different from control (Dunnetts test p<0.05 or Fisher exact test p<0.05); **: Significant trend (linear combination of dose ranks from ANOVA p<0.05)

External, visceral or skeletal examinations did not reveal findings attributable to treatment (see **table B.6.6.2.2-2).**

Table B.6.6.2.2-2: Developmental study of lenacil in rabbits ([redacted],1991) – developmental findings

Endpoints/dose : mg/kg bw/d	**0**	**50**	**200**	**1000**	**4000**
Incidence of malformations:					
N° examined (foetuses/litters)	79/15	84/16	88/14	89/13	75/13
External:					
N° affected: (foetuses/litters)	0/0	0/0	0/0	0/0	0/0
Visceral:					
N° affected: (foetuses/litters)	1/1	0/0	0/0	0/0	1/1
Head					
brain-hydrocephaly (foetuses/litters)	1/1	0/0	0/0	2/1	1/1
Skeletal:					
N° affected: (foetuses/litters)	1/1	0/0	0/0	0/0	0/0
Total # affected foetuses/litters	3 (4%) /3 (20%)	0/0	0/0	2 (2%) /1 (8%)	2 (3%) /2 (15%)
Incidence of variations:					
N° examined (foetuses/litters)	79/15	84/16	88/14	89/13	75/13
External:					
N° affected: (foetuses/litters)	0/0	0/0	0/0	0/0	0/0
Visceral:					
N° affected: (foetuses/litters)	0/0	0/0	0/0	0/0	0/0
Head					
N° affected: (foetuses/litters)	0/0	0/0	0/0	0/0	0/0
Skeletal:					
N° affected: (foetuses/litters)	0/0	1/1	1/1	1/1	1/1
Total n° affected (foetuses/litters)	0/0	1/1	1/1	1/1	1/1
Variations due to retarded development:					
N° examined	79/15	84/16	88/14	89/13	75/13
External:					
N° affected:	0/0	0/0	0/0	0/0	0/0
Visceral:					
N° affected:	0/0	0/0	1/1	0/0	0/0
Head					
N° affected:	0/0	0/0	0/0	0/0	0/0
Skeletal:					
N° affected:	18(7)	19(9)	25(9)	17(7)	16(5)
Hyoid bent	1/1	5/5	3/3	4/3	0/0
Sternebrae partially ossified	12/5	12/5	15/7	8/5	10/5
Unossified	4/3	2/2	6/3	5/2	5/3

Incidences expressed as #foetuses / #litters; *: Significantly different from control (Dunnetts test p<0.05 or Fisher exact test p<0.05); **: Significant trend (linear combination of dose ranks from ANOVA p<0.05)

Conclusion (main rabbit developmental study)

Oral administration of Lenacil technical to rabbits did not affect foetal parameters at any of the doses tested. Maternal toxicity (reduced body weight gain during gestation) was evident at a daily dose of 4000 mg/kg/day. Therefore, the No Observable Adverse Effect Level (NOAEL) was 1000 mg/kg/day for the dam and greater than 4000 mg/kg/day for the conceptus, as no malformations of other signs indicative for developmental toxicity were observed in the study.

Maternal **NOAEL** = **1000 mg/kg b.w./d**
Maternal LOAEL = 4000 mg/kg b.w./d, based on: ↓body weight gain and clinical signs (stained tail).

Developmental **NOAEL** = **4000 mg/kg b.w./d**

B.6.6.3 (CA 5.6.3) Summary of reproductive toxicity

Table B.6.6.3 – 1 **Summary of reproduction and developmental studies**

Results of fertility and developmental studies with lenacil are summarised below.

Type of test, test species, doses *(ppm)* - mg/kg b.w./d	**Batch n°, purity (%)**	**NOAEL (mg/kg b.w./d)**	**LOAEL, critical effect (mg/kg b.w./d)**	**Reference**
(B.6.6.1.1) 2G oral, ***pilot*** study, diet, Wistar rat, *(0, 10000, 25000, 50000 ppm)* ♂: 0, 749, 1952, 3840 mg/kg bw/d ♀: 0, 755, 2003, 4014 mg/kg bw/d	Batch No. 141712003, purity 98.6%	**MATERNAL** 25000 ppm = **1952 mg/kg bw/d** **OFFSPRING** 25000 ppm = **1952 mg/kg bw/d** **REPRODUCTIVE** 25000 ppm = **1952 mg/kg bw/d**	**MATERNAL** 50000 ppm = **3840 mg/kg bw/d,** based on: ↓body w, ↑clinical signs (alopecia) **OFFSPRING** 50000 ppm = **3840 mg/kg bw/d** **REPRODUCTIVE** 50000 ppm = **3840 mg/kg bw/d**	[redacted] 2002
(B.6.6.1.2) 2G oral, ***main*** study, diet, Wistar rat, *(0, 1000, 25000, 50000 ppm)* ♂: 0, 82, 817, 4279 mg/kg bw/d ♀: 0, 93, 935, 4787 mg/kg bw/d	Batch No. 141712003, purity 98.6%	**MATERNAL** 1000 ppm = **82 mg/kg bw/d** **OFFSPRING** 1000 ppm = **82 mg/kg bw/d** **REPRODUCTIVE** 50000 ppm = **4279 mg/kg bw/d**	**MATERNAL** 10000 ppm = **817 mg/kg bw/d**, based on: ↓body w, ↑clinical signs, ↑liver w, ↑thyroid w, ↑pituitary w, ↓spleen w, ↓thymus w, ↑dark thyroids, ↑thyroid cell necrosis **OFFSPRING** 10000 ppm = **817 mg/kg bw/d,** based on: ↓body weight (lactation), ↓spleen w, ↓thymus w **REPRODUCTIVE** >50000 ppm = **>4279 mg/kg bw/d**	[redacted] 2003
(B.6.6.2.2) Developmental oral, ***pilot*** study, gavage, CD Sprague Dawley rat, 0, 500, 1000, 4000 mg/kg bw/d	Batch No. DPX-B634091, 98.5%	**MATERNAL** **1000 mg/kg bw/d** **DEVELOPMENTAL** (n.a.)	**MATERNAL** **4000 mg/kg bw/d**, based on: ↓body w, ↑clinical signs (alopecia) **DEVELOPMENTAL** (n.a.)	[redacted] 1996
(B.6.6.2.3) Developmental oral, ***pilot*** study, gavage, CD Sprague Dawley rat,	Batch No. 141712003,	**MATERNAL** **300 mg/kg bw/d**	**MATERNAL** **1000 mg/kg bw/d**, based on: ↑clinical signs (staining)	[redacted] 2003b

Type of test, test species, doses *(ppm)* - mg/kg b.w./d	**Batch n°, purity (%)**	**NOAEL (mg/kg b.w./d)**	**LOAEL, critical effect (mg/kg b.w./d)**	**Reference**
0, 100, 300, 1000 mg/kg bw/d	purity 98.6%	**DEVELOPMENTAL 1000 mg/kg bw/d**	**DEVELOPMENTAL >1000 mg/kg bw/d**	
(B.6.6.2.4) Developmental oral, ***main*** study, gavage, CD Sprague Dawley rat, 0, 100, 300, 1000 mg/kg bw/d	Batch No. 141712003, purity 98.6%	**MATERNAL 100 mg/kg bw/d** **DEVELOPMENTAL 300 mg/kg bw/d**	**MATERNAL 300 mg/kg bw/d**, based on: ↑clinical signs (alopecia, staining) **DEVELOPMENTAL 1000 mg/kg bw/d**, based on: ↑skeletal variants (thickened rib, ossification delay vertebrae)	[redacted] 2003c
(B.6.6.2.5) Developmental oral, ***main*** study, gavage, NZW rabbit, 0, 50; 200, 1000, 4000 mg/kg bw/d	Batch No. 141712003, purity 98.6%	**MATERNAL 1000 mg/kg bw/d** **DEVELOPMENTAL 4000 mg/kg bw/d**	**MATERNAL 4000 mg/kg bw/d**, based on: ↓body weight gain, ↑clinical signs (tail staining) **DEVELOPMENTAL**, based on: **>4000 mg/kg bw/d**	[redacted] 1991

(n.a. developmental investigation not available)

In a ***preliminary reproduction study***, dietary administration of lenacil to rats at concentrations of 10000, 25000 or 50000 ppm was generally well-tolerated. Effects consisted of slightly low bodyweight gain prior to pairing for F_0 ♀ at top-dose and for all treated ♀during mid-lactation, and clinical signs. Mating performance, fertility and development of subsequent F_1 progeny, up to physical sexual maturation, showed no adverse effects of treatment. Dietary concentrations up to 50000 ppm were therefore considered suitable for use in the main two-generation study in this strain of rat.

In the ***main 2-generation reproduction study***, dietary administration of lenacil was assessed in rats at concentrations of 1000, 10000 or 50000 ppm. At 10000 ppm (817 mg/kg b.w./d) and 50000 ppm (4279 mg/kg b.w./d), maternal body weight was slightly altered, dams exhibited alopecia, and there was evidence of thyroid toxicity: increased weight, altered metabolism and histopathology. At these doses, dose-dependent decreases of spleen and thymus weight , and increased liver and pituitary weights were observed.

There were no effect on reproductive organs or reproductive performance at any of the dietary concentrations and offspring survival was not affected by treatment. There was no effect upon the physical and sexual development of the offspring.

At 10000 ppm and above, body weight gain of offsprings were reduced during lactation from d7 of age for the F_1 offspring and from d4 of age for the F_2 offspring. Whether treatment caused a reduction in milk production or quality, or whether the offspring was exposed to lenacil via milk could not be ascertained in this study.

Oral administration of lenacil in a ***developmental rat study*** at 100, 300 or 1000 mg/kg b.w./d did weakly affect maternal or foetal parameters at the mid- and top-doses tested. The lowest dose was identified as a NOAEL on the basis of clinical signs *i.e.* fore limb alopecia and yellow ventral staining in dams, and variations in ossification performance in the foetuses) at 300 mg/kg b.w./d and above.

Oral administration of lenacil in a ***developmental rabbit study*** at doses of 50, 200, 1000, or 4000 mg/kg b.w./d did not affect foetal parameters at any of the doses tested in the main study (although there were indications of slightly increased resorptions/litter in the preliminary rabbit development study, to be confirmed by detailed assessment of this study). Maternal toxicity (decreased body weight gain) was evident at a daily dose of 4000 mg/kg b.w./d. Therefore, the NOAEL was 1000 mg/kg/day for the dam and greater than 4000 mg/kg/day for the conceptus.

Overall, both reproduction and development studies are not indicative of fertility or developmental adverse findings, suggesting that, at least based on the investigated parameters, lenacil is unlikely to be an endocrine disrupting substance.

However, a developmental neurotoxicity study is lacking, and it should thus be discussed whether this constitutes a data gap in order to conclude on the inocuity of lenacil on the thyroid functionality, especially on the offspring.

B.6.7 (CA 5.7) Neurotoxicity

B.6.7.1 Repeated neurotoxicity studies

Lenacil is a uracil type herbicide. This class of compounds is devoid of any neurotoxic effects. Furthermore, the chemical structure of Lenacil has no structural relationships with any known neurotoxicants.

No evidence of clinical signs indicative of neurotoxicity was seen in the acute, subacute, subchronic (90-day) or long term toxicity studies, even at international regulatory limit dose levels. Furthermore, there were no neuropathological changes. Similarly, in the two generation reproduction toxicity study, no clinical signs of specific neurotoxicity were seen in either the F_1 or F_2 offspring or their parents.

For these reasons, acute, subchronic or developmental neurotoxicity studies were not triggered. Therefore none have been conducted.

However, a developmental neurotoxicity study is lacking, and it should thus be discussed whether this constitutes a data gap in order to conclude on the inocuity of lenacil on the thyroid functionality, especially on the offspring.

During renewal, notifier further waived the need to conduct a neurotoxicity study on the basis of following rationale:

"The chemical structure of lenacil has no structural relationships with any known neurotoxicants such as carbamates or organophosphates. Furthermore, there was no evidence of clinical signs indicative of neurotoxicty in the acute, subacute, subchronic (90-day) or long-term toxicity studies in rodents, even at if lenacil was administered at or above the limit dose levels as defined in the corresponding testing guidelines. Moreover, there were no neuropathological changes. Similarly, in the two generation reproductive toxicity study, no clinical signs indicative for neurotoxicity were seen in either the F1 or F2 offspring or their parents. For these reasons, acute, subchronic or developmental neurotoxicity studies were not triggered and are, therefore, not required.

Many neurotoxins share common chemical structural elements and therefore comparing the structure of lenacil with the structure of known neurotoxins will provide more information on the likelihood of lenacil being a neurotoxin. In neurotoxicity, there have been relatively few well-characterised structure activity relationships (SARs). There are examples where SARs have been derived but none are functionally or structurally related to simple uracils such as lenacil. Examples cited in the ECETOC Monograph (1992) include substances that induce neuropathy, produce myelinopathy or induce selective degeneration of distal axons with the central and/or peripheral nervous system following systemic exposure.

None of these substances structuraly bears any similarlity to lenacil. Other SARs that have been published are discussed in the WHO report (2001). Valproate analogues that cause developmental neurotoxic effects, hexacarbon diketones that cause peripheral neuropathy, organophosphorus compounds which induce delayed neurotoxicity, carbamates for cholinergic effects and organic solvents that cause narcosis. For such homologous groups of chemicals, SARs combined with knowledge of chemical or physical properties provide useful supporting information on the risk of acute neurotoxicity or narcotic effects. Some SARs have been encoded into expert systems such as Derek Nexus (LHASA Ltd). This knowledge-based system comprises several specific alerts that are useful to provide a positive indication of neurotoxic potential. The majority of the alerts have already been mentioned above. The alerts comprise gamma diketones, acrylamide, nitroimidazole, carbon disulphide, pyrethoids, lead and organophosphorus esters. Based on the available published SARs and those that have been encoded into expert systems such as Derek Nexus, there is no prior SAR knowledge that would suggest uracils are likely to be potential neurotoxins.

Furthermore, and most importantly, no evidence of clinical signs indicative of neurotoxicity was seen in the acute, subacute, subchronic (90-day) or long-term toxicity studies, even at international regulatory limit dose levels. Furthermore, there were no neuropathological changes. In addition, in the two-generation reproduction toxicity study, no clinical signs of toxicity were seen in either the F_1 or F_2 offspring or their parents. There was no indication for neurotoxicity and effects on behavioural landmarks in young animals as pre- weaning surface and air-righting reflexes was unaffected and all F_1 offspring displayed normal auditory and visual responses. Pairing of F_0 animals to produce the F_1 generation which was further used as F_1 parents to produce the F_2 generation, had no adverse effect on pubertal (neuro)development of juvenile animals in both generations. No

adverse effects on the following F_2 endpoints were seen: bodyweight, organ weight or macropathology. Overall, there were no signs that indicated that the thyroid effects observed in the repeated dose toxicity studies had a negative impact on neurological development.

Moreover, lenacil and other uracil compounds are herbicides and therefore their pesticidal mode of action is designed to target physiological processes unique to plants and not designed to interact with the nervous system as is the case for some insecticides. An evaluation of the data available for other uracil compounds supports the conclusion that this class of herbicides does not possess the potential to be neurotoxic.

Based on all of the the above and considering the findings from the two generation reproductive toxicity study in particular, there is no evidence to suggest that lenacil will present a potential hazard or exert adverse effects on neurodevelopment. Therefore, further studies to investigate the potential developmental neurotoxicity are not required and not justified based on the weight-of-the-evidence from all available studies. Finally, if lenacil would have affects on neurodevelopment this would have become evident especially in the two-generation reproductive study. If there would have been subtle effects on neurodevelopment they are not regarded to be adverse considering the results of the RDT, reproductive and developmental toxicity studies."

Cited reference:

ECETOC Monograph 18, Evaluation of the Neurotoxic Potenial, september 1992.

RMS considers this waiver justified as regards the specific neurotoxic potential of lenacil.

B.6.7.2 Delayed neurotoxicity studies

Delayed neurotoxicity following acute exposure

A delayed neurotoxicity study was not required for lenacil as the substance does not belong to the family of organophosphates. None of the subchronic and chronic studies conducted with lenacil, in rats, mice, or dogs, demonstrated any effects indicative of delayed neurotoxicity. The results of acute and subchronic (90-day) toxicity studies substantiate the absence of a primary effect on the nervous system. Therefore, no further studies were required.

B.6.8 (CA 5.8) Other toxicological studies

B.6.8.1 Toxicological studies on metabolites

B.6.8.1.1 Toxicological studies on groundwater metabolites

(Data and rationale submitted as confirmatory data, 05.2016)

B.6.8.2.1 Introduction and objective.

During the EU review process, lenacil was proposed for classification as Carcinogen Category 2 (CLP, Reg (EC) no 1272/2008).

Thus, the following specific provisions are included in Regulation (EU) No 540/2011:

"If a decision on the classification of lenacil under Regulation (EC) No 1272/2008 of the European Parliament and of the Council identifies the need for further information on the relevance of the metabolites IN-KE 121, IN-KF 313, M1; M2, M3, Polar B and Polars, the Member States concerned shall request the submission of such information. They shall ensure that the notifier provides that information to the Commission within six months from the notification of such a classification decision."

Based on the evaluation of the CLH report on the harmonised classification and labelling of lenacil by the RAC Committee of ECHA, a classification into category 2 for carcinogenicity according to the CLP regulation (Carc. 2; H351) has been concluded upon for the active substance. This classification of lenacil has been adopted by the 7th ATP to the CLP and as a result the substance is now being included in Annex VI of the CLP regulation. The carcinogenicity classification of lenacil as decided upon by RAC impacts the toxicological relevance assessment of the groundwater metabolites of lenacil.

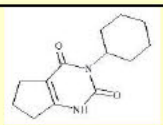
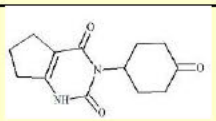
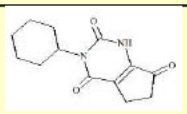
As a consequence, further toxicological information on the following metabolites is required considering the additional requirements set out in Regulation (EU) No 540/2011 as well as the provisions of Sanco/221/2000 –rev.10-final, 25 February 2003 ("Guidance Document On The Assessment Of The Relevance of Metabolites In Groundwater Of Substances Regulated Under Council Directive 91/414/EC") in parallel:

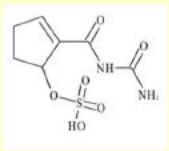
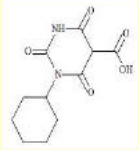
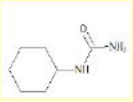
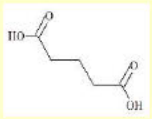
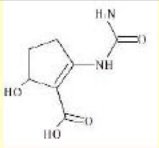
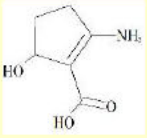
- IN-KE121; IN-KF313;
- Metabolite fractions M1, M2, M3. These fractions include Polar B and Polars which consists of the six polar metabolites IPM1 and PM1-PM5.

Hereinafter Polar B and Polars will be referred to as polar metabolites IPM1 and PM1-PM5.

In this context, information with regards to the elucidation of the potential carcinogenic mode of action (MOA) of lenacil and of the groundwater metabolites is of particular importance to assess the toxicological relevance of the metabolites.

Table B.6.8.2.1 Identifiers, structures and IUPAC names of discussed groundwater metabolites of Lenacil.

LENACIL	IN-KE121	IN-KF313
		
3-cyclohexyl-1H,2H,3H,4H,5H,6H,7H-cyclopenta[d]pyrimidine-2,4-dione	3-(4-oxocyclohexyl)-1H,2H,3H,4H,5H,6H,7H-cyclopenta[d]pyrimidine-2,4-dione	3-cyclohexyl-1H,2H,3H,4H,5H,6H,7H-cyclopenta[d]pyrimidine-2,4,7-trione

IDENTIFIED POLAR	PROPOSED #1	PROPOSED #2	PROPOSED #3	PROPOSED #4	PROPOSED #5
IPM1	PM1	PM2	PM3	PM4	PM5
					
{2-[(carbamoylamino) carbonyl] cyclopent-2-en-1-yl}oxidanesulfonic acid	1-cyclohexyl-2,4,6-trioxo-1,3-diazinane-5-carboxylic acid	1-cyclohexylurea	pentanedioic acid; glutaric acid	2-(carbamoylamino)-5-hydroxycyclopent-1-ene-1-carboxylic acid	2-amino-5-hydroxycyclopent-1-ene-1-carboxylic acid

As a consequence, according to the above mentioned guidance document on the assessment of the relevance of metabolites in groundwater, for parent active substances classified as category 2 carcinogens under the CLP (Carc. 2; H351), equivalent to category 3 carcinogens under the DSD (Carc.Cat 3; R40), convincing evidence must be provided that metabolites will not display the same carcinogenic potential as the parent molecule.

The CLH report on lenacil as prepared and submitted to the RAC committee by the RMS BE has been assessed. Due to mammary gland tumours seen in female rats of the long-term/carcinogenicity study the incidences of which were above the historical background range of the laboratory and which were considered relevant for humans, a classification of lenacil as a Category 2 carcinogen under the CLP (Carc. 2; H351) and as a Category 3 carcinogen under the DSD (Xn; R40) was decided upon and adopted by RAC.

The harmonised classification of lenacil has meanwhile been included in Annex VI of the CLP regulation (CLP 7th ATP. COM REG 2015/1221 published 24.07.2015).

It is of note that during the evaluation of the carcinogenicity of lenacil in rats, the RMS did not consider the incidences of mammary gland tumours to be of relevance for a classification of lenacil as a potential carcinogen. In particular, one set of historical control data covered the incidences of mammary gland observed in ♀rats, rendering this effect of questionable relevance for classification.

However, a different decision was made by the RAC at ECHA, based on a certain dose-response of the tumours.

As the MoA for the induction of mammary tumours in ♀rats is unknown, and as the absence of a genotoxic potential of lenacil does not suggest that mammary gland tumours are caused by a genotoxic mode of action, an epigenetic, (*possible*) endocrine-mediated MoA *could* be the cause for the induction of these tumours.

In order to give an ***indication*** as regards an endocrine-mediated MoA of carcinogenicity, the potential endocrine disrupting (ED) properties of lenacil have been investigated in a first step using the OECD toolbox, considering the profilers estrogen receptor (ER) binding and rtER Expert System ver. 1 – USEPA.

Similarly, for the assessment of the potential toxicity of the groundwater metabolites, a weight-of-evidence approach has been adopted, based on QSAR screening and structural alerts for endocrine disruption and carcinogenicity in the OECD toolbox. Carcinogenicity (genotoxic and non-genotoxic) alerts by ISS and oncologic primary classification profilers in the OECD Toolbox have been used to screen for carcinogenicity structural alerts.

The overall objective is to address the specific provisions as set out for lenacil in Regulation (EU) No 540/2011 by investigating the genotoxic and carcinogenic potential of the groundwater metabolites. In addition, the endocrine disrupting potential of both lenacil and its groundwater metabolites will be addressed.

B.6.8.1.1.1 Overview of the status of lenacil and its groundwater metabolites

In soil, under aerobic conditions, lenacil has been shown to form the major soil metabolites IN-KF313, IN-KE121, the polar metabolite IPM1, and, in addition, is ***proposed*** to form five further polar metabolites PM1-PM5 (Dixon, K. and Alderman, D., 2011).

The structures, SMILES and selected physiochemical characteristics of the 6 polar metabolites of lenacil are summarised in Table 1 of Appendix 2. The same information is provided for lenacil and its other metabolites IN-KE121 and IN-KF313 in Table 2 of Appendix 3. A summary of these are given in table B.6.8.2.1 hereabove.

The proposed pathway of the metabolism of lenacil in soil was previously submitted in a position paper (Goodyear, 2012) see confirmatory data B.8, Fate and Behaviour.

The results of the new microlysimeter study indicate that the fractions M1, M2 and M3 consist of a high number of polar individual compounds, up to **33 sub-fractions** are separated but mostly unidentified. Only one fraction could possibly be related to IPM1.

In the available lysimeter study with (^{14}C)-lenacil, neither the parent nor IN-KF313 were detected in the leachate at any time during the four-year study (Schnöder, F., 2004). The soil metabolite IN-KE121 was also not present in the leachate.

Polar metabolites found in groundwater were described by the notifier as M1, M2 and M3, and were postulated to consist of the six polar metabolites IPM1 and PM1-PM5. The polar structure IPM1 (“identified polar metabolite 1”) has been identified in soil, and evaluated at the occasion of the submission of confirmatory data and acknowledged in the EFSA Conclusion (EFSA, 2013).

However, the other five polar metabolites (PM1, PM2, PM3, PM4 and PM5) have not been elucidated nor confirmed yet.

It was agreed (see LoEP) that

- the a.s. lenacil was **not** present in the groundwater at >0.1 µg/L (FOCUS: 0/9 Pearl scenario; not found in the lysimeter leachate).
- Metabolite IN-KE 121 was **not** present in the groundwater at >0.1 µg/L (FOCUS: 0/9 Pearl scenario; no information in the lysimeter leachate).
- Metabolite IN-KF 313 was **not** present in the groundwater at >0.1 µg/L (FOCUS: Non reliable information indicated that in geoclimatic regions represented by Piacenza groundwater scenario, contamination of groundwater >0.1 µg/L, cannot be excluded; not found in the lysimeter leachate).

However, some radioactive components were detected in the leachate fractions M1, M2 and M3 which were of a generally polar nature, but they could not be conclusively identified despite mass spectrum analysis. Although the composition of leachate fractions M1, M2 and M3 could not be identified at that time, the available information suggested that these polar metabolites are likely to be fragments of the parent molecule resulting from opening of the cyclopentapyrimidine ring and/or low molecular weight fragments incorporated into natural products.

RMS agreed with this viewpoint.

During the Peer Review at the occasion of the structural elucidation of the groundwater metabolites, following remark was made (15.03.2013):
EFSA: "We consider that the position paper does not address in any way the requirement for 'information on the identity and characterisation of' 'metabolites M1, M2, and M3 which occurred in lysimeter studies'. The applicant has made a valiant attempt to present information that infers that the three lysimeter metabolites are the same three components characterised in the new soil incubation (termed Polar 1, Polar 2 and Polar 3, Polar 3 having had a structure ascribed, but Polars 1 and 2 being only characterised by their chromatographic behaviour). However, the information presented is wholly insufficient to conclude that M1, M2 and M3 are the same as Polar 1, Polar 2 and Polar 3. Even if you were to be generouse and accept this inference of the applicant, we only have a tentative identification of one of the components. With the structure of the lysimeter leachate metabolites M1, M2 and M3 remaining unknown, it is impossible to conclude that these compounds are residues that are 'non relevant and carries no toxicological concern.'

RMS (March 2013):
Whilst there is limited evidence to confirm the exact nature of polar metabolites in lysimeter leachate, the nature of the polar residue can be assessed and proposed on certain facts observed in the soil degradation studies.
The primary oxidation products IN-KE121 and IN-KF313 do not form part of the polar material formed in soil studies and do not occur in leachate from the lysimeter study. Instead, evidence from soil degradation studies has shown that polar material contains metabolites that result from a more extensive breakup of the lenacil molecule. This involves loss of the cyclohexyl ring and/or opening of the central lenacil pyrimidine ring structure. The resulting polar fragments are chemically unstable which leads to further degradation in soil resulting in numerous low molecular weight metabolites. The evidence for this further degradation is the very high level of CO_2 that is formed in soil degradation studies, reaching up to 54% by Day 30 and up to 77% by Day 120 in a recent study (Hurst, 2012).
As shown in the degradation pathway, a proportion of the polar fragments formed are low molecular weight carboxylic acids and these substances have the potential to be incorporated into naturally occurring amino acids. Similarly, low molecular weight amino acids may be formed directly as lenacil degradation products.
It was proposed that molecules of this type will be polar in nature and represent the most likely nature of the residue found in lysimiter leachate. The presence of such polar molecules is an indication that polar material observed in the lysimeter leachate have no 'alerting structure'.

In the EFSA conclusion, it was stated: "*Regarding the lysimeter leachate metabolites ascribed as M1, M2 and M3, the available information from the lysimeter study indicated a potential for leaching to vulnerable shallow groundwater, consequent to the representative use assessed, in annual average recharge concentrations above the parametric drinking water limit of 0.1 µg/L that applies to relevant metabolites. Whilst these metabolites remain unidentified, an assessment of their relevance according to European Commission (2003) guidance could not be finalised.*"

In the meanwhile one of the polar structures (IPM1) has been identified (Dixon and Alderman, 2011) and assessed by EFSA and the Member States in the EFSA Conclusion (ESFA, 2013).

The proposed structures of the five other polar metabolites (PM1, PM2, PM3, PM4 and PM5) could not be elucidated with certainty, and remain to be confirmed yet.

However, a proposal was made and the putative structures are visualised in table B.6.8.2.1 above.

Key characteristics of the lenacil metabolites are found in Appendix 2 (polar metabolites) and Appendix 3 (non-polar metabolites).

Reference: conclusion on pesticide peer review: Conclusion on the peer review of the pesticide risk assessment of confirmatory data submitted for the active substance lenacil, EFSA Journal 2013;11(9):3354.

B.6.8.1.1.2 SCREENING OF THE GENOTOXICITY POTENTIAL OF THE GROUNDWATER METABOLITES

In a submitted position paper (Tier, G.T. and Serex, T.L., 2014, reproduced in Appendix 6), the genotoxic potential of the identified polar metabolite IPM1 has been addressed in depth and notifier concluded that it carries "*low potential for genotoxicity*". However, **RMS** would be more precautionous in this conclusion (see below).

The detailed rationale submitted to support this conclusion for the genotoxicity of IPM1 and other polar metabolites is reproduced hereunder, and further details can be retrieved in the mentioned position paper in Appendix 6.

The main conclusion of the notifier, based on read-across data from other structural analogue substances (Leadscope analogues) possessing the α,β-unsaturated amide functionality and other similar functionalities, plus data from the OECD Toolbox profilers, TOPKAT and TIMES, on the genotoxicity of IPM1 as stated in their position paper was that IPM1 was not mutagenic in Ames and to has a low potential to cause clastogenic effects. This conclusion is supported by the high hydrophilicity ($logP_{ow}$ approx.–3) which renders IPM1 readily excretable and less likely to cause a genotoxic effect.

Thus notifier estimates that the risk of actually being genotoxic is significantly lowered by the high hydrophilicity of the molecule because the internal exposure time can be reasonably expected to be low.

RMS sees a point in the rationale of the notifier, but considers that such a prediction should be underpinned by a valid genotoxicity assay for IPM1, to be provided under AIR-3. The sole fact that the metabolite is easily excreted does not detract from a potential clastogenic effect, and further data concerning this potential effect will have to be submitted (see below).

Electrophilicity is well known to be an important factor in driving mutagenicity and carcinogenicity (Miller and Miller, 1977). They found that it was possible to rationalise the activity of a large majority of animal carcinogens at the time on the basis of their electrophilic potential.

-For predictive purposes, electrophilic features are readily encoded as structural alerts (SA) in expert tools such as the OECD Toolbox v3.1.

-In addition to structural alerts or SARs, global QSAR models exist which aim to provide mutagenicity estimations for diverse sets of chemicals. Such (Q)SARs are typically implemented into expert systems such as TOPKAT which empirically makes predictions for Ames mutagenicity (Serafimova *et al*, 2010).

-Other expert systems notably TIMES attempt to provide clear mechanistic meaning through the use of SA which addresses the reactivity towards DNA and/or proteins. TIMES also includes 3D QSARs to underpin some of the available SA (Serafimova *et al*, 2007).

All the aforementioned SARs or QSARs are derived on the basis of Ames mutagenicity data.

-The OECD Toolbox v3.1 was used to profile the 6 polar metabolites on the basis of a number of different profiling schemes to characterise DNA binding alerts (OECD and OASIS) and potential *in vitro* mutagenicity (ISS alerts) (Benigni and Bossa, 2008). The DNA Binding alerts for OASIS and OECD characterise potential for genotoxicity based on organic chemistry reaction principles. The endpoint schemes for these same alerts, together with the ISS alerts, are substantiated by experimental toxicity data, typically Ames data. Predictions were also made using the expert system TOPKAT and TIMES.

B.6.8.1.1.2.1 Genotoxicity of metabolites IN-KF313 and IN-KE121

The structures of both IN-KF313 and IN-KE121 (see Table 2 of Appendix 3) reveal they are likely to be formed as a result of carbon oxidation in the cyclopentyl and cyclohexyl rings of lenacil. Both metabolites are ketones, and on comparison of the structural features of the ketone metabolites with the parent active molecule, it is apparent that IN-KF313 and IN-KE121 are closely related to lenacil from the structural point of view and for this reason there are no additional toxicological concerns expected.

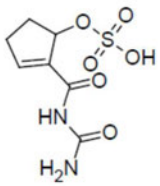
IN-KF313 in particular demonstrates a comparable $logP_{ow}$ with lenacil (3.11 *vs.* 3.09 as calculated by EpiWin) and is thus considered the most representative when compared to lenacil itself. As a consequence, the ketone soil metabolites were compared to the metabolites observed in the rat metabolism study with lenacil, in order to assess whether their toxicity potential can be adequately evaluated based on the toxicology studies performed with the parent active substance. The metabolites identified in the rat metabolism study with lenacil were formed by hydroxylation of the cyclopentyl and cyclohexyl rings to give simple mono- and/or dihydroxylated compounds that are readily excretable based on polarity considerations. The rat was able to enzymatically hydroxylate these metabolites in the cyclohexyl ring to greater enhance the hydrophilicity and, thus, enhance the elimination of the metabolites.

Being ketones, IN-KF313 and IN-KE121 are related to the hydroxylated metabolites observed in the rat metabolism study with lenacil. It is known and well accepted that in biological systems alcohols and ketones are often in equilibrium since their formation is reversible in the presence of oxido-reductase enzyme systems. Reduction of cycloalkylketones to the corresponding alcohols is a common process in the rat and it can be expected that these ketones would be readily metabolised to the alcohols and excreted as such or after conjugation in a phase 2 biotransformation reaction. In fact, it is very likely that ketones would have been formed as minor metabolites/intermediates in the rat metabolism study with lenacil but were not detected as significant components in excreta due to their subsequent reduction to the observed mono-/dihydroxylated species.

For these reasons, notifier concluded that the ketone metabolites would be readily excreted by mammals and would therefore not present any toxicity not previously observed for the parent active substance. IN-KF131 and IN-KE121 are therefore not expected to be of higher toxicity due to their structural similarity to the parent active substance.

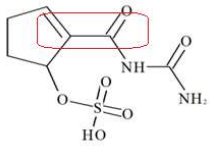
Since in addition, the primary oxidation products IN-KE121 and IN-KF313 do not form part of the polar material formed in soil studies and do not occur in leachate from the lysimeter study, and as also discussed in the first version of the DAR and subsequent addenda, **RMS** agrees with this viewpoint.

B.6.8.1.1.2.2 Genotoxicity of IPM1



IPM1 triggers the DNA Binding alert for urea derivatives – the hypothesis being metabolic activation of the urea derivation could result in the formation of an electrophilic nitrenium ion as an active species. The DNA binding by OECD highlights an α, β-unsaturated amide that could undergo a Michael addition mechanism. The ISS alerts flag an unsaturated carbonyl group as potentially responsible for eliciting a Michael addition reaction. Compounds with an α,β-unsaturated carbonyl are bis-electrophiles reactive molecules that may interact with electron-rich biological macromolecules. Because of conjugation with the carbonyl group, the *b*-carbon is

positively polarised and becomes the preferred site of nucleophilic attack, as is in a classic Michael type addition (Koleva *et al,* 2008). In spite of a common structural feature, α,β-unsaturated carbonyl compounds can undergo different interactions with DNA, which lead to different genotoxic and mutagenic responses. The following genotoxic mechanisms are conceivable: formation of cyclic adducts, frameshift interaction, strand breaks, and crosslinking. In addition to direct interactions, other metabolic activations are conceivable, such as metabolic epoxidation and formation of radicals (Eder *et al,* 1990).



Notifier highlighted that α,β-unsaturated carbonyl compounds trigger a potential Michael addition alert due to the negative mesomeric effect induced by a carboxy moiety and stressed that the potential negative mesomeric effect is mediated by a carboxyamide group, which is itself further substituted. Carboxyamide structures are *per se* mesomerically more stable and thus less reactive than carboxy moieties because of a difference in electronegativity [oxygen (3.44) > nitrogen (3.04)].

However, the training data set obviously contains structures containing carboxyamides that are positive for genotoxic effects. **RMS** recognises that those structures contain either only an unsubstituted carboxy amide moiety or substituted carboxy amide structures with functional groups that increase the overall negative mesomeric effect, such as carboxy moieties.
The carboxyamide structure in IPM1 is embedded in a larger mesomeric complex, which will further diminish the overall negative mesomeric effect on the α,β-unsaturated moiety. Thus, it may be reasoned that the alert for potential Michael addition could be considered somewhat outside the applicability domain.

The α,β-unsaturated amide alert was explored in more detail.

A search using Leadscope was made to identify other substances with this functionality. A selection of acrylamide-containing substances was found with experimental genotoxicity data using Leadscope. The structures are shown in appendix Figure 11 and a table of results are shown in Table 12. The results are quite variable indicating that acrylamide type substances are electrophilic, and do result in **positive and negative** outcomes in Ames, *in vitro* chromosome aberration and micronucleus assays. A similar search was conducted using the OECD Toolbox. Analogues containing an acrylamide-type functionality were identified with Ames data.

A qualitative **read-across** found IPM1 to be non-mutagenic in Ames. The read-across is shown in Figure 12. The prediction is subject to some uncertainty since the IPM1 is outside of the $LogK_{ow}$ defining the scope of the read-across but the very low estimated $LogK_{ow}$ is likely to limit bioavailability.

A similar read-across was attempted exploring the available *in vitro* chromosome aberration data. Tentatively based on the analogues identified which were many of the same acylic acrylamide type substances gave rise to a mixed *in vitro* chromosome aberration profile.

RMS: the read across compared ames-results from 5 “nearest neighbours”, which were negative, although one molecule came out equivocal, and one positive. It is therefore not completely excluded that IPM1 could theoretically give rise to a positive response.

Notifier indicated that QSAR analyses performed for lenacil revealed an alert for potential genotoxicity hazard which was not proven in the genotox test battery. IPM1 is more polar than lenacil and the other metabolites which renders IPM1 more easily excretable, either directly or after conjugation, than lenacil and the other metabolites. As stated before the internal exposure time of IPM1 in the body can be reasonably expected to be low and therefore low genotoxcity potential can be concluded.

The notifier brought also under the attention that, whilst this is a plausible route for activity to be observed, the extent to which this will happen will be influenced (hindered) by

- the presence of the remaining functional group and by
- the fact that IPM1 is so hydrophilic.

Whilst no α, β-unsaturated ureas could be identified, a handful of urea derivatives shown in Table 13:

- carbamide: Ames positive, Mouse lymphoma positive;
- biuret: Ames positive;
- bromodiethylacetylurea: Ames negative, *in-vitro* CA positive)

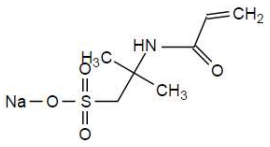
were found, which again exhibited a **mixed genotoxic profile**.

These analogues served to evaluate the extent of the other alerting group identified (urea derivative). The outcomes do suggest that a **positive response for *in vitro* chromosome aberration** might be possible for IPM1 but with it are uncertainties about the similarity of the analogues relative to IPM1 and to what extent the hydrophilicity and cyclic nature may play a role.

For this, predictions were made using the 2 expert systems TOPKAT and TIMES.

- A prediction was made by TOPKAT which gave rise to **a negative result for Ames**.
- TIMES were then used and gave rise to a negative outcome for Ames but a **positive outcome for *in vitro* chromosome aberration and *in vivo* micronucleus**. The predictions were scrutinised in more detail to understand the reasoning for why overall positive responses were predicted for these 2 endpoints.
- The OECD Toolbox had flagged the alerting group as a potential for why IPM1 might be **positive in genotoxicity assays** but these largely fail to take into account the environment around the alert in most cases.

Evaluating the metabolites synthesised, it was further postulated by the notifier that IPM1 itself was not contributing to the positive response but that downstream metabolites were formed such as carbamates and simple substituted acrylamides, which could drive a positive response.



Examples of such substances are noted in Table 14 and 15. For the *in vivo* micronucleus endpoint, only the carbamate was flagged as active for effects. Figure 14 shows the pathway simulated for IPM1 and in yellow are highlighted those metabolites that are predicted as active. The probabilities of these metabolites being formed are stated "quite low" by the notifier, which lowers the likelihood of IPM1 actually being positive in a study if tested.

One of the "analogues" cited by the notifier was CAS 5165-97-9, a highly hydrophilic substance which was associated with mixed outcomes in the *in vitro* chromosome aberration assay with positive and negative responses as reported in the OECD Toolbox. The structure corresponds to sodium 2-acrylamido-2-methylpropane-1-sulfonate (the acid is investigated in REACH, with notification of CAS 15214-89-8).

RMS: the relevance of abovementioned substance in the QSAR considerations of IPM1 was further commented by the notifier, as this example is not a a good structural analogue of IPM1. Notifier explained that, whilst sodium 2-acrylamido-2-methylpropane-1-sulfonate is by itself not a good structural analogue of IPM1, it is however a highly hydrophilic substance like IPM1, and was associated with mixed outcomes in the *in vitro* chromosome aberration assay with positive and negative responses as reported in the OECD Toolbox. However, in the REACH disseminated

dossier for sodium 2-acrylamido-2-methylpropane-1-sulfonate, an *in vivo* chromosome aberration assay (OECD 475) is available and which found the substance to be non-clastogenic in rat bone marrow cells under the condition of the assay. This data overrules the mixed outcomes in the *in vitro* chromosome aberration assay with positive and negative responses as reported in the OECD Toolbox for 2-acrylamido-2-methylpropane-1-sulfonate and supports the argument that IPM1 which is also a highly hydrophilic substance will have low gentoxicity potential due to its low systemic bioavilability.

RMS notes that for the time being, no such study is fully available in the context of this submission, and the hypothesis of no *in-vivo* genotoxicity cannot be confirmed. The finding has some illustrative value on the variability of true genotoxic responses in case of the presence of suspected genotoxic alerts, but is not essential in the context of genotoxicity assessment of lenacil groundwater metabolites.

Note: the cited study is summarised in http://echa.europa.eu/de/registration-dossier/-/registered-dossier/15200/7/7/3

Notifier comes to the conclusion that a further metabolisation of IPM1 would be unlikely. The notifier's assumption is that, based on the chemical properties of IPM1, a fast elimination of the systemic compartment can be reasonably assumed. Hence, neither further metabolisation nor potential toxic effects that would require transport in cells are likely or would occur at a relevant level in the exposure time. Since this assumption is speculative for the RMS, and somewhat contradictory as regards former assumptions, a further investigation seem necessary to exclude the potential clastogenicity of the substance IPM1.

RMS acknowledges that a toxicological relevance assessment of such potential 'metabolites' of IPM1 would only be required when the predicted metabolite is actually formed in soil and subsequently detected in ground water.

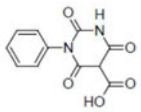
Conclusion RMS:

Based on the read-across from the OECD Toolbox, the TOPKAT and TIMES estimate and the other Leadscope analogues, IPM1 is **not *expected* to be mutagenic in Ames**. However, alerting groups identified coupled with predictions from TIMES also suggest **a *potential* exists for clastogenic effects**. While notifier has a point in the rationale that:

- lenacil also shows a QSAR-alert for clastogenicity whilst displaying no *in-vivo* clastogenicity in valid studies, and
- IPM1 is more polar than lenacil (and the other metabolites) which renders IPM1 more easily excretable, and potentially of low systemic bioaviliability,

an intrinsic potential for clastogenic effects is probably low, but not excluded.

B.6.8.1.1.2.3 Genotoxicity of PM1



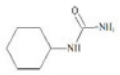
No prediction could be made for PM1 using TOPKAT since it was structurally out of the applicability domain. Toolbox DNA binding alerts revealed the urea derivative alert suggesting formation of a nitrenium ion. This is an alert that was flagged for IMP1 and is also in common with that of Lenacil itself. A prediction in TIMES for Ames gave rise to a negative outcome. This is consistent with the prediction derived for Lenacil itself. Experimental data on Lenacil identified in the OECD Toolbox itself shows it to be negative in an Ames study. In this study, only TA97, TA98, TA100 and TA102 strains were tested with metabolic activation. A prediction for *in vitro* chromosome abberration however gives rise to a **positive** outcome for PM1. A prediction for *in vivo* micronucleus also gives rise to a **positive** outcome for PM1. The alert driving the response in this case is that for a pyrimidine. The genotoxicity of pyrimidines and purines have

been studied by the reverse mutation assay in bacteria and the chromosomal aberration test in cultured Chinese hamster lung (CHL/IU) cells. The chemicals including Fluorouracil, Tegafur, Caffeine, Theophylline, 4-Amino-1-pentofuranosyl-2(1H)-pyrimidinone, Enocitabine, 6-Mercaptopurine, Disodium 5'-guanylate induced chromosomal aberrations in *in vitro* assay in CHL cells without metabolic activation.

However, the same outcomes were predicted for Lenacil as a result **of the same alerting group**. An EFSA peer review pesticide final risk assessment document states that Lenacil is unlikely to be genotoxic based on a set of adequately conducted *in vitro* and *in vivo* assays. Whilst the predictions for Ames agree with the limited Ames data actually found, the TIMES predictions for *in vitro* chromosomal aberration and *in vivo* micronucleus differ from the experimental data reported for Lenacil. Based on the commonality of the alerting group driving these effects in both Lenacil and PM1 – the expectation is that PM1 experimentally would not give rise to positive outcomes. PM1 is not expected to be genotoxic.

RMS can agree with this position, since the same alert as for lenacil itself (pyrimidine) is present, and while lenacil's output in this prediction is positive, the actual lenacil genotoxicity in the DAR studies is overall negative, overruling the possible alert for PM1.

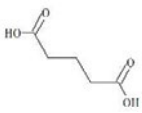
B.6.8.1.1.2.4 Genotoxicity of PM2



TOPKAT found PM2 to be non-mutagenic in Ames. Predictions using TIMES for all 3 models gave rise to negative outcomes. The only alert identified for DNA binding was for the urea derivative. Indeed, based on the analogues identified for IPM1, a potential may exist for the urea to give rise to a positive outcome as was evidenced for (57-13-6) itself. PM2 is not expected to present a genotoxic concern but available data for other ureas such as 57-13-6 give rise to some uncertainty regarding the predictions made.

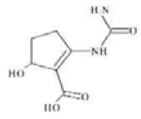
RMS can agree with this conclusion.

B.6.8.1.1.2.5 Genotoxicity of PM3



TOPKAT found PM3 to be non-mutagenic in Ames. Predictions using TIMES for all 3 models gave rise to negative outcomes. This was consistent with expectations given PM3 contains no electrophilic features and did not trigger any alerting groups. Data was identified within the OECD Toolbox for the PM3 structure, specifically dicarboxylic acid C4-C6 [68603-87-2]. The Ames test was found to be negative; a negative outcome was also reported in an *in vivo* chromosome aberration study. A qualitative read-across using the OECD Toolbox identified other analogues (including adipic acid, malic acid, methyl succinic acid) with available Ames information. PM3 was predicted to be negative in Ames. The read-across is depicted in Figure 15 (appendix 6).
RMS can agree with this conclusion.

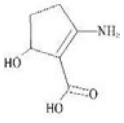
B.6.8.1.1.2.6 Genotoxicity of PM4



TOPKAT found PM4 to be non-mutagenic in Ames. TIMES found PM4 to be Ames negative. Carbamate metabolites simulated in the TIMES *in vitro* chromosome aberration model propose a **positive** outcome. TIMES *in vivo* micronucleus found PM4 to be negative. On account of the negative *in vivo* estimate, PM4 is not considered to pose a concern for genotoxicity.

RMS can agree with this conclusion.

B.6.8.1.1.2.7 Genotoxicity of PM5



TOPKAT predicted PM5 to be **positive** in Ames. Alerts within the Toolbox flagged the urea derivative and the heterocyclic primary amine (although strictly, PM5 is not predicted to be a heterocyclic amine).

TIMES predicted PM5 to be **positive** in *in vitro* chromosome aberration and *in vivo* micronucleus on account of the primary amine – though the alert description made reference to only aromatic amines such as aniline or phenylene diamines. The relevance of these predictions is therefore questionable. Notifier highlighted that aryl groups have a positive mesomeric effect, as do phenylamines. Hence, the free electron pair of the primary

amine is the relevant functunal entity. As the present amine is linked with a carboxy group via α,β- unsaturated carbonyls, the negative mesomeric effect will translocate the electrons and make the free electron pair less available for potential genotoxic effects. Hence, the TIMES alert can be reasonably considered to be outside of the applicability domain and no further investigation is warranted.

RMS can agree with this conclusion: the outcome of the predictions is considered undecided; further investigation in other models is warranted.

Further evidence should have been provided to the RMS with the EU Renewal dossier. RMS remarks that no genotoxicity assays were submitted, impeding a full assessment of the genotoxicity of groundwater metabolites.

In the context of the assessment of the potential genotoxicity/carcinogenicity of the identified polar metabolite IPM1, it is of particular note that QSAR expert systems (TIMES predictions for *in vitro* chromosomal aberration and *in vivo* micronucleus) indicated an alert for genotoxicity *in vitro* and *in vivo* for lenacil whilst Ames tests, however, were negative for lenacil. In the available genotoxicity/mutagenicity studies submitted as part of the dossier established according to Directive 91/414/EC, lenacil itself showed evidence of clastogenic activity in human lymphocytes *in vitro* in the absence of S9 mix only. However, no clastogenic activity was observed in the presence of S9 mix which is considered to be more relevant as it more closely mimics in-life conditions. Moreover, in the *in vivo* studies, lenacil technical did not show any evidence of causing chromosome damage or bone marrow cell toxicity when administered orally to mice. Lenacil is concluded not to be genotoxic or a mutagen which is in agreement with the conclusions drawn in the EFSA peer review pesticide final risk assessment document, where it is stated that lenacil is unlikely to be genotoxic based on a set of adequately conducted *in vitro* and *in vivo* assays.

Notifier took the following position: "*Based on a refined assessment of the chemical properties of the present functional groups in the respective structures IPM1 and PM5 the QSAR alerts can be considered to be out of the applicability domain of the models. Hence, QSAR does not provide evidence for toxicological concern for these metabolites."*

Thus, the notifier suggested, based on the current available information, that the polar groundwater metabolites are *not* toxicologically relevant. **RMS** would take a more precautionous approach, and thinks that a further confirmation of the absence of mutagenicity/clastogenicity for the polar water metabolites, especially IPM1, is scientifically justified. However, notifier confirmed that polar metabolite IPM1 is extremely unstable and impossible to synthetise. Therefore, further data are not requested.

General conclusion on genotoxicity.

RMS is of the opinion that, to be on the safe side, a full genotoxicity battery on this groundwater metabolite, or ideally for the pool of IPM1+polar metabolites would be the best way to demonstrate non-genotoxicity. However, it is acknowledged that the amounts of groundwater metabolites formed *in-situ* (as also predicted in the groundwater models) are all extremely low, precluding the purification, let alone the testing of these metabolites in practice.

Since IPM1 has been elucidated, and is prone to synthesis, it would be necessary to test this substance in an *in-vitro* or *in-vivo* test. RMS takes note that further evidence should have been submitted in the AIR-3 dossier, awaited June 2016. RMS remarks that no genotoxicity assays were submitted, impeding a full assessment of the genotoxicity of groundwater metabolite IPM1. According to the notifier this metabolite IPM1 is very unstable and synthesis is impeded because of this reason. If correct, the substance cannot be tested.

B.6.8.1.1.3 Screening of the carcinogenicity potential of the groundwater metabolites

In order to assess the carcinogenic potential of the groundwater metabolites of lenacil, the OECD Toolbox (v3.3.5) was used to screen the groundwater metabolites for structural alerts for carcinogenicity. The metabolites IN-KF131, IN-KE121 and the 6 polar metabolites were evaluated using the two following profiling schemes: carcinogenicity (genotoxic and non-genotoxic) alerts by ISS and oncologic primary classification. Further information and explanations on the two profiling schemes used can be found in Appendix 3, along with a summary of the results.

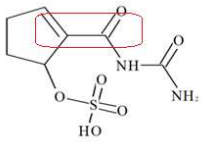
Briefly, the profiler carcinogenicity (genotoxic and non-genotoxic) alerts by ISS are based on a list of **55 known structural alerts** for genotoxic and/or non-genotoxic carcinogens. The structural alerts for carcinogenicity are molecular functional groups or sub-structures known to be linked to the carcinogenic activity of chemicals. If one or more structural alerts embedded in a molecular structure are recognised, the system flags the potential carcinogenicity of the chemical. According to the notifier, "*the oncologic primary classification profiler is not appropriate for predicting the potential carcinogenicity of the chemical, but instead allows for categorisation of chemicals according to the U.S. Environmental Protection Agency's OncoLogic Cancer Expert System. Thus, oncologic primary classification profiler is used for classification purposes only*".

Lenacil and its metabolites IN-KE121 and IN-KF313

Lenacil and its metabolites IN-KE121 and IN-KF313 revealed no structural alerts for genotoxic or non-genotoxic carcinogenicity in the ISS profiler. Furthermore, the oncologic primary classification profiler found no classification for any of the compounds.

Polar metabolites

(i) ISS profiler



From the 6 polar groundwater metabolites, the 5 non-identified polar metabolites PM1-PM5, revealed no structural alerts for genotoxic carcinogenicity in the ISS profiler. However, **IPM1 revealed structural alerts for genotoxic carcinogenicity** in the ISS profiler. IPM1 was shown to have a structural alert for genotoxic carcinogenicity due to the presence of an α, β-unsaturated carbonyl function in the molecule (see on structure).

According to the notifier, this is based on a debateable assessment which disregards the chemical properties of the functional groups as discussed above.

(ii) OncoLogic profiler

The oncologic primary classification profiler on the other hand concluded all 6 polar metabolites, including IPM1, to have no classification for carcinogenicity.

RMS observes that this is a valid rationale *per se*, but notes that Lenacil was finally classified as at Cat.2 carcinogen by ECHA, and asks what the outcome of the QSAR would have been, including Lenacil as a carcinogenic compound in this expert system. Notifier is of the opinion that Lenacil is not considered to be carcinogenic but is classified as a "*suspected human carcinogen with limited evidences in rats substantiating that classification".* Hence, inclusion of Lencacil as a "carcinogenic" compound in QSAR analysis would not increase the robustness of a prediction for carcinogenicity.

For the **RMS**, this would at best indicate that the metabolites are not genotoxic carcinogens, but the position indicates that there are no valid predictions to exclude Cat.2 carcinogenicity.

As noted under B.6.8.2.3, the hypothesis that there would indeed be no genotoxic, and thus genotoxic carcinogenic hazard, should be further confirmed by a study (on IPM1).

B.6.8.1.1.4 Screening for endocrine disrupting properties

As the MoA for the induction of mammary tumours observed in the carcinogenicity study with lenacil is unknown, and as the absence of a genotoxic potential of lenacil does not suggest that mammary gland tumours are caused be a genotoxic mode of action, an epigenetic, endocrine-mediated MoA *could* be the cause for the induction of these tumours.

In the meanwhile, Level 2 *in-vitro* assays were conducted on lenacil in order to investigate interaction with the oestrogenic or androgenic receptors, and a level 3-study (uterotrophic assay) to assess the *in-vivo* effect of lenacil on the uterus. None of these studies provide evidence of an effect of lenacil on the oestrogenic or androgenic pathway.

Nevertheless, the rationale of the notifier on the basis of QSAR considerations in order to provide *in-silico* information on endocrine effects are given below (as submitted at the occasion of the request of confirmatory data).

In order to give an indication of an endocrine-mediated MoA of carcinogenicity, the potential endocrine disrupting (ED) properties of lenacil have been investigated in a first step using the OECD toolbox, considering the profilers oestrogen receptor (ER) binding and rtER Expert System ver. 1 – USEPA.

Furthermore, the ED properties of the groundwater metabolites of lenacil were also investigated in a similar manner.

The OECD Toolbox (v3.3.5) was used to screen lenacil and its groundwater metabolites for structural alerts regarding ED properties. Lenacil, IN-KF131, IN-KE121 and the 6 polar metabolites (IPM1 and PM1-PM5) were evaluated using the two profiling schemes, which are:

a) oestrogen receptor (ER) binding and

b) rtER Expert System ver. 1 – USEPA.

Further information on the two profiling schemes used is provided in Appendix 5.

RMS observes that the approximation for an EDS potential only relies on the theoretical potential of the metabolites to bind to ER-receptors. Taking into account that the observed mammary tumours induced by lenacil are of unknown etiology, the restriction to ER-based effects is speculative. However, was accepted, pending further data on the EDS potency of lenacil itself.

In the meanwhile, OECD Conceptual Framework level-2 *in-vitro* studies and one level-3 *in-vivo* study was submitted for the a.s. itself, confirming the predicted ED-negativity (cfr 6.8.3)

Notifier provided following explanation:

"Data from a scientific paper investigating the in vitro ER/AR activity of lenacil will be included in the renewal dossier (Kojima, H., 2004) and will provide further re-assurance of the absence of an ED potential of lenacil. These data were not submitted in the position paper.

Lenacil did not show any oestrogenic or androgenic transcriptional activity at concentrations $\leq 10^{-5}$ M via the two human estrogen receptor (hER) subtypes, hERα and hERβ, and via the human androgen receptor (hAR). Therefore, lenacil is concluded to have no ERα, ERβ or AR agonistic activities, in vitro.

In addition to the above mentioned data, in the renewal dossier, further information on the interaction of lenacil with the following receptors has been included:
- Pregnane X receptor (PXR) - Kojima, H. 2011
- Peroxisome proliferator-activated receptors (PPARs): mouse PPARα and PPARγ - Takeuchi, S. 2006
- The aryl hydrocarbon receptor (AhR) - Takeuchi, S. 2008
Based on the above receptor-based in vitro investigations, lenacil was concluded to have no PXR, mPPARα, mPPARγ or AhR agonistic activities at concentrations of ≤10⁻5 M.
The overall conclusion from these investigations is that based on the weight of the evidence from all receptor-based studies performed, lenacil is unlikely to possess agonistic or antagonistic activities on the different receptor (sub-)types examined. The results substantiate that lenacil neither induces the ER nor AR receptors which provides further evidence that lenacil does not interact with the endocrine system and is, therefore, unlikely to be an endocrine disruptor.
An involvement of hormones via the ER, by oestrogenic compounds, can be expected for a large amount of breast cancer cases in humans. Activation of ER alpha leads to a proliferation of breast cancer cells. Hence, an involvement of ER in the development of breast cancer is per se likely and plausible. The prediction for the absence of ER binding for the molecules therefore suggests a non-endocrine-mediated mode of action. In light of the present knowledge of breast cancer etiology this assessment is not speculative but is - in contrast and based on the available data- the most likely explanation.
Further, the notifier did not submit measured data on nuclear receptor binding for lenacil in this assessment but will do so in the renewal process: the data shows an absence of ER and AR binding for Lenacil and thus suggest the absence of the –at least- most likely hormonal mode of action for potential cancerogenicity.

Cited references:
1) Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K., and Kobayashi, K. (2004); Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. Environmental health perspectives, 112:524-531
2) Kojima, H., Sata, F., Takeuchi, S., Sueyoshi, T., and Nagai, T. (2011); Comparative study of human and mouse pregnane X receptor activity in 200 pesticides using in vitro gene assays. Toxicology, 280:77-87
3) Takeuchi, S., Matsuda, T., Kobayashi, S., Takahasi, T and Kojima, H. (2006); In vitro screening of 200 pesticides for agonistic activity via mouse peroxisome proliferator-activated receptor (PPAR)α and PPARγ and quantitative analysis of in vivo induction pathway. Toxicology and Applied Pharmacology, 217: 235-244
4) *Takeuchi, S., Iida, M., Yabushita, H, Matsuda, T and Kojima, H. (2008); In vitro screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, DR-EcoScreen cells, and in vivo mouse liver cytochrome P450-1A induction by propanil, diuron and linuron. Chemosphere, 74:155-165."*
RMS remains of the opinion that a final demonstration of the non-relevance of lenacil (and its metabolites) as regards endocrine activity would be provided if the substance would at least be tested with the Level 2-studies of the OECD Conceptual Framework, which offers a toolbox for endocrine interaction, but considers the predictions below as complementary information.

However, **RMS** considers that a QSAR analysis in isolation is insufficient to exclude a potential endocrine effect. QSAR estimations are so far not sufficiently evolved and validated to make strong statements other than acute or genotoxicity effects. Therefore, further data were requested and submitted to further underpin the absence of endocrine effects of lenacil in mammalian cells. It is acknowledged that no specific studies on the metabolites themselves have been submitted, and that the absence of ED properties of the metabolites can only be inferred taking into account the data on the parent compound. Although this may be accepted by the RMS in a first step, a further rationale is requested to bridge the existing ED studies from the a.s. to its metabolites.

Notifier commented provide data when they know which metabolites exceed the groundwater trigger, and if they had mechanism to address.

Note: Explanation of the prediction tools for endocrine activity:

-The ER binding profiling scheme which is based on structural and parametric rules extracted from literature sources and supported by experimental data. The ER-binding profiler classifies chemicals as non-binders or binders depending on both molecular weight (MW) and structural characteristics of the chemicals (profiler "A").

-The rtER Expert System ver.1-USEPA profiler ("rainbow trout Oestrogen Receptor" (profiler "B")) consists of molecular definitions which mimic the structural criteria of chemical classes with potential estrogen receptor-binding covered by US EPA Estrogen Receptor Expert System (ERES). The ERES profiler is an effects-based automated

system used to predict estrogen binding affinity. In the OECD Toolbox, the rtER Expert System ver.1 –USEPA profiler is used for the purpose of categorisation based on the structural definitions of the original ERES chemical classes. It was highlighted that the Expert System ver 1-USEPA profiler is introduced for categorisation purposes and not for predicting relative binding affinity (RBA). As a first tier, this is acceptable to the **RMS**.

Profiler "A" (oestrogen receptor binding).
Neither the parent compound lenacil, nor the 2 metabolites (very similar to lenacil) IN-K121 and IN-KF313 revealed structural alerts for ED properties in the rtER Expert System ver. 1 – USEPA profiler and was found to be a non-binder, without OH or NH_2 group in the ER binding profiler.

For the proposed groundwater metabolites IPM1, PM1 and PM2, the same conclusion, namely: "*non-binder, without OH or NH_2 group*" holds.

The identity of polar metabolite PM3 is assumed to be glutaric acid. Glutaric acid is naturally produced in the body during metabolism of some amino acids, including lysine and tryptophan. Due to the fact that glutaric acid is naturally produced in the body, it was proposed to be a non-relevant metabolite and a further assessment was not considered to be required by the notifier. PM3 was classified "non-binder, non-cyclic structure".

PM4 and PM5 were found to be moderate and weak binders with OH groups, respectively, in the ER binding profiler.

Profiler "B" (Expert System ver 1-USEPA).
With regards to the groundwater metabolites, none were found to have clear structural alerts for ED properties in the rtER Expert System ver. 1 – USEPA profiler.

Conclusion RMS:
Based on the outcome of the Oestrogen receptor binding OECD Toolbox screen, it can be concluded that lenacil and its groundwater metabolites are not *likely* to have ED properties. An endocrine-mediated MoA for the induction of mammary tumours observed in the carcinogenicity study with lenacil is therefore not expected and appears to be unlikely.
However, a final demonstration of the non-relevance of lenacil (and its metabolites) as regards endocrine activity would be provided if the substance would at least be tested with the Level 2-studies of the OECD Conceptual Framework, which offers a toolbox for endocrine interaction.

Applicant indicated the following:
"Hormonal interaction and human breast cancer (as inference from mammary gland tumors in rats) usually occurs via the ER (alpha). Hence, the most likely target of a potential endocrine action would be the ER. The notifier did not submit measured data on nuclear receptor binding for lenacil in this assessment but will do so in the renewal process (Kojima, 2004): the data shows an absence of ER and AR binding for lenacil and thus suggest the absence of the –at least- most likely hormonal mode of action for potential cancerogenicity. Therefore the presumably most relevant Level-2-study has been performed for lenacil and will be available in the AIR-3 process."
RMS: it is of note that not only data from open literature are needed, but complete GLP-studies, run according the accepted OECD guidelines or EC test methods, and with the a.s. having the specification for which authorisation is sought.

B.6.8.1.1.5 Summary of the potential genotoxic, carcinogenic and endocrine disrupting properties of the groundwater metabolites of lenacil

On the next page, the table B.6.8.2.2 summarises the outcome (alerts and possible MoA relevant for genotoxicity, carcinogenicity or ED potential) for the various lenacil groundwater metabolites.

General conclusion of RMS BE (06.2016) regarding the hazard assessment of the potential groundwater metabolites of Lenacil, using QSAR expert systems provided by the notifier.

RMS has to report that a final evidence, excluding the relevance of the groundwater metabolites of lenacil, classified Carc. Cat.2, has not been provided.

Notifier: That is correct. We are waiting on the E-fate/modelling to provide final exposure/modelling information.

As regards the **genotoxicity**, PM1-PM4 are considered overall negative. For PM5, although alerts were flagged based on aminobenzene moieties, PM5 itself has no aromatic amine part, but a substituted cyclopentene ring, rendering any comparison with aromatic amines rather void.

Metabolite IPM1 has a mixed genotoxicological outcome. Although unlikely genotoxic, the final predictions of IPM1 and PM5 is undecided.

As regards the **carcinogenicity**, the models predict the metabolites being negative in almost all cases, except for IPM1 showing an equivocal result (+ for ISS but – for OnoLogic). Therefore, the outcome of IPM1 is equivocal for carcinogenicity.

As regards the **endocrine activity**, the models provide in general a negative prediction for most metabolites, but contradictory results for PM4 and PM5. Therefore, the outcome of these 2 metabolites is also considered equivocal.

However, taking into account the fact that the total amount of polar groundwater metabolites in the leachate study is about 1.048 µg/L lenacil equivalent, and that at least 6, but probably much more different substances could be resolved, the average concentration of each metabolite separately would be about 0.17 µg/L, which is barely above the trigger of 0.1 µg/L.

Notifier announced following study: *"The results of the new microlysimeter study (Hein, W., 2016), suggests that a high number of polar individual compounds, up to 33 sub-fractions are present in the leachate, which supports our previous assumption that the individual substances are likely to be <0.1µg/L.*

Reference: Hein, W (2016).Microlysimeter study with ^{14}C-Lenacil for the characterisation of metabolite pattern in leachates from two different soil types.". The study was submitted and evaluated under Vol. 3, B.8

Table B.6.8.1.1:

Overview of the outcome (alerts and possible MoA relevant for genotoxicity, carcinogenicity or ED potential) for the various lenacil groundwater metabolites

Metabolite /endpoint	IPM1	PM1°	PM2	PM3	PM4	PM5
DNA binding	[1]urea derivative (nitrenium formation) [2]unsaturated carbonyl (+)	[1]urea derivative (nitrenium formation); Pyrimidine alert (?)	[1]urea derivative (nitrenium formation);			[1]urea derivative, heterocyclic primary amine
Ames	[3]non-mutagenic (–) [4]non-mutagenic (–) [5]non-mutagenic (–)	[4]non-mutagenic (–)	[3]non-mutagenic (–) [4]non-mutagenic (–)	[1]non-mutagenic (–) [3]non-mutagenic (–) [4]non-mutagenic (–) [5]non-mutagenic (–)	[3]non-mutagenic (–) [4]non-mutagenic (–)	[3]amino alert: mutagenic (+??)
In-vitro CA	[4]clastogenic (+) [5]clastogenic: mixed + and –	[4]clastogenic (+?)			[4]carbamate: clastogenic (+)	[3]amino alert: clastogenic (+??)
In-vivo MN	[4]clastogenic (+)	[4]clastogenic (+?)		[1]non-clastogenic (–)	[4]carbamate: non-clastogenic (–)	[3]amino alert: clastogenic (+??)
Carcinogenicity	[6]Possibly carcinogenic [7]Not carcinogenic	[6]Not carcinogenic [7]Not carcinogenic	[6]Not carcinogenic [7]Not carcinogenic	[6]Not carcinogenic [7]Not carcinogenic	[6]Not carcinogenic [7]Not carcinogenic	[6]Not carcinogenic [7]Not carcinogenic
ED potential	[8]Not EDS [9]Not EDS	[8]Not EDS [9]Not EDS	[8]Not EDS [9]Not EDS	[8]Not EDS [9]Not EDS	[8]Moderate EDS [9]Not EDS	[8]Weak EDS [9]Not EDS
WoE genotox **WoE carcino** **WoE EDS**	**Undecided** **Undecided** **Negative**	**Negative** **Negative** **Negative**	**Negative** **Negative** **Negative**	**Negative** **Negative** **Negative**	**Negative** **Negative** **Equivocal**	**Equivocal** **Negative** **Negative**

Genotoxicity: [1]: OECD toolbox; [2]: Toxtree; [3]: Topkat; [4]: TIMES; [5]: Read-across; **Genotoxic carcinogenicity** profilers: [6]: ISS (OECD toolbox); [7]: EPA OncoLogic
Endocrine Disrupting potential profilers OECD Toolbox (v.3.3.5) [8]: Oestrogen Receptor Binding ; and [9]: rtER Expert System v.1-USEPA

°: not entirely predictable in TOPKAT because of 'Out Of Domain' (not enough structurally related data in the training set, making predictions not very reliable)
?: prediction discutable, taking into account that Lenacil, carrying the same pyrimidine alert, was pve *in vitro* -S9, but nve +S9, and nve in the *in-vivo* micronucleus assay
??: prediction discutable, taking into account that the training set is mainly composed of aromatic amines, behaving differently than the amino-hydroxycyclopentene derivative for PM5.

B.6.8.1.1.6 Quantification of potential polar groundwater metabolites

In the available lysimeter study with (^{14}C)-lenacil (Schnöder, F., 2004), neither lenacil nor the known metabolites IN-KF313 and IN-KE121 were found in the leachates.

However, based on the results obtained, the annual average concentrations of the unidentified metabolite fractions M1, M2 and M3 each of which is composed of several unidentified metabolites present in the leachate were above 0.1 µg/L.

Due to the concentration of these metabolite fractions being above the parametric drinking water limit of 0.1 µg/L, the polar metabolite fractions M1, M2 and M3 are regarded as potentially relevant.

In the first year of monitoring, the two polar fractions (M1 and M2) and the less polar fractions M3 exceeded 0.1 µg/L whilst only the mean concentration of M1 (and M3 in one of the lysimeters with 0.104 µg/L) was found to be above 0.10 µg/L in the second year of monitoring.

No individual fraction exceeded 0.10 µg/L in the third and fourth monitoring years. It is of note that each of these fractions consists of several individual metabolites and for this reason it can be reasonably assumed that the concentration of individual metabolites in each of these fractions is well below the maximum permissible concentration of 0.1 µg/L for groundwater and would, therefore, not be significant for a further assessment.

The concentrations of the respective polar fractions during the first year of the lysimeter study are depicted in the following:

M1 = 0.238 to 0.256 µg lenacil equivalents/L

M2 = 0.489 to 0.519 µg lenacil equivalents/L

M3 = 0.200 to 0.273 µg lenacil equivalents/L

Furthermore, previous and new FOCUS/PELMO groundwater modelling has predicted concentrations in groundwater for lenacil, IN-KE-121 and IN- KF313 to be less than 0.1 µg/L and, therefore, these metabolites are not significant, and thus not relevant for a further assessment. This is in agreement with the results of the lysimeter study of Schnöder (2004) where neither lenacil nor IN-KE-121 and IN- KF313 were detected in the leachates.

The full PEC reports has been provided to the RMS with the EU Renewal dossier.

Currently, a new microlysimeter study with (^{14}C)-lenacil is being conducted to re-confirm the levels of the polar fractions in the leachate of the previously study performed in 2004 (see announced study, Hein, 2016 above). In this study, lenacil was applied under real-life conditions and the objective is to demonstrate that lenacil and its groundwater metabolites are below the parametric drinking water limit of 0.1 µg/L and therefore non-significant.

The full study report has been provided to the RMS with the EU Renewal dossier.

B.6.8.1.1.6.1 Exposure and risk assessment – threshold of toxicological concern approach

Notifier proposed following approach for the esposure and risk assessment:

"For substances of unknown structure with no alerts for carcinogenicity/mutagenicity the Scientific Committee on Plants (Munro, I., 1999) proposed a toxicological threshold of concern (TTC) of 1.5 µg/person/day or 0.02 µg/kg bw/d (75 kg/person) and 0.025 µg/kg bw/day (60 kg/person) which is in line with the threshold developed by the US-FDA. Assuming a consumption of 2L of water/day for an adult, all of which comes from the upper soil layer, such an acceptable exposure level relates to an acceptable estimated upper limit of 0.75 µg/L for a metabolite.

The maximum estimated total metabolite concentration in groundwater of 0.519 µg/Lwhich is based on the measured concentration in the lysimeter leachate for fraction M2 is well below 0.75 µg/L and, thus, below the toxicological threshold of concern of 1.5 µg/person/day.

Furthermore, the theoretical ingestion by a person drinking 2L of water per day would be equivalent to 0.512, 1.038 and 0.546 µg for metabolite fractions M1, M2 and M3 respectively, corresponding to 0.0085, 0.0173 and 0.0091 µg/kg bw/d for M1, M2 and M3 (assuming a default weight of 60 kg/person). The calculated exposure values are below the toxicological threshold of concern of 0.02 -0.025 µg/kg bw/day or 1.5 µg/person/day as set by the Scientific Committee on Plants and are in line with the threshold developed by the US-FDA for substances of unknown structures.

Taking into account the conservative threshold of toxicological concern approach, it can be concluded that the estimated groundwater concentration of polar fractions M1, M2 and M3, respectively, present no unacceptable health risk to consumers via drinking water consumption. It is further noted that this approach is additionally conservative in that in the absence of knowledge on the composition of the metabolite fractions, the groundwater concentrations of the whole fractions were taken into account for this assessment as a worst case".

RMS prefers to use the latest approach adopted by the EFSA Scientific Committee (2012), as depicted in figure B.6.8.2-1 below.

As only one groundwater metabolite (IPM1) was considered « undecided » as regards genotoxicological potency, based upon QSAR-models, among in total 6 polar groundwater metabolites, one could consider to conduct an exposure assessment with the "average" value of 0.17 µg/L per identified/putative metabolite, as defined under B.6.8.2.6. As shown in table B.6.8.2.3, this would lead to an expected exposure of 0.006, 0.02 or 0.03 µg/kg b.w./d for adult, toddler or bottle-fed infants, respectively, thus theoretically breaching the "genotoxicity" threshold of 0.0025 µg/kg b.w./d for all consumer groups.

In the reasonable assumption that much more metabolites are present among the polar "fraction", around possibly 30 substances, the likelihood that IPM1 would breach this TTC threshold will be meaningfully lower.

In such a scenario, an average concentration of 0.035 µg/L would be obtained, leading to an exposure of 0.001, **0.004** or **0.005** µg/kg b.w./d, which would be acceptable for adults, but not necessarily for toddlers or infants.

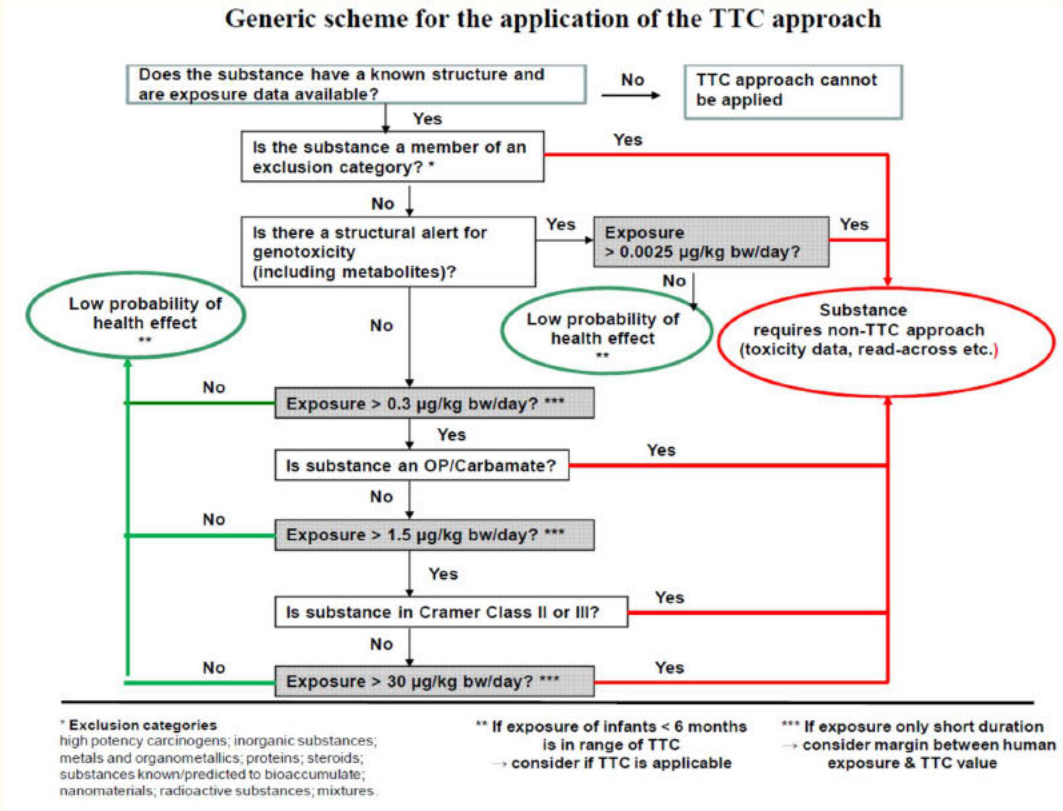
Since the values are only slightly above the threshold value, and it is for the moment only speculative whether gw metabolite would be genotoxic or not, **RMS** proposed to await the studies requested in june 2016, where notifier should unequivocally exclude any doubt as regards the genotoxicity of this compound on the basis of experimental data.

RMS: Please notifier give some indication if such studies are planified, and when these would be submitted.

Notifier: We plan to conduct the appropriate genetic toxicity studies, as soon as we know what compounds are exceeding the groundwater trigger values.

Notifier proposed: "*Based on the QSAR results, considering the high hydrophilicity of IPM1 and the negative genotoxicity of parent lenacil, there is presently no conclusive evidence to suggest that IPM1 is genotoxic when applying a weight-of-evidence approach. Therefore, the threshold of concern (TTC) value of 1.5 µg/person/day or 0.025 µg/kg bw/day (60 kg/person), 0.15 µg/kg bw/day (10 kg/person) and 0.30 µg/kg bw/day (5 kg/person) is deemed to be appropriate for the risk assessment of the polar metabolites and shows no unacceptable health risk to consumers via drinking water consumption.*"

Figure B.6.8.2-1:



Reference: EFSA Scientific Committee; Scientific Opinion on Exploring options for providing advice about possible human health risks based on the concept of Threshold of Toxicological Concern (TTC).

EFSA Journal 2012;10(7):2750 [103 pp.] doi:10.2903/j.efsa.2012.2750.

B.6.8.1.1.6.2 Exposure and risk assessment – comparison with the ADI of lenacil

Notifier proposed:

"Based on the results of the QSAR screens for genotoxicity and carcinogenicity and when applying a weight-of-evidence approach, it can be concluded that based on their structural similarity with lenacil, the polar metabolites or metabolite fractions are not expected to reveal a more severe toxicity profile and to be toxicologically more relevant than the parent active substance which is classified as a category 2 carcinogen under the CLP (Carc. 2; H351). Since all metabolites are closely related to lenacil from the structural point of view, did not reveal an alert for endocrine disrupting properties in the QSAR screen for estrogen receptor binding and considering the logPow values of these

metabolites which are all comparable to or even lower than the logPow of lenacil, a comparable toxicity profile to lenacil can be assumed. As a result, it is justified to perform a strictly human health-based risk assessment and to consider for this purpose the reference values as derived for lenacil. For the purpose of a strictly human health-based exposure and risk assessment, the maximum annual average concentrations of polar fractions M1, M2 and M3 have been compared with the ADI of 0.12 mg/kg bw/day as derived for lenacil in the context of the plant protection dossier.

The theoretical ingestion by a person drinking 2 liters of water per day would be 0.512, 1.038 and 0.546 µg for M1, M2 and M3 respectively, corresponding to 0.0085, 0.0173 and 0.0091 µg/kg bw/d for M1, M2 and M3 (assuming a default weight of 60 kg/person). Compared to the ADI of 0.12 mg/kg bw/d, the theoretical ingestion of M1, M2 and M3 via the groundwater would represent 0.007, 0.014 and 0.008 % of the ADI.

Based on these results it is not anticipated that either of the polar fractions M1, M2 and M3 or a combination of these will represent an unacceptable health risk for consumers via drinking water consumption."

RMS prefers to use again the default values proposed in the latest opinion of EFSA.

Table B.6.8.2.3: Overview of the exposure and % of ADI for the 3 different groundwater fractions of Lenacil based upon leachate study)

	GW level	ADI	Exposure					
	PEC_{gw}		Adult		Toddler		Infant	
Metabolite	**µg/L**	**µg/kg bw/d**	**µg/kg bw/d[a]**	**% of ADI**	**µg/kg bw/d[b]**	**% of ADI**	**µg/kg bw/d[c]**	**% of ADI**
M1	0.256	400	0.00853	0.0021%	0.02560	0.0064%	0.03840	0.0096%
M2	0.519	400	0.01730	0.0043%	0.05190	0.0130%	0.07785	0.0195%
M3	0.273	400	0.00910	0.0023%	0.02730	0.0068%	0.04095	0.0102%
Total	*1.048*	*400*	*0.03493*	*0.0087%*	*0.10480*	*0.0262%*	*0.15720*	*0.0393%*
Average*	0.170	400	0.00567	0.0014%	0.01700	0.0043%	0.02550	0.0064%
Average**	0.035	400	0.00117	0.0003%	**0.00350**	0.0009%	**0.00525**	0.0013%

TTC "genotoxicological" threshold is 0.0025 µg/kg b.w./d

Exposure (PECgw, µg/L) based on average in a lysimeter, calculated from the total maximal concentration for the 3 fractions /6 metabolites (*)

or /30 metabolites (**), assuming consumption of [a]2L, [b]1L, or [c]0.75L of contaminated groundwater per day by a person of [a]60kg, [b]10kg, or [c]5kg bodyweight.

It is obvious that, even taking into account the maximal value of 1.048 µg/L for the sum of M1+M2+M3, and estimating the intake for respectively adults, toddlers or bottle-fed infants respectively, that the obtained estimates amount 0.03, 0.10 or 0.16 µg/kg b.w./d, thus below the threshold value of 0.3 µg/kg b.w./d, and in any case far below the established ADI of the parent compound, taken as a surrogate value for its metabolites (maximally 0.04%).

In conclusion, if the notifier could exclude the genotoxicological hazard of metabolite IPM1, the refined exposure assessment as described below would be reconforting as regards the risk for the consumers.

General conclusion.

Notifier was requested to investigate the intrinsic characteristics of the polar metabolites of lenacil, lately classified Carc. Cat. 2 by ECHA.

(i) In the EFSA conclusion, it was noted that Lenacil and the major soil metabolites IN-KF313 and IN-KE121 were not observed in any leachate during the four years of the study.

(ii) On the contrary, and as evaluated before, and rediscussed in this DRAR, lysimeter studies indicated that the polar metabolites, M1, M2 and M3, corresponding with amongst others, polar metabolites, including IPM1 and PM1-PM5 could be present above 0.1 µg/L, but below 0.75 µg/L. The structural characteristics could only be revealed for IPM1, while there was a characterisation of the polar metabolites PM1-PM5, with a proposal of the tentative structure.

(iii) A final direct demonstration of non-relevance of these metabolites as regards potential carcinogenic properties was not provided.

(iv) However, notifier did submit a first circumstantial indication, by assessing the genotoxic, carcinogenic and endocrine potential *via* various QSAR-models, which is considered by RMS the most reasonable approach possible for metabolites present at low level, precluding even straightforward structural elucidation overall.

These models indicate that overall, no major concern would exist with these metabolites, with one exception: IPM1. A further attempt to identify the polar metabolites would be necessary, including a final conclusion on the basis of other QSAR models for these polar metabolites. Although unlikely, RMS cannot entirely disregard the possibility of a clastogenic potency of the metabolite IPM1, and it is of note that further data about the clastogenic activity should have been provided during the current AIR-3 evaluation.

Also, a further reassurance in order to dismiss potential ED activity of lenacil itself is considered compulsory. This has been addressed under B.6.8.3.

The reason why RMS considers the present data **package only partly fulfilled** is that the IPM1 structure gives a 'mixed' predicted outcome for clastogenicity.

However, the anticipated PEC_{gw} for this molecule is still unclear; taking into account that this molecule will be only part of the global M1+M2+M3 fraction, it is far from clear if this molecule would appear at levels ≥ 0.1µg/L. Taking into account that potentially about 30 metabolites are present in this fraction, this should be further elucidated.
RMS considers the issue to be addressed by the notifier.

Notifier:

(i) These results have not changed. There is not a new 4-year lysimeter study available. Thus, the notifier confirms that Lenacil and the major soil metabolites IN-KF313 and IN-KE121 were not observed in any leachate during the 4 years of the lysimeter study by Schnöder (2004). A new microlysimeter study was performed by Hein (2016) which comprised leaching with soil columns of 28 cm and a duration of 135 days. This study was performed to generate polar metabolites for identification purposes. It cannot be used for quantitative determination of the individual compounds in the leachate. However, the qualitative results for the representative sugar beet soil (2.7% organic carbon, 48% sand and 34% silt)

were similar to the results from the outdoor lysimeter study (1.3% organic carbon, 76.4% sand) with no residues of Lenacil, IN-KF313 or IN-KE121 found at any lower depth to raise concern for leaching.

(ii) The study on the structural elucidation of Lenacil metabolites is on-going. Preliminary non-audited data suggests 22 potential structures. Data from chromatograms of the non-purified leachate and the purified fractions A and B are available in a summary by Schnitzler (2018).

(iii) As one of the criteria for relevance is the exceedance of 0.1 ppb in the groundwater, it is likely that no one metabolite will be in exceedance.

(iv) IPM1 is highly unstable in water and synthesis of this compound has not been possible. Thus, one can be reasonably certain that it would not be a relevant metabolite in groundwater.

Cited references:

- *Sriranjan Kurubaran "Assessment of the toxicological relevance of the groundwater metabolites of lenacil and proposal with a view to a human health-based risk assessment", Position Paper: DuPont de Nemours (Deutschland) GmbH (2015).*
- *Tier G, Serex T "A non-testing approach to evaluate the relevance of specific groundwater metabolites of lenacil" Position paper: E. I. du Pont de Nemours and Company Wilmington, Delaware 19898 U.S.A. (2014)*

Following stement of notifier is reproduced below:

"*In soil, under aerobic conditions, lenacil has been shown to form the identified polar metabolite IPM1 (Dixon and Alderman, 2011, DuPont Report No. 8224956 in Lenacil EU Dossier, Document M-CA, Section 7, DuPont-43894 EU) and is proposed to form five further polar soil metabolites PM1-PM5 (Goodyear, 2012, Position Paper in Lenacil EU Dossier, Document M-CA, Section 7, DuPont-43894 EU). The polar metabolites are likely to be fragments of the parent molecule resulting from opening of the cyclopentapyrimidine ring and/or low molecular weight fragments incorporated into natural products.*

The identified metabolites of lenacil in soil arise from carbon oxidation in the cyclopentyl and cyclohexyl rings. There are no structural features about these metabolites, which present any concerns due to differences compared to the parent molecule.

The metabolites identified in the rat metabolism study (HLR 62-94 cited in Point CA 5.1.1) with lenacil were formed by hydroxylation of the cyclopentyl and cyclohexyl rings to give simple mono- and/or dihydroxylated compounds that are readily excretable based on polarity considerations. The precise position of the hydroxyl group in the cyclopentyl ring was not ascertained. The rat was able to enzymatically hydroxylate these metabolites in the cyclohexyl ring to greater enhance the hydrophilicity and, thus, enhance the elimination of the metabolites. The polar metabolites PM1-PM5 including IPM1 are related to the hydroxylated metabolites observed in the rat metabolism study with lenacil. In biological systems alcohols and ketones are often in equilibrium since their formation is reversible in the presence of oxido-reductase enzyme systems. It is known and well accepted that reduction of cycloalkylketones is a common process in the rat and it can be expected that these metabolites would be readily metabolized to the alcohols and excreted.

In fact, it is very likely that ketones are formed as minor metabolites/intermediates in the rat metabolism study with lenacil but were not detected as significant components in excreta due to their subsequent reduction to the observed mono-/ dihydroxylated alcohol structures. For these reasons, it can be concluded that the ketone metabolites would be readily excreted by mammals and would therefore not present any toxicity not previously observed for the parent active substance. Taking into account also polarity/lipophilicity considerations the polar metabolites are therefore not expected to be of higher toxicity due to their structural similarity to the parent active substance.

In the available lysimeter study with (^{14}C)-lenacil, neither the parent nor IN-KF313 were detected in the leachate at any time during the four-year study (Lenacil EU Dossier, Document M-CA, Section 7, DuPont-43894 EU). The soil metabolite IN-KE121 was also not present in the leachate. However, some radioactive components were detected in the leachate fractions M1, M2 and M3 which were of a generally polar nature. Although the composition of leachate fractions M1, M2 and M3 could not be identified at that time, the available information suggested that these polar metabolites are likely to be polar fragments of the parent molecule resulting from opening of the cyclopentapyrimidine ring and/or low molecular weight fragments incorporated into natural products. As these polar fragments are chemically unstable they are likely to further degrade in soil resulting in numerous low molecular weight metabolites. This was confirmed by the very high level of CO_2 formed in the soil degradation studies, reaching up to 54% by day 30 and up to 77% by day 120 (see Lenacil EU Dossier, Document M-CA, Section 7, DuPont-43894 EU). To support the conclusion that the polar residue fractions consist of several individual metabolites a microlysimeter study was recently conducted (see Lenacil EU Dossier, Document M-CA, Section 7, DuPont-43894 EU). The results of the microlysimeter study indicates that the fractions M1, M2 and M3 consist of a high number of polar individual compounds, up to 33 sub-fractions were separated. Furthermore, one fraction could possibly be related to IPM1.

One of the polar structures (IPM1) has been identified and accepted by EFSA and the Member States in the EFSA Conclusion (ESFA, 2013[2]). The proposed structures of the five polar metabolites (PM1, PM2, PM3, PM4 and PM5) could not be elucidated and confirmed yet. However, PM1 and PM2 are predicted to be 1-cyclohexylurea (CAS 698-90-8) and glutaric acid (CAS 110-94-1), respectively.

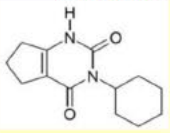
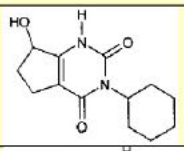
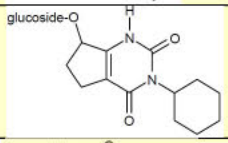
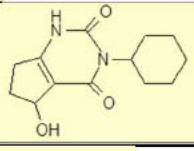
In the expert statement on the assessment of the toxicological relevance of the groundwater metabolites of lenacil (Kurubaran, S., 2016 summarised in point CA 5.8.1), the potential endocrine disrupting (ED) properties of lenacil and its polar groundwater metabolites as well as the carcinogenicity properties of the polar groundwater metabolites of lenacil were investigated in a first step using the OECD toolbox, considering the following profilers: estrogen receptor (ER) binding, rtER Expert System ver. 1 – USEPA, carcinogenicity (genotoxic and non-genotoxic) alerts by ISS and oncologic primary classification.

The QSAR system used did not reveal structural alerts regarding ED properties of both lenacil and its groundwater metabolites. With respect to the carcinogenicity potential of the groundwater metabolites, all polar metabolites (PM1-PM5) except IPM1 were found to have no structural alerts for either genotoxic or nongenotoxic carcinogenicity. Due to the presence of an alpha, beta-unsaturated carbonyl function, IPM1 revealed a structural alert for genotoxic carcinogenicity. However, the genotoxic potential of the identified polar metabolite IPM1 has been addressed in depth in another previously submitted position paper and it was concluded to have low potential for genotoxicity (Tier, G.T. and Serex, T.L., 2014). Based on the QSAR results, considering the high hydrophilicity of IPM1 and the negative genotoxicity of parent lenacil, there is presently no conclusive evidence to suggest that IPM1 is a genotoxic carcinogen when applying a weight-of-evidence approach.

Overall, when applying a weight-of-the-evidence approach taking into account the QSAR results obtained for polar metabolites PM1-PM5 and based on polarity/lipophilicity considerations, it can be concluded that based on their structural similarity to lenacil, the groundwater metabolites are not expected to be of higher toxicity as compared to the parent active substance."

B.6.8.1.2 Other metabolites

The compounds shown below were found in one or more studies involving the metabolism of lenacil. The parent compound structure of lenacil (DPX-B0634) is shown first as the reference.

Code Number (Synonyms) Found in…	Description (IUPAC, common name, CAS RN)	Structure
Lenacil DPX-B0634	**Chemical name (IUPAC):** 3-cyclohexyl-1,5,6,7-tetrahydrocyclopentapyrimidine-2,4(3H)-dione **Common name:** Lenacil **CAS number**: 2164-08-1	
IN-KC943 Plant (sugar beet)	**Chemical name (IUPAC):** 3-cyclohexyl-6, 7-di hydro-7-hydroxy-1 H-cyclopentapyrimidme-2,4(3H,5H)-dione **CAS number**: Not available	
IN-KC943-glucoside Plant (sugar beet)	**CAS number**: Not available	
IN-KQ961 Water (aquatic photolysis at alkaline pH)	**CAS number**: Not available	

RMS observes that all these metabolites have no particularly concerning reactive moieties that are not present in the parent compound itself.

Both IN-KC943 and IN-KQ961 are hydroxylated derivatives of lenacil at the cyclopentene ring, thereby enhancing detoxicifation and rapid excretion. The glycosylated derivative of IN-KC943 likely represents the product of a even stronger detoxification step, which however can reform the aglycone. In addition, it has been demonstrated that most of these hydroxylated metabolites are part of the rat degradation pathway, and are thus in part covered toxicologically.

Overall, RMS accepts that the structures are quite comparable to the parent compound, and are unlikely to be of higher toxicity than lenacil itself.

B.6.8.2 Supplementary studies on the active substance

B.6.8.2.1 Lenacil technical Investigation into potential effects on thyroid function after 20 weeks of treatment in female Han Wistar rats using the "perchlorate discharge test" ([redacted] 2004) - DuPont Report No.: ACD 060/033946

REPORT AMENDMENT n°1 ([redacted] 2007) - DuPont Report No.: ACD 060/033946

Guidelines: not stated in the study report

The thyroid function study in ♀Han Wistar rats was originally submitted under EU Rev8 Point IIA 5.8.2 and has been conducted with lenacil technical. The guidelines according which the study was performed were not reported. A review of this study indicates that no EU or OECD guidelines are available for this non-standard special investigation. A first assessment of the thyroid histopathology was already performed as extention of a 90d-rat subchronic test (see B.6.3.2.1.2), ([redacted] 2007).

GLP status: yes

Materials and Methods

2 groups of 18 ♀rats received Lenacil technical (Batch No. 141712003, purity 98.6%) by the dietary route at dosages of 250 or 50000 ppm over an entire period of 20 weeks. A similarly constituted negative (untreated) control was included. The positive control received Propylthiouracil (PTU, Batch No. 32K2526, purity 99%), an inhibitor of iodide organification (and a direct thyrotropic agens), at a dosage of 200 mg/kg b.w./d by gavage for 2 weeks (weeks 19 and 20).

Test principle. The efficiency of the thyroid iodide organification mechanism can be monitored by the perchlorate discharge test. Perchlorate is a competitive inhibitor of thyroidal iodide transport (competes with I^- for the sodium/iodide symporter or NIS), and if free iodide is concentrated within the thyroid cells following perchlorate administration, there is a diffusional discharge of iodide . In case of direct antithyroid effect, free iodide will diffuse out of the thyroid, and subsequently in the blood stream.

During the study, clinical condition, detailed physical observation, bodyweight, food consumption, blood chemistry, organ weight and macropathology investigations were undertaken in addition to the terminal metabolic investigations of the perchlorate discharge test. The accuracy of the test formulations was confirmed by periodic chemical analysis of the diets prepared for administration.

Deviations from protocol:

1. In order to obtain a suitable formulation for administration of the Propythiouracil a modified method to that given in the protocol was used;
2. Statistical analysis of organ weight data was performed.

Notifier clarified ([redacted] 2007) that the results of the predose rT_3, T_3, T_4 and TSH analyses were considered unreliable as the original raw data has not been retained.
The results of the Week 19 T_3 analysis were considered unreliable as the data has been reprocessed without documented justification. These data are therefore removed from this report and are not used for interpretation purposes. The absence of the Week 19 T_3 data is considered not to affect the scientific integrity of this study. Thyroid hormone estimations were included to investigate whether the data for the Lenacil treated animals was more similar to untreated controls or propylthiouracil treated animals. The absence of T_3 data in Week 19 does not preclude this assessment as valid data are still available for rT_3, T_4 and TSH.
Taking this new element into consideration, **RMS** relies also on the new study of [redacted] (2009), which did however not cover the high dose in the current study.

The study is accepted.

Findings

Lenacil treated rats:

There were no unscheduled deaths.

Clinical signs: a higher incidence of hairloss, poor grooming and brown stained tails was recorded at top dose.

Body weight: mean body weight gain was marginally lower at top dose without attaining statistical significance.

Food intake was unaffected.

Blood chemistry:

T_4 level was lower in the animals given either 250 or 50000 ppm lenacil when compared to that of controls in week 10 and were then higher in week 19 than in week 10.

~~T_3 and~~ TSH values were similar to those of controls throughout the study. Lower rT_3 values seen for rats receiving 250 or 50000ppm lenacil during week 19.

Notifier: "*The lower rT_3 values seen for rats receiving 250 or 50000 ppm lenacil during week 19 are not considered to be toxicologically significant since rT_3 is biologically inactive. No biological importance attaches to this finding. There was no disruption of rT_3 occurred following administration of the positive control.*"

T_3 and T_4 levels:

At both 250 ppm and 50000 ppm, mean T_4 was statistically lower in week 10. This change was not accompanied by lower T_3 nor rise in TSH values and was no longer evident in week 19.

Thyroid weights: Mean thyroid weight was increased.

^{125}Iodide uptake: There was no clear reduction in the ability of the thyroid to take up and accumulate ^{125}Iodide.

^{125}Iodide displacement: The ability of thyroid peroxidases to convert the ^{125}Iodide to organic compounds was unaffected by treatment.

Propylthiouracil treated rats

Propylthiouracil is a compound that exerts a direct toxic effect on the thyroid by inhibition of the thyroidal peroxidase enzymes and is used here as the positive control.

Clinical signs: Rats had salivation with paddling of forepaws. Irritable behaviour was noted in rats during the treatment periods of weeks 19-20.

Body weight and food intake: was not affected.

Typical and statistically significant differences from control rats were as follows:

T_3 and T_4 levels: There was a large reduction in circulating T_3 and T_4 levels (attributable to the direct effect of PTU on the thyroid leading to decreased production of T_3 and T_4), accompanied by marked elevation of mean TSH levels (due to the resulting negative feedback).

Thyroid weights: A large increase in mean thyroid weight was noted, consistent with TSH-mediated hypertrophy.

^{125}Iodide uptake: The ability of the thyroid to take up and accumulate ^{125}Iodide was reduced.

^{125}Iodide displacement: About 80% of thyroid radioactivity was displaced by perchlorate, when PTU/saline treated rats were compared with PTU/perchlorate treated animals. The large amount of free ^{125}Iodide present in the thyroids of propylthiouracil treated animals is a consequence of the inhibition by PTU of the thyroid peroxidases that would normally convert the ^{125}Iodide to organic compounds. This is in contrast to the control rats, were little free ^{125}Iodide was present.

Thyroid: blood concentration ratio:

The reduced ability of the thyroid to take up and metabolise ^{125}Iodide was further demonstrated by the much lower thyroid: blood concentrations ratio in propylthiouracil treated animals. Lenacil did not disrupt iodide organification in the thyroid. The slightly and non-statistically significant lowered T:B in the lenacil dose-groups when compared to the control was not deemed severe enough to be considered evidence of functional loss of uptake/organification, since in addition, absolute values are unchanged and T_3 hormone levels remain unmodified.

RMS accepts notifier's consideration that lenacil technical at doses up to 50000 ppm did not affect the ability of the thyroid to take-up and organify ^{125}Iodine in rats. Therefore, lenacil is not an inhibitor of deiodinase or peroxidase which converts T_4 to T_3. Lenacil is concluded not to be not a directly acting thyroid toxin. The concurrently used positive control PTU was shown to cause a significant reduction in T_3 and T_4 and a marked increase in TSH accompanied by a thyroid weight increase which is consistent with a TSH-mediated hypertrophy.

RMS: The effects of lenacil on thyroid function can be summarised as follows: slight reduction of T_4 and rT_3 while TSH is not altered, at high doses in the ♀. From the ADME studies it appeared that radioactivity was identified in the thyroid. Lenacil does not to act through deiodinase or peroxidase inhibition. In ♀, there was a treatment-related increase in the incidence and severity of Schmorl's positive staining and a slight increase in the severity of this finding in ♂ at 50000 ppm. At the end of the recovery period, the incidence and severity of staining was higher than controls in ♀at top dose and in ♂ the severity was marginally higher than controls. Thyroid hypertrophy was reported.

Changes in serum concentrations of thyroid hormone can be caused by chemicals that inhibit thyroid hormone synthesis, release, and transport, and by chemicals that increase metabolism of various thyroid hormones (*e.g.* deiodinases, UDPGTs). In the case of lenacil, no sufficient information is provided for interpreting changes in hormone levels in term of mechanisms of toxicant action or potential adverse effects, but based. The reason for the observation of black thyroids is not clear.

The notifier was of the opinion that the there is a profound difference in the thyroid homeostasis between rats and humans and rats demonstrate a far higher turnover in the thyroid than humans. In particular, due to differences in protein binding (TBG -thyroid binding globulin-is lacking in rats), rats are more susceptible towards effects on plasma T_3/T_4 levels than humans. For this reason, notifier considered thyroid effects in rats may be of questionable relevance for humans.

RMS considers the black thyroids (reported in the guideline 90d rat study) toxicologically relevant. Indeed, the considerations of species-specific behaviour of thyroid hormone transport (presence of globulin as a human-specific carrier not present in the rats) may be invoked in case of thyroid tumours, and subsequently as a rebuttal to disregard the finding for classification issues, but not necessarily for all toxicological observation reflecting a potential adverse effect.

Therefore, RMS considers that these effects should be taken into account for the setting of NOAELs (being < 250 ppm equivalent to 21 mg/kg b.w./d in this study).

This mechanistic study NOAEL<21 mg/kg b.w./d does not compromise the most relevant subchronic NOAEL, which was established on the basis of a more robust 90d rat study (NOAEL=41 mg/kg b.w./d, on the basis of ↑Schmorl's$^+$ pigment in thyroid cells), conducted at intermediate doses of 500 ppm and 5000 ppm, additionally to the low-dose (250 ppm) and top-dose (50000 ppm) of the current study.

Conclusion

There was no evidence to suggest that Lenacil technical at dosages of up to 50000 ppm was affecting the ability of the thyroid to take-up and organify ^{125}Iodide. Measurements of T_3 made during the study also indicate that the test substance is not acting as an inhibitor of the deiodinase which convert T_4 into T_3, but a definite MoA remains undetermined, and notifier was invited to further explain the adverse thyroid findings. Notifier claimed to have generated preliminary data and will share details as soon as the report is available. Even with the submission of a new mechanistic study ([redacted] 2019) no clear explanation is provided.

Table B.6.8.2.1-1: Potential effects of lenacil on thyroid, comparison with propylthiouracil ([redacted] 2004)

Endpoints/dose		**Control Saline**	**lenacil**		***Propylthiouracil***
Dose (ppm)		**0**	**250**	**50000**	
Achieved dose mg/kg bw/d		**0**	**21.2**	**4421.1**	***200***
Clinical signs:					
Irritable		0	0	0	*5/18*
Hairloss	head	1	0	**5**	*10*
	dorsal body surface	4	**9**	**11**	*13*
Tail staining		0	0	7	*0*
Body weight :	Week 19			↓3%	
Bw gain	Week 0-19			↓8%	*↑3%*
Food consumption			↑2%	↑2%	*↑1%*
Blood chemistry: before treatment[§]					
~~rT_3~~	~~nmol/L~~	~~0.29~~	~~0.27~~	~~0.29~~	~~*0.27*~~
~~T_3~~	~~total nmol/L~~	~~1.18~~	~~1.12~~	~~1.01~~	~~*1.20*~~
~~T_4~~	~~total nmol/L~~	~~33~~	~~31~~	~~27~~	~~*33*~~
~~TSH~~	~~ng/mL~~	~~4.0~~	~~5.2~~	~~4.3~~	~~*4.1*~~
Blood chemistry: week 10					
rT_3	nmol/L	0.17	0.16 (↓6%)	0.16 (↓6%)	*0.17*
T_3	total nmol/L	1.03	0.97	1.04	*1.17*
T_4	total nmol/L	32	**21* (↓34%)**	**20* (↓38%)**	*31*
TSH	ng/mL	5.2	5.0	**5.8 (↑12%)**	*5.0*
Blood chemistry: week 19[§]					
rT_3	nmol/L	0.23	**0.19* (↓17%)**	**0.19* (↓17%)**	*0.27*
~~T_3 total~~	~~nmol/L~~	~~1.34~~	~~1.19 (↓11%)~~	~~1.22 (↓9%)~~	~~*0.87* (↓)*~~
T_4 total	nmol/L	28	**23 (↓18%)**	**25 (↓11%)**	*12*(↓)*
TSH	ng/mL	6.6	5.7 (↓14%)	6.0 (↓9%)	*27.5*(↑)*
Macroscopy of thyroid					
Enlarged		0	0	0	*6*
dark		0	0	**6**	*6*
Thyroid weight		0.0129	0.0159 (↑23%)	**0.0159* (↑23%)**	*0.0599* (↑364%)*
Radioactivity (total % dose)					
	in whole blood	7.21	7.48	7.72	*6.37 (↓12%)*
	in thyroid	8.01	8.55	9.15	*8.80*
Thyroid/blood radioactivity ratio		1522	1393 (↓8%)	1257 (↓17%)	*432* (↓72%)*

* Statistically significantly different from control.

[1]: the measurements were not relied upon

B.6.8.2.2 – Summary of additional studies on the active substance lenacil

Two additional studies were performed to clarify the effects of lenacil on thyroid.

From the first 90d-study (**B.6.3.2.1.2 -** ACD 055/024499) it is concluded that oral administration of lenacil at a concentration of 50000 ppm to Han Wistar rats via the diet caused an increase in the incidence and severity of Schmorl's-positive staining in ♀ and a slight increase in the severity of this finding in ♂. In view of the nature of the staining reaction applied in this highly specific study, it was not possible to establish evidence for any significant recovery after four weeks respite from treatment. RMS proposed a NOAEL of 500 ppm = taking into account the slightly increased incidence of staining of lipofuscin in the follicular epithelium of thyroids of ♀ at 5000 ppm. Black thyroid is rare and pigment accumulation in normal tissue could occur by inhibition of thyroid peroxidase.

A NOEL was proposed by the notifier at 5000 ppm. Notifier: *"The notifier agrees that whilst effects of lenacil at 5000 ppm were possible in terms of lipofuscin staining, there are no findings in the study to support the postulated causes of minor thyroid changes. In the absence of any such evidence, the slight changes noted at 5000 ppm were not adverse, and thus, the NOAEL of this specialised study is set at 5000 ppm."*

The results of the second mechanistic study (**B.6.8.2.1 -** ACD 060/033946 cited) provided no evidence that lenacil technical at dosages of up to 50000 ppm was affecting the ability of the thyroid to take-up and organify ^{125}Iodide. Measurements of T_3 made during the study also indicate that the test substance is not acting as an inhibitor of the deiodinase which converts T_4 into T_3.

Changes in serum concentrations of thyroid hormone can be caused by chemicals that inhibit thyroid hormone synthesis, release, and transport, and by chemicals that increase metabolism of various thyroid hormones (*e.g.* deiodinases, UDPGT's). In the case of lenacil, no sufficient information is provided for interpreting changes in hormone levels in term of mechanisms of toxicant action or potential adverse effects. The cause of the black thyroids is not clear.

In table B.6.8.2-1, the special thyroid study is summarised:

Type of test, test species, doses *(ppm)* - mg/kg b.w./d	**Batch n°, purity (%)**	**NOAEL (mg/kg b.w./d)**	**LOAEL, critical effect (mg/kg b.w./d)**	**Reference**
20-weeks oral, diet, ♀Wistar rat, *(0, 250, 50000 ppm)* ♂: n.a. ♀: 0, 21, 4421 mg/kg bw/d	Batch No. 141712003, purity 98.6%	<250 ppm **<21 mg/kg bw/d**	50000 ppm No effects on ability of thyroid to take-up and organify iodide; ↑alopecia, ↓T_4 and rT_3, Top-dose: ↑thyroid weight	[redacted] 2004 [redacted] 2007

B.6.8.3 Endocrine disrupting properties

- During renewal, notifier brought in experimental studies (**B.6.8.3.1)** in order to put regulators in a position to formulate a reasoned opinion on the basis of facts rather than on QSAR-models (*in-silico* approaches – see B.6.8.1.1.4).

- Next, a number of articles form the open scientific literature were reviewed (**B.6.8.3.2**) in order to complete the findings evaluated under B.6.8.3.1.

- Notifier was also requested to compile the existing data (**B.6.8.3.3**), taking into account the updated Appendix E of the finalised Guidance Document https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2018.5311
  See under Appendix E – Excel template for reporting the available information relevant for ED assessment. It is based upon the template in the zip file 'EDGD_Appendix-E.zip'

  The rationale of the notifier is found in the position paper:
  Position paper of notifier FMC: lenacil (B0634) technical: assessment of experimental data to characterise evidence of endocrine disrupting potential" (Wohlman *et al,* 2019). (Report: FMC Corporation - FMC Agricultural Solutions Stine Research Center Newark, Delaware 19711 USA, Project Identification Number FMC-51816).

  The text of this document was reproduced in italics, and RMS comments are added where necessary.
  Please note that the establishment of the NOAEL's are only to be found in the evaluation in the DRAR, at the appropriate places.

- Conclusions are drafted under **B.6.8.3.4**.
  The ED assessment isevaluated and comments or additional tables are brought in by the **RMS**.

B.6.8.3.1 New studies provided by the notifier:

The notifier provided, on request of the **RMS**, a series of assays examining various potential endocrine-modulating or metabolic inducing properties of lenacil, which were submitted in 09.2018 and 04.2019. These studies are:

- **B.6.8.3.1.1** Lenacil (DPX-B0634) technical: oestrogen receptor binding assay using rat uterine cytosol (ER-RUC) (Nabb D.L., 2018a);

- **B.6.8.3.1.2** Evaluation of the oestrogenic agonist and antagonist activity of lenacil (DPX-B0634) technical using the stably transfected human oestrogen receptor-α transactivation assay (hERα-HeLa-9903 cell line) (Rijk J.C.W., 2018a);

- **B.6.8.3.1.3** Lenacil (DPX-B0634) technical: Androgen receptor binding assay using rat prostate cytosol (AR-RPC) (Nabb D.L., 2018b);

- **B.6.8.3.1.4** Evaluation of the androgenic agonist and antagonist activity of lenacil (DPX-B0634) technical using the stably transfected human androgen receptor transcriptional activation assay (AR EcoScreenTM) (Rijk J.C.W., 2018b);

- **B.6.8.3.1.5** Lenacil (DPX-B0634) technical: 6-day uterotrophic assay for detecting oestrogenic activity in ovariectomised rats ([redacted] 2018);

- **B.6.8.3.1.6** Lenacil (DPX-B0634) technical : *in vitro* aromatase inhibition using human recombinant microsomes (Rijk, J.C., 2019);

- **B.6.8.3.1.7** Screening Lenacil (DPX-B0634) technical for modulation of steroidogenesis using the human H295R adeno-carcinoma cell line (Verkaart S., 2019);

- **B.6.8.3.1.8** Lenacil (DPX-B0634) technical: thyroid mechanistic 14-day feeding study in rats ([redacted] 2019).

These studies and further specific hormone measuremùents performed in former studies are summarised and avaluated below under B.6.8.3.1.

B.6.8.3.1.1

Lenacil (DPX-B0634) technical: Estrogen receptor binding assay using rat uterine cytosol (ER-RUC) (Nabb D.L., 2018) - DuPont Report No.: 49349

Guidelines:
U.S. EPA, OPPTS 890.1250: Oestrogen receptor binding assay (Rat Uterine Cytosol), Endocrine Disruptor Screening Program Test Guidelines (2009)
GLP: yes (no attest of the competent authority)

Materials and Methods
The ability of Lenacil (DPX-B0634-108, lot number: 047303003, purity 99.33%) Technical (lenacil) to bind to the oestrogen receptors by competing with a [^{3}H]-ligand was determined using Sprague-Dawley ♀ rat (ovariectomised 7 to 10 days prior to being humanely euthanised) uterine cytosol as a source of the receptor.
Lenacil was formulated in DMSO and diluted (20µL to a 0.5 mL final assay volume) to final concentrations ranging from 1×10^{-10} to 1×10^{-3} M, each tested in 3 independent runs.
The competing ligand was [^{3}H]-17β-estradiol (Perkin-Elmer, NET517, lot number: 2236119, radioactive purity > 97%).
Control substances, used to verify test system performance, were as follows: the strong positive control was 17β-oestradiol (Sigma-Aldrich, lot number SLBP6339V, purity 100%), the weak positive control was norethindrone (Sigma-Aldrich, lot number SLBK8397V, purity 100%), the negative control compound was octyltriethoxysilane (Sigma-Aldrich, lot number SHBG5008V, purity 97.5%).
Performance criteria
The following criteria were applied to assess the performance of the competitive binding assays. Increased concentrations of unlabelled 17β-oestradiol displace [3H]-17β-oestradiol from the receptor in a manner consistent with one-site competitive binding. Ligand depletion was no greater than 15%. The parameter values (top, bottom, and slope) for 17β-oestradiol and the concurrent positive control (19 norethindrone) were within the tolerance bounds provided. The solvent control did not alter the sensitivity or reliability of the assay. The negative control substance (octyltriethoxysilane) did not displace more than 25% of the radioligand from the oestrogen receptor on average across all concentrations. The test substance was tested over a concentration range that fully defined the top of the curve (i.e., a range that showed that a top plateau was achieved), and the top is within 25% of either the solvent control or the value for the lowest concentration of the oestradiol standard for that run.
The study is accepted

Findings

Oestrogen receptor saturation binding assay:

Three independent oestrogen receptor saturation binding runs were completed using [^{3}H]-17β-oestradiol as the radioligand and radioinert 17β-oestradiol as the ligand.

The K_d values for each of the three runs were 0.332, 0.330, and 0.403 nM [^{3}H]-17β-oestradiol with an average of 0.355 ± 0.024 nM [^{3}H]-17β-oestradiol.

The B_{max} values for each of the 3 runs were 106.96, 105.3, and 107.74 fmol/100 µg protein with an average of 106.6 ± 7.06 fmol/100 µg protein.

Confidence in these numbers was considered high as the adjusted coefficient of determination (adjusted R^2) was 0.9687, 0.9749, and 0.9721 for each of the respective runs, with small variations between runs. The K_d and B_{max} were within the range provided by the test guideline (**table B.6.8.3.1.1-1**).

Table B.6.8.3.1.1-1 Lenacil: oestrogen receptor binding assay using rat uterine cytosol (Nabb, 2018). Saturation binding assay: K_d and B_{max} values for uterine cytosol.

Parameter	Run S1[a]	Run S2[a]	Run S3[a]	Mean[b]	S.E.[b]
Adjusted R^2 (unweighted)	0.969	0.975	02.972	0.969-0.975	-[c]
B_{max} (nM)	0.074	0.073	0.093	0.08	0.007
B_{max} (fmol/100 µg protein)	106.9	105.3	107.7	106.6	7.06
K_d (nM)	0.332	0.330	0.403	0.355	0.024
Adjusted specific activity on date of run (Ci/mmol)	115.750	115.696	115.607	-[c]	-[c]
Percent NSB of TB	<10	<10	<10	-[c]	-[c]

[a]: N=3; [b]: mean for all 3 runs; [c]: value not determined. R^2 = goodness of fit for curve calculated for specific binding; B_{max}: maximal binding capacity; K_d: dissociation constant; NSB: non-specific binding; TB: total binding.

Oestrogen receptor competitive binding assay:

Three independent oestrogen receptor competitive binding runs (C1 to C3) were completed using [^{3}H]-17β-oestradiol as the radioligand and radioinert 17β-oestradiol as the reference standard, 19-norethindrone as the positive control, octyltriethoxysilane as the negative control, or the test substance lenacil (**table B.6.8.3.1.1-2**).

Table B. 6.8.3.1.1-2 Lenacil: oestrogen receptor binding assay using rat uterine cytosol (Nabb, 2018): Competitive binding assay

Test substance	Log Conc. (M)	run C1[a]		run C2[a]		run C3[a]	
		Mean specific binding (%)	S.D.	Mean specific binding (%)	S.D.	Mean specific binding (%)	S.D.
17β-oestradiol	-7	0.0	0.3	0.0	0.0	0.0	0.1
	-8	10.5	1.6	8.9	0.4	10.4	0.3
	-8.5	27.1	0.3	25.1	0.2	28.3	0.5
	-9	54.4	1.3	50.5	1.1	55.8	1.1
	-9.5	78.0	2.1	78.0	1.5	83.2	1.7
	-10	90.4	1.8	92.8	0.9	96.4	1.5
	-11	97.8	2.8	96.5	5.3	104.0	0.3
Octyltriethoxysilane	-3	109.2	2.0	89.2	2.6	91.9	0.7
	-4	106.8	0.9	96.5	1.1	99.5	1.7
	-5	100.5	3.9	100.5	1.8	103.6	1.8
	-6	100.7	1.2	104.3	5.2	104.8	1.0
	-7	101.6	1.9	99.9	1.0	103.5	3.4
	-8	100.3	0.6	102.3	1.0	104.1	2.5
	-9	100.7	0.5	101.4	0.8	104.2	1.1
	-10	99.4	0.2	100.5	1.0	103.6	1.0
Norethindrone	-4	0.2	0.2	0.5	0.1	0.5	0.1
	-4.5	3.4	0.1	3.8	0.1	4.3	0.1
	-5.5	34.9	0.7	34.5	0.9	36.8	0.4
	-6	61.5	0.8	63.2	1.4	66.8	0.8
	-6.5	84.7	0.6	84.8	1.4	89.4	1.5
	-7	93.6	0.9	96.1	1.0	99.9	0.8
	-7.5	97.9	0.8	99.4	2.1	102.5	1.1
	-8.5	100.7	1.7	101.6	1.5	105.5	1.2
Lenacil technical	-3	202.0[b]	37.2	178.2[b]	9.0	240.7[b]	6.4
	-4	99.6	1.5	101.4	3.0	100.8	2.1
	-5	98.4	7.3	103.1	0.7	104.6	1.4
	-6	102.8	0.7	100.1	0.4	104.0	0.3
	-7	102.0	0.8	103.1	1.0	104.7	1.1
	-8	101.5	1.2	100.9	1.5	103.3	0.9
	-9	118.2	7.6	102.8	1.4	103.3	1.8
	-10	118.7	15.1	102.0	1.0	104.9	1.1

Values are mean ± standard error of the % [^{3}H]-17β-oestradiol bound for each concentration
[a]: N=3; [b]: data were not used due to apparent precipitation after assay incubation.

The logIC_{50} values for 17β-oestradiol for each of the three runs were -8.90, -8.95, and 8.92 logM [^{3}H] 17β oestradiol with an average of -8.92 logM [^{3}H]-17β-oestradiol (**Table B.6.8.3.1.1-3**).

The logIC_{50} values for 19 norethindrone for each of the three runs were -5.77, -5.77, and -5.75 logM [^{3}H]-17β-oestradiol with an average of -5.76 logM [^{3}H]-17β-oestradiol (**Table B.6.8.3.1.1-3**).

As expected, neither a logIC_{50} nor relative binding affinity (RBA) were able to be determined for octyltriethoxysilane, as no competitive binding was observed.

A logIC_{50} was also not determined for the test substance lenacil, since there were no test substance-related effects on the oestrogen receptor binding up to the concentration of 1 mM, which represents the highest concentration required by the test guideline (**Table B.6.8.3.1.1-3**).

Table B.6.8.3.1.1-3 Lenacil: oestrogen receptor binding assay using rat uterine cytosol (Nabb, 2018): competitive binding assay (LogIC_{50} values, relative binding affinity, performance standards, and assay drift).

Competitor	Parameter	Lower limit[a]	Upper limit[a]	Run C1[b]	Run C2[b]	Run C3[b]	Mean[c]	S.E.[c]
Radioinert 17β-oestradiol	Top (%)	94	111	98.46	98.36	104.98	100.6	3.79
	Bottom (%)	-4	1	-1.31	-0.23	-1.13	-0.89	0.58
	Slope	-1.1	-0.7	-0.98	-1.06	-0.99	-1.01	0.04
	Log_e (S_{yx})	NA[d]	2.35	0.44	0.80	-0.01	0.41	0.41
	Log (IC_{50}) (M)	NA	NA	-8.90	-8.95	-8.92	-8.92	0.03
19-norethindrone	Top (%)	90	110	100.47	101.69	105.14	102.43	2.42
	Bottom (%)	-5	1	-2.10	-1.38	-1.24	-1.57	0.46
	Slope	-1.1	-0.7	-0.96	-0.99	-1.01	-0.99	0.025
	Log_e (S_{yx})	NA	2.60	-7.53	0.13	-0.11	-2.50	4.35
	Log (IC_{50}) (M)	NA	NA	-5.77	-5.77	-5.75	-5.76	0.01
Lenacil[e]	Top (%)	NA	NA	118.33	101.86	104.13	NA	NA
	Bottom (%)	NA	NA	100.46	-49115*	98.81	NA	NA
	Slope	NA	NA	-2.78	f	-4.19	NA	NA

[a]: suggested acceptable range according to test guideline; [b]:N = 3; [c]: mean and S.E. for all three runs; [d]: not applicable;
[e]: parameter was outside the performance criteria; [f]; the slope on run 2 was slightly positive, therefore, not applicable.
*: unparametrisable curve (no reaction)

Assay performance (**Table B.6.8.3.1.1-3**):

The performance parameters for radioinert 17β-oestradiol were within the acceptable ranges as specified in the test guideline.

The performance parameters for 19-norethindrone were within the acceptable ranges as specified in the test guideline. All runs were within the suggested performance criteria, and therefore the assay was considered valid.

Confidence in these numbers was considered high due to the small variation between runs. Precipitation was observed in the test substance assay tubes at 10^{-3}M after overnight incubation, consistent with the high values obtained for percent of total ligand bound at 10^{-3}M in all three runs.

Conclusion

Under the conditions of the study, the test substance, lenacil, did not competitively bind to the oestrogen receptor when tested up to a maximum concentration of 1 x 10^{-4} M.

Lenacil is considered a non-inhibitor in the oestrogen receptor binding assay.

B.6.8.3.2

Evaluation of the oestrogenic agonist and antagonist activity of lenacil (DPX-B0634) technical using the stably transfected human oestrogen receptor-α transactivation assay (hERα-HeLa-9903 cell line) (Rijk J.C.W., 2018a) - DuPont Report No.: 49351

Guidelines: OECD TG 455 "Stably transfected Human oestrogen receptor-α transcriptional activation assay for detection of oestrogenic agonist-activity of chemicals" (2016)
Deviations:
*Remarks **RMS***

- *4 ER agonist and 5 ER antagonist experiments were initially performed. However, 2 ER agonist and 3 ER antagonist experiments were rejected by the notifier (luciferase response considered too low or the acceptance criteria were not met). The data of the rejected experiments are not included in the report but are stated to be included in the study file.*
  Since 2 indepedent runs remained and were valid (2 runs give comparable and therefore reproducible results), it is not necessary to conduct a 3rd run, and RMS considers that the rejected runs do not compromise the outcome of the study overall.
- *Summary tables 27 and 29, as well as table 41 of the study report n°49351 mention erroneous values PC/IC_{xx} values and their log transformations were switched; these have been corrected in the summary tables B.6.8.3.1.2-1 ,-2 and -4 of this DRAR).*

GLP: yes (certifying NL authority specified)

Materials and Methods
This study investigated the ability of Lenacil (047303003, purity 99.33%) Technical (lenacil) to either activate (agonism) or block (antagonism) human oestrogen receptor alpha (hERα) signalling, using the human hERα-HeLa-9903 cell line. The hERα-HeLa-9903 cell line is a cell-based reporter assay, expressing human ERα and an ERα inducible luciferase reporter gene resulting in oestrogen-dependent luminescence. For both the ER agonist and ER antagonist assay, two independent experiments were performed.

Solubility analysis was conducted before the two independent experiments in order to identify a suitable maximum concentration of Lenacil for use in the ERTA assay. The final concentrations ranged from 10 pM to 10 µM in the ER agonist assay, and from 100 pM to 10 µM in the ER antagonist assay.

Cytotoxicity was assessed using the MTT assay method. No cytotoxicity (≥20% reduction in cell viability) was observed for the test item when tested up to the maximum concentration (10 µM).

ER agonist assay:
Within each ER agonist experiment, the test item was tested at 7 concentrations (10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM and 10 pM) together with vehicle controls, positive controls, and complete concentration-response curves of the reference items 17ß-oestradiol (E2), 17α-oestradiol, 17α-methyltestosterone and corticosterone (resp. strong, weak, very weak and negative agonist).

Results were expressed as PC_{50}: concentration inducing 50% of the maximum level of the positive control; PC_{10}: concentration inducing 10% of the maximum level of the positive control; EC_{50}: the half maximal effective concentration of a test chemical; RPC_{max}: maximum level of response induced by the compound, expressed as % of the response induced by 1 nM E2.

Dose response curves were fitted for the positive reference items E2 and 17α-oestradiol and the log EC_{50} and Hill slope was determined. The log EC_{50} and Hill slope values obtained for E2 and 17α-oestradiol were within the acceptability criteria. The reference items were correctly classified as positive (E2, 17α-oestradiol and 17α-methyltestosterone) or negative (corticosterone). Within each ER antagonist assay experiment, the reference items tamoxifen and flutamide were included. The individual concentration-response curves of these reference items were determined. Log IC_{50} and log IC_{30} values obtained for the reference items.
Therefore, both ER agonist experiments were considered to be valid.

ER antagonist assay: Within each ER antagonist experiment, the test item was tested at 6 concentrations (10 µM, 1 µM, 100 nM, 10 nM, 1 nM and 100 pM) together with vehicle controls, reference items and complete concentration-response curves of the reference items tamoxifen (positive control) and flutamide (negative control).

Results were expressed as IC_{50} and IC_{30}: concentration inhibiting by 50% or 30% of the maximum of the activity of the reference items; RTA_{max}: maximum level of relative transcription activity induced by the compound, expressed as % of the response.

The log IC_{50} obtained for tamoxifen in experiment 2 was within the acceptance criteria. However, no log IC_{50} was obtained for tamoxifen in the 1st experiment. Notifier clarified that, since the log IC_{30} values for tamoxifen were almost identical between both experiments and the relative transcriptional activity (RTA) values for the positive control for antagonism PC_{ATG} (1 µM OHT) were 37.1% and 33.6%, meeting the met the acceptability criterion (<40.6%) in experiment 1 and 2, respectively, the cells were considered to be sufficiently ER antagonistic responsive in experiment 1.
No log IC_{50} could be determined for flutamide in both experiments, which was in agreement with the acceptance criteria. The reference items were correctly classified as positive (tamoxifen) or negative (flutamide) in the ER antagonist assay.
Therefore, both ER antagonist experiments were considered to be valid.

The study is accepted.

Findings

Solubility test:

Lenacil dissolved at a concentration of 10 mM in 0.1% DMSO and 333-fold dilution of this 10 mM solution in exposure medium resulted in a clear solution. Therefore, this concentration was used as the highest test item concentration in the main experiments (final concentration in the well: 10 µM).

Cytotoxicity test:

Potential cytotoxicity of the test item in hERα-HeLa-9903 cells was determined using the MTT test. Lenacil did not exhibit cytotoxicity when tested up to the highest concentration (10 µM).

ER agonist assay:

Assay acceptability parameters for the positive and negative control were met and therefore, both ER agonist experiments were considered valid.

For the first independent run, mean and s.d. values of relative transcriptional activity data are mentioned for 3 reference items (17α-methyltestosterone, corticosterone, 17α-oestradiol and E2) in **Table B.6.8.3.1.2-1;** corresponding values are mentioned for lenacil**.** The RPC_{max} (maximum level of response) obtained for lenacil was 7.4% (± 2.4%).

Along the same line, the values obtained in the second independent run are given in **Table B.6.8.3.1.2-2** The RPC_{max} obtained for lenacil was 3,2% (± 1.3%).

Since the RPC_{max} values were below 10% in two out of two experiments (7.4% and 3.2%, respectively), lenacil was considered to be negative in the ER agonist assay.

See tables B.6.8.3.1.2-1 and -2 Evaluation of the oestrogenic agonist activity of lenacil (DPX-B0634) technical using the stably transfected human oestrogen receptor-α transactivation assay (hERα-HeLa-9903 cell line) (Rijk J.C.W., 2018)

ER antagonist assay:

Assay acceptability parameters for the positive and negative control were met and therefore, both ER antagonist experiments were considered valid.

For the first independent run, mean and SD values of relative transcriptional activity data are mentioned for 2 reference items (tamoxifen, flutamide) and for lenacil in **table B.6.8.3.1.2-3**. Along the same line, the values obtained in the second independent run are given in table **B.6.8.3.1.2-4**

A log IC_{30} could not be determined for the test item in the two independent experiments. As such, lenacil was considered to be negative in the ER antagonist assay.

See tables B.6.8.3.1.2-3 and -4 Evaluation of the oestrogenic antagonist activity of lenacil (DPX-B0634) technical using the stably transfected human oestrogen receptor-α transactivation assay (hERα-HeLa-9903 cell line) (Rijk J.C.W., 2018)

Conclusions:

Lenacil did neither show oestrogenic agonist nor antagonist activity in the hERα-HeLa-9903 cell line when tested up to a maximum concentration of 10^{-5} M.

Table B.6.8.3.1.2-1 and -2: Oestrogenic agonism of lenacil in the stably transfected human oestrogen receptor-α transactivation assay (Rijk, 2018a):

Experiment 1														
17α-Methyltestosterone			Corticosterone			17α-Oestradiol			17β-Oestradiol			Lenacil		
Log Conc. (M)	Mean (%)	±SD	Log Conc. (M)	Mean (%)	±SD	Log Conc. (M)	Mean (%)	±SD	Log Conc. (M)	Mean (%)	±SD	Log Conc. (M)	Mean (%)	±SD
-5	26.8	3.9	-4	-4.4	0.4	-6	80.8	14.5	-8	98.9	25.2	-5	7.4	2.4
-6	2.3	1.5	-5	-2.1	0.8	-7	75.5	8.9	-9	80.9	15.1	-6	-2.3	1.0
-7	-0.1	0.5	-6	0.0	0.9	-8	68.7	15.8	-10	71.3	1.5	-7	-0.7	0.2
-8	0.6	0.1	-7	0.7	0.6	-9	15.5	1.0	-11	14.7	4.8	-8	-0.7	1.8
-9	0.0	0.5	-8	0.1	0.3	-10	0.8	0.9	-12	0.9	1.5	-9	-1.9	2.1
-10	-0.3	0.4	-9	0.4	0.4	-11	0.4	0.6	-13	1.1	0.6	-10	-2.2	1.4
-11	-0.1	0.5	-10	0.5	1.0	-12	0.8	1.2	-14	-0.4	0.4	-11	-0.9	1.4
RPC_{max} (%)	26.8%			0.7%			80.8%			98.9%			7.4%	
PC_{50} (M)	-			-			4.45×10^{-9}			4.21×10^{-11}			-	
PC_{10} (M)	2.07×10^{-6}			-			4.23×10^{-10}			4.59×10^{-12}			-	
Log PC_{50} (M)	-			-			-8.35			-10.38			-	
Log PC_{10} (M)	-5.68			-			-9.37			-11.34			-	

Experiment 2														
17α-Methyltestosterone			Corticosterone			17α-Oestradiol			17β-Oestradiol			Lenacil		
Log Conc. (M)	Mean (%)	±SD	Log Conc. (M)	Mean (%)	±SD	Log Conc. (M)	Mean (%)	±SD	Log Conc. (M)	Mean (%)	±SD	Log Conc. (M)	Mean (%)	±SD
-5	21.4	5.3	-4	-3.7	0.3	-6	82.9	6.3	-8	102.9	2.9	-5	3.2	1.3
-6	1.8	2.0	-5	-3.1	0.3	-7	80.1	6.9	-9	102.0	0.7	-6	-1.9	0.2
-7	-0.2	0.4	-6	-1.1	0.4	-8	76.7	6.1	-10	76.0	16.4	-7	0.6	1.5
-8	0.2	1.5	-7	-0.1	1.0	-9	14.7	1.7	-11	27.6	10.0	-8	-0.3	1.1
-9	0.2	0.6	-8	-0.1	0.5	-10	3.6	0.2	-12	3.6	0.2	-9	-1.7	1.5
-10	-1.4	1.2	-9	0.3	0.6	-11	0.5	0.2	-13	-0.5	0.5	-10	-2.3	1.0
-11	-2.5	1.1	-10	-0.6	0.4	-12	0.4	1.4	-14	1.1	0.6	-11	-2.8	0.7
RPC_{max} (%)	21.4%			0.3%			82.9%			102.9%			3.2%	
PC_{50} (M)	-			-			3.71×10^{-9}			2.90×10^{-11}			-	
PC_{10} (M)	2.61×10^{-6}			-			3.77×10^{-10}			1.85×10^{-12}			-	
Log PC_{50} (M)	-			-			-8.43			-10.54			-	
Log PC_{10} (M)	-5.58			-			-9.42			-11.73			-	

- = no value obtained

Table B.6.8.3.1.2-3 and -4: Oestrogenic antagonism of lenacil in the stably transfected human oestrogen receptor-α transactivation assay (Rijk, 2018a):

Experiment 1								
Tamoxifen			**Flutamide**			**Lenacil**		
Log Conc. (M)	**Mean (%)**	**±SD**	**Log Conc. (M)**	**Mean (%)**	**±SD**	**Log Conc. (M)**	**Mean (%)**	**±SD**
-5	53.9	4.5	-5	92.5	10.0	-5	107.5	7.5
-6	54.6	5.3	-6	116.4	19.7	-6	109.4	12.9
-7	81.0	15.9	-7	110.6	7.0	-7	106.6	15.2
-8	96.5	3.5	-8	100.9	12.0	-8	103.1	16.9
-9	100.9	8.3	-9	105.4	4.4	-9	104.3	8.3
-10	121.3	8.3	-10	105.3	27.8	-10	107.6	15.4
RTA_{max} (%)	121.3%			116.4%			109.4%	
IC_{50} (M)	-			-			-	
IC_{30} (M)	2.62×10^{-7}			-			-	
Log IC_{50} (M)	-			-			-	
Log IC_{30} (M)	-6.58			-			-	

Experiment 2								
Tamoxifen			**Flutamide**			**Lenacil**		
Log Conc. (M)	**Mean (%)**	**±SD**	**Log Conc. (M)**	**Mean (%)**	**±SD**	**Log Conc. (M)**	**Mean (%)**	**±SD**
-5	36.0	6.6	-5	80.7	15.5	-5	98.9	12.7
-6	39.3	2.7	-6	94.6	2.0	-6	106.4	8.0
-7	96.2	14.1	-7	108.2	6.6	-7	118.7	19.1
-8	137.0	6.9	-8	112.3	13.7	-8	111.0	22.9
-9	106.9	6.2	-9	106.3	6.4	-9	100.0	19.3
-10	122.1	29.5	-10	107.0	9.2	-10	119.1	18.2
RTA_{max} (%)	137.0%			112.3%			119.1%	
IC_{50} (M)	6.48×10^{-7}			-			-	
IC_{30} (M)	2.89×10^{-7}			-			-	
Log IC_{50} (M)	-6.19			-			-	
Log IC_{30} (M)	-6.54			-			-	

- = no value obtained

B.6.8.3.3

Lenacil (DPX-B0634) technical: androgen receptor binding assay using rat prostate cytosol (AR-RPC) (Nabb D.L., 2018b)

DuPont Report No.: 49367

Guidelines: U.S. EPA Health Effects Test Guideline OPPTS 890.1150 (2009)
Deviations: None
GLP: yes (no attest of the competent authority)

Materials and Methods

The ability of Lenacil (DPX-B0634-108, lot number: 047303003, purity 99.33%) Technical (lenacil) to bind to the androgen receptors by competing with a [^{3}H]-ligand was determined using castrated Sprague-Dawley male rat ventral prostates (Charles River, as a source of the receptor. Tissues were collected 22-26 hours after castration at an age of 90 days. Lenacil was formulated in DMSO and diluted (10µL to a 0.3 mL final assay volume) to final concentrations ranging from 1 x 10^{-10} to 1 x 10^{-3} M, each tested in three independent runs. The competing ligand was [^{3}H]-R1881 ('Metribolone', Perkin-Elmer, catalog number: NET590, lot number: 2051473, radioactive purity > 97%). Control substances, used to verify test system performance, were as follows: the strong positive control was R1881 (Sigma-Aldrich, lot number: 055M4614V, purity 99.8%), the weak positive control was dexamethasone (Sigma-Aldrich, lot number BCBM4557V, purity 99.2%).

The study is accepted

Findings

The suitable top concentration of the test substance for use in the assay was initially thought to be 10^{-3} M but after incubations in the assay a very slight turbidity could be observed at the mM concentrations. This appeared to be consistent with protein precipitation rather than compound insolubility. Therefore, the top suitable concentration of the test substance was 10^{-4} M.

A summary of data obtained for R1881, dexamethasone and lenacil for all thre runs at the different concentrations are shown below. All data were within the suggested upper and lower limits for parameters in competitive binding assay curves for the positive standards outlined in the OPPTS 890.1150 guideline and within "in house" historical data from the company performing the assay (Cyprotex US, LLC – USA). Therefore all three runs were considered valid and definitive.

Androgen receptor saturation binding assay:

Three independent androgen receptor saturation binding runs were completed using [^{3}H]-R1881 as the radioligand and radioinert R1881 as the ligand. The K_d values for each of the three runs were 1.216, 0.8506, and 0.7915 nM [^{3}H] R1881 with an average of 0.9527 nM [^{3}H] R1881. The B_{max} values for each of the three runs were 8.2930, 7.5470, and 6.8270 fmol/100 µg protein with an average of 7.5557 fmol/100 µg protein.

Confidence in the assay results was high as the adjusted coefficient of determination (adjusted R^2) was 0.9718, 0.9898, and 0.9809 for each of the respective runs, with small variations between runs (**Table B.6.8.3.1.3-1**).

Table B.6.8.3.1.3-1 Lenacil: androgen receptor binding assay using rat prostate cytosol (Nabb, 2018b): Saturation binding assay (K_d and B_{max} values).

Parameter	Run S1[a]	Run S2[a]	Run S3[a]	Mean[b]	S.E.[b]
Adjusted R^2 (unweighted)	0.9718	0.9898	0.9809	0.9808	0.0090
B_{max} (nM)	0.02530	0.02302	0.02082	0.0230	0.0022
B_{max} (fmol/100 µg protein)	8.2930	7.547	6.8270	7.5557	0.7330
K_d (nM)	1.216	0.8506	0.7915	0.9527	0.2299
Adjusted specific activity on date of run (Ci/mmol)	83.846	83.829	83.752	c	c

[a]: N=3; [b]: mean for all three runs; c: value not determined. R^2 = goodness of fit for curve calculated for specific binding; B_{max}: maximal binding capacity; K_d: dissociation constant.

Androgen receptor competititve binding assay:

Three independent androgen receptor competitive binding runs were completed using [^{3}H]-R1881 as the radioligand and radioinert R1881 as the strong positive control, dexamethasone as the weak positive control, or the test substance (**Table B.6.8.3.1.3-2**).

The logIC_{50} values for radioinert R1881 for each of the three runs were -8.96, -9.01, and -8.99 logM [^{3}H] R1881 with an average of -8.99 logM [^{3}H]-R1881. The logIC_{50} values for dexamethasone for each of the three runs were -4.60, -4.68, and -4.62 logM [^{3}H]-R1881 with an average of -4.63 logM [^{3}H]-R1881.

A logIC_{50} was not determined for the test substance since there were no test substance-related effects on androgen receptor binding up to the concentration of 1 x 10^{-4} M, which represents the highest concentration (**Table B.6.8.3.1.3-3**).

Table B.6.8.3.1.3-2 Lenacil: androgen receptor binding assay using rat prostate cytosol (Nabb, 2018b): Competitive binding assay

		Run C1[a]		Run C2[a]		Run C3[a]	
Competitor	**Log Conc. (M)**	**Mean specific binding (%)**	**S.D.**	**Mean specific binding (%)**	**S.D.**	**Mean specific binding (%)**	**S.D.**
R1881	-6	0.0	0.8	0.0	1.0	0.0	0.6
	-7	1.4	0.5	-0.4	2.0	1.0	0.5
	-8	9.0	1.0	7.6	0.6	8.8	1.4
	-9	53.2	1.2	49.9	1.3	52.5	0.6
	-10	91.3	2.4	93.8	2.1	95.2	3.1
	-11	101.0	2.0	100.7	1.4	102.5	1.5
Dexamethasone	-3	4.2	0.9	3.1	0.5	4.3	0.9
	-4	22.3	1.1	20.4	0.8	21.6	1.3
	-5	72.0	1.1	71.7	1.0	73.4	1.4
	-6	98.3	2.0	115.7	34.2	101.1	3.9
	-7	100.5	3.3	99.8	1.7	104.2	0.4
	-8	102.6	1.2	100.2	2.4	101.6	1.0
	-9	99.4	3.0	100.5	2.0	101.1	1.1
	-10	99.0	1.3	101.3	3.2	100.8	1.3
Lenacil technical	-3	109.1[b]	11.3[b]	131.6[b]	50.1[b]	135.7[b]	23.5[b]
	-4	97.3	0.6	96.0	4.5	100.7	0.3
	-5	99.7	1.8	101.4	1.7	102.2	2.1
	-6	99.8	0.7	101.8	1.1	101.4	2.1
	-7	98.6	4.4	90.1	146.6	99.2	1.0
	-8	100.3	1.9	101.5	1.4	100.5	2.6
	-9	96.8	1.6	101.8	2.1	100.9	0.6
	-10	99.7	1.4	99.8	1.0	99.8	2.1

Values are mean ± standard deviation of the percent [^{3}H]-R1881 bound for each concentration;
[b]: data that was not used due to apparent protein precipitation after assay incubation.

Androgen receptor competititve binding assay performance:

The performance parameters for radioinert R1881 were within the acceptable ranges as specified in the test guideline. The performance parameters for dexamethasone were within the acceptable ranges as specified in the test guideline. All runs were within or very close to the suggested performance criteria, and therefore the assay was considered valid and definitive (**table B.6.8.3.1.3-3**).

Confidence in the assay results was high due to the small variation between runs. Slight precipitation was observed in the test substance assay tubes at 1 × 10^{-3}M after overnight incubation, therefore, the top suitable concentration of the test substance was 1 × 10^{-4}.

Table B.6.8.3.1.3-3 Lenacil: androgen receptor binding assay using rat prostate cytosol (Nabb, 2018b): competitive binding assay ($LogIC_{50}$ values, relative binding affinity, performance standards, and assay drift).

Competitor	Parameter	Lower limit[a]	Upper limit[a]	Run C1[b]	Run C2[b]	Run C3[b]	Mean[c]	S.E.[c]
Radioinert R1881	Top (%)	82	114	101.18	101.55	103.41	102.05	1.195
	Bottom (%)	-2.0	2.0	-0.21	-0.31	0.01	-0.17	0.159
	Slope	-1.2	-0.8	-0.99	-1.08	-1.05	-1.04	0.046
	Log (IC_{50}) (M)	NA[d]	NA	-8.96	-9.01	-8.99	-8.99	0.025
Dexamethasone	Top (%)	87	106	100.74	103.54	102.38	102.22	1.407
	Bottom (%)	-12	12	1.92	4.51	2.72	3.05	1.326
	Slope	-1.4	-0.6	-1.00	-1.22	-1.05	-1.09	0.155
	Log (IC_{50}) (M)	NA	NA	-4.60	-4.68	-4.62	-4.63	0.042
Lenacil[e]	Top (%)	NA	NA	99.057	99.404	100.663	99.71	0.845
	Bottom (%)	NA	NA	97.710	96.021	100.531	98.09	2.279
	Slope	NA	NA	-66.98	-15.51	-57.70	-46.73	27.433
Percent ligand depletion		No ligand displacement response lower than 90.1%						

[a]: suggested acceptable range according to test guideline; [b]: N = 3; [c]: Mean ± SE for all three runs; [d]: not applicable;
[e]: lenacil did not bind to the androgen receptor when tested up to a maximum concentration of 1 ± 10^{-4} M

Conclusions:

Under the conditions of the study, lenacil did not competitively bind to the androgen receptor when tested up to a maximum concentration of 10^{-4} M.

Lenacil is considered a non-inhibitor in the androgen receptor binding assay.

B.6.8.3.4

Evaluation of the androgenic agonist and antagonist activity of lenacil (DPX-B0634) technical using the stably transfected human androgen receptor transcriptional activation assay (AR EcoScreen™) (Rijk J.C.W., 2018b)

DuPont Report No.: 50113

Guidelines: OECD 458 : "Stably transfected human androgen receptor transcriptional activation assay for detection of androgenic agonist and antagonist activity of Cchemicals (29 July 2016). (2016)
Deviations: None
GLP: yes (certifying NL authority specified)

Materials and Methods

Lenacil (DPX-B0634-108, Lot n° 047303003, purity 99.33%) Technical (lenacil) was evaluated for its ability to act as either an agonist or antagonist of the human androgen receptors (hAR) using the AR-EcoScreen™ cell line, a engineered stable transfected CHO-K1 line expressing androgen receptor and AR-inducible luciferase reporter genes.

Preliminary assessments of solubility were conducted in order to identify suitable top concentrations for use in the transcriptional activation assays. Dimethyl sulfoxide (DMSO) was selected as the vehicle for the test item, and was not shown to have a significant effect on the assay. For the test item, two independent experiments were performed for both the AR agonist and AR antagonist assay.

The cytotoxicity of lenacil was determined simultaneously with the AR antagonist assay. Cytotoxicity was determined by evaluating the Renilla luciferase activity by the test items in the assay. All doses that exhibited a reduction in luciferase activity of >20% were excluded for further evaluation.

AR agonist assay:

Within each AR agonist experiment, the test item was tested at seven concentrations (1 µM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM and 1 pM) together with vehicle controls, positive controls, and a complete concentration-response range for the reference items 4,5α dihydrotestosterone (DHT, Bio-connect, lot number 17916, purity > 98.0%), mestanolone (Sigma-Aldrich, lot number SLBN3810V, purity 97%), and di(2-ethylhexyl)phthalate (DEHP, Sigma-Aldrich, lot number SZBB167XV, purity 99.7%). The final testing concentrations of lenacil were 1 pM to 1 µM.

AR antagonist assay:

Within each AR antagonist experiment, the test item was tested at six concentrations (1 µM, 100 nM, 10 nM, 1 nM, 100 pM and 10 pM) together with vehicle controls, positive controls, and complete concentration-response range of the reference items hydroxyflutamide (HF, Sigma-Aldrich, lot number 105M4763V, purity 98.5%), bisphenol A (BPA, Sigma-Aldrich, lot number MKBS0991V, purity 99.9%), and di(2-ethylhexyl)phthalate (DEHP, Sigma-Aldrich, lot number SZBB167XV, purity 99.7%). The final testing concentrations of lenacil were 10 pM to 1 µM.

The study is accepted.

Findings:

Solubility test:

Lenacil dissolved at a concentration of 10 mM in DMSO. A 10-fold dilution of this solution in exposure medium resulted in the precipitation of the test item. A 10-fold dilution of a 1mM solution of the test item in exposure medium resulted in a clear solution. Therefore, this concentration 10^{-4} M was used as the highest test item vehicle solvent concentration in the experiments (final concentration in the well: 1 µM).

Cytotoxicity test:

Cytotoxicity was evaluated by determining the *Renilla* luciferase activity in the AR antagonist assay experiments. No cytotoxicity (>20% reduction in *Renilla* luciferase activity) was observed for lenacil when tested up to a concentration of 1 µM.

AR agonist assay:

Assay acceptability criteria for the positive and negative controls were met and therefore both AR agonist experiments were considered valid.

For the first independent run, mean and SD values of relative transcriptional activity data are mentioned for the reference items (DHT, mestanolone, DEHP) and for lenacil in **table B.6.8.3.1.4-1.** The RPC_{max} (maximum level of response) obtained for lenacil was 0.46% (±0.2%).

Along the same line, the values obtained in the second independent run are given in **table B.6.8.3.1.4-2.**

The RPC_{max} obtained for lenacil was 0.26% (±0.5%).

Table B.6.8.3.1.4-1. Androgenic agonism of lenacil in the stably transfected human androgen receptor transcriptional activation assay(Rijk, 2018b): Experiment 1.

DHT			Mestanolone			DEHP			Lenacil		
Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd
-6	95.6	3.1	-6	88.8	2.5	-5	0.1	0.3	-6	0.46	0.2
-7	101.7	4.7	-7	96.9	3.0	-6	0.0	0.3	-7	0.01	0.4
-8	99.2	2.3	-8	93.4	2.1	-7	0.1	0.5	-8	-0.05	0.2
-9	98.7	3.7	-9	91.6	1.3	-8	0.0	0.6	-9	0.03	0.2
-10	92.0	3.3	-10	86.3	4.2	-9	0.3	0.7	-10	-0.14	0.6
-11	40.3	2.1	-11	50.2	3.3	-10	-0.4	0.1	-11	-0.14	0.5
-12	2.6	0.5	-12	2.4	0.4	-11	-0.5	0.4	-12	-0.20	0.1
RPC_{max}	101.7			96.9			0.25			0.46	
PC_{50} (M)	1.54×10^{-11}			9.89×10^{-12}			-			-	
PC_{10} (M)	1.57×10^{-12}			1.44×10^{-12}			-		-	-	
Log PC_{50} (M)	-10.81			-11.00			-		-	-	
Log PC_{10} (M)	-11.80			-11.84			-		-	-	

Mean and SD Values of Relative Transcriptional Activity Data (in %) of AR Agonist Assay;
- = no value obtained; RPC_{max} = maximum level of response.

Table B.6.8.3.1.4-2. Androgenic agonism of lenacil in the stably transfected human androgen receptor transcriptional activation assay(Rijk, 2018b): Experiment 2.

DHT			Mestanolone			DEHP			Lenacil		
Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd
-6	95.6	3.1	-6	88.8	2.5	-5	0.1	0.3	-6	0.46	0.2
-7	101.7	4.7	-7	96.9	3.0	-6	0.0	0.3	-7	0.01	0.4
-8	99.2	2.3	-8	93.4	2.1	-7	0.1	0.5	-8	-0.05	0.2
-9	98.7	3.7	-9	91.6	1.3	-8	0.0	0.6	-9	0.03	0.2
-10	92.0	3.3	-10	86.3	4.2	-9	0.3	0.7	-10	-0.14	0.6
-11	40.3	2.1	-11	50.2	3.3	-10	-0.4	0.1	-11	-0.14	0.5
-12	2.6	0.5	-12	2.4	0.4	-11	-0.5	0.4	-12	-0.20	0.1
RPC_{max}	101.7			96.9			0.25			0.46	
PC_{50} (M)	1.54×10^{-11}			9.89×10^{-12}			-			-	
PC_{10} (M)	1.57×10^{-12}			1.44×10^{-12}			-		-	-	
Log PC_{50} (M)	-10.81			-11.00			-		-	-	
Log PC_{10} (M)	-11.80			-11.84			-		-	-	

Mean and SD Values of Relative Transcriptional Activity Data (in %) of AR Agonist Assay;
- = no value obtained; RPC_{max} = maximum level of response.

Since the RPC_{max} values were below 10% in two out of two experiments (0.46 and 0.26%, respectively), lenacil was considered to be negative in the AR agonist assay.

AR antagonist assay:
Assay acceptability criterial for the positive and negative controls were met and therefore both AR antagonist experiments were considered valid.

For the first independent run, mean and SD values of relative transcriptional activity data are mentioned for the reference items (HF, bisphenol A, DEHP) and for lenacil in **Table B.6.8.3.1.4-3.** Along the same line, the values obtained in the second independent run are given in **Table B.6.8.3.1.4-4.**

Table B.6.8.3.1.4-3. Androgenic antagonism of lenacil in the stably transfected human androgen receptor transcriptional activation assay(Rijk, 2018b): Experiment 1.

Hydroxyflutamide			Bisphenol A			Bis-(2-ethylhexyl)-phthalate			Lenacil		
Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd
-5	6.1	0.3	-5	16.9	1.6	-5	110.6	3.6	-6	116.5	5.6
-6	4.9	0.5	-6	81.8	4.1	-6	110.5	2.0	-7	112.6	4.3
-7	41.5	2.0	-7	100.9	4.3	-7	108.1	0.7	-8	107.8	3.4
-8	95.9	5.2	-8	105.3	4.4	-8	107.6	2.0	-9	104.3	5.4
-9	96.1	4.1	-9	103.2	3.5	-9	102.3	3.0	-10	102.6	5.3
-10	98.6	2.5	-10	102.9	6.9	-10	103.0	3.0	-11	102.4	6.6
RTA_{max}	98.6			105.3			110.6			116.5	
IC_{50} (M)	6.98×10^{-8}			3.09×10^{-8}			-			-	
IC_{30} (M)	2.99×10^{-8}			1.52×10^{-8}			-		-	-	
Log IC_{50} (M)	-7.16			-5.51			-		-	-	
Log IC_{30} (M)	-7.52			-5.82			-		-	-	

Mean and SD Values of Relative Transcriptional Activity Data (in %) of AR Antagonist Assay;
- = no value obtained; RPC_{max} = maximum level of response.

Table B.6.8.3.1.4-4. Androgenic antagonism of lenacil in the stably transfected human androgen receptor transcriptional activation assay(Rijk, 2018b): Experiment 2.

Hydroxyflutamide			Bisphenol A			Bis-(2-ethylhexyl)-phthalate			Lenacil		
Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd	Log Conc. (M)	Mean	±sd
-5	6.1	0.3	-5	16.9	1.6	-5	110.6	3.6	-6	116.5	5.6
-6	4.9	0.5	-6	81.8	4.1	-6	110.5	2.0	-7	112.6	4.3
-7	41.5	2.0	-7	100.9	4.3	-7	108.1	0.7	-8	107.8	3.4
-8	95.9	5.2	-8	105.3	4.4	-8	107.6	2.0	-9	104.3	5.4
-9	96.1	4.1	-9	103.2	3.5	-9	102.3	3.0	-10	102.6	5.3
-10	98.6	2.5	-10	102.9	6.9	-10	103.0	3.0	-11	102.4	6.6
RTA_{max}	98.6			105.3			110.6			116.5	
IC_{50} (M)	6.98×10^{-8}			3.09×10^{-8}			-			-	
IC_{30} (M)	2.99×10^{-8}			1.52×10^{-8}			-		-	-	
Log IC_{50} (M)	-7.16			-5.51			-		-	-	
Log IC_{30} (M)	-7.52			-5.82			-		-	-	

Mean and SD Values of Relative Transcriptional Activity Data (in %) of AR Antagonist Assay;
- = no value obtained; RPC_{max} = maximum level of response.

A log IC_{30} could not be determined for the test item in the two independent experiments. As such, lenacil was considered to be negative in the ER antagonist assay.

Conclusions:
Lenacil did not show an androgen agonist response in two independent experiments (RPC_{max} ≤10%).
In both androgen antagonist experiments, no log IC_{30} could be determined for the test item.
Under the conditions of this study, the AR agonist and AR antagonist assay experiments were valid and lenacil did not show any androgenic agonist or antagonist activity in a stable transfected CHO-K1 cell line.

B.6.8.3.1.5

Lenacil (DPX-B0634) technical: 5-day uterotrophic assay for detecting estrogenic activity in ovariectomised rats ([redacted] 2018)

DuPont Report No.: 49350

Guidelines: EC Test Method B.54 of Reg (EC) no 440/2008, equivalent to OECD TG 440 (2007)
U.S. EPA Health Effects Test Guidelines OPPTS 890.1600 (2009)

Deviations from the EC test Method:

GLP: yes (no attest of competent authority)

Materials and Methods

Groups of young adult ovariectomised ♀Crl:CD(SD) rats (15 animals/group) received orally 0 (negative control), 500 or 1000 mg/kg b.w./d of lenacil (DPX-B0634-148, lot n° 036402003, purity 99.33%) in 0.5% methylcellulose by gavage for 5 consecutive days. The homogeneity and concentration (including the stability for up to 14d) of lenacil in the dosing formulations were checked by analyses using ultra high performance liquid chromatography with UV detection.

Two separate positive control groups were included. One positive control group was administered 0.1 mg/kg/day of the oestrogen receptor agonist 17α-ethinyl oestradiol (CAS 57-63-6, Sigma-Aldrich, purity ≥ 98%) dissolved in vehicle (corn oil with 1% ethanol), and the second positive control group was administered 2 mg/kg b.w./d of the dopamine (D2) receptor antagonist haloperidol (CAS 52-86-8, Sigma-Aldrich, purity ≥98%) suspended in vehicle (0.5% methylcellulose).

Body weights, food consumption and clinical observations were recorded daily. Vaginal cytology was evaluated daily (d4-d6) to assess the potential of the test substance to induce cytological changes consistent with those observed with the 17α-ethinyl oestradiol positive control.

Approximately 24h after the last dose, animals were sacrificed by exsanguination under isofluorane anaesthesia. Blood was collected by cardiac puncture at the time of necropsy and placed on ice for preparation of serum for hormonal measurements. Serum prolactin concentrations were evaluated, but not quantifiable due to methodological limitations, and were not reported. Gross examinations were performed on all main study animals. Organs that were collected are uterus (including uterine horns and cervix), vagina and ovarian stumps. Relative organ weights (percent of terminal body weight) were calculated. At scheduled euthanasia, uterine weights were collected to assess the ability of the test substance to induce uterine growth. Uterus, vagina, and ovarian stumps were collected and placed in 10% neutral buffered formalin for possible histopathological analysis, but analysis of these tissues was deemed unnecessary by the notifier.

Statistics: body weight (gain, food consumption and efficiency, and organ weight were evaluated using Levene's test for homogeneity and Shapiro Wilk test for normality. If not significant, one way Anova followed by Dunnett's test was performed. If homogeneity and normality tests were significant, data were transformed to achieve normality and variance homogeneity. The order of transforms attempted was log, square root, and rank order. If the log and square root transforms failed, the rank order was used.

The study is accepted

Findings

All animals from all treatment groups survived to scheduled sacrifice. No test substance-related effects on body weight or clinical signs were observed in either dose group in rats administered lenacil. Slight decreases in food consumption was observed in top-dose animals attaining statistical significance compared to control ($p<0.05$) on d2 (-7.7%) and d6 (-4.4%). Cumulated food consumption differences (d1-6) were not significantly diferent from controls. Body weight gains were statistically different from controls on d2 and d3, but not on other days, including when integrated over the whole treatment period.

All animals in all lenacil treatment groups remained in dioestrus for the duration of the study (**table B.6.8.3.1.5-1**). There were no test substance-related effects on uterine weight (**table B.6.8.3.1.5-2**) or gross observations (except that uterine fluid was observed in oestradiol-treated animals– **table B.6.8.3.1.5-3**), indicating proper response of the animals.

There were no clinical signs observed in rats administered 17α-ethinyl oestradiol. Final mean body weight, overall mean body weight gain, overall mean food consumption and overall mean food efficiency were all lower ($p < 0.05$) compared to control. Oestrous stage effects were observed in all 15 animals administered 17α-ethinyl oestradiol with cytological markers indicative of either proestrus or oestrus observed in all rats by test day 4 (**table B.6.8.3.1.5-1**). Absolute uterine wet weight and blotted

wet weight were 265% and 254% of vehicle control, respectively. Relative (to final body weight) uterine wet weight and blotted wet were 288% and 272% of vehicle control, respectively (**table B.6.8.3.1.5-2**). The results with 17α-ethinyl oestradiol are consistent with an oestrogen receptor agonist.

Table B.6.8.3.1.5-1 Lenacil 5d-uterotrophic assay in ovariectomised rats (2018): oestrous cycle stages

Treatment/ Dose	Test days																	
	1			2			3			4			5			6		
	D	E	P	D	E	P	D	E	P	D	E	P	D	E	P	D	E	P
Negative control (0.5% methylcellulose)	*15*	*0*	*0*	*15*	*0*	*0*	*15*	*0*	*0*	*15*	*0*	*0*	*15*	*0*	*0*	*15*	*0*	*0*
Lenacil 500 mg/kg bw/d	15	0	0	15	0	0	15	0	0	15	0	0	15	0	0	15	0	0
Lenacil 1000 mg/kg bw/d	15	0	0	15	0	0	15	0	0	15	0	0	15	0	0	15	0	0
Positive control (17α-ethinyl oestradiol)	*15*	*0*	*0*	*15*	*0*	*0*	*13*	*0*	***2***	*0*	*12*	***3***	*0*	*15*	*0*	*0*	***15***	*0*
Positive control (haloperidol)	*15*	*0*	*0*	*15*	*0*	*0*	*15*	*0*	*0*	*15*	*0*	*0*	*15*	*0*	*0*	*15*	*0*	*0*

N=15; D: dioestrus; E: oestrus; P: pro-oestrus. First day of lavage on test day 1 occurred prior to test substance administration.

There were no clinical signs observed in rats administered haloperidol. Final mean body weight, overall mean body weight gain, overall mean food consumption and overall mean food efficiency were all lower ($p < 0.05$) compared to control. All animals remained in diestrus for the duration of the study (**table B.6.8.3.1.5-1**). There were no treatment-related effects on uterine weight (**table B.6.8.3.1.5-2**) or gross observations. The results with haloperidol are consistent with a dopamine (D2) receptor agonist.

Table B.6.8.3.1.5-2 Lenacil 5d-uterotrophic assay in ovariectomised rats (2018): body and uterus weigth (mean ± SD).

Treatment/ Dose	Terminal body weigth (BW, g)	Uterus weigth – wet (g)	Uterus wet/ terminal BW (%)	Uterus weigth – blotted (g)	Uterus blotted/ terminal BW (%)
Negative control (0.5% methylcellulose	*301.4 ± 17.5*	*0.0763 ± 0.0130*	*0.025 ± 0.004*	*0.0749 ± 0.0128*	*0.025 ± 0.004*
Lenacil 500 mg/kg bw/d	299.6 ± 18.6	0.0714 ± 0.0099	0.024 ± 0.004	0.0700 ± 0.0092	0.023 ± 0.003
Lenacil 1000 mg/kg bw/d	301.8 ± 14.6	0.0743 ± 0.0076	0.025 ± 0.003	0.0718 ± 0.0090	0.024 ± 0.003
Positive control (17α-ethinyl oestradiol)	*280.2 ± 16.0 (a)*	*0.2024 ± 0.0505 (b) (↑165%)*	*0.072 ± 0.018 (c) (↑188%)*	*0.1903 ± 0.0312 (b) (↑154%)*	*0.068 ± 0.011 (b) (↑172%)*
Positive control (haloperidol)	*271.2 ± 22.9 (a) (↓10%)*	*0.0747 ± 0.0162*	*0.028 ± 0.005 (↑12%)*	*0.0700 ± 0.0141*	*0.026 ± 0.005*

(a): Dunett 2-sided $p<0.05$; (b): Dunnett 1-sided positive (Treatment > Control) $p>0.05$; (c): Dunnett non-parametric 1-sided positive (Treatment > Control) $p>0.05$

Table B.6.8.3.1.5-3 Lenacil 6d-uterotrophic assay in ovariectomised rats (2018): Incidence of gross observations in female rats

Treatment		Negative control	Lenacil	Lenacil	*Positive control oestradiol*	*Positive control haloperidol*
Dose	**(mg/kg b.w./d)**	**0**	**500**	**1000**	***0.1***	***2***
	No visible lesion (N)	15	15	15	*8*	*15*
	Present (N)	0	0	0	*7*	***0***
Uterus	Fluid	-	-	-	***7***	-

Conclusion

Under the conditions of this study, lenacil did not induce changes in parameters associated with oestrogen receptor agonism in ovariectomised adult female rats administered up to 1000 mg/kg b.w./d for 6 consecutive days.

B.6.8.3.1.6

Lenacil (DPX-B0634) technical : *in vitro* aromatase inhibition using human recombinant microsomes (Rijk, J.C., 2019)
Report No. FMC-51364

Guideline(s): US EPA OPPTS guideline 890.1200

GLP: yes

Materials and methods:
The aromatase inhibition assay measured the conversion of androgen to oestrogen in presence of microsomes containing aromatase (CYP19) and cytochrome P450 (CYP) reductase. In brief, radioactive substrate (^{3}H-androstenedione, batch no. 2447553, radiochemical purity >97%, chemical purity not indicated), mixed with "cold" ASDN (batch no. BCBS3965V, purity 99.4%), and NADPH were added to microsomes and reductase complex (Human CYP19 + P450 Reductase Supersomes™, Discovery Labware, Inc., Woburn, MA, USA). During the conversion of androstenedione (ASDN) to oestrone, ^3H_2O was released, which was quantified as a direct measurement of aromatase activity per unit reaction time.

Competitive inhibition of aromatase activity by lenacil (DPX-B0634) Technical (Batch no. 047303003, purity 99.33%) was detected by serial reaction tubes containing increasing concentrations (final: 31.6, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 µM) - in triplicates- , of the substance. After incubation at 37 ± 1°C for 15 ± 1 minutes in 1 mL mixtures containing 500 µL CYP19 supersomes (20 µg/mL), the enzymatic reaction was stopped by the addition of 1 mL methylene chloride, followed by mixing for approximately 5" and placing the tubes on ice for 5'. Subsequently, the tubes were vortexed for an additional 20-25", and centrifuged for 10'at 200 g and 4°C. The bottom layer was removed using a Pasteur pipette and discarded.
The aqueous layers were extracted again with 1 mL methylene chloride, vortexed for 20-25", and centrifuged for 10' at 200 g (4°C) and the methylene chloride layer (bottom layer) was removed with a Pasteur pipet and discarded. This procedure was repeated once more (3 extraction procedures in total). Radioactivity in the aqueous layer was then measured in a liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA, USA).
Four independent assays were performed. The aromatase inhibitor formestane (4-hydroxy-androstenedione, 4-OH-ASDN, batch no. MKCC3385, purity 100%) was used as positive control.

The study is accepted.

Design of main experiments

For each independent assay, the composition of incubation mixture was as followed:

Constituent (final concentration)	Full activity control	Background activity control	Positive control - Formestane	Test item - Lenacil
	(to determine maximum aromatase activity)	*(to determine non-specific binding when aromatase is not activated by the cofactor NADPH)*	*(concentration curve run in triplicates – 3 replicates / concentration, 8 concentrations)*	*(8 concentrations, each concentration in triplicates)*
Buffer	X	X	X	X
Propylene glycol (5%)	X	X	X	X
Microsomal protein (0.004 mg/mL)	X	X	X	X
[^{3}H]-ASDN (100 nM)	X	X	X	X
NADPH (0.3 mM)	X	-	X	X
Vehicle (1%)	X	X	-	-
Formestane (from 0.1 to 10000 nM)	-	-	X	-
Lenacil	-	-	-	X
Number of tubes	4*	4*	24	24

*2 tubes at the beginning, 2 tubes at the end of each run

Findings

A total of 4 independent aromatase assay experiments were performed. Since the full enzyme activity in the first experiment (0.11 nmol/mg protein/min) was close to the acceptance criteria (≥ 0.1 nmol/mg protein/min, see **table B.6.8.3.1.6-1**), the final protein concentration in the incubations was raised and three experiments were performed under the similar conditions. In each experiment, the test item was tested at eight concentrations ranging from 0.00001 µM to 31.6 µM, together with complete dose response curves for the positive control inhibitor 4-OH-ASDN.

Results for the Full Activity and Background Activity Controls

The results obtained for the full activity control and background activity controls are summarised in **table B.6.8.3.1.6-1.**

Table B.6.8.3.1.6-1 Aromatase Inhibition Test (Rijk, 2019) : Mean full enzyme activity control and mean background activity control values

Experiment	Mean full enzyme activity control (nmol/mg protein/min)	Mean background activity control (% of full activity control)
1	0.11	6.23
2	0.18	1.59
3	0.39	0.89
4	0.67	0.38

In each of the 4 aromatase assay experiments the mean aromatase activity in the absence of inhibitor (the full enzyme activity control) was above 0.1 nmol/mg protein/min and the mean of the background activity controls was ≤ 10% and therefore met the acceptance criteria.

Results for Positive Control 4-OH-ASDN

Within each independent experiment, a dose response curve for the positive control 4-OH-ASDN was included at eight concentrations in triplicate. A plot with combined dose response curves for 4-OH-ASDN across experiments is presented in **figure B.6.8.3.1.6-1**. The curve fit parameters obtained for 4-OH-ASDN are presented in **table B.6.8.3.1.6-2**.

Figure B.6.8.3.1.6-1 Aromatase Inhibition Test (Rijk, 2019) : Dose response curves for the positive control inhibitor 4-OH-ASDN combined across experiments.

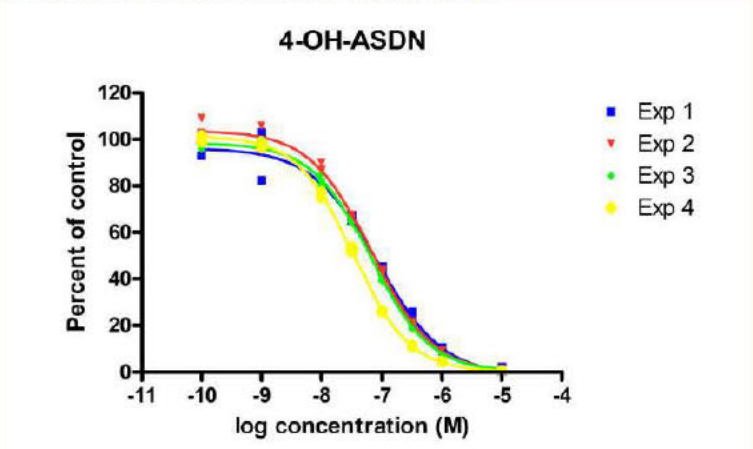


Table B.6.8.3.1.6-2 Aromatase Inhibition Test (Rijk, 2019) : Curve fit parameters and performance criteria for 4-OH-ASDN.

Parameter	Exp. 1	Exp. 2	Exp. 3	Exp. 4	performance criteria (based on laboratory HCD)	
					Lower limit	Upper limit
Slope	-0.8	-0.9	-0.9	-0.9	-1.2	-0.8
Top (%)	96	104	98	101	90	111
Bottom (%)	-1.5	-0.4	0.1	0.1	-5	6
Log IC_{50} (M)	-7.1	-7.1	-7.2	-7.5	-7.5	-7.0
IC_{50} (nM)	72.3	71.9	62.8	34.4	NA	
R^2 (unweighted)	0.9881	0.9965	0.9991	0.9994		

NA: Not Applicable.
Since all results obtained for the full activity controls, the background controls and the positive control inhibitor 4-OH-ASDN were within the acceptance criteria, the results of all four aromatase assay experiments were accepted.

Results for Test Item Lenacil

A plot with combined dose response curves for the test item across experiments is presented in **figure B.6.8.3.1.6-2**. Dose response curves for the test item and positive control inhibitor 4-OH-ASDN combined per experiment are presented in **figure B.6.8.3.1.6-3**. A summary of the results and the classification of the test item response is presented in **table B.6.8.3.1.6-3**.

Figure B.6.8.3.1.6-2 Aromatase Inhibition Test (Rijk, 2019) : Dose response curves for lenacil combined across experiments.

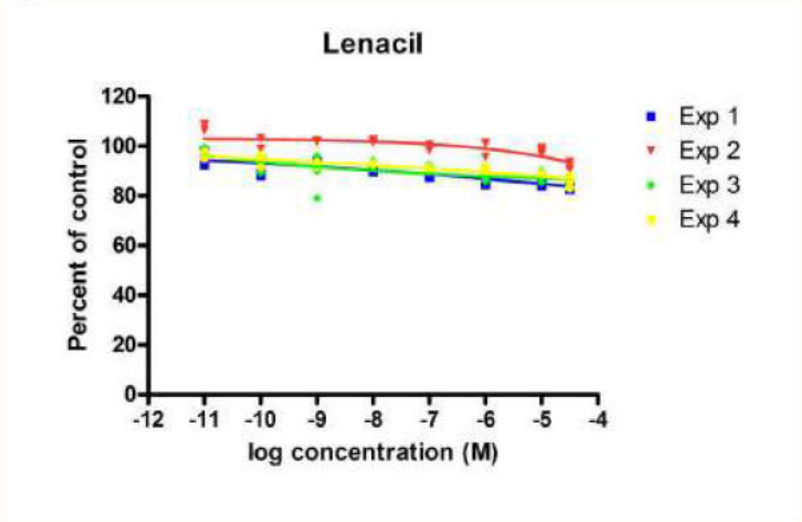


Figure B.6.8.3.1.6-3 Aromatase Inhibition Test (Rijk, 2019) : Dose response curves for the positive control inhibitor 4-OH-ASDN and lenacil combined per experiment.

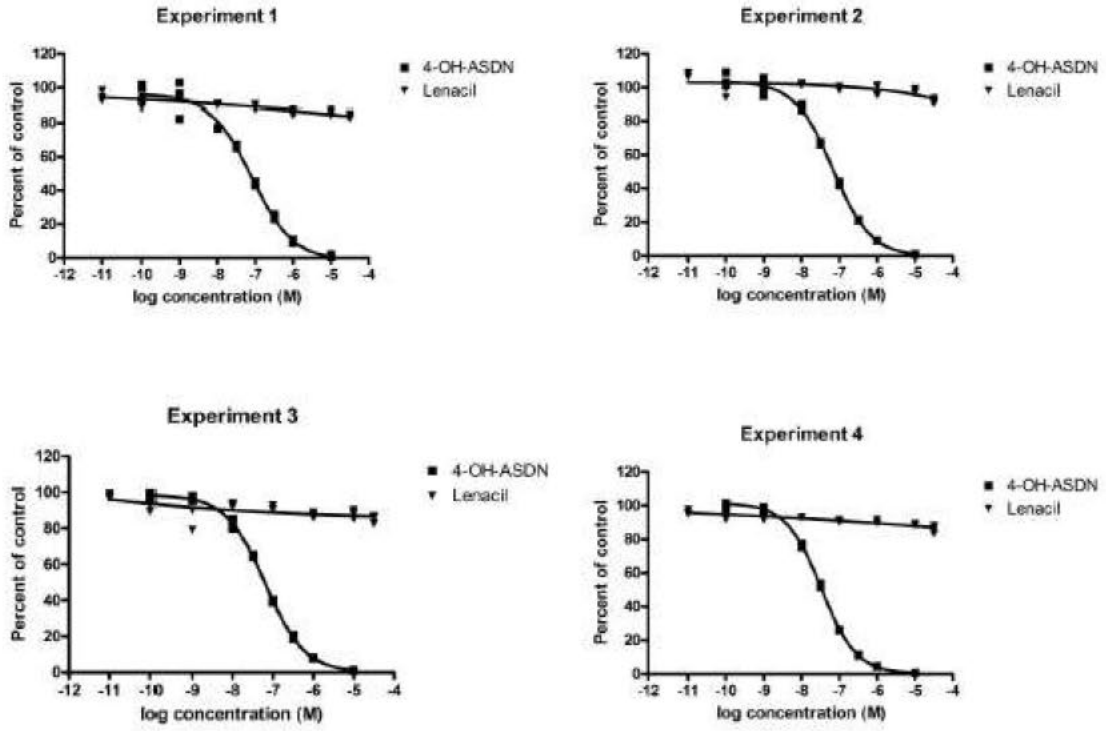

Experiment 1
Percent of control
log concentration (M)
4-OH-ASDN
Lenacil
Experiment 2
Percent of control
log concentration (M)
4-OH-ASDN
Lenacil
Experiment 3
Percent of control
log concentration (M)
4-OH-ASDN
Lenacil
Experiment 4
Percent of control
log concentration (M)
4-OH-ASDN
Lenacil

Table B.6.8.3.1.6-3 Aromatase Inhibition Test (Rijk, 2019) : Lowest point on dose response curve, Log IC_{50} and classification of lenacil

Parameter	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Average	Classification
Lowest point on dose response curve (% of control activity)	83.5%	92.1%	84.9%	85.8%	86.6%	Non-inhibitor
Log IC_{50} (M)	-	-	-	-	NA	

Exp.: Experiment; NA: Not Applicable: -: no value obtained

For the test item, no log IC_{50} value was obtained in any of the four aromatase assay experiments. The average lowest portion of the curves across runs was 86.6%. Since this is > 75%, the test item Lenacil was classified as a non-inhibitor in the aromatase assay.

Conclusion
All four aromatase assay experiments were valid and the test item lenacil was classified as a non-inhibitor in the aromatase assay.

B.6.8.3.1.7

Screening Lenacil (DPX-B0634) technical for modulation of steroidogenesis using the human H295R adenocarcinoma cell line (Verkaart S., 2019)
Report No. FMC-51365

Guideline(s): EC test method B.57 of Council Regulation (EC) No 440/2008, equivalent to OECD 456
GLP: yes

Materials and methods:
H295R human adenocarcinoma cells (ATCC CRL-2128) were exposed for 48 hours to lenacil (DPX-B0634) technical (batch no. 047303003, purity 99.33%) at concentrations of 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 µM (experiment 1); 3.16, 1, 0.1, 0.001, 0.00316, 0.0001 and 0.00001 µM (experiment 2); 3.16, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 µM (experiment 3). Steroidogenesis inducer forskolin (Sigma-Aldrich, batch no. SLBP3308V, purity 98%) and inhibitor prochloraz (Sigma-Aldrich, batch no. BCBW4694, purity 98.7%) were used as positive controls. After exposure, the viability of the cells was determined using the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The concentration of oestradiol and testosterone in the exposure medium was determined using commercial Enzyme-Linked Immuno Sorbent Assays (Cayman Chemical Company, Ann Arbor, MI, USA).

The study is accepted.

Findings

Steroidogenesis Assay – Experiment 1 (lenacil concentrations: 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 µM)
Oestradiol measurements.
The mean ± SD basal oestradiol production in the DMSO solvent control wells on the test item plate was 154 ± 14 pg/mL. The average oestradiol concentrations in medium from H295R cells exposed to the test item varied from 116 pg/mL to 157 pg/mL. The average relative change of test item exposed wells compared to the solvent treated control wells varied from 0.76 to 1.02. Statistical analysis showed that lenacil significantly ($p \leq 0.05$) decreased oestradiol synthesis in H295R cells at a concentration of 0.0001 µM (**table B.6.8.3.1.7-1**). Since the effect observed for oestradiol was not dose-related, *i.e.* no statistically significant effect was observed at two (or more) adjacent concentrations, the decrease at 0.0001 µM in the first steroidogenesis experiment was considered to be spurious by the notifier.
RMS produced the chart with the individual and average oestradiol levels in this *in-vitro* assay. Strikingly, it would appear that the dose-response is not linear, but exhibits an initial drop of the oestradiol concentrations up to and including 10^{-4} µM lenacil, while the levels increased on average up to the top-concentration of 1 µM, suggesting a non-monotonic dose response superposing both an inhibition and an induction of the oestradiol biosynthesis.
While it remains evident that the effect size is borderline (and showing only statistical significance in one point), when compared with overt disruptors like forskolin and prochloraz, the trend seems obvious and an effect cannot be ignored. However, this dose respons seems not to be replicated when other intermediate concentrations were tested in experiments 2 and 3, and thus the toxicological relevance of this observation remains doubtful.

Table B.6.8.3.1.7-1 Screening Lenacil technical for modulation of steroidogenesis using the human H295R adenocarcinoma cell line (Verkaart, 2019): experiment 1 - oestradiol level.

		Oestradiol (pg/mL)				%
		Well 1	Well 2	Well 3	mean ± s.d.	mean ± s.d. (relative)
Blank		176	133	151	153 ± 22	1.29 ± 0.18
DMSO (SC)		116	116	125	119 ± 5	1.00 ± 0.04
Forskolin	1 µM	1050	935	1214	1067 ± 140	8.97 ± 1.18
	10 µM	2000	2379	1249	1876 ± 575	15.78 ± 4.84
Prochloraz	0.1 µM	75	77	90	80 ± 8	0.68 ± 0.07
	1 µM	28	38	43	36 ± 8	0.31 ± 0.06
Lenacil (µM)	0	147	144	170	154 ± 14	1.00 ± 0.09
	0.00001	137	124	118	126 ± 10	0.82 ± 0.06
	0.0001	134	102	112	**116* ± 17**	0.76 ± 0.11
	0.001	103	122	129	118 ± 13	0.77 ± 0.09
	0.01	119	120	146	128 ± 15	0.84 ± 0.10
	0.1	150	148	119	139 ± 17	0.90 ± 0.11
	1	174	134	162	157 ± 20	1.02 ± 0.13

SC = solvent control; SD = Standard deviation: *significant ($p \leq 0.05$, Dunnett's multiple t-test)

Figure B.6.8.3.1.7-1a Screening Lenacil technical for modulation of steroidogenesis using the human H295R adrenocortical cell line (Verkaart, 2019): experiment 1 - oestradiol level; oestradiol concentration (pg/mL)

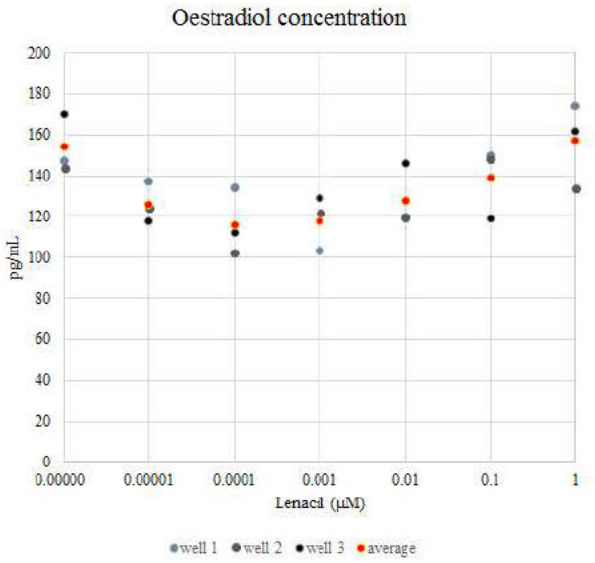
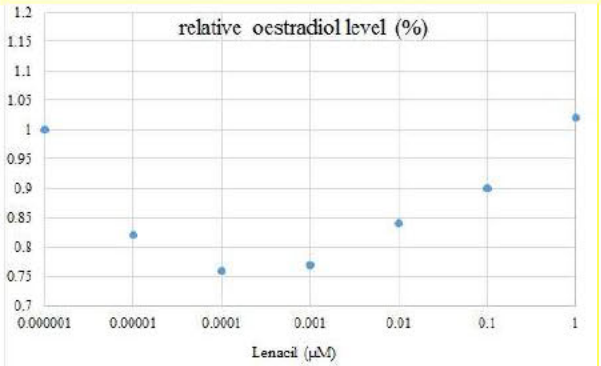


Figure B.6.8.3.1.7-1b Screening Lenacil technical for modulation of steroidogenesis using the human H295R adrenocortical cell line (Verkaart, 2019): experiment 1 - oestradiol level; average (relative oestradiol level, % of control)



Testosterone measurements.

The mean ± SD basal testosterone production in the DMSO solvent control wells on the test item plate was 2765 ± 233 pg/mL. The average testosterone concentrations in the medium from the H295R cells exposed to the test item varied from 2362 pg/mL to 2821 pg/mL. The average relative change of test item exposed wells to solvent treated control wells varied from 0.85 to 1.02 (see **table B.6.8.3.1.7-2**). Statistical analysis showed that Lenacil had no significant effect on testosterone synthesis in H295R cells.

Table B.6.8.3.1.7-2 Screening Lenacil (DPX-B0634) technical for modulation of steroidogenesis using the human H295R adenocarcinoma cell line (Verkaart, 2019): experiment 1 - Testosterone level.

		Testosterone (pg/mL)				%
		Well 1	**Well 2**	**Well 3**	**mean ± s.d.**	**mean ± s.d. (relative)**
Blank		2740	2493	2637	2623 ± 124	1.19 ± 0.06
DMSO (SC)		2314	2080	2211	2202 ± 117	1.00 ± 0.05
Forskolin	1 µM	3874	3644	4786	4101 ± 604	1.86 ± 0.27
	10 µM	5374	4885	5086	5115 ± 246	2.32 ± 0.11
Prochloraz	0.1 µM	697	800	799	765 ± 59	0.35 ± 0.03
	1 µM	256	439	356	350 ± 92	0.16 ± 0.04
Lenacil (µM)	0	2984	2520	2792	2765 ± 233	1.00 ± 0.08
	0.00001	2737	2752	2975	2821 ± 133	1.02 ± 0.05
	0.0001	3098	2685	2631	2804 ± 256	1.01 ± 0.09
	0.001	2371	2129	2588	2362 ± 230	0.85 ± 0.08
	0.01	2606	2693	2605	2635 ± 51	0.95 ± 0.02
	0.1	2612	2463	2480	2518 ± 82	0.91 ± 0.03
	1	2802	2401	2310	2504 ± 262	0.91 ± 0.09

SC: solvent control; s.d.: standard deviation

Steroidogenesis Assay – Experiment 2 (lenacil concentrations: 3.16, 1, 0.01, 0.001, 0.000316, 0.0001, 0.00001 µM)

Oestradiol measurements.

Based on the results obtained in experiment 1, ½-log concentration dilutions were included in the second experiment next to the concentration that elicited an effect (0.0001 µM) and at the end of the concentration range, as 10 µM could not be analysed due to test item precipitation. This was done to better characterise any potential effect of the test item on oestradiol and testosterone synthesis. The concentrations tested in experiment 2 were: 0.00001, 0.0001, 0.000316, 0.001, 0.01, 1 and 3.16 µM.

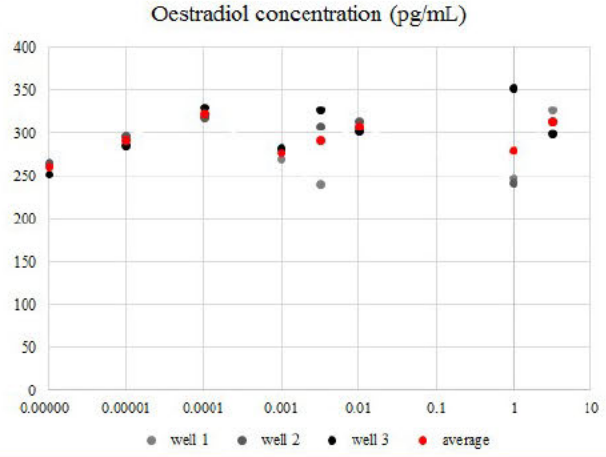
The mean ± s.d. basal oestradiol production in the DMSO solvent control wells on the test item plate was 261 ± 7 pg/mL. The average oestradiol concentrations in medium from H295R cells exposed to the test item varied from 278 pg/mL to 323 pg/mL. The average relative change of test item exposed wells compared to the solvent treated control wells varied from 1.07 to 1.24 (**table B.6.8.3.1.7-3**). Statistical analysis showed that lenacil had no significant effect on oestradiol synthesis in H295R cells. **RMS**: the non-monotonic dose response observed in the first experiment is not replicated in the second experiment (see **figure B.6.8.3.1.7-2**).

Table B.6.8.3.1.7-3 Screening Lenacil (DPX-B0634) technical for modulation of steroidogenesis using the human H295R adenocarcinoma cell line (Verkaart, 2019): experiment 2 - oestradiol level.

		Oestradiol (pg/mL)				%
		Well 1	Well 2	Well 3	mean ± s.d.	mean ± s.d. (relative)
Blank		260	238	226	241 ± 17	1.27 ± 0.09
DMSO (SC)		172	198	198	189 ± 15	1.00 ± 0.08
Forskolin	1 µM	2121	1515	2382	2006 ± 445	10.59 ± 2.35
	10 µM	2986	2921	2798	2901 ± 95	15.31 ± 0.50
Prochloraz	0.1 µM	123	119	125	123 ± 3	0.65 ± 0.02
	1 µM	43	56	60	53 ± 9	0.28 ± 0.05
Lenacil (µM)	0	265	266	253	261 ± 7	1.00 ± 0.03
	0.00001	296	297	287	293 ± 5	1.12 ± 0.02
	0.0001	320	319	331	323 ± 6	1.24 ± 0.02
	0.000316	241	309	328	293 ± 46	1.12 ± 0.17
	0.001	270	282	283	278 ± 7	1.07 ± 0.03
	0.01	307	314	303	308 ± 6	1.18 ± 0.02
	1	248	243	352	281 ± 61	1.07 ± 0.24
	3.16	328	314	300	314 ± 14	1.20± 0.05

SC: solvent control; s.d.: standard deviation.

Figure B.6.8.3.1.7-2 Screening Lenacil technical for modulation of steroidogenesis using the human H295R adrenocortical cell line (Verkaart, 2019): experiment 2 - oestradiol level; oestradiol concentration (pg/mL)



Testosterone measurements.

The mean ± SD basal testosterone production in the DMSO solvent control wells on the test item plate was 3126 ± 301 pg/mL. The average testosterone concentrations in the medium from the H295R cells exposed to the test item varied from 2516 pg/mL to 3288 pg/mL. The average relative change of test item exposed wells to solvent treated control wells varied from 0.80 to 1.05 (**table B.6.8.3.1.7-4**). Statistical analysis showed that lenacil had no significant effect on testosterone synthesis of H295R cells.

Since no statistically significant effects on oestradiol and testosterone synthesis were observed, lenacil was considered to be negative in steroidogenesis experiment 2.

Table B.6.8.3.1.7-4 Screening Lenacil (DPX-B0634) technical for modulation of steroidogenesis using the human H295R adrenocortical cell line (Verkaart, 2019): experiment 2 - Testosterone level.

		Testosterone (pg/mL)				%
		Well 1	Well 2	Well 3	mean ± s.d.	mean ± s.d. (relative)
Blank		3728	3131	2836	3231 ± 454	1.16 ± 0.16
DMSO (SC)		2783	2745	2816	2781 ± 35	1.00 ± 0.01
Forskolin	1 µM	6877	4770	6380	6009 ± 1101	2.16 ± 0.40
	10 µM	8450	6896	6955	7434 ± 881	2.67 ± 0.32
Prochloraz	0.1 µM	1061	1017	1108	1062 ± 45	0.38 ± 0.02
	1 µM	432	522	522	492 ± 52	0.18 ± 0.02
Lenacil (µM)	0	3358	3235	2787	3126 ± 301	1.00 ± 0.10
	0.00001	3049	3045	3145	3080 ± 57	0.99 ± 0.02
	0.0001	3354	3126	3293	3258 ± 118	1.04 ± 0.04
	0.000316	2450	3941	3473	3288 ± 762	1.05 ± 0.24
	0.001	2573	2377	2742	2564 ± 183	0.82 ± 0.06
	0.01	3520	2740	2929	3063 ± 407	0.98 ± 0.13
	1	2294	1910	3548	2584 ± 857	0.83 ± 0.27
	3.16	2721	2629	2196	2516 ± 280	0.80 ±0.09

SC: solvent control; s.d.: standard deviation

Steroidogenesis Assay – Experiment 3 (lenacil concentrations: 3.16, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 µM)

Oestradiol measurements.

Since no statistically significant changes in oestradiol synthesis were observed in the second experiment, in contrast to the first experiment, a third experiment was conducted using the test concentrations of the second experiment. The concentrations tested in experiment 3 were: 0.00001, 0.0001, 0.001, 0.01, 0.1, 1 and 3.16 µM.

The mean ± SD basal oestradiol production in the DMSO solvent control wells on the test item plate was 238 ± 18 pg/mL. The average oestradiol concentrations in medium from H295R cells exposed to the test item varied from 267 pg/mL to 285 pg/mL. The average relative change of test item exposed wells compared to the solvent treated control wells varied from 1.13 to 1.20 (**table B.6.8.3.1.7-5**). Statistical analysis showed that lenacil had no significant effect on oestradiol synthesis in H295R cells.

RMS: as for the second experiment, the non-monotonic dose response observed in the first experiment is not replicated in the third experiment. On the basis of this confirmatory third experiment, it seems reasonable to postulate that the results of the first experiment were fortuitous.

(see **figure B.6.8.3.1.7-3**).

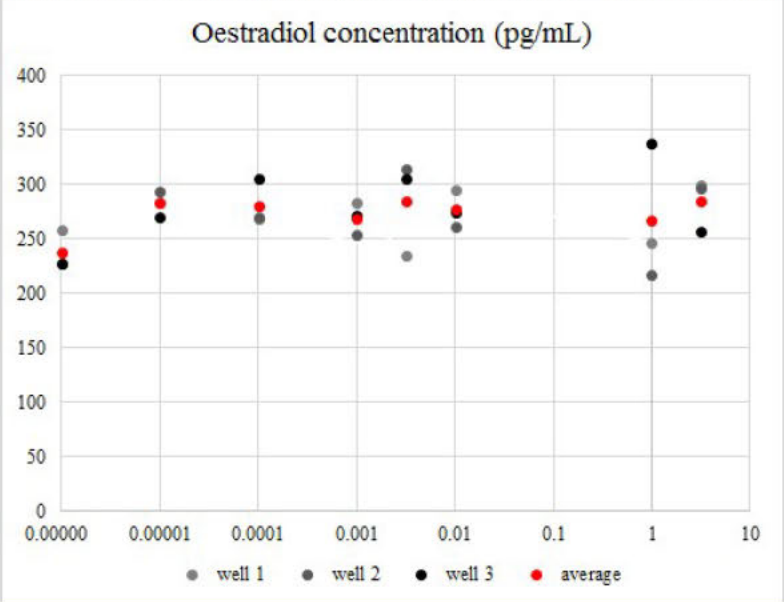
Table B.6.8.3.1.7-5 Screening Lenacil (DPX-B0634) technical for modulation of steroidogenesis using the human H295R adrenocortical cell line (Verkaart, 2019): experiment 3 - Oestradiol level.

		Oestradiol (pg/mL)				%
		Well 1	Well 2	Well 3	mean ± s.d.	mean ± s.d. (relative)
Blank		171	161	207	180 ± 24	1.12 ± 0.15
DMSO (SC)		169	169	142	160 ± 15	1.00 ± 0.10
Forskolin	1 µM	1377	983	1372	1244 ± 226	7.78 ± 1.41
	10 µM	2049	1652	2005	1902 ± 218	11.89 ±1.36
Prochloraz	0.1 µM	87	81	85	84 ± 3	0.53 ± 0.02
	1 µM	28	31	38	32 ± 5	0.20 ± 0.03
Lenacil (µM)	0	259	227	228	238 ± 18	1.00 ± 0.08
	0.00001	284	294	270	283 ± 12	1.19 ± 0.05
	0.0001	269	270	305	281 ± 20	1.18 ± 0.09
	0.001	235	315	306	285 ± 44	1.20 ± 0.18
	0.01	283	254	271	269 ± 15	1.13 ± 0.06
	0.1	295	261	275	277 ± 17	1.17 ± 0.07

	1	247	217	338	267 ± 63	1.12 ± 0.26
	3.16	300	297	257	285 ± 24	1.20 ± 0.10

SC: solvent control; SD: standard deviation

Figure B.6.8.3.1.7-3 Screening Lenacil technical for modulation of steroidogenesis using the human H295R adrenocortical cell line (Verkaart, 2019): experiment 3 - oestradiol level; oestradiol concentration (pg/mL)



Testosterone measurements.

The mean ± SD basal testosterone production in the DMSO solvent control wells on the test item plate was 3258 ± 310 pg/mL. The average testosterone concentrations in the medium from the H295R cells exposed to the test item varied from 2718 pg/mL to 3549 pg/mL. The average relative change of test item exposed wells to solvent treated control wells varied from 0.83 to 1.09 (**Table B.6.8.3.1.7-6**). Statistical analysis showed that lenacil had no significant effect on testosterone synthesis in H295R cells.

Since no statistically significant effects on oestradiol and testosterone synthesis were observed, lenacil was considered to be negative in steroidogenesis experiment 3.

Table B.6.8.3.1.7-6 Screening Lenacil (DPX-B0634) technical for modulation of steroidogenesis using the human H295R adrenocortical cell line (Verkaart, 2019): experiment 3 - Testosterone level.

		Testosterone (pg/mL)				%
		Well 1	**Well 2**	**Well 3**	**mean ± s.d.**	**mean ± s.d. (relative)**
Blank		2271	2288	2745	2435 ± 269	1.07 ± 0.12
DMSO (SC)		2302	2512	2029	2281 ± 242	1.00 ± 0.11
Forskolin	1 µM	4333	2765	4435	3844 ± 936	1.69 ± 0.41
	10 µM	5832	4883	5869	5528 ± 559	2.42 ± 0.25
Prochloraz	0.1 µM	818	673	664	718 ± 86	0.31 ± 0.04
	1 µM	117[a]	252	286	269 ± NA[b]	0.12 ± NA[b]
Lenacil (µM)	0	3604	3162	3007	3258 ± 310	1.00 ± 0.10
	0.00001	3523	3614	3199	3446 ± 218	1.06 ± 0.07
	0.0001	3798	3485	3365	3549 ± 223	1.09 ± 0.07
	0.001	3185	3784	3484	3484 ± 300	1.07 ± 0.09
	0.01	3112	2930	3003	3015 ± 92	0.93 ± 0.03
	0.1	3578	3177	3022	3259 ± 287	1.00 ± 0.09
	1	2653	2183	3478	2771 ± 655	0.85 ± 0.20
	3.16	2963	2618	2573	2718 ± 213	0.83 ± 0.07

NA: not applicable; SC: solvent control; s.d.: standard deviation

[a]: value excluded in calculation average; [b]: not applicable as only 2 values were used for calculation of average.

Discussion

While in the first experiment, a peculiar non-monotonic dose-response was observed with a decrease and subsequent increase of the oestradiol levels, the absolute oestradiol concentrations were also approximately 2× lower than those measured in the second and the third experiment. Furthermore, the results in the 2nd and 3rd experiment, assayed at a top-concentration even higher than that of the first run, did exhibit neither a decreasing nor an increasing trend of oestradiol with the dose. As the results in the first oestradiol experiment could not be confirmed in the two subsequent experiments (both negative), the results of the first experiment were consequently considered to be spurious. Therefore, it was considered that overall, oestradiol levels remained unaffected in this study.
The testosterone levels remained unaffected in all runs of the study.

Overall, lenacil did not significantly alter oestradiol or testosterone synthesis in H295R cells. The No Observed Effect Concentration (NOEC) is 3.16 µM, the highest concentration tested and the maximum concentration to be assayed according to EC test method B.57, equivalent to OECD Test Guideline 456.

Conclusion

The test item lenacil did not alter oestradiol or testosterone synthesis in H295R cells and therefore RMS considered lenacil to be negative under the conditions of the steroidogenesis assay.

B.6.8.3.1.8

Lenacil (DPX-B0634) technical: thyroid mechanistic 14-day feeding study in rats ([redacted] 2019)
Report No. 49352

Guideline(s): not applicable
GLP: no.

Materials and methods:
-Four groups of young adult ♂rats Crl:CD(SD) (15/sex/group) were administered diets that contained 0, 2500 (equivalent to 189 mg/kg bw/d), 12500 (equivalent to 923 mg/kg bw/d)), or 25000 ppm (equivalent to 1841 mg/kg bw/d) lenacil (DPX-B0634) technical ([redacted] numbers 32157 (lot -151) and 32158 (lot -152), purity 98.8%) for 14 days. Body weights, food consumption, and clinical observations were evaluated weekly and acute clinical observations were evaluated daily. Blood was collected at sacrifice (test day 15) for analysis of serum concentrations of thyroid hormones [thyroxine (T_4), triiodothyronine (T_3), reverse T3 (rT_3), and thyroid stimulating hormone (TSH)] using commercially-available radioimmunoassay kits. At necropsy, liver and thyroid glands were collected, weighed, and saved for microscopic evaluation. A section of liver was collected and microsomes were prepared by differential centrifugation for evaluation of UDP-glucuronyltransferase (UDPGT) activity. A separate section of liver was flash frozen and prepared for cytochrome P450 gene expression.
-Thyroid peroxidase inhibition was evaluated *in vitro* using porcine thyroid microsomes. Thyroid glands from untreated microswine were purchased (CR Laboratories Inc. (Kingston, New York, USA), and microsomes were prepared by differential centrifugation, and microsomal protein content was measured before analyses.
Chemical chemistry:
- The T_4 RIA kit (catalog number 06B-254029) was purchased from MP Biomedicals (Orangeburg, New York, U.S.A.).
- The T_3 RIA kit (catalog number 06B-256447) was purchased from MP Biomedicals (Orangeburg, New York, U.S.A.).
- The TSH RIA kit (catalog number 07RK-554) was purchased from MP Biomedicals (Eschwege, Germany).
- The reverse T_3 (rT_3) kit (catalog number 38-RT3HU-R125) RIA kit was purchased from Alpco (Salem, New Hampshire, U.S.A.).
- UDPGT activity was measured spectrophotometrically, using ρ-nitrophenol as the substrate, according to the methods of McClain et al/ (1989).
- Reverse transcription of RNA to cDNA was performed (Applied Biosystems) and quantitative real-time PCR (7500 real time PCR system, Applied Biosystems) using SYBR green and primers specific to CYP450 1A1, 2B1, 2E1, 3A1, 4A1 completed using the housekeeping gene B2M for normalisation. Fold-change in CYP450 isozyme gene expression was expressed relative to the average of vehicle control treated animals and statistical significance identified.
- Thyroid peroxidase activity was measured spectrophotometrically according to the methods of Astroff and Safe (1990).

Statistical analysis included Dunnett's test.
Cited references

- *McClain, RM., Levin, AA., Posch, R., and Downing, JC. . Effect of phenobarbital on the metabolism and excretion of thyroxine in rats. Toxicol. Appl. Pharmacol. 99, 216-228, 1989.*
- *Astroff, B., and Safe, S. 2,3,7,8-Tetrachlorodibenzo-p-dioxin as an antiestrogen: effect on rat uterine peroxidase activity. Biochem. Pharmacol. 39, 485-488, 1990*

The study is accepted.

Findings:

***Body weight, body weight gain*:**
The body weight gain during wk 1 of treatment was decreased to 86% of controls at 25000 ppm. No other effect was observed (**table B.6.8.3.1.8-1**).

Table B.6.8.3.1.8-1 Lenacil: thyroid mechanistic 14d feeding study in ♂rats (Munley, 2019): body weight (gain).

Dose (ppm)	0	2500	12500	25000
mg/kg b.w./d	0	189	923	1841
Body weight				
d1	261.1 ± 18.1	162.4 ± 17.9	261.2 ± 19.5	261.6 ± 17.3
d9	315.9 ± 22.3	316.6 ± 25.3	312.9 ± 20.4	309.0 ± 19.2
d15	358.7 ± 26.2	358.8 ± 30.6	356.4 ± 23.5	351.3 ± 24.7
Body weight gain				
d1-8	54.8 ± 11.3	54.1 ± 10.2	51.7 ± 5.8	**47.4 ± 8.5 (↓14%)**
d8-15	42.7 ± 5.6	42.3 ± 6.9	43.5 ± 5.0	42.4 ± 8.5
d1-15	97.6 ± 14.5	96.4 ± 15.9	95.2 ± 6.3	89.8 ± 14.7

N=15 rats/dose; Results expressed in average ± s.d.

***Food consumption, food efficiency*:**
No adverse effect was seen. Overall (day 1-15) mean food consumption in the top-dose group was 94% of control.

***Clinical observations, mortality*:**
No clinical signs were observed in any study group. All animals survived until the scheduled terminal sacrifice.

Organ weight:
A small significant increase in relative (to terminal b.w.) liver weight was observed at 12500 and 25000 ppm (increases of 7.2 and 6.8% above control, respectively), and not associated with statistically significant changes in absolute liver weight.
Nonetheless, based on correlative changes of minimal hepatocellular hypertrophy noted microscopically, the minimal weight increases were considered to be test substance-related.
A non-significant and non dose-related increase (higher at 12500 than at 25000 ppm) was observed in the thyroid.

Table B.6.8.3.1.8-2 Lenacil: thyroid mechanistic 14d feeding study in ♂rats (Munley, 2019): organ weight.

Dose (ppm)	0	2500	12500	25000
mg/kg b.w./d	**0**	**189**	**923**	**1841**
Terminal body weight (g)	358.7 ± 26.2	358.8 ± 30.6	356.4 ± 23.5	351.3 ± 24.7
Liver absolute (g)	13.990 ± 1.285	14.519 ± 1.827	14.875 ± 0.970 (↑6%)	14.630 ± 1.343 (↑5%)
Liver relative (%)	3.899 ± 0.190	4.038 ± 0.279	**4.179* ± 0.194 (↑7%)**	**4.163* ± 0.235 (↑7%)**
Thyroid absolute (g)	0.016 ± 0.002	0.016 ± 0.004	0.018 ± 0.003 (↑10%)	0.017 ± 0.003 (↑3%)
Thyroid relative (%)	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.001 (↑9%)	0.005 ± 0.001 (↑5%)

N=15 rats/dose; Results expressed in average ± s.d., statistically significant modification: * p<0.05, Dunnett 2-sided

Gross observations
Bilaterally small testes and epididymides were observed in 1/15 top-dose male. Similar findings were not observed in any other animals in this group and these isolated gross observations were considered by the notifier to be spurious findings known to occur as a background lesions in rats of this strain and age.
RMS: notes that a unique case of unilaterally small testes and epididymides (n = 9-10 animals) was observed at top-dose (50000 ppm) also in the rat 13 weeks study ([redacted] 2002, **B.6.3.2.1.1**), while unilaterally small testis occurrences were higher at top-dose (1223.2 mg/kg bw/d) in the combined chronic toxicity and carcinogenicity study of lenacil in rats over 104 weeks ([redacted] 2003, **B.6.5.1.1**). Along the same line, 1/28 animal showed flaccid/blue testes at top-dose (50000 ppm) in the 2G study with lenacil in Wistar rats ([redacted] 2003, **B.6.6.1.2**), while no animal (/28) exhibited this feature in control and at lower doses.
The RMS is thus not fully convinced that the "spurious findings" are not treatment-related. However, as the incidence is low, and the effect is only observed at systemically toxic doses, the relevance in terms of primary endocrine effect is probably weak.

Table B.6.8.3.1.8-2 Lenacil: thyroid mechanistic 14d feeding study in rats (Munley, 2019): gross observations

Dose (ppm)	0	2500	12500	25000
mg/kg b.w./d	**0**	**189**	**923**	**1841**
No visible lesion	15	15	15	**14**
Present	0	0	0	**1**
Epididymides				
Small	0	0	0	**1**
Testes				
Small	0	0	0	**1**

N=15 rats/dose;

Microscopic findings:
In table B.6.8.3.1.8-4, the histopathological lesions in the liver and the thyroid are reported.

Minimal hepatocellular hypertrophy was observed in 9/15 and 14/15 animals at 12500 and 25000 ppm. Hypertrophy was characterised primarily by slight enlargement and pale eosinophilic cytoplasmic staining of hepatocytes in the centrilobular areas. Low incidences of increased mitotic figures (1/15 and 3/15, respectively) were also present in these groups. The microscopic lesions, corroborating the organ weight changes in the liver, were not associated with other degenerative changes, and were considered secondary to induction of liver metabolising enzymes as seen by the CYP2B1 gene expression by the notifier. These changes were considered by the notifier to be "non-adverse" However, the combination of significant (although moderate) liver weight increase and centrilobular hypertrophy (with in addition, increased mitotic figures) is commonly considered adverse, and any setting of a NOAEL should thus take such changes into account. The precise MoA for the observed mitogenicity (or mitotic block?) remained unexplained.
There were no test-substance-related microscopic findings in the thyroid gland at any of the dietary concentrations tested. The outcome is different from that of the [redacted] (2004) study, but this does not change the conclusions, as the 2004 study was conducted up to 5000 0ppm (4000 mg/kg b.w./d), where thyroid blackening has been reported.

Table B.6.8.3.1.8-4 Lenacil: thyroid mechanistic 14d feeding study in rats (Munley, 2019): microscopic findings

Dose (ppm)	**0**	**2500**	**12500**	**25000**
mg/kg b.w./d	**0**	**189**	**923**	**1841**
Liver				
No visible lesions	13	13	5	1
Hepatocellular hypertrophy	0	0	**9**	**14**
Mononuclear infiltrate	2	2	4	3
Mitotic figures	0	0	**1**	**3**
Thyroid gland				
No visible lesions	15	15	15	15

N=15 animals; all hepatic lesions were characterised as "minimal"

Thyroid hormone evaluation:
After two weeks of test substance administration, serum "reverse T_3" concentration was minimally, but statistically significantly higher at 12500 and 25000 ppm, while being also higher (↑16%, but not s.s) at 2500 ppm. Serum T_3 and TSH concentrations were statistically significantly lower in the 25,000 ppm group (78 and 75 % of control, respectively) compared to control. TSH level was also reduced, although not s.s, to 88% of control at 12500 ppm). No statistically significant alterations were observed in serum T_4 concentration.

Table B.6.8.3.1.8-5 Lenacil: thyroid mechanistic 14d feeding study in rats (Munley, 2019): hormone levels.

Dose (ppm)	**0**	**2500**	**12500**	**25000**
mg/kg b.w./d	**0**	**189**	**923**	**1841**
Reverse T_3 (rT_3) (ng/mL)	0.057 ± 0.013 (N=14)	**0.066 ± 0.013 (↑16%)**	**0.069* ± 0.010 (↑22%)**	**0.070* ± 0.013 (↑23%)** (N=13)
T_3 (ng/dL)	63.110 ± 9.383	62.744 ± 14.045	58.950 ± 13.425 (↓7%)	**49.075* ± 6.914 (↓22%)**
T_4 (µg/dL)	4.174 ± 0.505	4.075 ± 0.606	4.032 ± 0.428	4.188 ± 0.792
TSH (ng/mL)	8.237 ± 3.963	7.719 ± 2.677 (↓6%)	**7.243 ± 3.487 (↓12%)**	**6.215# ± 4.223 (↓25%)**

N= 15 animals/dose, unless stated otherwise; Statistically significant modification *Dunnett 2-sided, $p<0.05$; #Dunett non-parametric 2-sided, $p<0.05$

Notifier highlighted that there was no effect of lenacil on thyroid gland weight, morphology and serum T_4 concentrations. Serum TSH and T_3 concentrations were minimally decreased and serum rT_3 concentrations were minimally increased. According to the notifier, these data demonstrate that there was no evidence of an adverse effect on thyroid hormone "economy" (physiological regulation of thyroid function). In their view, the minimal changes in serum TSH, T_3 and rT_3 concentrations *may have represented biological variation, and/or subtle adaptive changes in hormonal regulation*.

RMS considers yet that there is an obvious involvement of the treatment with lenacil , taking into account the dose-dependency, and the coherence with thyroid hormone modifications in other studies. A decrease of the inactive rT_3 has uncertain toxicological implications; it is a conversion product of T_4 and an increase could indicate an accelerated

metabolism, leading to more rT_3, but in a regular metabolic event, it remains uncertain by what cause the peripheral degradation to rT_3 and free T_3 formation display different directions when originating from T_4.
In any case, the weak decrease of the biologically most active T_3 is an *potentially* adverse effect, even if T_4 levels remain unaltered. The finding is not entirely in line with what has been observed in the [redacted] 2004 study, for which a comparison remains difficult as the latter was a 13wk-study, while the Munley study is restricted to 2 weeks, and in addition does not test the highest dose of 50000 ppm (~4000 mg/kg b.w./d) as in the [redacted] 2004 study. In addition, the comparison is even more complicated because two different rat strains were used in the two experiments (Wistar in the 10/20 wk-[redacted] study and CD in the 2-wk Munlay study).
It should however be acknowledged that the observed hormonal changes are indeed weak, and without meaningful effect on developmental parameters so far, although it is noted that no NDT study is present to exclude any developmental NT effect. Further discussion is neede to sort out the need for such a DNT study.

***In vitro thyroid peroxidase inhibition*:**
It was of note that the results do not pertain to findings in rat hepatocytes, but a microsome assay was set up with thyroidal cells of microswines.
The positive control, PTU, caused an expected dose-related decrease in thyroid peroxidase activity. The IC_{50} value was 12.2 ± 0.36 µM.
Lenacil, when tested up to a concentration of maximum solubility in the assay system (200 µM), showed no evidence of thyroid peroxidase inhibition. As a result, an IC_{50} value could not be determined.

Table B.6.8.3.1.8-6 Lenacil: thyroid mechanistic 14d feeding study in rats (Munley, 2019): thyroid peroxidase inhibition.

Final concentration (µM)	Propylthiouracil (PTU)	Lenacil
0	0.249 ± 0.0023	0.249 ± 0.0023
0.5	0.209 ± 0.0054	-
1	0.195 ± 0.0130	-
2	0.182 ± 0.0160	0.254 ± 0.0070
5	0.145 ± 0.0049	0.240 ± 0.0045
10	0.108 ± 0.0092	0.254 ± 0.0089
20	0.079 ± 0.0000	0.251 ± 0.0194
50	0.040 ± 0.0025	0.255 ± 0.0066
100	-	0.252 ± 0.0082
200	-	0.244 ± 0.0058
500	-0.009 ± 0.029	-

-: no data at this concentration; peroxidase activity expressed as absorbance/min/mg protein (mean ± s.d.)

***UDPGT activity evaluation*:**
Hepatic UDPGT activity was statistically significantly higher at12500 and 25000 ppm (127 and 138% of control, respectively).

Table B.6.8.3.1.8-7 Lenacil: thyroid mechanistic 14d feeding study in rats (Munley, 2019): UDGPT activity.

Dose (ppm)	**0**	**2500**	**12500**	**25000**
mg/kg b.w./d	**0**	**189**	**923**	**1841**
N	*10*	*8*	*8*	*10*
UDPGT (µmol/mg×min)	0.0675 ± 0.0122	**0.0852 ± 0.0259 (↑26%)**	**0.0842* ± 0.0139 (↑25%)**	**0.0934* ± 0.0134 (↑38%)**

N= number of rats, *Dunnett 2-sided, $p<0.05$

***Cytochrome P450 enzyme gene expression*:**
Dietary exposure to lenacil was associated with changes in hepatic cytochrome P450 enzyme activity and gene expression similar to those seen with constitutive androstane receptor (CAR) activation, with statistically significant changes occurring in CYP2B. Hepatic CYP2B1 gene expression was statistically significantly higher in all treatment groups compared to control with difference of about 9, 12 and 15-fold relative to control at 2500, 12500 and 25000 ppm, respectively. There were no statistically significant or dose-dependent differences in CYP1A1, CYP3A1, CYP4A1, or CYP2E1 gene expression in any treatment group compared to control.

Table B.6.8.3.1.8-8 Lenacil: thyroid mechanistic 14d feeding study in rats (Munley, 2019): cytochrome P450 enzyme gene expression.

Dose (ppm)	0	2500	12500	25000
mg/kg b.w./d	0	189	923	1841
N	*9*	*10*	*10*	*10*
CYP1A1	1.00 ± 0.47	1.45 ± 1.00	1.31 ± 0.90	1.49 ± 0.65
CYP2B1	1.00 ± 0.45	**8.81* ± 3.30**	**11.90* ± 2.40**	**14.75* ± 5.88**
CYP3A1	1.00 ± 0.40	1.05 ± 0.35	0.96 ± 0.33	1.19 ± 0.28
CYP4A1	1.00 ± 0.97	0.57 ± 0.34	0.45 ± 0.35	0.49 ± 0.28
CYP2E1	1.00 ± 0.62	0.62 ± 0.36	0.56 ± 0.44	0.58 ± 0.36

N= number of rats Activity expressed as fold change (mean ± SD); Statistically significant modification *Dunnett 2-sided, $p<0.05$

Conclusions:
There was no effect of lenacil on thyroid gland weight, morphology and serum T_4 concentrations. Serum T_3 (at top-dose) and TSH (at 12500 ppm and above) concentrations were decreased and serum rT_3 concentrations were increased at all doses.
Lenacil caused increases in hepatic centrilobular hypertrophy, mitotic figures and UDP-glucuronyl transferase (UDPGT) activity in the at 2500 ppm and above. The latter effect was also seen (although not s.s), in the 2500 ppm group (↑26%).
Lenacil caused a dose-dependent increase in hepatic microsomal CYP2B1 enzyme gene expression in all treatment groups (~2-.18× above control). Notifier stated that this pattern was consistent with CAR activation, which is possible but no MoA study was present to underpin this opinion.
The effects on hepatic enzymes at dietary concentrations of lenacil at 12500 ppm and above also correlated with increases in liver weight and liver hypertrophy.
Finally, a satellite experiment assaying the effect of lenacil on thyroid peroxidase on porcine thyroid microsomes, indicated that the a.s. was devoid of any inhibiting activity, suggesting that there was no direct effect of the a.s. on the organification of iodide.

According to the notifier, in the absence of anatomic pathology evidence of hepatic cellular injury, the changes noted in biochemical parameters were considered test substance-related but were consistent with *"an adaptive response of increased metabolism due to exposure to xenobiotics"*.

RMS notes that enzyme induction may indeed well be associated with adverse thyroidal effects. In the case of lenacil it remains debatable to attribute the finding *only* to liver CYP450 enzyme induction. The pattern of effects produced by lenacil (↑rT_3, ↓T_3, no effect on T_4) is not typical for an inductive phenomenon (↓T_4 would be expected), but further mechanisms have not been explored.

In conclusion, it seems plausible that lenacil strongly induces the CYPB1 isoform, and moderately induces UDPGT. While this type of substances may cause induction of certain metabolic enzymes in the liver, this normally results in increased clearance of T_4 by induction of T_4-UDPGT, which is suggestive of increased clearance of THs with concomitant reduction in circulating T_4, Subsequently, this may result in an increase of TSH that, in turn, would stimulate thyroid growth manifested by follicular cell hypertrophy/hyperplasia/neoplasia. While some features are in line with this hypothesis, the hormonal measurements are not *completely* in line with it, leaving the possibility that some other mechanism may play a role, currently incompletely revealed for lenacil.

NOAEL (♂ rats 14d mechanistic thyroid study) = 2500 ppm = **189 mg/kg b.w./d**
LOAEL = 12500 ppm = 923 mg/kg b.w./d, based upon ↑centrilobular hypertrophy, ↑mitoses, ↓T_3, ↑TSH.

B.6.8.3.1.9 Summary and conclusion on the mechanistic studies performed with lenacil.

Since lenacil exhibited effects on the thyroid, uterus and mammary tissue in a number of guideline studies, notifier conducted 7 level 2 *in-vitro* studies and one level-3 *in-vivo* study, according to the OECD Conceptual Framework for testing and assessment of endocrine disrupters. Specific measurements of circulating thyroid hormones (T_3, T_4, TSH) were performed in 2 repeated toxicity studies (10-20 wk and 52 wk), and another non-guideline assay included the assessment of iodine organification with a perchlorate discharge test.

The results were as followed *in-vitro*:

- Lenacil did not competitively bind to the oestrogen receptor in rat uterine cytosol when tested up to a maximum concentration of 10^{-4} M, and was thus considered a non-inhibitor in the oestrogen receptor binding assay.
- Lenacil did neither show oestrogenic agonist nor antagonist activity in the hERα-HeLa-9903 cell line when tested up to a maximum concentration of 10^{-5} M.
- Lenacil did not competitively bind to the androgen receptor when tested up to a maximum concentration of 10^{-4} M, and was thus considered a non-inhibitor in the androgen receptor binding assay.
- Lenacil did not show any androgenic agonist or antagonist activity in a stable transfected CHO-K1 cell line when tested up to a maximum concentration of 10^{-4} M.
- Lenacil did not show any aromatase inhibiting activity in human recombinant microsomes, when tested up to a maximum concentration of 3.16×10^{-5} M.
- Lenacil did not did not alter oestradiol or testosterone synthesis in a steroidogenesis assay in the adrenocorcital H295R cell line, when tested up to a maximum concentration of 3.16×10^{-6} M.
- Lenacil did not inhibit peroxidase in porcine thyrocytes when tested up to a maximum concentration of 5×10^{-4} M.

Further results *in-vivo*:

- Lenacil did not induce changes in uterine parameters associated with oestrogen receptor agonism in ovariectomised adult ♀SD-rats administered by gavage up to 1000 mg/kg b.w./d for 5 consecutive days.
- Lenacil did not inhibit the thyroidal deiodinase/peroxidase in a perchlorate discharge test in adult ♀Wistar-rats administered in the diet up to 4421 mg/kg b.w./d for 20 consecutive weeks.
- Lenacil did not meaningfully alter the circulating thyroid hormone (T_3, T_4) levels in the interim (1yr) sacrificed rats assayed in a 2yr-dietary study up to 1200/1700 mg/kg b.w./d. A weak n.s.s. increase (28-33%) of TSH was observed, which was not considered of high concern in the view of the no-effect on the thyroidal hormones.
- Lenacil did significantly decrease the T3- and TSH-levels in ♂SD rats treated during 2-week in a dietary assay, but the effect was not reproduced in the 10wk- or 52 wk dietary study. T4 levels remained unaltered, while slight modifications of uncertain toxicological significance were found in the 10wk- 20wk- and 52 wk-phases. The thyroid hormone measurements were inconsistent, and also difficult to interpret given the variable experimental set-up.

As complementary information:

- Lenacil did strongly induce the hepatic CYPB1 isoform, and moderately induces UDPGT in ♂SD rats treated in the abovementioned 2-week dietary assay. While this type of substances may cause induction of certain metabolic enzymes in the liver, this normally results in increased clearance of T_4 by induction of T4-UDPGT, which is suggestive of increased clearance of THs. Subsequently, this may result in an increase of TSH that, in turn, would stimulate thyroid growth manifested by follicular cell hypertrophy/hyperplasia/neoplasia. While some features are in line with this hypothesis, the hormonal measurements are not completely in line with it, leaving the possibility that some other mechanism may play a role, currently incompletely revealed for lenacil.

It could thus be concluded that lenacil was devoid of any oestrogenic, anti-oestrogenic, androgenic or anti-androgenic activity, tested at appropriate levels as recommended. Inconsistent effects were noted on the thyroid hormone-levels, but from the guideline studies is became clear that lenacil may be thyrotoxic, however at doses associated with relatively high systemic toxicity, and mainly at doses exceeding the accepted limit dose of 1000 mg/kg b.w./d.

While no reprotoxic effects were reported, it could be discussed whether a DNT study would be desirable, in the case that developing young animals would possibly be more sensitive for neurodevelopmental endpoints.

B.6.8.3.2 Open scientific literature

As previously discussed some publications were highlighted by the notifier to underpin the absence of ED properties of lenacil. The publications are considered to provide complementary information only.

Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. Kojima H, Katsura E, Takeuchi S, Niiyama K, Kobayashi K. Environ Health Perspect. 2004 Apr;112(5):524-31.

Abstract:

"We tested 200 pesticides, including some of their isomers and metabolites, for agonism and antagonism to two human estrogen receptor (hER) subtypes, hERalpha and hERbeta, and a human androgen receptor (hAR) by highly sensitive transactivation assays using Chinese hamster ovary cells. The test compounds were classified into nine groups: organochlorines, diphenyl ethers, organophosphorus pesticides, pyrethroids, carbamates, acid amides, triazines, ureas, and others. These pesticides were tested at concentrations < 10-5 M. Of the 200 pesticides tested, 47 and 33 showed hER- and hERbeta-mediated estrogenic activities, respectively. Among them, 29 pesticides had both hERalpha and hERbeta agonistic activities, and the effects of the organochlorine insecticides beta-benzene hexachloride (BHC) and delta-BHC and the carbamate insecticide methiocarb were predominantly hERbeta rather than hERalpha agonistic. Weak antagonistic effects toward hERalpha and hERbeta were shown in five and two pesticides, respectively. On the other hand, none of tested pesticides showed hAR-mediated androgenic activity, but 66 of 200 pesticides exhibited inhibitory activity against the transcriptional activity induced by 5alpha-dihydrotestosterone. In particular, the antiandrogenic activities of two diphenyl ether herbicides, chlornitrofen and chlomethoxyfen, were higher than those of vinclozolin and p,p -dichlorodiphenyl dichloroethylene, known AR antagonists. The results of our ER and AR assays show that 34 pesticides possessed both estrogenic and antiandrogenic activities, indicating pleiotropic effects on hER and hAR. We also discussed chemical structures related to these activities. Taken together, our findings suggest that a variety of pesticides have estrogenic and/or antiandrogenic potential via ER and/or AR, and that numerous other manmade chemicals may also possess such estrogenic and antiandrogenic activities."

***In vitro* screening of 200 pesticides for agonistic activity via mouse peroxisome proliferator-activated receptor (PPAR)alpha and PPARgamma and quantitative analysis of in vivo induction pathway. Takeuchi S, Matsuda T, Kobayashi S, Takahashi T, Kojima H. Toxicol Appl Pharmacol. 2006 Dec 15;217(3):235-44.**

Abstract:

"Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors and key regulators of lipid metabolism and cell differentiation. However, there have been few studies reporting on a variety of environmental chemicals, which may interact with these receptors. In the present study, we characterized mouse PPARalpha and PPARgamma agonistic activities of 200 pesticides (29 organochlorines, 11 diphenyl ethers, 56 organophosphorus pesticides, 12 pyrethroids, 22 carbamates, 11 acid amides, 7 triazines, 8 ureas and 44 others) by in vitro reporter gene assays using CV-1 monkey kidney cells. Three of the 200 pesticides, diclofop-methyl, pyrethrins and imazalil, which have different chemical structures, showed PPARalpha-mediated transcriptional activities in a dose-dependent manner. On the other hand, none of the 200 pesticides showed PPARgamma agonistic activity at concentrations <or =10(-5) M. To investigate the in vivo effects of diclofop-methyl, pyrethrins and imazalil, we examined the gene expression of PPARalpha-inducible cytochrome P450 4As (CYP4As) in the liver of female mice intraperitoneally injected with these compounds (< or =300 mg/kg). RT-PCR revealed significantly high induction levels of CYP4A10 and CYP4A14 mRNAs in diclofop-methyl- and pyrethrins-treated mice, whereas imazalil induced almost no gene expressions of CYP4As. In particular, diclofop-methyl induced as high levels of CYP4A mRNAs as WY-14643, a potent PPARalpha agonist. Thus, most of the 200 pesticides tested do not activate PPARalpha or PPARgamma in in vitro assays, but only diclofop-methyl and pyrethrins induce PPARalpha agonistic activity in vivo as well as in vitro."

In vitro screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, DR-EcoScreen cells, and in vivo mouse liver cytochrome P450-1A induction by propanil, diuron and linuron. Takeuchi S, Iida M, Yabushita H, Matsuda T, Kojima H. Chemosphere. 2008 Dec;74(1):155-65.

Abstract:

"The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that regulates genes involved in xenobiotic metabolism, cellular proliferation and differentiation. In this study, we have developed a highly sensitive AhR-mediated reporter cell line, DR-EcoScreen cells, which are mouse hepatoma Hepa1c1c7 cells stably transfected with a reporter plasmid containing seven copies of dioxin-responsive element. Using these DR-EcoScreen cells, we performed the reporter gene assay and characterized the AhR agonistic activities of 200 pesticides (29 organochlorines, 11 diphenyl ethers, 56 organophosphorus pesticides, 12 pyrethroids, 22 carbamates, 12 acid amides, 7 triazines, 6 ureas, and 45 others). Eleven of the 200 pesticides (acifluorfen-methyl, bifenox, chlorpyrifos, isoxathion, quinalphos, chlorpropham, diethofencarb, propanil, diuron, linuron, and prochloraz) showed AhR-mediated transcriptional activity. In particular, three herbicides (propanil, diuron, and linuron) have a common chemical structure and showed more potent agonistic activity than other pesticides. To investigate the in vivo effects, we examined the gene expression of AhR-inducible cytochrome P450 1As (CYP1As) in the liver of female C57BL/6 mice intraperitoneally injected with these three herbicides (300 mg kg(-1)) by quantitative RT-PCR, resulting in induction of significant high levels of CYP1A1 and CYP1A2 mRNAs. This indicates that propanil, diuron and linuron possess AhR-mediated transactivation effect in vivo as well as in vitro. Through the present study, we demonstrated that DR-EcoScreen cells are useful for sensitive, rapid and simple identification of AhR agonists among a large number of environmental chemicals."

Comparative study of human and mouse pregnane X receptor agonistic activity in 200 pesticides using in vitro reporter gene assays. Kojima H, Sata F, Takeuchi S, Sueyoshi T, Nagai T. Toxicology. 2011 Feb 27;280(3):77-87.

Abstract

"The nuclear receptor, pregnane X receptor (PXR), is a ligand-dependent transcription factor that regulates genes involved in xenobiotic metabolism. Recent studies have shown that PXR activation may affect energy metabolism as well as the endocrine and immune systems. In this study, we characterized and compared the agonistic activities of a variety of pesticides against human PXR (hPXR) and mouse PXR (mPXR). We tested the hPXR and mPXR agonistic activity of 200 pesticides (29 organochlorines, 11 diphenyl ethers, 56 organophosphorus pesticides, 12 pyrethroids, 22 carbamates, 12 acid amides, 7 triazines, 7 ureas, and 44 others) by reporter gene assays using COS-7 simian kidney cells. Of the 200 pesticides tested, 106 and 93 activated hPXR and mPXR, respectively, and a total of 111 had hPXR and/or mPXR agonistic activity with greater or lesser inter-species differences. Although all of the pyrethroids and most of the organochlorines and acid amides acted as PXR agonists, a wide range of pesticides with diverse structures also showed hPXR and/or mPXR agonistic activity. Among the 200 pesticides, pyributicarb, pretilachlor, piperophos and butamifos for hPXR, and phosalone, prochloraz, pendimethalin, and butamifos for mPXR, acted as particularly potent activators at low concentrations in the order of 10^{-8}-10^{-7} M. In addition, we found that several organophosphorus oxon- and pyributicarb oxon-metabolites decreased PXR activation potency compared to their parent compounds. These results suggest that a large number of structurally diverse pesticides and their metabolites possess PXR-mediated transcriptional activity, and their ability to do so varies in a species-dependent manner in humans and mice."

MINIREVIEW Endocrine-disrupting Potential of Pesticides via Nuclear Receptors and Aryl Hydrocarbon Receptor. Hiroyuki Kojima, Shinji Takeuchi, Tadanori Nagai. J. Health SCiVolume 56 (2010) Issue 4 Pages 374-386

Abstract

"Nuclear receptors (NRs) and the aryl hydrocarbon receptor (AhR) form a ligand-dependent transcription factor that regulates the genes involved in key physiological functions such as cell growth and differentiation, development,

homeostasis, and metabolism. These receptors are potential targets of endocrine-disrupting chemicals (EDCs). To date, many studies have shown that EDCs, such as plasticizers, pesticides, and dioxins, can function as ligands of NRs and AhR. In this review, we focus on recent studies showing that a variety of pesticides, intentionally released into the environment, have agonistic and/or antagonistic activity against NRs and AhR, and present our transactivation assay-based screening results for 200 pesticides against estrogen receptors (ERs), androgen receptor (AR), thyroid hormone receptors (TRs), pregnane X receptor (PXR), peroxisome proliferator-activated receptors (PPARs), and AhR. Our studies have shown that a number of pesticides possess ERα, ERβ, and PXR agonistic activity as well as AR antagonistic activity, whereas none of the pesticides affect the TRα1, TRβ1, and PPARγ-mediated signaling pathways. In addition, several of the 200 tested pesticides were found to have PPARα and AhR agonistic, and ERα and ERβ antagonistic activity. Although the activities of each of these compounds were weak compared to those of endogenous hormone or dioxins, the endocrine-disrupting potential of pesticides, particularly those which function against ERα/β, AR, and PXR, may reflect that of numerous environmental chemicals."

Conclusion:

These high-throughput assays did not particularly focus on the a.s. lenacil under investigation in this DRAR. Therefore, the impact on the discussion regarding the endocrine effect of lenacil is limited.

RMS identified following document, which is considered to provide complementary information:

"*Extended impact assessment study of the human health and environmental criteria for endocrine disrupting substances proposed by HSE, CRD (A Ewence, P Rumsby and I Johnson, 2013).*

The general objective of the study was: "to determine which active substances from the PPP Approved List can be regarded as EDs more likely to pose a risk, which substances require further information, which substances are considered EDs less likely to pose a risk and which substances are not EDs".
Since the PPP Approved List contains over 400 active substances (in 2013), it was agreed that the project would be achieved most effectively by adopting a staged approach, namely:

1) Stage 1 – Conduct of a feasibility study to:
- Initially evaluate the effectiveness of the assessment approach with 20 substances, from different regulatory sources, that have been identified in consultation with HSE.
- Identify any issues that need to be addressed before the evaluation of a wider group of substances is conducted. The knowledge gained from the feasibility study was used to modify the approach adopted in Stage 2 whilst maintaining its scientific rigour.

2) Stage 2 – Application of the finalised and modified methodology to address a larger group of substances in a cost-effective manner. This involved:
*a) Carrying out human health assessments of a further group of approximately 78 substances that were selected by HSE. **Lenacil** was part of this group.*
b) Carrying out detailed ecotoxicological assessments of 20 substances selected by HSE and WRc.

*Results from Stage 2 (**toxicological assessment**):*
The criteria adopted for the human health assessments were able to discriminate the 78 substances into four groups:

- *Group A substances (Substances requiring further information) represented 28.2% (22 of 78) of all the substances evaluated. **Lenacil** was in this group (see **Table 1**, reporting on Table B.46 of the original document)*
- *Group B (Endocrine disrupters more likely to pose a risk) represented 3.8% (3 of 78) of substances.*
- *Group C (Endocrine disrupters less likely to pose a risk) represented 11.5% (9 of 78) of substances*
- *Group D substances (Substances not considered to be endocrine disrupters) were found to be the major group, being 56.4% (44 of 78) of all the substances evaluated.*

Table 1 (reporting on Table B.46 of the original document) : Human Health Endocrine Disruption Evaluation for Lenacil

Table B.46 Human Health Endocrine Disruption Evaluation for Lenacil

Substance details	
Substance Name	**Lenacil**
Substance Synonyms	3-Cyclohexyl-6,7-dihydro-1H-cyclopentapyrimidine-2,4-(3H,5H)-dione
Substance CAS Number	2164-08-1
Substance EC Number	
Data Source(s)	European Union Draft Assessment Report (2007)

Data on the classification of the substance		
Legislation	**Hazard class/classification**	**Hazard statement/risk phrase**
Classification of the substance: Directive 67/548/EEC	-	-
Regulation (EC) No 1272/ 2008	-	-
Is the substance already classified as CMR Category 1A or 1B under the CLP Regulation?	**No**	

Mammalian toxicology data for the evaluation of the endocrine disrupting properties of the substance (informative studies)

Study	Reliability of the data	Adverse effects	Mechanistic information	Reported NOAEL (mg/kg bw/day)	Reported LOAEL (mg/kg bw/day)	Remarks
90-day rat oral study	1	Leucopenia, increased excretion of urinary proteins; lipofuscin staining in thyroid follicular epithelium	No information reported	40	412	Thyroid effects could be due to endocrine disruption.
90-day dog oral study	1	Increased relative liver weight in female dogs, increased relative thyroid and parathyroid weight, centrilobular/midzonal hepatocyte hypertrophy	No information reported	44	221	Thyroid effects could be due to endocrine disruption.
2-year rat oral long-term toxicity and carcinogenicity study	1	Reduced bodyweight gain. Reduced motor activity, organ weight effects, thyroid discolouration, increased thyroidal luminal concretions, centrilobular hepatocyte hypertrophy and vacuolation, mammary gland tumours.	No effect on ability of thyroid to take up and organify iodide. Slight decrease in T4 and T3.	139	1390	Thyroid effects and mammary gland tumours could be due to endocrine disruption.
2-year mouse oral long-term toxicity and carcinogenicity study	1	Hepatocellular adenomas, lung alveolar tumours.	No information reported	332	1358	No evidence of an endocrine effect.
2-generation rat oral reproduction study	1	Parental thyroid toxicity. Decreased offspring bodyweight during lactation. Altered lactation at top dose.	No information reported	Systemic 81 Offspring 89 Reproduction 1727	Systemic 810 Offspring 897 Reproduction 8635	Thyroid effects could be due to endocrine disruption.
Rat oral developmental and teratogenicity study	1	No effects reported	No information reported	Maternal - Developmental -	Maternal - Developmental -	No evidence of an endocrine effect.
Rabbit oral developmental and teratogenicity study	1	Clinical signs and altered bodyweight changes in dams.	No information reported	Maternal 1000 Developmental 4000	Maternal 4000 Developmental -	No evidence of an endocrine effect.

Evaluation of the available mammalian toxicology data for the grouping of the substance regarding its endocrine disrupting properties

Question	Response (Yes/No)	Summary
Are there adverse effects potentially[3] related to endocrine disruption in intact organisms in acceptable studies?	Yes	Thyroid effects and mammary gland tumours could be due to an endocrine mechanism of action.
Does the available evidence[4] demonstrate that an endocrine disruption mode of action in animals is plausible?	No	Mechanistic studies to show conclusively that the thyroid function has been altered or to establish and endocrine disrupter mode of action for the mammary gland tumours are not available.
Are the effects judged to be relevant to humans?	Yes	On the basis of the available evidence, the relevance to humans of the effects on the thyroid and mammary gland cannot be excluded. However, the evidence is insufficient to establish the substance as an endocrine disrupter.

An additional assessment was conducted on the group A substances (substances requiring further information):

- *The substances were assumed to have mechanistic data showing them to be EDs*

- *The toxicity apical data were reassessed and a LOAEL relevant to endocrine-related adverse effects determined – more than one LOAEL may be derived based on different regulatory tests (e.g. 90-days, 2-years and reproduction)*
- *Where there was no relevant LOAEL based on endocrine-related adverse effets in standard toxicity tests, a LOAEL (or LOEL) from an endocrine activity/disruption in vivo screening assay was used in the assessment.*
- *The LOAEL values and the severity of the effects at the LOAELs were compared to the STOT-RE cat. 1 guidance values and the substances ranked as EDs more or less likely to pose a risk. For the overall conclusion for each substance, the lowest LOAEL identifying the highest level of concern was used*

Based on this additional assessment, lenacil was considered ED less likely to pose a risk ("likelihood low").

Table 2 (excerpt): likelihood of lenacil posing a risk, based on the assessment of mammalian toxicity apical data, assuming positive endocrine mechanistic data.

	Lenacil	Yes	**90-day dog oral study** ↑relative liver weight in female dogs, ↑relative thyroid and parathyroid weight, centrilobular/midzonal hepatocyte hypertrophy	221	Low	Thyroid and parathyroid effects could be due to endocrine disruption.
			2-year rat oral long-term toxicity and carcinogenicity study ↓bodyweight gain. ↓motor activity, organ weight effects, thyroid discolouration, ↑thyroidal luminal concretions, centrilobular hepatocyte hypertrophy and vacuolation, mammary gland tumours.	1390	Low	Thyroid effects and mammary gland tumours could be due to endocrine disruption.
			2-generation rat oral reproduction study Parental thyroid toxicity. ↓offspring	810 (systemic)	Low	Thyroid effects could be due to endocrine disruption.

Substance type	Substance	Substance ED grouping (more or less likely to pose a risk) based on the assessment of mammalian toxicology apical data, assuming positive endocrine mechanistic data				Comments
		Further information required	Adverse effects potentially related to an endocrine MoA (underlined)	LOAEL mg/kg bw/day	Likelihood of posing a risk (</>STOT RE 1)	
			bodyweight during lactation. Altered lactation at top dose.			

*Results from Stage 2 (**ecotoxicological assessment**):*

A group of 100 substances (including lenacil) was identified for a more extensive toxicological ED assessment based on a discussion between WRc and HSE. For the identification of appropriate plant protection substances three independent regulatory and non-governmental lists of potential ED have been reviewed to identify those which occur most frequently and, therefore, can be considered to be of greater value to this evaluation. These lists were:

- *European Union List of Potential Endocrine Disrupters as indicated in the EDS 2003 DHI2006 database*
- *The TEDX List of Endocrine Disruptors, which is maintained by The Endocrine Disruption Exchange*
- *United States Environmental Protection Agency Endocrine Disruption Screening Program List*

Lenacil was absent from all three lists.

RMS considers the report also as a source of complementary information, since no new data have been presented; the cited studies are the guideline studies already evaluated in the current DRAR.

B.6.8.3.3 Evaluation taking into account the ED Guidance document

The position paper with the considerations of notifier FMC (Wohlman *et al,* 2019) as regards appendix E of the GD was reproduced here below in italics for information. As stated above, only the NOAELs and final evaluation of the **RMS** at the appropriate places of this DAR should be taken into consideration for further discussion. Relevant parts of the summary and conclusions were considered by the RMS under **B.6.8.3.4** ("Conclusions") and further commented in order to draw final conclusions on the endocrine properties of lenacil.

The paper is based on Appendix E (the excel-file produced to support the evaluation of the various endocrine findings). The XL-file also contains the tab pages with the "Lines Of Evidence, LoE), which lists the effects per endpoint.

In table **B.6.8.3.3-1** RMS indicates which guideline toxicology studies in mammals supported the data of the XL-file.

Table B.6.8.3.3-1 Overview of the guideline mammalian toxicity studies listed in Appendix E for lenacil

Study ID	Year	Study Principle	Species	Doses tested	Dose unit
1	2001	Repeated dose 28-day oral toxicity study in rodents	Rat	0; 5000; 10000; 20000/50000	ppm
2	2002	Repeated dose 90-day oral toxicity study in rodents	Rat	0; 500; 5000; 50000	ppm
3	2004	Repeated dose 90-day oral toxicity study in rodents	Rat	0; 500; 5000; 50000	ppm
4	2003	Combined chronic toxicity/carcinogenicity studies	Rat	0; 250; 2500; 25000	ppm
5	2003	Two-generation reproduction toxicity test	Rat	0; 1000; 10000; 50000	ppm
6	1978	Prenatal developmental toxicity study	Rat	0; 500; 2500; 5000	ppm
7	1996	Prenatal developmental toxicity study	Rat	0; 500; 1000; 4000	mg/kg bw/day
8	2003	Prenatal developmental toxicity study	Rat	0; 100; 300; 1000	mg/kg bw/day
9	1991	Repeated dose 90-day oral toxicity study in rodents	Mouse	0; 100; 1000; 5000; 10000	ppm
10	1994	Carcinogenicity	Mouse	0; 100; 2500; 7000	ppm
11	2001	Repeated dose 28-day oral toxicity study in non-rodents	Dog	0; 5000; 20000; 50000	ppm
12	2002	Repeated dose 90-day oral toxicity study in non-rodents	Dog	0; 1000; 5000; 25000	ppm
13	1991	Prenatal developmental toxicity study	Rabbit	0; 50; 200; 1000; 4000	mg/kg bw/day
14	2018	AR Binding Assay	Rat	0; 0.0001; 0.001; 0.01; 0.1; 1; 10; 100; 1000	µM
15a	2018	Stably Transfected Human AR Transactivation Assay (AR STTA)	Human	0.00001; 0.0001; 0.001; 0.01; 0.1; 1	µM
15b	2018	Stably Transfected Human AR Transactivation Assay (AR STTA)	Human	0.000001; 0.00001; 0.0001; 0.001; 0.01; 0.1; 1	µM
16	2018	ER Binding Assay	Rat	0; 0.0001; 0.001; 0.01; 0.1; 1; 10; 100; 1000	µM
17a	2018	Stably Transfected Human ERα Transcriptional Activation Assay (ER STTA)	Human	0; 0.00001; 0.0001; 0.001; 0.01; 0.1; 1; 10	µM
17b	2018	Stably Transfected Human ERα Transcriptional Activation Assay (ER STTA)	Human	0.0001; 0.001; 0.01; 0.1; 1.0; 10	µM
18	2018	Uterotrophic assay	Rat	0; 500; 1000	mg/kg bw/day
19	2004	Repeated dose 10/20 wk oral toxicity study in rodents ("*Mammals Not in list*")	Rat	0; 250 50000	ppm
22*	2019	Repeated dose 14-day oral toxicity study in rodents	Rat	0; 2500; 12500; 25000	ppm

*: added by **RMS** (not yet in submitted Appendix E document)

Position paper of notifier FMC: lenacil (B0634) technical: assessment of experimental data to characterise evidence of endocrine disrupting potential (Wohlman et al, 2019).

(Report: FMC Corporation - FMC Agricultural Solutions Stine Research Center Newark, Delaware 19711 USA, Project Identification Number FMC-51816).

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LIST OF ABBREVIATIONS

ADME	*Absorption, Distribution, Metabolism, Excretion*
AhR ALP	*Aryl hydrocarbon receptor alkaline phosphatase*
AR	*androgen receptor*
ARTA	*androgen receptor transactivation assay*
AST	*aspartate transaminase*
BW	*body weight*
BWG	*body weight gain*
CF	*conceptual framework*
CTL	*control*
CYP450	*cytochrome P450*
Dev	*developmental*
DHT	*dihydrotestosterone*
EATS	*Oestrogen, Androgen, Thyroid, Steroidogenesis*
EC	*European Commission*
ED	*endocrine disruptor*
ER	*oestrogen receptor*
ERTA	*oestrogen receptor transactivation assay*
EU	*European Union*
EChA	*European Chemicals Agency*
EFSA	*European Food Safety Authority*

FC	*food consumption*
FSH	*follicle stimulating hormone*
GD	*gestational day*
GnRH	*gonadotropin releasing hormone*
HB	*haemoglobin*
HCD	*historical control data*
hCG	*human chorionic gonadotropin*
HT	*haematocrit*
LH	*luteinizing hormone*
LHRH	*luteinizing hormone-releasing hormone*
NA	*not applicable*
NOAEL	*No Observed Adverse Effect Level*
NOEL	*No Observed Effect Level*
OECD	*Organization for Economic Co-operation and Development*
PND	*post-natal day*
RBC	*red blood cells*
SDH	*sorbitol dehydrogenase*
TG	*test guideline*
TR	*thyroid receptor*
UDPGT	*uridine 5'-diphospho-glucuronosyltransferase*

LENACIL (B0634) TECHNICAL: ASSESSMENT OF EXPERIMENTAL DATA TO CHARACTERISE EVIDENCE OF ENDOCRINE DISRUPTING POTENTIAL

Irene M. Wohlman, Tessa Scown, Michael Battalora

1.0 SUMMARY

The potential of lenacil (B0634) to interact with the endocrine system in mammals and wildlife has been assessed based on the available data. Lenacil has been tested extensively in a wide range of both in vivo and in vitro studies for effects on mammalian and non-mammalian (ecotoxicology) endpoints, including those pertaining to the endocrine system.

Following a review of all available and relevant data from mammalian and non-mammalian toxicology studies and based on a weight of evidence evaluation, it is concluded that lenacil is not an "endocrine disruptor" according to the regulatory definition.

INTRODUCTION

Criteria for the identification of plant protection products as potential endocrine disruptors were recently adopted by the EU Commission (19 April 2018) and officially entered into force on 10 May 2018 (EU, 2018a). As of 10 November 2018 the criteria apply to all ongoing applications for plant protection products in the EU. The criteria are aligned with the definition of an endocrine disruptor (ED) as defined by the IPCS/WHO (2002). Based on a request by the European Commission, the European Food Safety Authority (EFSA) and the European Chemicals Agency (EChA) have developed a guidance document for identifying chemicals that may be potential endocrine disruptors. That document entitled Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 was published on 7 June 2018 (EU, 2018b).

Based on Commission Regulation (EU) No 2018/605, information on the potential for endocrine disruption of a substance should be based on a weight of evidence approach and can be obtained from existing data, read-across from structurally similar chemicals, in silico tools, in vitro and in vivo screening assays and/or from mechanistic studies (EU, 2018a).

Assays appropriate for use in the weight of evidence determination are discussed in the OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors (OECD, 2018b). Using combinations of Level 1- Level 5 assays, endocrine disruptors can be identified according to their adverse effects on apical endpoints, taking into account severity, reversibility, potency and consistency. Currently, the most complete testing battery exists for oestrogen, androgen, thyroid and steroidogenesis (EATS) modalities, while non-EATS modalities will require further development in the future to allow reliable assessments.

Using a weight of evidence approach, it is possible to determine if a substance meets the following criteria relevant to plant protection products within the EU framework. A substance is considered to have endocrine disrupting properties if all the below are true [underlining added by authors]:

"It shows an adverse effect in an intact organism or its progeny, which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;

It has an endocrine mode of action, i.e., it alters the function(s) of the endocrine system; and

The adverse effect is a consequence of the endocrine mode of action" (EU, 2018a).

The term 'endocrine mode of action' should be interpreted as endocrine activity since endocrine mode of action includes both endocrine activity and a biologically plausible link to an adverse effect. 'Endocrine activity' is the capacity to alter the function(s) of the endocrine system, and an adverse effect is a consequence of the endocrine activity in this context. As part of a hazard identification strategy, these criteria should be assessed for both humans and non-target organisms (EU, 2018b).

3.0 DATA ACQUISITION AND EVALUATION

Data Acquisition

Studies upon which this assessment are those included in the Draft (Renewal) Assessment Report, Lenacil, Volume 3-B.6 (AS) Toxicology and Metabolism and Volume 3-B.9 (AS) Ecotoxicology, both dated 21.Dec.2018. In addition, three endocrine studies that were recently conducted are also included.
In addition, a literature search and review was conducted in fulfilment of EU data points: KCA Section 9/KCP Section 11; Submission of scientific peer-reviewed open

literature under Regulation (EC) No 1107/2009. For a list of references, evaluations and reliability scoring of the individual items, please refer to the Literature Review Report in M-CA Section 9, Literature Data, DuPont-43896 EU of the dossier.

Data Reliability
This assessment report is based primarily upon the interpretation of data from OECD/EPA-approved guideline studies. Both guideline and non-guideline studies were performed in accordance with GLP principles apart from two in vitro studies. Each study was evaluated for reliability using the methods of Klimisch, et al. (1997) and was assigned a Reliability Score according to the criteria in Table 1 below:

TABLE 1KLIMISCH RELIABILITY CATEGORIES

RELIABILITY SCORE	*CATEGORY*
1	*Reliable without restriction*
2	*Reliable with restriction*
3	*Not reliable*
4	*Not assignable*

Reliable without Restriction: Data from guideline studies or literature reports conducted or generated using valid and/or nationally/internationally accepted guidelines and performed according to GLP. All parameters described should be comparable to a guideline method.
Reliable with Restrictions: Data from studies, literature or reports in which test parameters did not comply completely with specific test guidelines but were well-documented and are scientifically acceptable.
Not Reliable: Included in this category are studies or data from the literature and/or reports in which the measuring system and the test substance interfere, organisms/test systems were used which were not relevant to the exposure or were conducted or generated according to an unacceptable method. In addition, the documentation of the studies or data is insufficient for an assessment and unconvincing for expert judgement.
Not Assignable: Data or studies from the literature lacking experimental details and listed only in brief abstracts or secondary literature.
Studies considered both relevant and reliable with a Klimisch criteria score of either 1 or 2 are included for consideration in this assessment.

Data from Standard Studies
To assess the potential endocrine disruption activity of lenacil, results from studies listed in the OECD GD 150 Conceptual Framework (CF) for Testing and Assessment of Endocrine Disruptors, Annex II, were evaluated with a focus on endpoints that are potentially indicative of endocrine-mediated effects (OECD, 2018). Studies in the CF are grouped in Levels 1 – 5:
Level 1 comprises existing information on physical/chemical properties, QSARs, absorption-distribution-metabolism-excretion (ADME) model predictions;
Level 2 includes in vitro assays providing data on selected endocrine mechanisms/pathways;
Level 3 includes in vivo assays covering selected endocrine mechanisms/pathways;
Level 4 assays are in vivo studies providing data on adverse effects on endocrine- relevant endpoints; and
Level 5 assays comprise in vivo studies which provide more comprehensive data on endocrine-relevant endpoints over more extensive periods of an organism's life cycle (EU, 2018b).

In this assessment for lenacil, OECD Conceptual Framework Levels 1 – 5 analyses and studies with endpoints indicative of potential EATS-mediated activity were reviewed using a weight of evidence approach. A QSAR analysis of ER binding with evaluations from five models was presented as a Level 1 evaluation.
Level 2 and 3 studies include in vitro and in vivo mechanistic evaluations of androgen and oestrogen receptor interaction, steroid hormone biosynthesis (e.g. oestradiol and testosterone), thyroid hormone levels (T4, T3, rT3), TSH, thyroid iodide uptake, CYP450 content and UDPGT activity.
Level 4 mammalian studies include the repeat-dose 28-day and 90-day studies in rats, mice and dogs, prenatal developmental toxicity studies in rats and rabbits, and long-term toxicity studies in rats, mice and dogs.
The two-generation reproduction study in rats is a Level 5 study. The number of organs or tissues subject to evaluation (principally organ weight, and/or histopathological assessment) depended on the study type. Parameters evaluated included body weight, body weight gain, food consumption, food efficiency, clinical signs, haematology, clinical chemistry, urinalysis, ophthalmology, organ weights, and gross and microscopic pathology. Endocrine or endocrine-related organs weighed at necropsy included adrenals, liver, kidney, testes, thyroid (rat and dog) and uterus. Tissues collected and processed for histological evaluation included adrenals, epididymides, kidneys, liver, mammary glands, ovaries, pancreas, pituitary, prostate, seminal vesicles, testes, thyroid-parathyroid, uterus and vagina.
Level 4 studies conducted in non-target vertebrates and invertebrates include a fish early life stage study, an avian reproduction test with bobwhite quail and reproduction studies in daphnia and earthworms.

Data from Literature Review

Summaries of the relevant literature found in the review described in Section 3.1.1 have been included in Section 6.0 as supplemental information.

4.0 STUDIES ASSESSING MAMMALIAN ENDOCRINE ACTIVITY ENDPOINTS

OECD Level 1 Existing / Non-test information

4.1.1 High Level QSAR Analysis of Lenacil for Oestrogen Receptor Binding. DuPont- 47276. (Kurubaran, S., 2016).
Summary: Using the OECD toolbox, and considering the profilers for oestrogen receptor (ER) binding and the rtER Expert System ver. 1 (US EPA), lenacil revealed no structural alerts for ED properties in the rtER Expert System ver. 1 profiler. It was also found to be a non-binder, without OH or NH2 groups in the ER binding profiler. Based on the outcome of the OECD Toolbox screen, it can be concluded that lenacil is not expected to have oestrogen receptor binding properties.
Conclusion: Lenacil does not contain the pharmacophoric phenolic moiety and is predicted to not bind the oestrogen receptor.

OECD Level 2 In Vitro Mechanistic Studies Assessing Mammalian Endocrine Activity Endpoints

4.2.2 Oestrogen Modality
Lenacil (DPX-B0634) Technical: Oestrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC). DuPont-49349. (Nabb, D.L., 2018).
Guideline: U.S. EPA Health Effects Test Guidelines, OPPTS 890.1250 (2009)
GLP: Yes
Reliability Score: 1
Summary: Lenacil (DPX-B0634) was evaluated for its ability to competitively bind to the oestrogen receptor in rat uterine cytosol by measuring the binding of a radioligand to an oestrogen receptor with increasing concentrations of a competing test substance. A saturation binding assay measured the affinity of a radiolabelled oestrogen ligand, 17□-oestradiol ([^{3}H-]17□-oestradiol) for the oestrogen receptor (K_d) and the concentration of the oestrogen receptors (B_{max}) present in the cytosol, by measuring specific binding of increasing concentrations of radioligand under conditions of equilibrium. Three independent runs were performed using [^{3}H]-17□- oestradiol as the radioligand to characterise the rat uterine cytosol. The K_d was approximately 0.355 □□0.024 nM for [^{3}H]-17□-oestradiol, and the B_{max} was approximately 106.6 □□7.06 fmol/100 □g protein, which is consistent with the acceptable range in the test guideline.

Three independent runs were performed to evaluate lenacil for its ability to compete with [³H]-17□-oestradiol in binding to rat uterine oestrogen receptors in vitro.

Lenacil was evaluated at eight concentrations ranging from 1 □□10^{-10} to 1 □□10^{-3} M. Radioinert 17□-oestradiol, the oestrogen receptor agonist and a positive control, 19- norethindrone, an oestrogen receptor agonist used as the weak positive control and octyltriethoxysilane, a non-oestrogen receptor agonist used as the negative control, were used to verify test system performance.

Results:

Radioinert 17□-oestradiol and 19-norethindrone showed effects consistent with strong and weak competitive binding, respectively, and octyltriethoxysilane did not compete for binding to the oestrogen receptor in all three runs. The $logIC_{50}$ was determined to be approximately -8.92 □□0.03 and -5.76 □□0.01 for radioinert 17□- oestradiol and 19-norethindrone, respectively. A $logIC_{50}$ was not determined for the test substance since there were no test substance-related effects on oestrogen receptor binding up to the concentration of 1 □□10^{-3} M, which represents the highest concentration recommended by the test guideline.

Conclusion:

Under the conditions of the study, lenacil did not competitively bind to the oestrogen receptor when tested up to a maximum concentration of 1 □□10^{-3} M, and therefore, is classified as a non-inhibitor in the oestrogen receptor binding assay.

Evaluation of the Oestrogenic Agonist and Antagonist Activity of Lenacil (DPX- B0634) Technical Using the Stably Transfected Human Oestrogen Receptor-α Transactivation Assay (hER□-HeLa-9903 cell line). Report No. 49351. (Rijk, J., 2018).

Guideline: OECD Guideline for the Testing of Chemicals No. 455 (2016)

GLP: Yes

Reliability Score: 1

Summary:

Lenacil (DPX-B0634) was evaluated for its ability to act as either an agonist or antagonist of human oestrogen receptor alpha (hER□) using the human hER□-HeLa-9903 cell line. Preliminary assessments of cytotoxicity and solubility were conducted in order to identify suitable top concentrations for use in the transcriptional activation assays. Dimethyl sulfoxide (DMSO) was selected as the vehicle and was not shown to have a significant effect on the assay. Two independent experiments were performed for both the oestrogen receptor (ER) agonist and ER antagonist assay.

ER agonist assay: Within each ER agonist experiment lenacil was tested at seven concentrations together with complete concentration-response curves of the reference items 17ß-oestradiol (E2), 17α-oestradiol, 17α-methyltestosterone, and corticosterone. Lenacil was dissolved in DMSO at final testing concentrations of 10 pM to 10 µM. There was no cytotoxicity (≥20% reduction in cell viability) observed for the test items or the controls in the valid independent experiments.

ER antagonist assay: Within each ER antagonist experiment lenacil was tested at six concentrations together with complete concentration-response curves of the reference items tamoxifen and flutamide. Lenacil was dissolved in DMSO at final testing concentrations of 100 pM to 10 µM. There was no cytotoxicity (≥20% reduction in cell viability) observed for the test items or the controls in the valid independent experiments.

Results:

Lenacil did not show an oestrogen agonist response in two independent experiments (RPCmax ≤10%), and a log IC_{30} could not be determined for lenacil in two independent experiments in the antagonist assay, and as such, was considered negative for both ER agonist and ER antagonist effects.

Conclusion:

The assay was considered valid. Based on the results of this study, lenacil did not show any oestrogenic agonist or antagonist activity in the hERα-HeLa- 9903 cell line.

4.2.2 Androgen Modality

Lenacil (DPX-B0634) Technical: Androgen Receptor Binding Assay Using Rat Prostate Cytosol (AR-RPC). DuPont-49367. (Nabb, D.L., 2018).

Guideline: USEPA Health Effect Test Guidelines OPPTS 890.1150 (2009)
GLP: Yes
Reliability Score: 1
Summary:
Lenacil (DPX-B0634) was evaluated for its ability to bind to the androgen receptors in rat prostate cytosol. The affinity of a radiolabelled androgen ligand ([^{3}H]-R1881) (K_d) for the androgen receptor and the concentration of the androgen receptors (B_{max}) present in the cytosol were determined by measuring specific binding of increasing concentrations of radioligand under conditions of equilibrium. Three independent runs were performed using hexatritiated R1881 ([^{3}H]-R1881) as the radioligand to characterise the rat prostate cytosol.
In a competitive binding assay, three independent runs were performed to evaluate lenacil for its ability to compete with [³H]-R1881 in binding to rat prostate androgen receptors in vitro. Lenacil was evaluated at eight concentrations between 1 □□ 10^{-10} and 1 □□ 10^{-3} M. Radioinert R1881, the androgen receptor agonist positive control, and dexamethasone, a weak androgen receptor agonist used as the weak positive control, were used to verify test system performance.
Results:
Radioinert R1881 showed effects consistent with strong competitive binding, and dexamethasone showed effects consistent with weak competitive binding to the androgen receptor in all three runs. The logIC_{50} was determined to be approximately -8.99 (± 0.02) and -4.63 (± 0.04) for radioinert R1881 and dexamethasone, respectively. A logIC_{50} was not determined for the test substance since there were no test substance-related effects on androgen receptor binding up to the concentration of 1 □□10^{-3} M, which represents the highest concentration recommended to be tested according to the US EPA OPPTS guideline 890.1150. Slight precipitation was observed in the test substance assay tubes at 1 x10^{-3}M after overnight incubation, therefore, the top suitable concentration of the test substance was 1 □□10^{-4} M.
Conclusion:
Under the conditions of the study, lenacil did not competitively bind to the androgen receptor when tested up to a concentration of 1 □□10^{-4} M. Therefore, lenacil is classified as a non-inhibitor in the androgen receptor binding assay.
Evaluation of the Androgenic Agonist and Antagonist Activity of Lenacil (DPX- B0634) Technical Using the Stably Transfected Human Androgen Receptor Transcriptional Activation Assay (AR-EcoScreen□). FMC Report No. 50113. (Rijk,J. 2018).

Guidelines: OECD Guideline for the Testing of Chemicals No. 458
GLP: Yes
Reliability Score: 1
Summary:
Lenacil (DPX-B0634) was evaluated for its ability to act as either an agonist or antagonist of the human androgen receptors (hAR) using the AR- EcoScreen□□cell line. Preliminary assessments of solubility were conducted to identify suitable top concentrations for use in the transcriptional activation assays. Dimethyl sulfoxide (DMSO) was selected as the vehicle for the test item and was not shown to have a significant effect on the assay. For the test item, two independent experiments were performed for both the AR agonist and AR antagonist assay.
AR antagonist assay: Within each AR agonist experiment, the test item was tested at seven concentrations together with vehicle controls, positive controls, and a complete concentration-response range for the reference items 4,5α-dihydrotestosterone (DHT), mestanolone, and di(2-ethylhexyl)phthalate (DEHP). The final testing concentrations of lenacil were 1 pM to 1 µM.
AR antagonist assay: Within each AR antagonist experiment, the test item was tested at six concentrations together with vehicle controls, positive controls, and complete concentration-response range of the reference items hydroxyflutamide (HF), bisphenol A (BPA), and DEHP. The final testing concentrations of lenacil were 10 pM to 1 µM.
Results:
In the agonist assays, the maximum level of response induced by lenacil compared to the response induced by 10 nM DHT (the RPC_{max}) was below 10% in both independent experiments. A log IC_{30} could not be determined for the test item in the antagonist assay.
Conclusion:

Both assays were considered valid. Lenacil did not show any androgen receptor agonist or antagonist activity in the AR-EcoScreen cell line.

Thyroid Modality
There are no validated Level 2 studies on thyroid-related endpoints currently available. However, assays assessing iodination of thyroid hormone were included in a mechanistic in vivo thyroid study in rats presented in section 4.3.2.2 ([redacted] 2004), where the ability of the thyroid to take up and accumulate ^{125}Iodide and the ability of thyroid peroxidases to convert the ^{125}Iodide to organic compounds was assessed, and found not to be affected by lenacil administration. In addition, there was no evidence of an effect on thyroid peroxidase in vitro, as assessed in porcine thyroid microsomes presented in section 4.3.2.1 ([redacted] 2019).

Steroidogenesis Modality
Lenacil (DPX-B0634) Technical: In Vitro Aromatase Inhibition using Human Recombinant Microsomes. Report No. FMC-51364. (Rijk, J.C., 2019).
Guideline: U.S. EPA Health Effects Test Guidelines, OPPTS 890.1200 (2009)
GLP: Yes
Reliability Score: 1
Summary:
Lenacil (DPX-B0634) was evaluated for its ability to inhibit the catalytic activity of human recombinant microsomal aromatase (CYP19) in an in vitro $^{3}H_2O$- aromatase assay. A radioactive substrate (^{3}H-androstenedione) and reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) are added to human microsomes containing the aromatase (CYP19) and reductase complex. $^{3}H_2O$ released during the conversion of androstenedione (ASDN) to estrone is quantified as a direct measurement of aromatase activity per unit reaction time. A total of four independent experiments were performed in triplicate. Within each experiment, the test item was evaluated at eight concentrations (from 0.00001 to 31.6 µM) together with a complete dose response curve of the positive control inhibitor 4-hydroxy-androstenedione (4- OH-ASDN). Ethanol (EtOH) was used as the vehicle and the concentration of vehicle in the incubations was kept constant at 0.1% (v:v).
Results:
The solubility of the test item in EtOH was assessed up to 100 mM. The highest dissolvable concentration of the test item in EtOH was 3160 µM. No precipitate or cloudiness was observed in this stock solution. Therefore, this concentration was used to prepare the top exposure concentration in the first aromatase assay experiment (1:100 dilution for final concentration in the tube of 31.6
µM). No precipitation of the test item was observed in any of the incubation mixtures.
In each of the four aromatase assay experiments the mean aromatase activity in the absence of inhibitor (the full enzyme activity control) was above 0.1 nmol/mg protein/min and the mean of the background activity controls was ≤ 10% and therefore met the acceptance criteria. The average of the background activity controls was within the acceptable range of -5% to 6% in each of the four experiments. The average of the full enzyme activity controls was within the acceptable range of 90% to 110% in each of the four experiments, demonstrating that the conditions throughout the individual aromatase experiments were consistent. For the positive control runs, all curve fit parameters were within the performance criteria. The mean IC_{50} value obtained for 4-OH-ASDN across runs was 60.3 ± 17.9 nM (mean ± SD).
For lenacil, no log IC_{50} value was obtained in any of the four aromatase assay experiments. The average lowest portion of the curves across runs was 86.6%. Since this is > 75%, the test item was classified as a non-inhibitor in the aromatase assay.
Conclusions:
All four aromatase assay experiments were valid, therefore the test item lenacil was classified as a non-inhibitor in the aromatase assay.

Screening Lenacil (DPX-B0634) Technical for Modulation of Steroidogenesis using the Human H295R Adreno-carcinoma Cell Line. Report No. FMC-51365 Revision 1. (Verkaart, S., 2019).

Guideline: OECD Guideline for the Testing of Chemicals No. 456 (2011)
GLP: Yes
Reliability Score: 1
Summary:
The potential of lenacil to modulate the steroidogenic pathway, beginning with the sequence of reactions from cholesterol through the production of testosterone and estradiol, was investigated using the human H295R cell line. Three valid steroidogenesis assay experiments were performed whereby the test item was tested at seven concentrations (up to 3.16 µM) together with the positive control inducer, forskolin, and positive control inhibitor, prochloraz.
Dimethylsulfoxide (DMSO) was used as the vehicle and the concentration of vehicle in the incubations was kept constant at 0.1% (v:v). H295R cells were exposed for 48 hours to the vehicle, lenacil and positive controls. After exposure, the viability of the cells was determined using the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The concentration of estradiol and testosterone in the exposure medium was determined using commercial Enzyme-Linked Immuno Sorbent Assays (ELISAs).
Results:
The assay acceptability criteria (basal hormone production) within plate coefficient of variation (CV) for the DMSO solvent controls, hormone measurement sensitivity, induction of hormone production upon exposure of 10 µM forskolin exposure and inhibition of hormone production upon exposure of 1 µM prochloraz, were all met for each experiment. Therefore, all steroidogenesis assay experiments were considered valid.
In experiment 1, estradiol synthesis was significantly decreased at 0.1 nM while there was no significant effect on testosterone synthesis. Since no dose-related effect was observed for estradiol (no statistically significant effect was observed at two or more adjacent concentrations) the results of the first steroidogenesis experiment were considered spurious.
In experiments 2 and 3, Lenacil had no significant effect on either estradiol or testosterone synthesis in H295R cells up to 3.16 µM, and the results of the second and third steroidogenesis experiment were considered to be negative.
Conclusion:
Since the spurious result for estradiol synthesis in the first experiment could not be confirmed in the two subsequent experiments, overall the results of the were considered to be negative. Lenacil did not alter estradiol or testosterone synthesis in H295R cells and therefore was considered to be negative in the steroidogenesis assay.

OECD Level 3 In Vivo Mechanistic Studies Assessing Mammalian Endocrine Activity Endpoints

4.3.1 Oestrogen Modality
4.3.1.1 Lenacil (DPX-B0634) Technical: 6-Day Uterotrophic Assay for Detecting Oestrogenic Activity in Ovariectomized Rats. Report No. 49350. ([redacted] 2018).
Guidelines: USEPA Health Effects Test Guidelines OPPTS 890.1600 (2009); OECD Guideline for the Testing of Chemicals (Part 440) (2007)
GLP: Yes
Reliability Score: 1
Summary:
The objective of this study was to evaluate the potential oestrogenic effects of lenacil when administered by oral gavage to ovariectomized rats for 5 days. Five groups of young-adult ovariectomized Crl:CD(SD) rats (15/group) were administered either test substance or positive control substance once daily by oral gavage for 5 consecutive days. Lenacil was administered at 0, 500 or 1000 mg/kg/day. Rats in the negative control group received vehicle only (0.5% methylcellulose). Two separate positive control groups were included. A positive control group was administered 0.1 mg/kg/day of the oestrogen receptor agonist 17α- ethynyl estradiol dissolved in vehicle (corn oil with 1% ethanol). The test substance was homogeneous, at targeted concentrations and stable in the vehicle. The dose formulations for the positive control groups were not evaluated. Body weight, food consumption and clinical observations were recorded daily. Vaginal cytology was evaluated daily to assess the potential of the test substance to induce cytological changes consistent with those observed with the 17α-ethynyl estradiol positive control. At scheduled euthanasia, uterine weights were collected to assess the ability of the test substance to induce uterine growth.
Results:

There were no mortalities, no test substance-related effects on body weight, nutritional parameters or clinical signs. All animals in both test substance treatment groups remained in diestrus for the duration of the study. There were no test substance-related effects on uterine weight or gross observations.
There were no clinical signs observed in rats administered 17α-ethynyl estradiol. Final mean body weight, overall mean body weight gain, overall mean food consumption and overall mean food efficiency were all lower ($p < 0.05$) compared to control. Estrus stage effects were observed in all 15 animals administered 17α- ethynyl estradiol with cytological markers indicative of either proestrus or oestrus observed in all rats by test day 4. Absolute uterine wet weight and blotted wet weight were 265% and 254% of vehicle control, respectively. Relative (to final body weight) uterine wet weight and blotted wet were 288% and 272% of vehicle control, respectively. The results with 17α-ethynyl estradiol are consistent with an oestrogen receptor agonist.
Conclusion:
Under the conditions of this study, lenacil did not induce changes in parameters associated with oestrogen receptor agonism in ovariectomized adult female rats administered up to 1000 mg/kg/day for 5 consecutive days.

4.3.2 Thyroid/HPT Axis
The following study is available upon request.
RMS: the study was provided to the RMS and evaluated

Lenacil (DPX-B0634) Technical: Thyroid Mechanistic 14-Day Feeding Study in Rats. Report No. 49352. ([redacted] 2019).
Guideline: Not applicable
GLP: Yes
Reliability Score: 1
Summary:
Potential mechanisms for effects on thyroid weight/histopathology with DPX-B0634 were evaluated. Four groups of young adult male rats, Crl:CD(SD) (15/sex/group) were administered diets containing 0, 2500, 12,500 or 25,000 ppm DPX-B0634 for 14 days. The test substance was homogeneous, at targeted concentrations, and stable in the diet. The overall mean daily intake of DPX-B6034 in the 2500, 12,500 or 25,000 ppm groups was 189, 923 and 1841 mg/kg/day, respectively. Body weights, food consumption, and clinical observations were evaluated weekly and acute clinical observations were evaluated daily. Blood was collected at sacrifice (test day 15) of test substance administration for analysis of serum concentrations of thyroid hormones [thyroxine (T4), triiodothyronine (T3), reverse T3 (rT3), and thyroid stimulating hormone (TSH)] using commercially- available radioimmunoassay kits. At necropsy, liver and thyroid glands were collected, weighed, and saved for microscopic evaluation. A section of liver was collected and microsomes were prepared by differential centrifugation for evaluation of UDP-glucuronyltransferase (UDPGT) activity. A separate section of liver was flash frozen and prepared for cytochrome P450 gene expression.
All animals survived to scheduled sacrifice and there were no test substance-related clinical observations. No adverse test substance related effects on body weight or nutritional parameters were observed.
Increased liver weight (compared to control) and minimal hepatocellular hypertrophy were present at dietary concentrations of 12,500 and 25,000 ppm. Hepatic UDP- glucuronyl transferase activity was higher in the 12,500 and 25,000 ppm treatment groups and hepatic cytochrome P450 2B1 (CYP2B1) gene expression was higher in all treatment groups compared to control. There were no test substance-related anatomic pathological effects on the thyroid gland in any treatment group. Serum rT3 concentration was minimally, but statistically significantly higher in the 12,500 and 25,000 ppm groups and serum T3 and TSH concentrations were minimally, but statistically significantly lower in the 25,000 ppm group compared to control. No statistically significant alterations were observed in serum T4 concentration. Additionally, there was no evidence of an effect on thyroid peroxidase, as assessed in porcine thyroid microsomes in vitro. Consistent with an adaptive response, hepatic UDP-glucuronyl transferase (UDPGT) activity and hepatic microsomal CYP2B1 gene expression were increased in a test substance-related manner. Although hepatic UDP-glucuronyl transferase activity was higher in the 12,500 and 25,000 ppm treatment groups, no difference was observed in serum T4 concentration in either treatment group. Furthermore, there were no effects on thyroid organ weights, no evidence of thyroid follicular cell hypertrophy and thyroid stimulating hormone was not elevated.
Conclusion:

There was no effect of lenacil on thyroid gland weight, morphology and serum T4 concentrations. The minimal changes in serum TSH, T3 and rT3 concentrations may have represented biological variation, and/or subtle adaptive changes in hormonal regulation. These data demonstrate that there was no evidence of an adverse effect on thyroid hormone economy. Lenacil caused minimal increases in hepatic microsomal hypertrophy and hepatic UDP-glucuronyl transferase activity in the 12,500 and 25,000 ppm treatment groups. Although hepatic UDP-glucuronyl transferase activity was increased, there was no effect on serum T4 and no increase in serum TSH concentrations in either treatment group. Lenacil caused an increase in hepatic microsomal CYP2B1 enzyme gene expression in all treatment groups consistent with CAR activation. The effects on hepatic enzymes at dietary concentrations of 12,500 and 25,000 ppm also correlated with the increases in liver weight and liver hypertrophy. In the absence of anatomic pathology evidence of hepatic cellular injury, the changes noted in biochemical parameters were considered test substance-related and were consistent with an adaptive response of increased metabolism due to exposure to xenobiotics.

Lenacil Technical Investigation into Potential Effects on Thyroid Function After 20 Weeks of Treatment in Female Han Wistar Rats Using the "Perchlorate Discharge Test". DuPont Report No. ACD 060/033946. ([redacted] 2004).
Lenacil Technical Investigation into Potential Effects on Thyroid Function After 20 Weeks of Treatment in Female Han Wistar Rats Using the "Perchlorate Discharge Test". DuPont Report No. ACD 060/033946, Report Amendment No. 1. ([redacted] 2007).
Guideline: None
GLP: Yes
Reliability Score: 1
Summary: Two groups of 18 female Han Wistar rats received lenacil (Batch No. 141712003, purity 98.6%) in the diet at dosages of 250 or 50,000 ppm over a period of 20 weeks. A similarly constituted untreated control was included as a negative control. A positive control group received propylthiouracil (Batch No. 32K2526, purity 99%), an inhibitor of iodide organification, at a dosage of 200 mg/kg/day by gavage for the last 2 weeks only (weeks 19 and 20).

Clinical and detailed physical observations, bodyweight, food consumption, blood chemistry, organ weight and macropathology investigations were made in addition to the investigations on perchlorate discharge at the end of the study. The accuracy of the test formulations was confirmed by analysis of the diets prepared for administration.

Results:
Lenacil-treated rats: Mean daily intakes of lenacil for the 250 and 50,000 ppm groups were 21 and 4421 mg/kg bw/day. There were no unscheduled deaths. An increased incidence of hair loss, poor grooming and brown stained tails was recorded at top dose. There were no changes in body weight. Mean body weight gain was marginally lower (8%) at the top dose but not statistically significant. Food intake was unaffected by treatment.

T4 concentrations were statistically lower in the animals at 250 and 50,000 ppm of lenacil (↓34 and 38%, respectively) compared to controls at week 10, but there was no dose response for this finding despite the dramatic difference in dietary concentrations, nor was this finding reproducible at week 19, and there was no consequent increase in TSH. In addition, there was no decrease in serum T3. rT3 was not changed on week 10 but was lower on week 19 in rats receiving 250 and 50,000 ppm lenacil (both ↓17% and statistically significant).

There were no statistically significant increases in mean liver weight reported, but there appeared to be a trend for increased adjusted liver weight with dose (↑11% at 50,000 ppm). There were no gross changes in thyroid size noted in the lenacil treated groups, but the mean absolute thyroid weight was statistically significantly increased at 50,000 ppm (↑23%). Darkened thyroids were observed in 6/6 animals at 50,000 ppm but were not observed in the controls or at 250 ppm (0/6 in both cases).

There was no reduction in the ability of the thyroid to take up and accumulate ^{125}Iodide, and the ability of thyroid peroxidases to convert the ^{125}Iodide to organic compounds was unaffected by treatment with lenacil.

Propylthiouracil (PTU)-treated rats: Rats had salivation with paddling of forepaws. Irritable behaviour was noted in rats during the treatment periods of weeks 19-20. Body weight and food intake was not affected. There was a large reduction in circulating T3 and T4 levels, accompanied by a marked elevation of mean TSH levels.
Gross observations described the thyroids as enlarged and darkened. Thyroid weights were dramatically increased (↑364%) by PTU treatment.
The effect of administering perchlorate, as compared to saline alone, was to decrease the amount of radioactivity due to ^{125}Iodide accumulation into the thyroid by approximately 80%. The reduced ability of the thyroid to take up and metabolise ^{125}Iodide was demonstrated by the lower thyroid: blood radioactivity ratio in propylthiouracil + perchlorate treated animals.
Conclusions:
There was no evidence to suggest that lenacil at dosages of up to 50,000 ppm was affecting the ability of the thyroid to take-up and organify ^{125}Iodide. Measurements of T3 made during the study also indicate that the test substance is not acting as an inhibitor of the deiodinase which converts T4 into T3.

4.4 OECD Level 4 EATS-Mediated Parameters - In Vivo Studies Assessing Mammalian Endocrine Activity Endpoints

4.4.1 Rat
4.4.1.1 Lenacil Technical. Preliminary Study by Dietary Administration to Han Wistar Rats for 4 Weeks. Laboratory Study No. ADC001/010098. ([redacted] P.M., 2001).
Guideline: Not fully in compliance with Dir. EEC 96/54/EEC Annex IV D or 92/69- 84/449 or OECD test guideline n° 407 (1995-81).
GLP: No
Reliability Score: 2, suitable as a range finding study, missing haematology, clinical chemistry and histopathology
Summary:
Three groups of five male and five female Han Wistar rats received lenacil in the diet for four weeks. One group received 5000 ppm throughout the treatment period. The other dose groups received 10,000 or 20,000 ppm for the first two weeks, and since there was a lack of treatment-related findings, were increased to 30,000 or 50,000 ppm, respectively, during weeks 3 and 4. A control group (5/sex) received basal diet only. Endocrine organs and endocrine-related organs were weighed and included adrenals, epididymides, kidneys, liver, ovaries, testes, thyroid with parathyroids, and uterus with cervix. Tissue samples of these organs were preserved, but not processed histologically.
Results:
There were no mortalities or signs related to treatment. Body weights, food consumption and efficiency of food utilization were not affected by treatment. The overall mean daily intakes for males and females receiving 5000 ppm was 571 and 631 mg/kg bw/day, respectively. During the first two weeks of treatment the mean daily intake at 10,000 ppm was 1269 and 1288 mg/kg bw/day for males and females, respectively; and at 20,000 ppm it was 2545 and 2643 mg/kg bw/day for males and females, respectively. For the males and females receiving 30,000 ppm on Weeks 3 and 4, it was 2978 and 3576 mg/kg bw/day for males and females, respectively; and for the males and females receiving 50,000 ppm it was 5029 and 5913 mg/kg bw/day, respectively.
According to the report, no macroscopic findings were observed after 4 weeks treatment with lenacil. Liver weights were increased compared to controls in females receiving the highest dietary concentrations (relative to body weight ↑13%; statistically significant). The incidence of fluid distention of the uterus in the control, low, middle and highest dietary concentrations were 1/5, 1/5, 3/5, and 3/5, respectively. This finding was not noted as abnormal by the laboratory performing the study and may only indicate that rats were at different stages of the oestrus cycle. Support for the lack of significance of this finding comes from its relatively low incidence in the 13-week rat study described below.
Conclusion:
Dietary administration of lenacil to Han Wistar rats for 4 weeks produced an increase in liver weight in females at the highest dietary concentrations. Increased fluid distension was also observed in the uterus, which was of questionable significance. Dietary concentrations of up to 50,000 ppm were considered suitable for use in a 13-week toxicity study in Han Wistar rats.

4.3.1.2 Toxicity study by dietary administration to rats for 13 weeks followed by a 4-week recovery period. DuPont Report No.: ACD 002/013903. ([redacted] P.M., 2002).
Guideline: EEC Directive 88/302/EEC, EEC Directive 92/69/EEC, EEC Directive 96/54/EEC equivalent to OECD 408.
Additional Histopathological Investigations to a Toxicity study by dietary administration to rats for 13 weeks followed by a 4-week recovery period. DuPont Report No.: ACD 055/024499. ([redacted] P.M., 2004).
Guideline: U.S. EPA OPPTS 870.3100 (1998), OECD 408, Directives 88/802/EEC; 92/69/EEC; 96/84/EEC
GLP: Yes
Reliability Score: 2
Summary:
Lenacil (Batch No. 141712003, purity 98.6%) was incorporated into the diet. Homogeneity and stability investigations were carried out and confirmed for dietary concentrations at 50 and 50,000 ppm before the start of treatment.
Concentration analyses were performed in weeks 1, 6 and 12 of treatment. The actual concentration average range was 97.2% (101-92.8%).
Groups of 10 male and ten female Han Wistar rats received lenacil for 13 weeks via the diet at 0, 500, 5000 or 50,000 ppm. A similarly constituted control group received the basal diet only. A further five males and females were assigned to the control group and the group receiving 50,000 ppm. These animals were treated for 13 weeks, followed by a four-week recovery period without treatment. Endocrine and endocrine-related organs were weighed at necropsy and included adrenal glands, kidneys, liver and testes. Endocrine and endocrine-related tissues examined microscopically included adrenals, epididymides, kidneys, liver, mammary glands, ovaries, pancreas, pituitary, prostate, seminal vesicles, testes, thyroid-parathyroid, uterus and the vagina.
An additional histopathological re-assessment of the thyroids was performed and reported in Huntingdon Life Sciences Report No. ACD 002/013903.
Results:
One male in the 50,000 ppm group was euthanized in extremis during Week 11, however the death of this animal was considered unrelated to treatment. According to the report, body weight gain, food consumption and food efficiency were considered unaffected by treatment; however, in males, there appeared to be lower weight gain at 5000 and 50,000 ppm. The report mentions that two control males (Animal Nos. 47 and No. 50) gained an enormous amount of weight and skewed the final body weight and body weight gain of the control group. The study reported that contemporary control groups from the laboratory had a mean weight gains of ~214 g (range 157 to 290 g) versus 262 ± 40 g in the current study. The dosed groups in this study all had average gains around 230 g, which are closer to the contemporary controls. (Also note that there was no change in in body weight or body weight gain in 2-year rat study at 13 weeks, albeit only went up to 25,000 ppm.)

Mean daily intakes for animals receiving 500, 5000 or 50,000 ppm were 40.6, 412.0 and 4356.9 mg/kg/day for males; 44.7. 467.6 and 4892.9 mg/kg/day for females.
There were no behavioural findings which were attributed to treatment with the lenacil for the in-the-hand, in-the-arena or in manipulation investigations performed during treatment. All changes were attributed to normal biological variation. No ocular findings attributed to the test substance treatment.
There were statistically significant decreases in mean white blood cell counts (↓19%) and lymphocytes (↓26%) in males at 50,000 ppm. Females at 5000 and 50,000 ppm also had lower mean white blood cell (↓26/26%), lymphocyte (↓31/27%), monocyte (↓36/45%), and leucocyte (↓33/33%) counts, and at 50,000 ppm eosinophils (↓25%) were also decreased. Partial or full recovery from these changes was reported by the end of the 4-week recovery period.
Statistically significantly lower phosphorous concentrations were reported in females at 5000 ppm (↓21%) and in both sexes at 50,000 ppm (↓6 and 18% in males and females). Slightly lower potassium (↓11%) was noted in high dose females. A slightly higher mean plasma creatinine (↑10%) value was noted in females at 50,000 ppm, as well as higher urinary specific gravity and protein content; however, there were no correlative changes in kidney weight or histology to indicate changes in kidney function. All clinical chemistry changes showed full recovery by the end of the recovery period.
There were no treatment-related macroscopic findings. There were changes in uterine (including cervix) weight, but they were not dose dependent or statistically significant. The incidences of uterine fluid distension observed at the 0, 500, 5000, and 50,000 ppm dietary concentrations were 2/10, 4/10, 4/10, and 3/10, respectively. The lack of a dose response in this study suggests that the response observed in the 4- week study was not test substance-related. Absolute and relative

(bw) mean liver weights in females at 50,000 ppm were increased (↑22 and 21%, respectively compared to control and statistically significant) at week 13. Increases were also noted at 50,000 ppm in males (10% rel. bw) and at 5000 ppm in females (22% rel. bw) but were not statistically significant. No marked changes were observed at the end of the recovery. Changes in liver weight at 13 weeks correlated with an increased incidence of centrilobular hepatocellular hypertrophy in both sexes at 50,000 ppm (5/9 males, 4/10 females); however, this change was no longer observed after the 4- week recovery period. Spleen weights were increased in males and thymus weights were decreased (absolute); however, neither of these changes were statistically significant.

Relative thyroid weights were increased in males (21%) and females (13%); however, neither change was statistically significant. No changes related to treatment with lenacil were observed in hematoxylin and eosin-stained sections of the thyroid. (HLS Report No. ACD 002/013903). However, other studies indicated some type of change. Thus, re-examination of thyroid sections with Schmorl's staining was undertaken. This staining showed an increased incidence and of Schmorl's-positive staining in females at 50,000 ppm, and an increased intensity of staining in males, also at 50,000 ppm (Schmorl's positive staining at 13 weeks: incidence in males, control 9/10 versus 8/10 at 50,000 ppm; incidence in females, control 1/10 versus 7/10 at 50,000 ppm (also 3/10 at 5000 ppm not statistically significant); Intensity of staining: in male controls 7 minimal & 2 slight versus 2 minimal, 5 slight and 3 moderate at 50,000 ppm pituitary, prostate, seminal vesicles, testes, thyroid and parathyroids, and uterus plus cervix.).

At the end of recovery, a similar finding was noted, suggesting that no significant decrease in staining had occurred.

Conclusion:

Dietary administration of lenacil at concentrations up to 50,000 ppm for 13 weeks caused increases in liver weight and haematological changes which were reversible after a four-week recovery period. The NOAEL may be considered 500 ppm in females (equivalent to intakes of 44.7 mg/kg bw/day) based on adverse or potentially adverse changes in haematological parameters occurring at ≥5000 ppm in females and in males at 50,000 ppm, as well as potentially adverse changes in liver weight (>20%) at ≥5000 ppm in females. Additionally, lenacil at 50,000 ppm via the diet caused an increase in the incidence of Schmorl's positive-staining in females and in the intensity of staining in males. No decrease in staining was seen after four weeks of recovery. The no-observed-effect level for increased Schmorl's staining was 5000 ppm. There was a low incidence in females at 5000 ppm which was not statistically significant.

4.3.1.4a Lenacil Technical Combined Chronic Toxicity and Carcinogenicity Study by Dietary Administration to Han Wistar Rats Over 104 Weeks. Interim Report: 0 – 52 Weeks. Laboratory Report No. ACD 045/024288. [redacted] 2003).

b Lenacil Technical Combined Chronic Toxicity and Carcinogenicity Study by Dietary Administration to Han Wistar Rats Over 104 Weeks. Laboratory Report No. ACD 045/042214. ([redacted] 2004).

Guideline: U.S. OPPTS 870.4300; OECD 453; EU Guideline 88/302/EEC, 92/69/EEC, 96/54/EC

GLP: Yes

Reliability Score: 1

Summary:

The carcinogenic and toxic potential of lenacil (Batch No. 141712003, purity 98.6%) to HsdBrl Han:Wist (Han Wistar) rats via dietary administration was assessed over a period of 104 weeks. Twenty rats/sex/dose were maintained on dietary concentrations of lenacil at 0, 250, 2500 or 25,000 ppm and were sacrificed after 52 weeks of treatment (toxicity phase). Fifty additional rats/sex/dose continued on the same doses to the end of 104 weeks (carcinogenicity phase).

Clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, bodyweight, food consumption, ophthalmic examination, haematology, blood chemistry, urinalysis, organ weight, macropathology and histopathology investigations were undertaken. Endocrine and endocrine-related organs weighed included adrenals, epididymides, kidneys, liver, ovaries, testes, thyroid with parathyroids, uterus with cervix. Organs examined histologically included adrenals, epididymides, kidneys, liver, mammary area, ovaries, pancreas, pituitary, prostate, seminal vesicles, testes, thyroid and parathyroids, and uterus plus cervix.

Results:

Survival was not affected by treatment with lenacil. No signs attributed to treatment with lenacil were observed at the physical examinations and arena investigations. However, females at 25,000 ppm did show higher incidences of yellow staining in the perigenital region and exfoliation of the tail. There were no treatment-related effects upon the group

distribution, multiplicity and mean time of onset of palpable swellings. There was no evidence of neurotoxicity from arena observations, assessment of sensory reactivity or grip strength. Motor activity (lower beam breaks) in week 50 in males receiving 2500 and 25,000 ppm was statistically significantly lower than controls at certain time points (2nd and 4th intervals) in the 60-minute assessment period. A statistically significant decrease was also observed at 25,000 ppm during the 3rd interval. This resulted in lower total low beam activity at the 2,500 and 25,000 ppm doses. There were no other indications of reduced motor activity (i.e. no changes in high beam values) and no changes in grip strength. Additionally, there were no changes occurring in adjacent sampling intervals (1st and 5th). Thus, these findings were not considered toxicologically significant. Support for this position comes from the fact that there were no marked changes in motor activity observed in the 90-day rat study, which used dietary concentrations double the current study (i.e. 50,000 ppm). Lastly, there were no corroborating clinical signs or histopathological findings to suggest a neurotoxic effect.

Overall (week 0 to 104) bodyweight was decreased compared to concurrent controls in females at 25,000 ppm (↓9%, statistically significantly), as well as body weight gain (↓13%, not statistically significant). Food consumption and food conversion efficiency were unaffected by treatment. During the 52- week Toxicity phase, mean daily intakes averaged 14.3, 139 and 1446 mg/kg/day for males, and 18.8, 189 and 1894 mg/kg/day for females receiving 250, 2500 and 25,000 ppm lenacil in the diet, respectively. The overall mean daily intakes for the Carcinogenicity phase (weeks 0-104) at 250, 2500 or 25,000 ppm were 12.0, 118 and 1223 mg/kg/day for males and 15.9, 160 and 1699 mg/kg/day for females, respectively.

Haematological and biochemical analysis of the blood were not reported to be affected by treatment. Transient haematology changes were observed, but were likely spurious findings given the lack of consistency over time and the differences in the direction of change. Likewise, some changes in clinical chemistry parameters occurred; however, given their transient nature, these changes may merely be incidental. Control and high dose blood samples taken at 52 were also analyzed for thyroid hormones. T3 and T4 were unchanged. TSH was slightly elevated, but the change was not statistically significant (male control: 6.3±1.12 ng/ml, 25,000 ppm: 8.4±3.58 ng/ml; female control 5.4±0.72 ng/ml, 25,000 ppm: 6.9±3.37 ng/ml; n = 20/sex/group).

Adrenal weights were not changed at 52 weeks but were increased in 25,000 ppm males at week 104 (↑147% for absolute and 116% for relative to bw, neither statistically significant). In females at 2500 ppm adrenals were increased by 10% (rel. to bw) and statistically significant, but at 25,000 ppm, where the weight was up 16% (absolute) and 30% (rel. to bw), these values were not statistically significant. The significance of these changes in geriatric animals is not clear.

Kidney weights (rel. to bw) were slightly higher (8%) after 52 weeks in females at 25,000 ppm. After 104 weeks they were slightly up in males and females (rel. to bw, ↑9 and 12%, respectively, both statistically significant). Liver weight (rel. to bw) was slightly increased (9%, statistically significant) after 52 weeks at 25,000 ppm in males only and increased in both sexes at 104 weeks (↑13 and 11% and statistically significant in males and females, respectively).

Relative thyroid+parathyroid weight was statistically significantly increased in males (↑23%) and females (20%) at 52 weeks at 25,000 ppm. They were also elevated at 104 weeks at 2,500 ppm in both sexes, but were not statistically significant; however, at 25,000 ppm the increases were marked and statistically significant (↑40 and 49% in males and females).

Testes and epididymal weights were not changed. Ovarian weights showed fluctuations in both directions. Uterine weights were increased, but without relationship to dose.

Spleen weight (rel.) was increased by 23% in top-dose females after 104 weeks, but the increase was not statistically significant. Thymus weights showed increases and decreases that were unlikely related to treatment.

Macroscopic examination of the thyroid showed a darkening after 52 weeks of treatment in 25% of males (5/20) and 52% of females (10/19) at 25,000 ppm versus no findings in either sex in controls. After 104 weeks, only a single male (2%) at 25,000 ppm had darkened thyroids, whereas 12 cases out of 50 (24%) were observed in females. None of the controls had the finding at 104 weeks. Given this pattern, the finding appears to be transient or at least something they appear to adapt to.

At 52 weeks, fluid distension in the uterus was noted at 25% (5/20 rats) in both the control and low dose group but only at 5% (1/19) in the high dose group. At 104 weeks there were no cases in the control, and only 12% (6/50) of the animals at 25,000 ppm were affected. Given this pattern there is no indication that this was test substance-related.

After 52-weeks of treatment an increased incidence of centrilobular hepatocyte hypertrophy (graded slight) occurred in males at the top dose (2/20 in control versus 15/20 at 25,000 ppm) but not in females. This finding was also noted at 104 weeks (11/50 in control, 26/50 at 25,000 ppm) at which time there was also an increase in hepatocyte vacuolation (16/50 in control, 28/50 at 25,000 ppm) suggesting frank toxicity to the liver. A possible test substance-related increase in hepatocellular hypertrophy was also noted in females, but only at 104 weeks (1/50 in controls, 4/50 at 25,000 ppm). A mechanistic study that has not yet been reviewed by the RMS on the rat liver has provided clear evidence for CYP2B1 enzyme gene expression consistent with CAR activation that correlated with increases in liver weight and liver hypertrophy, with no evidence of hepatotoxicity (See [redacted] 2019, section 4.3.2).

An increased incidence of luminal concretions was seen in the thyroids of males and females at 25,000 ppm in the carcinogenicity phase (male control 22%, 25,000 ppm 66%; female control 20%, 25,000 ppm 65%). There was no indication that this finding should be regarded as adverse.

There was no indication for an increase in neoplastic findings in males. According to the assessment by ECHA (Committee for Risk Assessment (RAC) Opinion proposing harmonised classification and labelling at EU level of Lenacil (ISO), 5 December 2013) , lenacil was considered to have caused an increase in mammary adenocarcinomas in rats, with incidences of 0, 4, 12 and 10% in the 0, 250, 2,500 and 25,000 ppm groups, respectively. While 12% fell within the laboratories historical control range of 0-22% (and was well within published ranges for this strain of rat), ECHA believed that the upper incidence of 22% observed in one study out of 19 was an outlier. The incidence of mammary acinar cell hyperplasia was 44, 50, 52 and 56% at 0, 250, 2,500 and 25,000 ppm, respectively; and thus, there was at best only a very weak trend with dose. The mammary adenocarcinoma response was not associated with a concurrent increase in fibroadenomas and drew support away from the tumors being test substance-related. Regardless, the NOAEL for carcinogenicity was considered 250 ppm, and ECHA took the position that the increase in adenocarcinomas warranted classification for limited evidence of carcinogenicity (Category 2).

ECHA also evaluated the C-cell thyroid tumor response in female rats (incidence of adenomas: 4, 4, 16 and 14% in the 0, 250, 2,500 and 25,000 ppm groups, respectively; with two carcinomas (4%) at the top dose). The incidence of these tumors was marginally above the historical control range. No corroborative microscopic treatment-related effects were reported in C-cells, i.e. there was no treatment-related increase in C-cell hyperplasia in the study. No effects on calcium homeostasis were reported in the 90-day study in rats at doses up to 50,000 ppm. Calcium levels were marginally decreased in males and only significantly increased in females at the week 104 sampling in the 2-year study, and without a dose-response, so there was no clear link to treatment. There are many indications that these tumors are a typical finding in aging rats (Thomas and Williams, 1999). Since the incidence of C-cell tumours in females was only marginally above the historical control range, ECHA considered the finding as equivocal evidence for carcinogenicity in the thyroid of the rat.
There were 3/50 (6%), 2/50, 2/49, and 5/50 (10%) follicular cell adenomas in males and 1/50 (2%), 0/50, 1/50, and 4/49 (8%) in females at 0, 250, 2,500 and 25,000 ppm, respectively. In addition, there were 0/50, 0/50, 1/49 and 1/50 follicular cell carcinomas in males, and 1/50, 0/50, 1/50 and 0/49 in females at 0, 250, 2,500 and 25,000 ppm, respectively. ECHA concluded that the incidence of follicular cell adenomas was significantly increased in high dose females but remained within the HCD range of the laboratory (0.0% - 11.7%). ECHA also considered that the incidence of carcinomas was not elevated with dose. The incidence of combined adenomas and carcinomas was within the HCD for adenomas only. However, ECHA concluded that there was no evidence that lenacil induced follicular cell tumours.
Uterine tissues were completely examined in the control and top dose groups only, as there was no consideration of test substance-related effects. The incidence of endometrial hyperplasia was 4% in the control versus 12% at 25,000 ppm. Uterine luminal dilation was 34% in the control versus 54% at 25,000 ppm.
<u>Conclusion:</u>
According to the report, the no-observed-adverse-effect level (NOAEL) was 2,500 ppm (118 and 160 mg/kg bw/day) based on non-specific toxicity in females at 25,000 ppm and adaptive and toxic findings in the liver. However, ECHA considered 250 ppm (15.9 mg/kg/day in females) the NOAEL based on the marginal increase in mammary adenocarcinomas at ≥2,500 ppm.
Based on mechanistic data available now which is summarized in this document, but not yet reviewed by the RMS, the liver hypertrophy observed in this study can be considered non-adverse, although the same cannot be concluded for

increased hepatocellular vacuolation at 25,000 ppm observed in males in the carcinogenicity phase of the study. Darkening of the thyroid was reported at 25,000 ppm in both sexes at 52 weeks. At 104 weeks it only occurred in females and at a lower incidence; thus, this finding appears to be reversible even under treatment. As there is no clear relationship of these findings to adversity and they can be reversible, there is no reason to conclude that they are adverse. Other findings were reported, many which were considered incidental in the report.

Embryotoxic and teratogenic study in rats with lenacil (INB-634). DuPont Report No.: HLR 405-78. ([redacted] 1978)
Guideline: None
GLP: No
Reliability Score: 2
Summary:
25 [redacted] CD rats/group were allocated to 4 experimental groups. Lenacil (Batch No. INB-634-61), purity approx. 100%) was administered continuously via the ground diet from GD 6 to 15 at 0, 500, 2500 or 5000 ppm. A fourth, control group received the basal diet without the test material. Rats were observed daily for clinical signs. Body weights were recorded on arrival and on days 6, 10, 16 and 21 of gestation. Food consumption was monitored and recorded throughout the study. Foetuses were collected via Caesarean section on GD 21 and corpora lutea, implantation sites, live and dead foetuses, resorptions, foetal weights, crown-rump length and gross abnormality recorded. Half of the foetuses were used for skeletal evaluation after Alizarin red staining, other half was subjected to visceral examination according to the razor blade Wilson technique.
Results:
There were no clinical signs attributable to treatment. All animals survived the test period. Body weights and food consumption were unaffected by treatment. There were no gross findings attributable to treatment.

Pregnancy and foetal parameters such as implantations, resorptions, body weight or crown-rump length were not affected by treatment. Skeletal and visceral examinations did not reveal findings attributed to treatment. The incidence of litters with early/partial resorptions was increased at the mid-dose, but not at the top-dose. A slight increase in the incidence of in anophthalmia/microphthalmia [3 litters (3 foetuses) in 21 litters (183 foetuses)] was noted at the top concentration of 5,000 ppm (equivalent to 486 mg/kg bw/day) versus 1 litter (1 foetus) out of 21 litters (172 foetuses) in the control]. While the incidence of anophthalmia/microphthalmia in this non-guideline, non-GLP study was outside the laboratories historical control range[3], this finding was not reproduced in the more recently conducted GLP rat developmental toxicity study that conformed to OECD TG 414 (EU B31) and tested up to 1000 mg/kg bw/day.
Conclusion:
Oral (dietary) administration of lenacil to rats did not affect maternal or foetal parameters at any dose level tested, therefore the NOEL was determined to be the top dose of 5000 ppm (485.7 mg/kg bw/d). Under the experimental conditions of this study, lenacil was not considered to be embryotoxic or teratogenic.

DPX-B634-91 (Lenacil): Pilot developmental toxicity study in rats. DuPont Report No.: HLR 996-96 ([redacted] 1996).
Guideline: None, used for dose setting purposes for other studies
GLP: No
Reliability Score: 2
Summary: A pilot study was performed using lenacil (batch n° DPX-B634091, 98.5%) in 0.5% methylcellulose administered to groups of 11 mated Crl:CD BR rats over day GD 7-16 at 0, 500, 1000, 4000 mg/kg bw/d by gavage. On day 22, all rats were euthanised and gross necropsy was performed. The foetuses were removed from the uterus and were weighed, sexed and examined for external alterations.
Results:
According to the authors of the study, there was no evidence of either maternal or developmental toxicity at any level tested. Alopecia was noted in 4/11 (36%) dams at 4000 mg/kg. Lower incidences (2/11, 18%) were noted at 500 and 1000 and one animal was affected in the control group (1/11, 9%). The incidence of alopecia at 4000 mg/kg was slightly over the laboratories historical control range.[4]
Conclusion:
Under the experimental conditions described for this study, the maternal and developmental NOEL was 4000 mg/kg.

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[3] *Historical control data, highest occurrence: 2 litters (2 foetuses) out of 25 litters (185 foetuses)*

[4] *Mean of 14%, range 0-28%, based on 25 studies from 1991 to 2001*

a Lenacil Technical: Preliminary Study of Effects on Embryo-Fetal Development in CD Rats Treated by Oral Gavage Preliminary Study. DuPont Report No.: ACD 057/030001. [redacted] 2003).
4.3.1.7b Lenacil Technical Study of Effects on Embryo-Fetal Development in CD Rats Treated by Oral Gavage Administration. DuPont Report No.: ACD 058/032316. ([redacted] 2003).
Guideline: US EPA OPPTS 870.3700, E.U. Guideline B31, OECD Guideline No. 414, Japanese MAFF 12 Nohsan No. 8147; Guideline 2-1-18
GLP: Yes
Reliability Score: 1
Summary:
In the preliminary study, lenacil (Batch No. 141712003, purity 98.6%) was administered once daily by oral gavage at dosages of 100, 300 or 1000 mg/kg/day to groups of 6 pregnant female CD rats from GD 1 to 19, inclusive.
Control animals received the vehicle, 0.5% w/v methylcellulose. The females were killed on GD 20 for examination of their uterine contents and foetuses were examined externally for abnormalities. Oral administration of lenacil to rats weakly affected maternal parameters (increased incidence of clinical signs, e.g. brown staining on the head) but did not affect foetal parameters. Therefore, the same concentrations were used in the main embryo-foetal toxicity study in rats.
In the main study, adult virgin female rats of the CD (Sprague-Dawley) strain were allocated to one control and three experimental dose groups (22 animals per group). The animals received lenacil (Batch No. 141712003, purity 98.6%) by gavage from GD 1 to 19 at 100, 300 or 1000 mg/kg body weight per day. The control animals received the vehicle, a 0.5% w/v methylcellulose suspension.
All maternal parameters were taken regularly throughout the study. Females were killed on GD 20 for examination of their uterine contents and foetuses were examined externally for abnormalities.
Results:
Body weights and food consumption were unaffected by treatment. Some clinical signs may have been related to treatment. At 1000 mg/kg forepaw alopecia was noted in 5 out of 22 dams (23%). In the control and at lower doses, the incidence of alopecia was 1 to 2 cases per 22 dams. Brown staining of the forelimbs was slightly increased at 1000 mg/kg (5/22 cases versus 1 to 3 cases in 21 to 22 dams in all other groups).
Treatment did not influence litter parameters such as corpora lutea, implantations, resorptions or live young, and the extent of pre-and post-implantation loss showed no evidence of an adverse effect of treatment. Foetal and placental weights were unaffected by treatment.
The incidence of major and minor abnormalities and skeletal variants showed no relationship to treatment with lenacil. At 300 and 1000 mg/kg there was a slightly higher number of thickened ribs, and incomplete ossifications of cervical and sacrocaudal vertebrae as compared to the control and 100 mg/kg dose; however, since the incidences of these findings were relatively low, they were considered background findings.
Conclusion:
Oral administration of lenacil to rats at dosages up to 1000 mg/kg bw/day did not affect maternal or foetal parameters at any of the doses tested. Minimal clinical findings occurred in the dams and minor changes in variations in the offspring that were considered background findings. Therefore, both the maternal and foetal NOAEL was considered to be 1000 mg/kg bw/day.

Mouse
Subchronic Oral Toxicity: 90-Day Study with DPX-B634-91 (Lenacil) Feeding Study in Mice. HLR 293-91. ([redacted] 1991).
Guideline: U.S. EPA 82-1, OECD 451
GLP: Yes
Reliability Score: 1
Summary:
Lenacil (DPX-B634-91, purity 98.2%) was incorporated into the ground diet to provide the required concentrations. Ten (10) CrL: CD-1(ICR) BR mice/sex/dose received lenacil orally, via the diet, at concentrations of 0, 100, 1000, 5000 and 10,000 ppm. Body weight and food consumption were determined weekly. Evaluation of haematology parameters was performed at 45 and 90 days. At termination, all mice were sacrificed, selected organs were weighed, and tissues examined microscopically.

Results:

Dietary intakes of 100, 1000, 5000 and 10,000 ppm resulted in mean daily intakes of 15.5 157, 787 and 1616 mg/kg bw/day in males and 20.2, 207, 1127 and 2150 mg/kg bw/day in females, respectively. No mortalities occurred during the course of the study. There were no effects reported on body weight or body weight gain, and neither food consumption nor food efficiency were affected. A compound- related effect on the incidence of clinical signs was not evident.

At the 45-day sampling, male mice administered 1000 ppm and above had mild, statistically significantly increased red cell mass parameters (RBC, HB, HCT), but all increases were below 15%. In affected groups, only one to two animals were outside the laboratories 95% reference interval. No similar increases were noted in males at the 90-day sampling. Male mice also had significantly decreased mean total leucocytes at ≥1000 ppm, and in all these cases, only 1-2 mice were below the laboratories 95% reference range. In contrast, two mice in the control group were above the reference range. For neutrophils, only a single male at the top dose was below the reference range; however, in the control, while no animal was above the 95% reference range, six of ten animals were above the mean value of the reference range. A similar pattern was seen with individual values for lymphocytes. For monocytes, nine of 10 control males had values above the reference range, skewing the decrements observed in the treated males. Thus, while many of the decreases were statistically significant, they were generally within the laboratories reference interval.

For white cell parameters in females, a similar trend was observed in ≥1000 ppm groups at the 45-day sampling, but rarely gaining statistical significance. Only a single control animal was above the laboratory reference range for leucocytes, but the majority of the control animals were above the mean of the reference range, and only a single treated female (at the top dose) was below it. While a single control was above the reference range for neutrophils, six of ten control values were above the mean of the range; and none of the females in the treated groups were below the reference range. A similar pattern was observed for lymphocytes and monocytes.

There were no changes in the red cell mass parameters in males at the 90-day sampling. The female HT was slightly elevated (8-10%) at ≥1000 ppm, but only one to two values out of 10 per group were above the laboratories reference range, and there was no marked increase in the number of red blood cells.

Leucocyte counts at 90 days were statistically significantly decreased in males at ≥1000 ppm. None of the control values were outside of the laboratory's 95% reference range, but two values were above the mean of the range. At 1000 ppm, only a single male was below the reference range, but at 5,000 ppm four of 10 animals were below the range and at 10,000 ppm two of 10 were below the range. Three of 10 control males were above the mean value of the reference range for neutrophils, but none of the treated animals were below the below the reference range. None of the control males were above the mean value of the reference range for lymphocytes, but at ≥1000 ppm, only one or two animals per group were below the reference range. None of the control monocyte counts were above the reference range, however 6 of 10 values were above the mean of the reference range, again, likely skewing the decreases seen in the treated groups. There were no statistically significant changes in leucocytes, neutrophils, lymphocytes or monocytes in females at the 90-day sampling. Eosinophil counts were down at ≥1000 ppm, with only the decrease at 1000 ppm being statistically significant; all values were within the laboratories reference range.

A statistically significant depression in platelet counts was seen in males at 1000 and 10,000 ppm, but not at 5000 ppm on day 45. At 90 days, a statistically significant decrease (20%) was only noted at 10,000 ppm. Mild decreases were seen in females at both samplings, but none of the changes were statistically significant.

The leucopenia observed in both sexes at ≥1000 ppm was considered to be potentially compound-related; however, the adversity of these findings should be considered in view of the degree of the changes, the consistency of the changes (e.g. day 45 versus day 90, and in comparison to the 13-week timepoint in the 18-month study) and how the changes relate to laboratory's reference range. In general, control values were higher than the mean values of the reference range which could skew the interpretation of the data. However, given the number of males below the reference range for leucocyte counts at the 90-day sampling at 5000 ppm, this finding should be noted.

Plasma proteins were slightly increased in males at 5000 and 10000 ppm. Other parameters were not measured.

Absolute, but not relative, liver weight was increased in males at 10,000 ppm (↑13%, not statistically significant). Relative liver (as wells as absolute) weight was increased in females at 5000 and 10,000 ppm (rel. to bw ↑14 and 17%, respectively). Given the lack of any correlative histological changes, these findings could be considered adaptive, and not adverse[5]. A higher incidence of extramedullary haematopoiesis was seen in high-dose female liver and spleen. Spleen weight was increased in high-dose females (↑43/36% absolute/rel. to bw) which may be related to the extramedullary

haematopoiesis. Other high-dose effects included lymphoid cell foci/hyperplasia and focal inflammation. Thyroid, thymus, uterus, ovaries, prostate and epididymides were not weighed.
Conclusion:
Oral administration of lenacil via the diet to CD-1 mice at concentrations up to 10,000 ppm for 13 weeks produced adaptive changes in the liver at 5,000 and 10,000 ppm (increased organ weight without concomitant histopathological changes, except that of haematopoiesis at 10,000 ppm), and increased spleen weight in females (possibly related to haematopoiesis). Based on adverse or potentially adverse decreases in leucocytes at ≥5000 ppm in males, the NOAEL is 1000 ppm, equivalent to 157 mg/kg bw/day.

Oncogenicity Study with DPX-B634-91(lenacil); Eighteen-Month Feeding Study in Mice. HLR 336-93. ([redacted] 1994)
Guidelines: OECD 451, U.S. EPA 83-2, EEC 1988
GLP: Yes
Reliability Score: 2
Summary:

Four groups each of 80 male and 80 female CRL-CD®-1(ICR)BR were fed diets containing 0, 100, 2500 or 7000 ppm of lenacil (DPX-B634-91, purity 98.2%, reanalysis 98.5%) for 18 months. Body weight and food consumption were measured and examinations for clinical signs were conducted weekly (first three months), or bi-weekly during the remainder of the study. Ophthalmoscopic examinations were performed during pre-test and at study end. Haematology and clinical chemistry analyses were conducted after 3, 6, 12 and 18 months. After 18 months, all survivors were sacrificed, selected organs weighed and tissues examined for the presence of gross or microscopic lesions.
Results: No compound-related mortality was observed. No clinical signs were attributed to the dietary administration of lenacil. Mean bodyweight and bodyweight gains in male and female mice were comparable to controls at all dose levels, as were food consumption and food efficiency. The overall mean daily intake for mice in the 100, 2500 and 7000 ppm groups was 13.8, 332 and 977 mg/kg bw for males and 19.6, 482 and 1358 mg/kg bw for females, respectively.
Some statistically significant changes in hematology parameters were observed (e.g.↓ lymphocytes at 3 months and neutrophils at 6 months in 2500 ppm males, ↓

See Hall, AP et al. (2012). Liver hypertrophy: A review of adaptive (adverse and non-adverse) changes-Conclusions from the 3rd International ESTP Expert Workshop. Toxicol. Pathol. 40:971-994.

lymphocytes in females at 6 months); however, generally, hematology changes did not show a dose-response, and were not consistent over time.

In high-dose males, there were non-statistically significant increases in mean absolute (↑15%) and relative (↑16%) liver weights which correlated with the centrilobular hepatocellular hypertrophy (7/80) at 7000 ppm. A statistically significant increase (↑7%) in mean relative liver weight was observed in high-dose females, but without a microscopic correlate. These effects were considered adaptive, and non-adverse. Decreases in mean absolute (13, 14 and 17%) and relative (12, 13 and 16%) kidney weight were observed in females at 100, 2500 and 7000 ppm, respectively. However, since they were not statistically significant and there was no histological correlate, these findings may be incidental. Non-statistically significant differences in spleen weights were noted in females. The group mean relative weights were down by 17, 32 and 35% in the 100, 2500 and 7000 ppm groups, respectively. This finding is influenced in part by several spleen weights that were outliers which magnified group differences. No correlative histological changes were observed.

Masses were observed in the liver and lungs of males at 7000 ppm only and correlated with microscopic changes. An increase in the incidence of hepatocellular adenomas and multiplicity of adenomas was observed microscopically, which was considered potentially compound-related and toxicologically significant (see below).

Leydig cell hyperplasia in the males was observed at rates of 7/80 (9%), 0/28, 3/21 (14%) and 12/80 (15%) in the 0, 100, 2500 and 7000 ppm groups, respectively. The difference between the control and top dose was not statistically significant and was considered incidental. Thus, the finding was not identified as a target, and therefore, was not investigated at the low and middle dose level. Preliminary data suggest that the incidence of Leydig cell hyperplasia in this study falls within the laboratories HCD range.

Pituitary cysts in males were observed at rates of 2/70, 0/17, 0/9 and 6/66 in the 0, 100, 2500 and 7000 ppm groups, respectively. The increase in this study at 7000 ppm was not statistically significant and was considered incidental given its low occurrence. Thus, the low and intermediate groups were not identified as targets.

A borderline statistically significant increase in combined incidence of alveolar adenomas and adenocarcinomas (26/80, 32%) was observed in the lungs of 7000 ppm males as compared to the controls (18/80, 22.5%). Since there was no focal hyperplasia of Type II alveolar cells, no decrease in alveolar tumor latency, no shift in tumor cell anaplasia and the incidences of adenomas and adenocarcinomas, taken separately, were not statistically increased, the alveolar lesions were considered unrelated to lenacil administration. No compound related pathological alterations were observed in males at 100 or 2500 ppm, or in any dose groups of female mice. In the RAC Opinion on lenacil (5.Dec.2013), ECHA considered this data and concluded:

"Overall, a significantly increased incidence of alveolar tumours is observed in male mice at the highest dose. The incidence is above laboratory historical control data. However, several studies in the literature provide evidence of the high incidence of bronchoalvealoar tumours in CD-1 male mice, up to 61.1% (Manenti, 2003), 43% (Fox, 2007) and 33.4% (Maita, 1988).

Besides, it is noted that lung is not a target organ of Lenacil toxicity and that the observed increase was restricted to males.

The link between the induction of bronchoalveolar tumours and Lenacil is therefore uncertain."

In addition to this, ECHA also considered the following regarding the liver tumors:

"No increase of liver single adenomas was observed. The incidence was similar in controls and high dose males.

A statistically significant increase of multiple adenomas was observed in high dose males.

Laboratory historical control data were not provided. Although of lower relevance, historical control data at [redacted] *were considered but the incidence of liver cell multiple adenoma reported in males at the highest dose (16%) is within the maximum range of historical control data at* [redacted] *(28%, single or multiple type not specified). Cumulative incidence of single and multiple adenomas at the high dose (30%) is slightly above this HCD.*

No increase of liver carcinomas was observed.

Incidence and historical control data for combined hepatocellular adenomas and carcinomas were not provided and no conclusion is possible on a combined analysis of tumours.

Considering the lack of effect observed on hepatic single adenomas and carcinomas and that only benign tumours were increased, the significance of the isolated increase of multiple adenomas is unclear. There is equivocal evidence of carcinogenicity of Lenacil in the mouse liver."

There was a marginal increase in Harderian gland adenomas in males, but it was not statistically significant (incidence: 6/80, 2/23, 2/16 and 9/80 at 0, 100, 2500 and 7000 ppm, respectively). As the increase was slight, it considered incidental by the pathologist, and thus, not further examined as a target in the low and middle dose groups. Preliminary data from the performing laboratory suggests that the incidence in this study at 7000 ppm falls within the laboratories HCD.
Conclusion:
The NOAEL for carcinogenicity in mice administered diets containing lenacil for 18 months was considered 2500 ppm (322 mg/kg bw/day) for males based on an equivocal increase in incidence of hepatocellular tumours at 7000 ppm. Bronchoalveolar tumours of uncertain significance were also observed at 7000 ppm in males. For females at 7000 ppm there were no toxicologically significant effects at any dose level. The sponsor notes that the NOAEL in the DAR of 2008 was 2500 ppm based on increased liver weight and associated hypertrophy in males at 7000 ppm, and the NOAEL for oncogenicity was 2500 ppm; however, the NOAEL for systemic toxicity was revised downward to 100 ppm (13.8 mg/kg bw/day) during the peer review.
Finally, based on mechanistic data now available which is summarized in this document, we now have evidence of CAR induction in rats (See [redacted] 2019, section 4.3.2). This mechanism is likely operable in mice. Given these data, it is plausible that the hepatocellular hypertrophy, increased liver weight and equivocal tumour response are related to a CAR-mediated mechanism, and thus, would not be relevant to humans.

Dog
Preliminary study by dietary administration to beagle dogs for 4 weeks. DuPont Report No.: ACD 003/013230. ([redacted] 2001).
Guideline: EEC Directive 92/69/EEC Method B.4, equivalent to OECD 404 (1992)
GLP: No
Reliability Score: 2
Summary:
Lenacil (Batch No. 141712003, purity 98.6%) was incorporated into the ground diet at concentrations of 5000, 20,000 and 50,000 ppm. A total of 3 male and 3 female pure-bred beagle dogs were used for the study. Clinical signs and mortality were assessed daily. Body weights were recorded twice weekly and food consumption once a week. Haematology and clinical chemistry investigations were performed at the beginning and at the end of the study. At terminal autopsy, macroscopic changes were recorded, and organs were preserved for possible future examinations.
Results:
Due to the small sample size and the lack of a control group, it is difficult to draw firm conclusions regarding test substance induced changes. Body weight gain decreases of more than 10% were observed in the males at the two highest doses and in the female at the top dose. Food consumption was also decreased in males at the two highest doses. Individual hematological parameters were variable but generally comparable between treatment groups and with pre-treatment values.
Slightly increased urea was noted for all treated animals in comparison with pre- treatment values, and when the highest dietary concentration was compared to the lowest concentration it was up by 91% in the male and 14% in the female. Creatinine was elevated relative to predosing by 24% and relative to the low dose male by 22% at 50,000 ppm. Alkaline phosphatase was elevated in the 20,000 ppm female by 61% relative to the low dose female and by 59% in the female at 50,000 ppm.
Relative liver weight was increased in mid and high dose females. Absolute and relative kidney weights were decreased in mid and high dose; relative kidney weight was decreased in mid and high dose females. Absolute and relative thymus weights were decreased in mid and high dose males but increased in females.
There were no findings at necropsy considered to be related to treatment.

Conclusion:
The study design was not suitable for setting reference values due to the lack of controls and the low number of animals per group. Based on the results of this study, dietary concentrations of up to 50,000 ppm could be considered tolerable for a 13-week study, although lower concentrations may be warranted based on the clinical chemistry findings.

4.3.3.2. Toxicity study by dietary administration to beagle dogs for 13 weeks. DuPont Report No.: ACD 022/014297. ([redacted] 2002)
Guideline: EEC Directive 96/54/EEC Method B.27, equivalent to OECD 409.

GLP: Yes
Reliability Score: 1
Summary:
Lenacil (Batch No. 141712003, purity 98.6%) was incorporated into the diet of pure-bred beagle dogs (4/sex/group) at dietary concentrations of 0, 1000, 5000, or 25,000 ppm for 13 weeks. Laboratory examinations were performed prior to the start of the study and at weeks 6 and 13. At terminal autopsy, macroscopic findings and organ weights were recorded and a broad spectrum of organs was subjected to histopathological examination from all animals.
Results:
Dietary concentrations of 0, 1000, 5000, or 25,000 ppm resulted in overall (Weeks 0-13) mean daily intakes of 0/0, 44/46, 221/225, or 1121/1102 mg/kg bw/day in males/females, respectively. There were no unscheduled deaths or clinical signs in noted due to treatment. Body weight gain was reduced by 13% in males and 15% in females at the top dose. While these changes were not statistically significant as compared to the control, this may be a result of the small group size. Lower mean body weight gain was also noted in females at 1000 ppm, however this effect was likely due to the abnormal low weight of one female. Food consumption was similar across all groups.
Many changes in haematology parameters were observed. In many cases, the differences that occurred during treatment were also observed during the pretreatment phase. Many of changes did not occur in a dose-responsive manner and could be related to the small group size. While decreases in reticulocyte counts of about 20% occurred pretreatment in males, during the treatment phase, decreases of 46 and 30% occurred at the 6-week sampling and of 34 and 41% occurred at the 13-week sampling at 5000 and 25,000 ppm, respectively. None of the decrements in the treated group males were below the laboratories reference range (98% range: 0.29-1.80, mean 0.76±0.29, studies from 1997-2002). In contrast, increases in reticulocyte counts were observed in females. A statistically significant increase (↑22%) in APTT was observed at the 13-week sampling at 25,000 ppm in males when compared to the control, but this group already had an increased time prior to dosing. Thus, when this mean is compared to its pre-dosing value, there is no increase. In fact, all APTT values were well within the laboratories reference range (98% range: 13.4-28.0, mean 21.0±3.2, studies from 1997-2002).
At week 6 and 13, higher and statistically significant mean alkaline phosphatase values occurred at 25,000 ppm in both sexes. As there were no increases in clinical chemistry parameters indicative of hepatobiliary damage (i.e. ALT, AST, GGT), this finding is likely related to the hepatocellular hypertrophy and not adverse (see below).[6] This position is consistent with the conclusions of the 3rd International European Society of Toxicologic Pathology working group on liver hypertrophy, which reported that increases in circulating ALP activity in the dog, with associated increased liver weight and histological hepatocellular hypertrophy but without hepatocellular degeneration could be interpreted as an adaptive, rather than an adverse response to chemical exposure (2012).[4]
Urinary pH was unremarkable across doses. High dose females exhibited significantly more proteins in their urine than control animals. Other parameters showed no meaningful differences from controls. Mean cholesterol values were sometimes elevated when compared to controls, but it is of note that in male treated groups, the highest value during treatment was only slightly above the highest pretreatment value (4.26 mmol/L at 25,000 ppm versus 4.11 mmol/L in a pretreatment male group) and in females, the highest mean value in a treated group was well below the highest value in a pretreatment group (4.35 mmol/L pretreatment at 5000 ppm versus 4.22 mmol/L at 6 weeks in the same group). Therefore, none of these differences in cholesterol values are likely to be test substance-related.
Mean absolute adrenal weights were increased in males at all doses (up by 13, 14, and 21% at 1000, 5000, and 25,000 ppm, respectively, but was unremarkable in females. These changes were not statistically significant, and there were no histological changes correlated to them; thus, their relationship to treatment was not clear.
Mean liver weight was slightly increased at 25,000 ppm in males in comparison to controls (adjusted ↑15%), though not statistically significant. This finding correlated with an increase in hepatocellular hypertrophy noted at ≥5000 ppm in males and the single case at 25,000 ppm in females.
There was a decrease in sperm in the epididymides at 25,000 ppm (2/4 males). This finding should be viewed in light of the age of the animals, which were approximately 4-5 months old at the start of treatment, and thus, would have been sacrificed at 7-8 months of age. In the literature it is recognized that if the evaluation of spermatogenesis is critical, incidental findings can be minimized by using males over twelve months of age.[7] In this lenacil study, it was noted that these dogs were immature, and this was supported by observations in the testes. Thus, this finding unlikely to be related to treatment.

Thyroid + parathyroid glands were weighed together. Some increased weights relative to the control were observed in both sexes. In males, both absolute and adjusted means were increased 11 and 15% at 5000 ppm, and by 20 and 23% at 25,000 ppm (23%, statistically significant). Thyroid + parathyroid weights were not adjusted in females, and mean absolute weights were increased relative to controls by 11 and 24% at 5000 and 25,000 ppm, respectively. Individual values often had overlap with the control values. In the absence of corroborative gross or histological changes, this increase in weight was not considered to be of toxicological significance.
Unadjusted absolute and adjusted mean spleen weights were decreased in males at 25,000 ppm (↓15 and 14%, respectively). In females, only absolute spleen weights were calculated, and they were decreased by 12, 34, and 29% at 1000, 5000, and 25,000 ppm, respectively. None of the changes in spleen weight were statistically significant. No correlative histological changes were noted.
Thymus weight was reduced at 5000 ppm (males) and 25,000 ppm (males and females) in comparison with controls, though statistical significance was not achieved and some degree of overlap of individual values between treated and controls was evident. There were increased incidences of involution/atrophy in the thymus in males at all doses, with an increase at the top-dose. In males, the incidences (grades) of thymic involution/atrophy were 0/4, 1/4 (minimal), 1/4 (slight), 2/4 (minimal), and in females 1/4 (minimal), 1/4 (slight), 1/4 (minimal), and 0/4 at dietary concentrations of lenacil of 0, 1000, 5000, 25,000 ppm, respectively. Females also had decreased mean thymus weight at 25,000 ppm, but no cases of involution/atrophy. Given that the grade of involution/atrophy did not correlate with dose, it is difficult to conclude that the 2/4 cases of involution/atrophy in males at 25,000 ppm is test substance- related.
Conclusion:
Lenacil caused increased liver weight in males at 25,000 ppm and hepatocellular hypertrophy (≥5000 ppm in males, one case at 25,000 ppm in females), with an associated increase in ALP (25,000 ppm, both sexes). No other correlative clinical chemistry markers indicating hepato/biliary toxicity were present, and consistent with current thinking in toxicologic pathology, this finding would be considered adaptive. Thyroid + parathyroid gland weights were marginally increased at 5000 ppm in both sexes and was up by ≥20% at 25,000 ppm. Spleen weights were decreased at 25,000 ppm in males, and possibly at ≥ 5000 ppm in females. Thymus weights were also decreased at 25,000 ppm in males but were variably decreased in females (no dose response). Minor changes were noted in various haematological, blood chemistry, urinalysis, organ weight and pathology parameters, but typically showed no dose relationship, no trends for increasing effect over time or with increasing dose and showed no consistency between the sexes. Dietary concentrations of 5,000 ppm lenacil (221 mg/kg bw/day) can be considered a NOAEL based on adverse or potentially adverse decrease in thymus weight and the involution/atrophy observed in at 25,000 ppm in males. This is in contrast to the assessment made during the first EU review (EFSA, 2009), that considered 1000 ppm (44 mg/kg bw/day) the NOAEL.

Rabbit
Teratogenicity study of DPX-B634-91 in rabbits. DuPont Report No.: HLR 626-91. ([redacted] 1991).
Guidelines: OECD 414, NohSan 59 No.4200, US EPA 83-3
GLP: Yes

Reliability Score: 1
Summary: Lenacil (Code DPX-B634-91, Batch No. 9038, purity 98.5%) was administered once daily by oral gavage as an aqueous solution of 0.5% w/v methyl cellulose at daily dose levels of 0, 50, 200, 1000 or 4000 mg/kg of bodyweight to pregnant rabbits (Hra:NZW) SPF on GD 7 to 19. While 20 does/group were artificially inseminated, this resulted in only 15-16 pregnancies/group. Control animals received the vehicle. The dosing volume was 10 mL/kg of body weight.
Body weights and food consumption were measured daily throughout treatment and on days 24 and 29. Dams were sacrificed on day 29 and their intrauterine content examined. Foetuses were removed, foetal parameters taken and all subjected to external, skull, visceral and skeletal examination for abnormalities.
Results: There were no deaths attributable to treatment. Only one animal aborted, that being a doe at 1000 mg/kg, which was not attributed to treatment. High-dose dams showed a non-significant increase in stained tail during dosing, that attained statistical significance post-dosing (5/20 in control versus 12/20 at the top dose, GD 20-29).
A significant trend towards reduction in maternal weight gain was seen from GD 13 to 16 as well as during the overall dosing period (days 7-20). This trend attained statistical significance at the highest dose level (body weight gain at 4000 mg/kg ↓74% compared to control over GD 13-16; ↓ 67% over GD 7-20). At the top dose a recovery of body weight gain was seen post-dosing (↑96% over GD 20-29).

There was no change during pre- and dosing period in mean maternal food consumption, but a significant upward trend was observed during post-dosing, attaining statistical significance at the highest dose level.
Treatment did not influence litter data such as number of live/dead foetuses, resorptions, corpora lutea, and implantations and mean foetal weights. The marginal variations of resorption rate or litter size at 1000 or 4000 mg/kg bw/d were considered of no toxicological relevance. External, visceral or skeletal examinations did not reveal findings attributable to treatment.
Conclusion:
Oral administration of lenacil to rabbits did not affect foetal parameters at any of the doses tested. Maternal toxicity (reduced body weight gain during gestation) was evident at a daily dose of 4000 mg/kg/day. Therefore, the NOAEL was 1000 mg/kg/day for the dams based on decreased body weight gain, and greater than 4000 mg/kg/day for developmental effects, as no malformations or other signs indicative of developmental toxicity were observed.

4.4 OECD Level 5 Sensitive to, but Not Diagnostic of EATS - In Vivo Studies Assessing Mammalian Endocrine Activity Endpoints

4.4.1a Lenacil Technical: Preliminary Study of Effects on Reproductive Performance in Han Wistar Rats by Dietary Administration. DuPont Report No.: ACD 019/010186. ([redacted] 2002).
4.4.1b Study of Reproductive Performance in Han Wistar Rats Treated Continuously Through Two Successive Generations by Dietary Administration. DuPont Report No.: ACD 020/023865. ([redacted] 2003).
Guidelines: EC test method B.35 (1999) equivalent to OECD 416 (1999), OPPTS 870.3800 (1998), JMAFF 12 Nohsan No. 8147 (2000).
GLP: Yes
Reliability Score: 2
Summary:
During the main study, the F0 generation comprised of 28 male and 28 female rats, received 0, 1000, 10,000, 50,000 ppm via the diet for 10 weeks before pairing, throughout pairing, gestation and lactation, until termination. F0 males were terminated after 17 weeks of treatment and the F0 females were terminated on day 28 post partum. The unselected F0 offspring were terminated at day 30 of age. Selected F0 rats, comprising 24 males and 24 females were exposed via the diet from weaning until they were paired for mating at approximately 14 weeks of age.
Results:
The mean daily intakes for animals receiving 1000, 10000 or 50,000 ppm prior to pairing were 81.9, 817.0 and 4278.8 mg/kg/day for the males and 92.5, 934.7 and 4787.6 mg/kg/day for the females. For females during gestation and lactation the mean daily intakes were 91.6, 917.9 and 4839.8 mg/kg/day, and 166.6, 1727.6 and 8659.3 mg/kg/day, respectively.
Parental animals: Mortalities were not considered treatment-related. At 50,000 ppm rats in both sexes showed a slightly increased incidence of hairloss on the dorsal surface in the F0 generation animals (2/28, 1/28, 3/28, 5/28 in males, 7/28, 3/28, 6/28, 10/28 in females, at 0, 1000, 10,000 and 50,000 ppm, respectively). A similar pattern was seen in the F1 generation with an increase also possibly occuring at 10,000 ppm in males (5/24 at 10,000 versus 1/24 in the control). A slight increase in skin encrustation (dorsal body surface and tail), and brown (head) or yellow (perigenital) staining were seen in the top-dose males; however, due to the low incidence and the lack of it being repeated between generations, the relationship of this to test substance treatment is not clear.
Overall bodyweight and body weight gain for F0 and F1 males was unaffected or only slightly decreased by treatment. F0 females showed only slight weight or weight gain decreases prior to mating; whereas F1 females had an overall decrease in body gain of 9% that was statistically significant at 50,000 ppm.
During gestation, body weight of F0 and F1 females was only slightly decreased (↓4- 7%) and the decreases were not statitstically significant. F0 females at ≥10,000 ppm and F1 females at 50,000 ppm showed minimal decreases in body weight gain (↓7 to 9% and statistically significant).

At the start of and during lactation, body weight of F0 dams at ≥10,000 ppm were slightly but not significantly (~5%) lower than controls. Maternal body weight gain was similar to controls or exceeded controls, except during lactation days 7-14 at 50,000 ppm, where gain was down by 38% (statistically significant) compared to the control. During the

same interval, body weight gain at 1000 and 10,000 ppm was down by 19% (not statistically significant). For F1 dams at the start of lactation, mean body weights were also slightly lower than controls at ≥10,000 ppm (↓6 and 7% at 10,000 and 50,000 ppm, not statistically significant). Body weight gain for treated F1 dams was similar to or exceeded controls except at ≥10,000 ppm during the day 4-7 interval where gains were down by 29% at both doses and the day 7-14 interval in which they were down by 12-18% (not statistically significant).

There were no statistically significant decreases in F1 or F2 offspring body weight during lactation. At 10,000 ppm, transient decreases in offspring body weight gain occurred during lactation at intakes equivalent to ~1727 mg/kg bw/day for F0 and F1 dams. At 50,000 ppm, F1 and F2 offspring weight gains were statistically significantly reduced by 6-9% and 8-12%, respectively, during several lactation intervals, such that overall reductions in gain were 6-7% for F1 pups and 10% for F2 pups, when dietary intakes for dams ranged from ~7000 to 10,000 mg/kg bw/day.

Food consumption and conversion efficiency in F0 and F1 animals was unaffected during the first 10 weeks of treatment (premating).

Oestrus cycle, precoital interval, mating performance, fertility index, gestation index and length, litter size, sex ratio and offspring survival were unaffected by lenacil.

Lenacil did not delay the return to normal oestrus cycle of the F0 and F1 females. All females showed oestrus before termination on Day 28 post partum. Sperm motility, morphology and concentration were unaffected by treatment.

Liver weight was increased (rel. bw 5-6%) in F0 and F1 parental rats at 10,000 ppm and 50,000 ppm (rel. bw 12-16%) and was generally statistically significant.

Thyroid+parathyroid weight was statistically significantly increased (rel. bw 12-19%) in F0 and F1 parental rats at the 50,000 ppm.

Pituitary weight was unaltered in the F0 animals, but showed an increase of 27 and 40% in relative weight (to bw) in F1 males at 10,000 and 50,000 ppm, respectively. Given that there were no histological correlates to this finding, it may merely reflect biological variability.

F1 females at 50,000 ppm had lower uterine weights (abs. 22%, rel. to bw 18%) on day 28 post partum. A comparison of individual uterine weights with the oestrus cycle stage suggested a correlation between the terminal oestrus cycle stage and uterine weight at termination.

Sex organ weights were unaffected by the treatment with lenacil.

Day 30 F1 male offspring absolute organ weights were mildly decreased at 50,000 ppm, in part, due to the decrease in termal body weight (7%, statistically significant). In this regard, absolute spleen and thymus weights were statistically significantly down by 14 and 13%, respectivly; however, spleen and thymus weights relative to body weight were not affected. A similar pattern was seen with the day 30 F2 male offspring absolute and relative spleen and thymus weights. Day 30 body weight at 50,000 ppm was down by 11% (statistically significant), and absolute spleen and thymus weights were down by 14 and 18%, respectively (statistically significant). When calculated as organ weights relative to body weight, spleen weight was not affected, but thymus weight was mildly decreased by 10% and statistically significant.

Day 30 F1 females offspring absolute spleen and thymus weights were down (19 and 14%, respectively) at 50,000 ppm. However, when calculated as relative weights only spleen weight was decreased (14%, statistically significant).

Absolute, but not relative spleen weights in day 28 post partum F1 females were slightly, but statistically significantly decreased at 10,000 and 50,000 ppm (9% in both cases). F1 female relative weights were only down by 4-5%. Similar findings were not seen in F0 females or F1 males.

Absolute and relative thymus weights in day 28 post partum F1 females were mildly decreased (abs. 21%, rel. bw 17%) at 50,000 ppm. No similar finding was seen in day 28 post partum F0 females, or in F0 or F1 week 17 males, and no correlative histological changes were observed in the thymus. Thus, the significance of this finding is unclear.

Histological examination of the control versus 50,000 ppm female uterus, uterus/cervical region and vagina did not reveal any statistically significant differences. A very minimal increase in some findings may have occurred, but given the minimal level of these findings they were considered incidental by the performing laboratory and were not further investigated. They are listed in the following table.

TABLE 2HISTOLOGICAL CHANGES IN THE UTERUS, UTERUS/CERVIX & VAGINA FROM CONTROL & 50,000 PPM FEMALESPOST-PARTUM DAY 28

Finding		*0 ppm*	*50,000 ppm*
Acute inflammatory infiltration of the uterine/cervical epithelium	*F0*	*0/25*	*0/27*
	F1	*2/22 (9%)*	*4/23 (17%)*
Uterine myometrial hyalinisation	*F0*	*17/25 (68%)*	*21/27 (77%)*
	F1	*12/22 (55%)*	*19/24 (79%)*
Uterine pigmented macrophage accumulation	*F0*	*14/25 (56%)*	*18/27 (67%)*
	F1	*10/22 (45%)*	*16/24 (67%)*
Acute inflammatory infiltration of vaginal epithelium:	*F0*	*0/25*	*2/27 (7%)*
	F1	*1/21 (5%)*	*6/24 (25%)*

After 17 weeks of treament one F0 male out of 27 showed a gross finding ofdark thyroid at 50,000 ppm versus no such finding in the control or in other dosed groups. On day 28 post-partum, F0 females also had gross findings of dark thyroids (0/25, 0/28, 1/27 and 25/27 at 0, 1000, 10,000 and 50,000 ppm). For F1 males, after 17 weeks of treament 0/24 cases of dark thyroid were found by gross examination at 0 and 1000 ppm, but 5/24 and 23/24 cases were observed at 10,000 and 50,000 ppm. In day 28 post-partum F1 females, dark thyroids were also seen (0/22, 0/23, 8/22 and 22/24 at 0, 1000, 10,000 and 50,000 ppm).
A few enlarged thyroids was also observed at the highest dose tested in F1 animals (2/24 males, 1/24 females) but not in the controls or in any other dose group.
In F0 male thyroids, there was an increased intensity of Schmorl's positive staining at ≥10,000 ppm (e.g. 1/20 cases of moderate staining in control and 1000 ppm versus 3/20 and 10/20 at 10,000 and 50,000 ppm). Additionally, there was an increase in follicular cellular debris (0/20 in control and 1000 ppm versus 6/20 and 15/20 at 10,000 and 50,000 ppm). Follicular cell hypertrophy was seen in 1/20, 0/20, 3/20 and 4/20 animals at 0, 1000, 10,000 and 50,000 ppm, respectively. A similar pattern was seen in F1 males, however, findings tended to only be observed at 50,000 ppm. A single follicular cell adenoma was observed in a high dose F1 male.
In F0 females at ≥10,000 ppm there was an increased incidence and degree of Schmorl's positive staining (incidence: 6/25, 9/28, 20/27 and 24/27 at 0, 1000, 10,000 and 50,000 ppm, respectively) of the thyroid, as well as an increase in follicular cell debris (0/25, 0/28, 5/27 and 25/27 at 0, 1000, 10,000 and 50,000 ppm, respectively). Follicular cell hypertrophy was only increased at 50,000 ppm (0/25 control versus 9/27 at 50,000 ppm). A similar pattern was seen in F1 females.

Liver histopathology was only examined in males after 17 weeks on study. In that regard, hepatocellular hypertrophy was not seen in controls for either parental group (F0: 0/28, F1: 0/24), but a marginal effect was seen at 50,000 ppm (F0: 1/28, F1: 3/24).
Pre-weaning surface and air righting reflex were unaffected and all offspring displayed normal auditory and visual responses. Physical sexual maturation of the selected rats, as assessed by the age and bodyweight at completion of balano-preputial separation and vaginal opening, was unaffected by treatment.

Conclusion:
Based on findings in the thyroid of cellular debris, darkening and increases in Schmorl's positive staining at ≥10,000 ppm, the NOEL can be considered 1000 ppm. Additionally, a mild increase in follicular cell hypertrophy occurred in F0 parental males at ≥10,000 ppm, and both sets of parental animals at 50,000 ppm.

Liver weight was increased in F0 and F1 parental rats at 50,000 ppm (rel. bw 12-16%) and was generally statistically significantly. Thyroid+parathyroid weight was statistically significantly increased (rel. bw 12-19%) in F0 and F1 parental rats at the 50,000 ppm.
No effect on fertility was observed at any dietary concentration of lenacil. Therefore, the NOEL for reproductive toxicity was 50,000 ppm, the highest dose tested. Oestrus cycle, precoital interval, mating performance, fertility index, gestation index and length, litter size, sex ratio and offspring survival were unaffected by lenacil. Sperm motility, morphology and concentration were unaffected by treatment.
The NOAEL for pups was 10,000 ppm based on decreases in pup weight during lactation at 50,000 ppm.

5.0 (ecotoxicity studies, see Vol.3, B.9)

6.0 LITERATURE STUDIES ASSESSING ENDOCRINE ACTIVITY ENDPOINTS

The following studies were identified through the literature search performed in support of registration (Document M-CA, Section 9, Literature Data. Report No. DuPont-43896 EU). Although the studies were not performed following OECD Guidelines or to GLP specifications, they are considered relevant and are presented as supporting information.

Screening for Oestrogen and Androgen Receptor Activities in 200 Pesticides by In Vitro Reporter Gene Assays Using Chinese Hamster Ovary Cells. MCA 5.8.3/01. (Kojima, H., 2004).

Guideline: None

GLP: No

Summary: Lenacil was tested in a group of 200 pesticides for agonism and antagonism to two human oestrogen receptor (hER) subtypes, hERα and hERβ, and a human androgen receptor (hAR) by highly sensitive transactivation assays using Chinese hamster ovary cells at concentrations < 10^{-5} M.

Agonistic activity was evaluated using relative activity expressed as REC20 (20% relative effective concentration) which was the concentration of test compound showing 20% of the activity of 10^{-10} M E2 for ERα, 10^{-9} M E2 for ERβ, or 10^{-9} M DHT for AR. When the activity of the test compound exceeded the REC20 within the concentration tested (~10^{-8} to 10^{-5} M), the chemical was considered positive. Chemical antagonistic activities were expressed as RIC20 (20% relative inhibitory concentration), or the concentration of the test substance showing 20% inhibition of activity induced by 10^{-11} M E2 for ERα, 10^{-10} M E2 ERβ or 10^{-10} M DHT for AR. If activity of the test substance exceeded the RIC20 within the concentration tested, the chemical was considered positive for inhibitory activity. Assays were performed at concentrations ≤ 10^{-5} M to avoid cell toxicity.

Results:

Results for the dose-dependent transactivation of ERα and ERβ by the positive control E2 showed maximal ERα activity was achieved at ≥ 10^{-10} M E2 (~10-fold that of the control solvent). The maximal activity induced for ERβ was 8.5-fold that of the solvent control at ≥10^{-9} M E2. E2 REC20 values for ERα and ERβ were reported as 2.5 × 10^{-12} M and 5.3 × 10^{-12} M, respectively. For the dose- dependent transactivation of AR by the positive control 5α-DHT, activity was detected from 10^{-11} M DHT to a maximum of 10^{-9} M DHT; the maximum induction was 21-fold of the control solvent. The REC20 value of DHT for AR was 3.1 × 10^{-11} M. Individual results were only reported for chemicals that were positive. No specific data was provided for lenacil.

Conclusion:

No details were provided on the performance of lenacil, therefore under the conditions of this study it was considered negative for both agonism and antagonism at the androgen and oestrogen receptors. These results are supported by the negative findings in the Level 2 OECD guideline-compliant ARTA and ERTA studies presented in Section 4.2 of this document.

In Vitro Screening for Aryl Hydrocarbon Receptor agonistic Activity in 200 Pesticides Using a Highly Sensitive Reporter Cell Line, DR-EcoScreen cells, and in vivo Mouse Liver Cytochrome P450-1A induction by Propanil, Diuron and Linuron. MCA 5.8.2/03. (Takeuchi, S., 2008).

Guideline: None

GLP: No

Summary:

Lenacil was tested in a group of 200 pesticides using DR-EcoScreen cells, a highly sensitive AhR-mediated reporter cell line consisting of mouse hepatoma Hepa1c1c7 cells stably transfected with a reporter plasmid containing seven copies of dioxin-responsive element, to characterize its AhR agonistic activity. The reporter gene assay was performed at concentrations ranging from 1 X 10^{-7} to 1 X 10^{-5} M, and the relative luciferase activity expressed as percentage induction with 100% activity defined as the AhR agonistic activity achieved by 10^{-10} M TCDD and expressed as 50% and 20% relative effective concentration, REC50 and REC20, respectively. If the activity of the test compound exceeded the REC20 within the concentration tested it was considered to be positive for activity.

Only three of the chemicals (Propanil, Diuron, Linuron) were further evaluated for AhR agonistic activity in vivo. Gene expression of AhR-inducible cytochrome P450 1As (CYP1As) in the livers of female C57BL/6 mice was examined by intraperitoneally injecting the chemicals then measuring the amount of mRNA induction using quantitative RT-PCR.
Results:

Luciferase activity induced by TCDD increased dose-dependently at concentrations from 10^{-13} M to 10^{-10} M, and incubation time-dependently from 3h to 24h. The minimal detection limit (MDL; means of control solvent + 10 X SD, n = 8) for TCDD was estimated to 0.003 pg well^{-1} (1 X 10^{-13} M). The maximal intensity of the AhR activity induced by 1 X 10^{-10} M TCDD was achieved at 18–24 h incubation time and was approximately 17-fold that of 0.1% DMSO, the control solvent.
The AhR ligands TCDD, PCB # 126, 3-MC, b-naphthoflavone and benzo[a]pyrene transactivated the receptor in a dose-dependent at low concentrations. The relative potency calculated from the REC20 values indicates that the AhR agonistic activity of PCB # 126, 3-MC, b-naphthoflavone and benzo[a]pyrene was about 21, 1250, 5900 and 10000-fold less than that of TCDD, respectively.
Percent relative luciferace activity for the 200 individual chemicals assayed in the DR-EcoScreen was reported graphically. Eleven of the chemicals, not including lenacil, induced AhR-mediated transcriptional activity greater than 20% of the 10^{-10} M TCDD-induced maximal activity. A value for lenacil activity could not be estimated by visual inspection of the graph; there was no bar or error bar present suggesting there was no or very minimal activity. No value was reported in any data tables, and lenacil was not reported as assayed in vivo.
Conclusion:
The authors concluded that 189 of the 200 pesticides assayed did not have AhR agonistic activity. Since lenacil was not listed as one of the eleven chemicals producing a positive response, under conditions of this study it can be considered negative for agonism of the AhR.

In Vitro Screening of 200 Pesticides for agonistic activity via mouse peroxisome proliferator activated receptor PPARα and PPAR γ and quantitative analysis of in vivo induction pathway. MCA 5.8.2/02. (Takeuchi, S., 2006).
Guideline: None
GLP: No
Summary:
Lenacil was tested in a group of 200 pesticides to characterize mouse PPARα and PPARγ agonistic activities by in vitro reporter gene assays using CV-1 monkey kidney cells. After CV-1 cells were transiently transfected with the expression plasmid for mouse PPARα or PPARγ, the PPAR-responsive luciferase reporter plasmid and control plasmid, cells were treated with the positive controls WY-14643 and pioglitazone for PPARα and PPARγ, respectively. To investigate the in vivo effects of positive-testing chemicals, the gene expression of PPARα-inducible cytochrome P450 4As (CYP4As) was examined in the liver of female mice intraperitoneally injected with these compounds (≤300 mg/kg), followed by RT-PCR analysis.
Results:
The PPARα agonistic activity of WY-14643 was approximately 17.8-fold over the vehicle control at the concentrations of 1×10^{-5} M. Maximal pioglitazone- induced PPARγ activity was 15.7-fold greater than that of the vehicle control at the concentrations ≥ 1×10^{-5}M. REC20 values of WY-14643 for PPARα and of pioglitazone for PPARγ were calculated from the dose-response curves to be 4.9×10^{-7} M and 1.6×10^{-7} M, respectively.
Most of the 200 pesticides tested (including lenacil) did not activate PPARα or PPARγ in the in vitro assay. A value for lenacil activity could not be determined by visual inspection of the graph and it was not reported in any data tables. Lenacil was not assayed in vivo.
Conclusion:
No details were provided on the performance of lenacil and it was not reported as tested in vivo. Visual inspection of the graph shows no bar or statistical data, therefore under the conditions of this study it can be considered minimally active or negative for activation of either PPARα or PPARγ in vitro.

Comparative Study of Human and Mouse Pregnane X Receptor Activity in 200 Pesticides using In Vitro Gene Assays. MCA 5.8.2/01. (Kojima, H., 2011).

Guideline: None

GLP: No

Summary:

Lenacil was tested in a group of 200 pesticides in order to characterize and compare the agonistic activities of a variety of pesticides against human PXR (hPXR) and mouse PXR (mPXR). The hPXR and mPXR agonistic activity of 200 pesticides was tested by reporter gene assays using COS-7 simian kidney cells. After a 3-h transfection period, cells were dosed with various concentrations of the test compounds or with 0.1% DMSO (vehicle control) in complete medium. screening assays for test chemicals was performed at concentrations from 1×10^{-7} to 1×10^{-5} M to avoid any cytotoxic effects. Luminescence intensity of the assay was presented as a dose–response curve, then the concentration of the compound equal to 20% of the maximal response of positive control (rifampicin - RIF, pregnenolone 16-α-carbonitrile - PCN) from the dose–response curve of the luminescence intensity was expressed as an REC20 (20% relative effective concentration) value. If the agonistic activity of the test compound exceeded the REC20 value for the concentration tested ($\leq1\times10^{-5}$ M), the chemical was considered positive for agonistic activity against hPXR or mPXR.

Results:

hPXR was preferentially activated at very low concentrations of RIF, but not activated even at 1×10^{-5}M of PCN. The maximal hPXR activity of RIF was 8.5-fold that of the vehicle control at 1×10−5 M. In contrast, Fig. 1B shows the dose–response transactivation of mPXR by RIF and PCN, indicating that mPXR is preferentially activated at very low concentrations of PCN, but not activated even at 1×10−5M of RIF. Again, the maximal mPXR activity of PCN was 8.5-fold that of the vehicle control at 1×10−5 M. From these dose–response curves, we estimated the REC20 values of RIF for hPXR and PCN for mPXR to be 4.3×10−7M and 5.7×10−8 M, respectively.

Results were only reported for individual chemicals that were positive. No specific data was provided for lenacil.

Conclusion:

Since no data was provided for lenacil, under the conditions of this study it was assumed to be negative for agonism at either hPXR or mPXR.

7.0 DISCUSSION

A weight of evidence approach using Level 1 - 5 studies as described in the OECD Guidance Document 150 (2012) was used in this assessment to evaluate the potential endocrine disrupting properties of lenacil. According to the current EU guidance, results from Level 1, Level 2 in vitro and Level 3-5 in vivo studies may be used to assess EATS-mediated activity (EU, 2018b). In vitro endpoints evaluated for possible endocrine disruption activity of lenacil included agonist or antagonist activity and transcriptional activation at androgen and oestrogen receptors, aromatase inhibition and steroidogenesis. In vivo endpoints relevant for assessing the potential endocrine disrupting properties of the substance evaluated in this report included organ weight, gross anatomic changes and microscopic histopathology from repeated dose sub- chronic and chronic studies conducted in rats, mice and dogs, developmental toxicity studies in rats and rabbits and a multigeneration reproduction study in rats. The format and content of this review has been conducted to align with the EFSA-EChA guidance in this area (EU, 2018b).

The following sub-sections consider a weight of evidence approach looking across the toxicology data package for lenacil for the determination of potential endocrine mediated effects in tissue/organ systems in one or more of the previously described toxicology studies.

TABLE 5OUTLINE OF THE LOGIC FLOW FOR THE LENACIL EDASSESSMENT

Activity	***Activity details***	***Result***
Gather information	*Guideline studies*	*Available data from guideline studies is presented*
	Other scientific data	*Obtained from the Literature Review (DuPont-43896).*
	Systematic review	*All data was reviewed, and studies updated based on available technical information.*
Assess the evidence	*All data was assembled and assessed*	*Data was organized by group based upon OECD GD 150 parameters, then assessed according to the method of Klimisch, et al. 1997. Values were assigned, and the information was included in this assessment if relevant and reliable (Klimisch score of 1 or 2). By exception, corroborative information was presented even though it was not Klimisch 1 or 2 (literature studies).*
Initial analysis of the evidence	*Has EATS mediated adversity been observed?*	*The initial analysis might be suggestive of an EATS- mediated MoA; this warranted further studies, some which have not been reviewed by the RMS, but are summarized in this paper.*
Mode of action analysis	*All data was examined, and various modes of action related to EATS mediated effects were addressed*	*Based on an assessment of all the data available, the initial suggestion for an EAS modality is ruled out. The data for T is inconsistent.*

The following sub-sections consider a weight of evidence approach looking across the toxicology data package for lenacil for the determination of potential endocrine mediated effects in tissue/organ systems in one or more of the previously described toxicology studies.

Lines of evidence for endocrine disrupting activity in various organs in mammalian toxicology studies by modality

EAS - Oestrogen, Androgen and Steroidogenesis

Reproduction and Development

The reproductive and developmental toxicity of lenacil was evaluated in rats and rabbits. Overall, the reproductive and developmental toxicity studies indicate that lenacil is not toxic to reproduction or development, and there was no indication of effects suggestive of an adverse effect on the endocrine system. No effects on offspring bodyweight occurred during lactation, however decreases in body weight gain occurred at doses well in excess of the guideline limit of 1000 mg/kg bw. Oestrus cycle, precoital interval, mating performance, fertility index, gestation index and length, litter size, sex ratio and offspring survival were unaffected by lenacil.

Lenacil did not delay the return to normal oestrus cycle of the F0 and F1 females. All females showed oestrus before termination on Day 28 post partum. Sperm motility, morphology and concentration were unaffected by treatment.

Reproductive Tract

Female Reproductive Organs

Except for the marginal response of mammary tumours and some slight changes in the uterus that were considered incidental in the 2-year rat study (e.g. endometrial hyperplasia of 4% in the controls versus 12% at 25,000 ppm; uterine luminal dilation in 34% of the control versus 54% at 25,000 ppm) there were no findings in the reproductive organs attributable to lenacil. These findings in the rat are not attributable to EAS mechanisms, based on the results of in vitro and in vivo endocrine assays, based both on guideline studies (see sections 4.2 and 4.3) and studies in the literature (see section 6).

Male Reproductive Organs

There were no indications of any changes in males in any species tested. There was no indication of an impact of lenacil on male fertility in the multigeneration reproduction study in rats, and no indication of an impact on any EAS-related parameters.

Thyroid

Thyroid/parathyroid gland

In some lenacil repeated dose rat studies a gross observation of dark was observed. Upon histological examination, increased pigmentation was noted. A special stain (Schmorl's) was used to further characterize these changes. Male controls in the 13- week study had a high frequency of Schmorl's staining; but with lenacil treatment at 50,000 ppm the intensity of staining increased. In females there was a very low incidence of Schmorl's positive staining in controls but the incidence and, to a degree, the intensity of staining increased at 50,000 ppm. There was possibly also a test substance-related increase in incidence at 5000 ppm in females. After a 4-week recovery period, Schmorl's staining in control males mostly resolved to control levels but not in females. Schmorl's stain is known to stain melanin and lipofuscin. The connection of these changes in pigmentation to potential thyroid hormone changes is unclear. These staining changes may be related to the increased thyroid concretions and cellular debris that was also increased with dose. It is likely this is what has been referred to as "altered colloid" in a recent publication of pathology nomenclature (Brändli-Baiocco, 2018).

Thyroid weight was increased in high dose rats. In addition, follicular cell hypertrophy was noted at ≥25,000 ppm in F0 parental rats in the multigeneration rat study and at a very low incidence in F1 parental animals at 50,000 ppm in both sexes. However, there was no marked increase in follicular cell hypertrophy with dose in the interim phase of the 2-year rat study. Finally, while the incidence of follicular cell adenomas was increased in high-dose females, it remained within the historical HCD range of the performing laboratory. The incidence of carcinomas was not elevated in a dose-dependent manner. ECHA (2013) concluded that there was no evidence that Lenacil induced follicular cell tumours.

Mechanistic studies show that lenacil does not inhibit the deiodinase that converts T4 to T3. The data also show that lenacil did not impair the uptake of iodine into the thyroid or the iodinification of organic compounds in the thyroid via thyroid peroxidases. Measurements of T4 gave an inconsistent picture (see table below). In the 20-week study with lenacil in female Han Wistar rats, there was a decrease in T4 at both 10 and 19 weeks. While these decreases were statistically significant, they occurred without an increase in TSH or a decrease in T3. Additionally, there was no dose response between lenacil dietary concentrations as disparate at 250 and 25,000 ppm. At the 52-week timepoint in the 2-year study in Han Wistar rats there were no marked changes in males or females for T3, T4 or TSH. In a recent study in male Sprague Dawley rats, while there was a dose-dependent induction of UDPGT, there was no typical pattern of T3, T4 or TSH changes. In all three mechanistic, rT3 was also examined and showed no consistent pattern of change. These data demonstrate that there were no consistent or adverse changes in thyroid hormone economy. The minimal changes may have represented biological variation or subtle adaptive changes in hormonal regulation.

There was a marginal increase in C-cell tumours in female rats. The finding of increased C-cell tumors in the female rats in this study has been suggested to be age- and sex-related, and unlikely related to lenacil treatment. These tumours are typically linked to changes in calcitonin. Calcitonin modulates calcium and phosphorus, and in the rat study database for lenacil, there were no consistent changes in calcium or phosphorus to suggest modulation by calcitonin. Finally, examination of the thyroid tissues on week 52 of the rat cancer study showed no treatment-related findings suggestive of precursor C-cell tumors. Finally, C-cell tumours are not mediated by classical thyroid hormones, thus, there would be no relationship between them and T3, T4 or TSH.

Considering all the data related to thyroid hormones, there was no consistent pattern suggestive of a clear impact on the T modality.

TABLE 6SUMMARY OF THYROID HORMONE LEVELS IN RAT STUDIES

Report/Design	*Duration of treatment (weeks)*	*ppm in diet*	*T3 nmol/L[b] Total*	*T4 nmol/L[b] Total*	*TSH nmol/mL[b]*	*rT3 ng/L[b]*
[redacted] *2004,* [redacted] *2007, Amend. 1 Wistar ♀; n=6, plasma*	*10*	*0*	*1.03 (0.155)*	*32 (6.7)*	*5.2 (0.46)*	*0.17 (0.011)*
		250	*0.97 (0.131)*	*21* (5.6)*	*5.0 (0.23)*	*0.16 (0.010)*
		50,000	*1.04 (0.158)*	*20* (3.9)*	*5.8 (0.75)*	*0.16 (0.008)*
	19	*0*	*_a*	*28 (7.9)*	*6.6 (0.62)*	*0.23 (0.037)*
		250	*_a*	*23 (6.5)*	*5.7 (1.75)*	*0.19* (0.022)*
		50,000	*_a*	*25 (7.1)*	*6.0 (0.89)*	*0.19* (0.012)*
			T3 pmol/L Free	*T4 pmol/L Free*	*TSH nmol/ml*	*rT3 ng/ml*
[redacted] *2004 Wistar ♂; n=20, plasma*	*52*	*0*	*1.1 (0.26)*	*12.4 (2.20)*	*6.3 (1.12)*	*n.d.*
		25,000	*1.2 (0.22)*	*13.0 (2.50)*	*8.4 (3.58)*	*n.d.*
[redacted] *2004 Wistar ♀; n=19-20, plasma*		*0*	*1.4 (0.42)*	*8.9 (2.94)*	*5.4 (0.72)*	*n.d.*
		25,000	*1.5 (0.34)*	*8.0 (2.29)*	*6.9 (3.37)*	*n.d.*
			T3 ng/dl	*T4 µg/dl*	*TSH ng/ml*	*rT3 ng/ml*
[redacted] *2019 Crl:CD(SD) ♂ n=13-15, serum*	*2*	*0*	*63.110 (9.383)*	*4.174 (0.505)*	*8.237 (3.963)*	*0.057 (0.013)*
		2,500	*62.744 (14.045)*	*4.075 (0.606)*	*(7.719) 2.677*	*0.066 (0.013)*
		12,500	*58.950 (13.425)*	*4.032 (0.428)*	*7.243 (3.487)*	*0.069* (0.010)*
		25,000	*49.075* (6.914)*	*4.188 (0.792)*	*6.215* (4.223)*	*0.070* (0.013)*

a: According to the report, this portion of the assay failed. b: Order of data presentation: Mean (S.D)
n.d.: not determined.
**: statistically significant from the control (see reports for degree of significance)*

Liver

Although not necessarily related to endocrine changes, increases in liver weights and hepatocellular hypertrophy could be associated with endocrine effects since the liver is the primary site of biotransformation of steroid and thyroid hormones. Increased hepatic metabolism may lead to alterations in circulating levels of hormones, which may in turn affect the target organs of these hormones (WHO, 2002; 2012). In this regard, increases in liver weight that usually correlated with hepatocellular hypertrophy were observed at the highest dose tested in all species (e.g. 90-day rat, 90-day dog, 2-year rat and 18-month mouse study.
In addition, a recently conducted 2-week study in rats has shown increased liver weight and minimal hepatocellular hypertrophy at dietary concentrations of 12,500 and 25,000 ppm lenacil. Consistent with an adaptive response, hepatic UDPGT activity and hepatic microsomal CYP2B1 gene expression were increased in a test substance-related manner. Although hepatic UDPGT activity was induced, there was no difference observed in serum T4 concentration in either treatment group.
Furthermore, there were no effects on thyroid organ weights, no evidence of thyroid follicular cell hypertrophy and TSH was not elevated.

TABLE 7SUMMARY OF ENDOCRINE-RELATED MECHANISTIC STUDIES

TYPE OF STUDY	*DOSES/ RANGE TESTED*	*FINDINGS*	*REFERENCE*
Oestrogen Receptor Binding Assay (ER-RUC)	*Range: 10^{-10} to 10^{-3} M*	*Negative - E*	*Nabb, D.L., 2018*
Oestrogen Receptor Agonism/Antagonism (hERα-HeLa-9903 cells)	*Ag: 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM and 10 pM; Antag: 10 µM, 1 µM, 100 nM, 10 nM, 1 nM and 100 pM*	*Negative - E*	*Rijk J.C.W., 2018*
Uterotrophic Assay	*0, 500, 1000 mg/kg/day*	*Negative - E*	[redacted] *2018*
Androgen Receptor Binding Assay (AR-RPC)	*Range: 10^{-10} to 10^{-3} M*	*Negative - A*	*Nabb, D.L., 2018*
Androgenic Agonism/Antagonism (AR EcoScreen™)	*Ag: 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM and 1 pM; Antag: 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM*	*Negative - A*	*Rijk J.C.W., 2018*
In vitro steroidogenesis in H295R cells	*Up to 3.16 µM*	*Negative - EAS*	*Verkaart, S., 2019*
In vitro aromatase in Human Recombinant Microsomes	*0.00001 to 31.6 µM*	*Negative - EAS*	*Rijk, J.C., 2019*
20 Week Thyroid Function in Female Han Wistar Rats – Perchlorate Discharge Test	*0, 250, 50000 ppm*	*Inconsistent - T*	[redacted] *2004*
Thyroid Mechanistic 14- Day Feeding Study in Rats	*0, 2500, 12500, 25000 ppm*	*Inconsistent - T*	[redacted] *2019*
52-week plasma analysis	*0 and 25000 ppm*	*Inconsistent - T*	[redacted] *2003*

Sensitive to, but not diagnostic of EATS

Pituitary

Minimal changes in the pituitary gland (pituitary cysts and weight) were observed that were most likely unrelated to lenacil treatment. This included a slight increase in the incidence of cysts in high-dose males in the 13-week dog study and 18-month mouse study, and weight changes in rats. It is noteworthy that the incidence of cysts in high dose male dogs was the same as the incidence in control females (2/4 animals), and the incidence in the high dose female dogs was the same as the male control (0/4 animals). Given this pattern, it doubtful that these findings are test substance-related. In high-dose male mice from the 18-month carcinogenicity study there were 6 cases of pituitary cysts versus 2 cases in controls. Pituitary cysts in rodents are non-proliferative lesions and typically represent either the remnants of the craniophyrangeal (Rathke's) pouch (Morton and Tekeli, 1997) or cysts or pseudocysts (Isobe et al. 2017). Given, the low numbers in this study and their lack of statistical significance, this finding is likely incidental, as supported by the interpretation of the study pathologist.

Pituitary weights were slightly increased in male parental F1 rats in the 2-generation reproduction study; however, the apparent statistically significant increased pituitary weights observed in the F1 generation males at 10,000 and 50,000 ppm lenacil after 17 weeks of treatment are most likely due to excessively lower pituitary weights in several animals in the F1 generation control group. In this regard, four males from the F1 generation controls had very low pituitary gland weights, as compared to the F0 generation. The lowest pituitary weight measured for the F0 generation control group males was a single animal weighing 0.05 grams. In contrast, for the F1 generation control group males, the lowest weight measured was 0.03 grams (animal

 # 2010) and three other males weighed 0.04 grams (animal numbers 2018, 2023 and 2024); in addition, four animals weighed 0.05 grams. Therefore, the statistically significant increases in absolute pituitary weight in the F1 generation males at 50,000 ppm, and relative weights at 10,000 and 50,000 ppm may, in part, be due to an untypically low mean control value. The fact that this finding was not consistent across the generations suggests it may not be related to treatment with lenacil and may merely be a spurious finding.

Kidney

There was no indication of a test substance related effect on kidneys in the 13-week dietary study in dogs. In rats at dose levels of ~4000 mg/kg bw/d (50,000 ppm) in the 13-week study changes in kidney function were suggested based on statistically significant decreases in blood electrolytes and increases in creatinine. Suggestive changes in urinary parameters were also observed but there was no correlation with dose. However, there were no effects on kidney weight or histopathology. In the chronic cancer rat study at 25,000 ppm (~1200-1900 mg/kg/d), males at 2-years and females at ≥52 weeks had minimal increases in relative kidney weight (8-12%); however, with no histological correlates. In the 18-month mouse study, relative (bw) kidney weight was minimally reduced in females but they were not statistically significant, and there was no histological correlate. Given the available results, there is no indication that these changes were related to modulation of any endocrine- related parameters.

Ecotoxicology: EATS in Avian, Fish and Non-target Organisms

The weight of evidence from the avian, fish and non-target organism studies conducted with lenacil indicate that no EATS-mediated effects were observed in the relevant studies, and that where any effects were observed at higher dose levels, the effects in the apical reproduction endpoints were general in nature and likely an indirect effect of systemic toxicity.

Lines of evidence for endocrine disrupting activity in in vitro /in vivo mechanistic studies according to EATS modality

Oestrogen/Anti-Oestrogen (E), Androgen/Anti-Androgen (A), Thyroid (T) and Steroidogenesis (S)

A mechanism to elucidate the slight increase in mammary tumors is currently unknown; however, based on the data generated to date, these tumors occur in the absence of genotoxicity, and there is no indication of a perturbation of E, A, or S modalities by lenacil. Therefore, it is likely that some non-EAS mechanism of action is operative. Alternatively, the finding of mammary tumors in the rat could be a spurious finding. Considering all the data related to thyroid hormones, there was no consistent pattern suggestive of a clear impact on the T modality.

In non-target vertebrates and invertebrates, no effects which could be directly attributed to of the E, A, T or S modalities were observed.

8.0 CONCLUSION

Following a review of all available relevant data and a weight of evidence evaluation, lenacil does not cause an EAS interaction. While a minimal increase in mammary tumours was observed in rats, the finding was initially considered spurious, but after ECHA classified lenacil for carcinogenicity, a series of mechanistic studies were initiated. Based on the data generated, there is no data available to support a role for E, A, or S modalities. Data for T are inconsistent, possibly due to lenacil effects being very weak at best. In this regard, the inconsistent changes occur at dose levels that exceed the current guideline maximum targeted dose. Given the lack of consistency in the thyroid database, the data suggest that lenacil does not adversely impact thyroid hormone economy based on the minimal nature of changes observed. Thus, it is concluded that lenacil is not an "endocrine disruptor" according to the regulatory definition and associated guidance.

TABLE 8SELECTION OF RELEVANT SCENARIO FOR EATS-MEDIATED EFFECTS/ADVERSITY

Adversity based on EATS-mediated parameters	*Positive mechanistic OECD CF level 2/3 Test*	*Scenario*	*Next step of the assessment*	*Scenario selected*
No (sufficiently investigated)	*Yes/No*	*1a*	*Conclude: ED criteria not met because there is no "EATS-mediated" adversity*	***X***
Yes (sufficiently investigated)	*Yes/No*	*1b*	*Perform MoA analysis*	
No (not sufficiently investigated)	*Yes*	*2a (i)*	*Perform MoA analysis (additional information may be needed for the analysis)*	
No (not sufficiently investigated)	*No (sufficiently investigated)*	*2a (ii)*	*Conclude: ED criteria not met because no EATS-mediated endocrine activity observed*	
No (not sufficiently investigated)	*No (not sufficiently investigated)*	*2a (iii)*	*Generate missing level 2 and 3 information. Alternatively, generate missing "EATS- mediated" parameters. Depending on the outcome move to corresponding scenario*	
Yes (not sufficiently investigated)	*Yes/No*	*2b*	*Perform MoA analysis*	

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B.6.8.3.4 Summary of the studies on endocrine properties of lenacil.

Notifier discussed the obtained results as follows:
"A weight of evidence approach using Level 1 - 5 studies as described in the OECD Guidance Document 150 (2012) was used in this assessment to evaluate the potential endocrine disrupting properties of lenacil.
According to the current EU guidance, results from Level 1, Level 2 in vitro and Level 3-5 in vivo studies may be used to assess EATS-mediated activity (EU, 2018b).
In vitro endpoints evaluated for possible endocrine disruption activity of lenacil included agonist or antagonist activity and transcriptional activation at androgen and oestrogen receptors, aromatase inhibition and steroidogenesis.
In vivo endpoints relevant for assessing the potential endocrine disrupting properties of the substance evaluated in this report included organ weight, gross anatomic changes and microscopic histopathology from repeated dose sub- chronic and chronic studies conducted in rats, mice and dogs, developmental toxicity studies in rats and rabbits and a multigeneration reproduction study in rats. The format and content of this review has been conducted to align with the EFSA-ECHA guidance in this area (EU, 2018b).
The following sub-sections consider a weight of evidence approach looking across the toxicology data package for lenacil for the determination of potential endocrine mediated effects in tissue/organ systems in one or more of the previously described toxicology studies."

Table B.6.8.3.4-1 Outline of the logic flow for the lenacil ED assessment (Wohlman, 2019)

Activity	Activity details	Result
Gather information	Guideline studies	Available data from guideline studies is presented
	Other scientific data	Obtained from the Literature Review
	Systematic review	All data was reviewed, and studies updated based on available technical information.
Assess the evidence	All data was assembled and assessed	Data was organised by group based upon OECD GD 150 parameters, then assessed according to the method of Klimisch, *et al.* 1997. **RMS**: the acceptability of the studies is evaluated at the appropriate places, and relevance is not essentially driven bu the proposed Klimisch score, although the appraisal of study relevance is not meaningfully different from that of the notifier.
Initial analysis of the evidence	Has EATS mediated adversity been observed?	The initial analysis might be suggestive of an EATS- mediated MoA; this warranted further studies. **RMS**: studies were submitted and evaluated. Notifier to update the EU-dossier accordingly.
Mode of action analysis	All data was examined, and various modes of action related to EATS mediated effects were addressed	Based on an assessment of all the data available, the initial suggestion for an EAS modality is ruled out. The data for T is inconsistent. **RMS**: as evaluated below, RMS accepts the submitted studies, and the relevance of the thyroid adverse effects, along with its endocrinological relevance etiology, is further discussed.

The following sub-sections consider a weight of evidence approach looking across the toxicology data package for lenacil for the determination of potential endocrine mediated effects in tissue/organ systems in one or more of the previously described toxicology studies. **RMS** considers the strategy sufficiently underpinned.

B.6.8.3.4.1 Lines of evidence for ED activity in various organs in mammalian toxicology studies with lenacil

B.6.8.3.4.1.1 EAS - Oestrogen, Androgen and Steroidogenesis

Reproduction and Development

The reproductive and developmental toxicity of lenacil was evaluated in rats and rabbits. Overall, the reproductive and developmental toxicity studies indicate that lenacil is not toxic to reproduction or development, and there was no indication of effects suggestive of an adverse effect on the endocrine system. Notifier considered that no effects on offspring bodyweight occurred during lactation, however **RMS** highlighted decreases in body weight gain occurred, albeit at doses in excess of the guideline limit of 1000 mg/kg bw/d.

Oestrus cycle, precoital interval, mating performance, fertility index, gestation index and length, litter size, sex ratio and offspring survival were unaffected by lenacil. **RMS** noted a delay of VO at the top-dose. In the absence of any other oestrogenic effect, the finding is considered associated with high top-dose toxicity, well above the limit dose of 1000 mg/kg b.w./d.

Lenacil did not delay the return to normal oestrus cycle of the F_0 and F_1 ♀. All ♀showed oestrus before termination on d28 *post partum.* Sperm motility, morphology and concentration were unaffected by treatment.

Reproductive Tract

-Female Reproductive Organs

Of note are the emergence of mammary tumours, as well as mainly top-dose related mammary acinar hyperplasia.

- Mammary gland adenoma of 0% in the controls *vs.* 6% at ~1700 mg/kg b.w./d (borderline in HCD);
- Mammary gland adenocarcinoma of 0% in the controls *vs.* 12% at ~160 mg/kg b.w./d and 10% at ~1700 mg/kg b.w./d (no clear DR, borderline in some HCD);
- mammary acinar hyperplasia in 44%% of the control *vs.* 56% at ~1700 mg/kg b.w./d;

RMS recommended the MCF-7 cell proliferation assay (testing for ER ant/agonism) considered by OECD as a sensitive level 2 *in vitro* assay which could provide complementary data about selected endocrine mechanism(s) / pathways(s). Notifier conducted oestrogenic agonism and antagonism in the stably transfected human oestrogen receptor-α transactivation assay, and has oestrogen receptor binding using rat uterine cytosol, and did not conduct the MCF-7 cell proliferation assay, as there is no OECD guideline.

Taking into account the uncertainty (0 tumour incidence in study control), a Carc. Cat 2 was concluded at ECHA. **RMS** is of the opinion that this classification is sufficient, and does not enhance the level of concern as regards a possible involvement of an endocrine MoA.

The observed rat tumours driving the C&L, mammary adenocarcinoma, are potentially endocrine sensitive, but in the absence of both genotoxic or endocrine activity, are likely to be explained by a still unravelled epigenetic MoA

Also, some changes in the uterus were seen, which were considered incidental by the notifier in the 2-year rat study.

- endometrial hyperplasia of 4% in the controls *vs.* 12% at ~1700 mg/kg b.w./d;
- uterine luminal dilation in 34% of the control *vs.* 54% at ~1700 mg/kg b.w./d;
- uterus endometrial gland hyperplasia in 4% of the control *vs.* 12% at ~1700 mg/kg b.w./d.

Uterine fluid distention was also seen in the 28d rat study, but was not replicated in the 90d rat study. In addition, the uterotrophic assay in ovariectomised rats turned out negative.

There were no other findings in the reproductive organs attributable to lenacil. The mechanistic studies (see results of *in vitro* and *in vivo* endocrine assays) demonstrated that these findings in the rat are most probably not attributable to EAS mechanisms. It is possible that the uterine and mammary findings are associated with top-dose toxicity.

For the mammary findings, it remains not completely excluded that other (epigenetic?) mechanisms could play a role.
The studies relied upon are both guideline studies and studies in the literature.

-Male Reproductive Organs

There were no consistent indications of adverse effects on ♂ reproductive organs in the species tested. There was a marginal increase Leydig cell hyperplasia (in the LT mouse assay) at ~1000 mg/kg b.w./d (15%) *vs.* study control (9%), however without concomitant emergence of Leydig cell adenoma. There was no indication of an impact of lenacil on ♂fertility in the multigeneration reproduction study in rats, and the spurious weight modifications or histopathological findings in the various repeated toxicity studies did not replicate. In addition, the results of *in vitro* endocrine assays (no *in vivo* mechanistic performed)are not necessarily indicative of a ♂ hormonal imbalance.

Thus, **RMS** is of the opinion that there is not enough indication of an impact on any EAS-related parameters.

An overview of uterine findings was produced in table **B.6.8.3.4.1.1-1**, and a more extended table with the LoE is reproduced in table **B.6.8.3.4.1.1-2.** The latter table contains the effects which are considered EAS-mediated and susceptible to but not diagnostic (STBNDO) of EAS parameters. In addition, the table reports systemic toxicity effects and (specific) target organ findings. The basis of this table is the tab *"E, A, S LOE"* of Appendix E (commented and amended by **RMS** where appropriate).

Table B.6.8.3.4.1.1-1: summary of uterine effects of lenacil in this DRAR

Study	Dose (ppm) + effect(s)				
Rat (Wistar) 4-wk (0, 5000, 10000/30000, 20000/50000 ppm)		10000/30000 ppm (~3500 mkd) Macropathology: **Fluid distention**		20000/50000 ppm (~5900 mkd) Macropathology: **Fluid distention**	
Rat (Wistar) 13-wk (0, 500, 5000, 50000 ppm)					50000 ppm (~4900 mkd) ↑ **weight** (a/r. – no DR)
Rat (Wistar) 13wk + rec. 4 wk (0, 50000 ppm)					50000 ppm (~4900 mkd) ↑ **weight** (abs. & rel.) Macropathology: **fluid distension** Histopathatology: **luminal dilatation**
Mouse (CD-1) 13-wk (0, 100, 1000, 5000, 10000 ppm)	**Uterus not weighed** Uterus pathology: **not examined**				
Dog (Beagle) 13-wk (0, 1000, 5000, 25000 ppm)	Uterus + cervix weight, pathology: **no change**				
Rat (Wistar) 52-wk, 104-wk (0, 250, 2500, 25000 ppm)	2500 ppm Macropathology: **fluid distention**		25000 ppm (~1700 mkd) Uterus + cervix weight: ↓**>10% (no DR)** Histopathology: **endometrial gland hyperplasia luminal dilatation**		
Mouse (CD-1) 18-months (0, 100, 2500, 7000 ppm)	**Uterus not weighed** Uterus pathology: **not examined**				
Rat (Han Wistar) 2-G (0, 10000, 25000, 50000 ppm)			25000 ppm (~800 mkd) Uterus and cervix weight (a/r.): **F_0 ↓10-13%** (no DR) **F_1 ↓4-8%** **F_2: no measurement** Macropathology: **no indication** Histopathology.: **no change**		50000 ppm (~4300 mkd) Uterus and cervix weight (a/r): **F_0 ↓8-9%** (no DR) **F_1 ↓18-22%** **F_2: no measurement** Macropathology: **no indication** Histopathology: **F_0: -Pigmented macrophage accumulation** **-Endometrial polyploid hyperplasia** **F1: -Myometrial hyalinisation** **-Pigmented macrophage accumulation** **-Glandular dilatation** (note: only marginal increase of histopath findings, and slight trend where effects $F_1>F_0$)

Table **B.6.8.3.4.1.1-2**: Lines of evidence on EAS-mediated parameters and on parameters susceptible to but not diagnostic of EAS (STBNDO)

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
14	*In vitro* mechanistic	Androgen receptor	Rat	20	H	Uptake from the medium		µM	No effect	Negative	No support for A	Not A
15a	*In vitro* mechanistic	Androgen receptor	Human	23	H	Uptake from the medium		µM	No effect			
15b	*In vitro* mechanistic	Androgen receptor	Human	22	H	Uptake from the medium		µM	No effect			
16	*In vitro* mechanistic	Oestrogen receptor	Rat	16-20	H	Uptake from the medium		µM	No effect	Negative	No support for E	Not E
17a	*In vitro* mechanistic	Oestrogen receptor	Human	21.5-22	H	Uptake from the medium		µM	No effect			
17b	*In vitro* mechanistic	Oestrogen receptor	Human	21.5-22	H	Uptake from the medium		µM	No effect			
18	*In vivo* mechanistic	Uterus histopathology (Uterotrophic assay)	Rat	5	D	Oral		mkd	No effect	Negative	No support for EAS	Not EAS
5	EATS-mediated	Age at PPS	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
5	EATS-mediated	Age at VO	Rat	17	W	Oral	50000	ppm	Increase	Slight ↑of age at VO (1d) at top-dose, NSS	equivocal for EAS	Not EAS
2	EATS-mediated	Epididymis histopathology	Rat	13	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
5	EATS-mediated	Epididymis histopathology	Rat	17	W	Oral		ppm	No effect			
9	EATS-mediated	Epididymis histopathology	Mouse	90	D	Oral		ppm	No effect			
10	EATS-mediated	Epididymis histopathology	Mouse	18	M	Oral		ppm	No effect			
12	EATS-mediated	Epididymis histopathology	Dog	90	D	Oral	25000	ppm	No effect			

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
1	EATS-mediated	Epididymis weight	Rat	4	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
2	EATS-mediated	Epididymis weight	Rat	13	W	Oral		ppm	No effect			
5	EATS-mediated	Epididymis weight	Rat	17	W	Oral		ppm	No effect			
11	EATS-mediated	Epididymis weight	Dog	4	W	Oral		ppm	No effect			
12	EATS-mediated	Epididymis weight	Dog	90	D	Oral		ppm	No effect			
4a	EATS-mediated	Epididymis weight	Rat	52	W	Oral		ppm	No effect			
4b	EATS-mediated	Epididymis weight	Rat	104	W	Oral		ppm	No effect			
5	EATS-mediated	Oestrus cyclicity	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
9	EATS-mediated	♀Mammary gland histopathology	Mouse	90	D	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
10	EATS-mediated	♀Mammary gland histopathology	Mouse	18	M	Oral		ppm	No effect			
12	EATS-mediated	♀Mammary gland histopathology	Dog	90	D	Oral		ppm	No effect			
4b5	EATS-mediated	♀Mammary gland histopathology	Rat	104	W	Oral	2500	ppm	Increase	Mammary adenocarcinoma: 0, 4, **12, 10%** at 0, 250, 2500 & 25000 ppm.	EAS unlikely based on mechanistic data	Not EAS
4b6	EATS-mediated	♀Mammary gland histopathology	Rat	104	W	Oral		ppm	Increase	Mammary acinar cell hyperplasia: 44, 50, 52, **56%** at 0, 250, 2500 and 25000 ppm.		
12	EATS-mediated	♂Mammary gland histopathology	Dog	90	D	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
2	EATS-mediated	Ovary histopathology	Rat	13	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
5	EATS-mediated	Ovary histopathology	Rat	17	W	Oral		ppm	No effect			
9	EATS-mediated	Ovary histopathology	Mouse	90	D	Oral		ppm	No effect			
10	EATS-mediated	Ovary histopathology	Mouse	18	M	Oral		ppm	No effect			
12	EATS-mediated	Ovary histopathology	Dog	90	D	Oral		ppm	No effect			
1	EATS-mediated	Ovary weight	Rat	4	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
2	EATS-mediated	Ovary weight	Rat	13	W	Oral		ppm	No effect			
5	EATS-mediated	Ovary weight	Rat	17	W	Oral		ppm	No effect			
11	EATS-mediated	Ovary weight	Dog	4	W	Oral		ppm	No effect			
12	EATS-mediated	Ovary weight	Dog	90	D	Oral		ppm	No effect			
4a	EATS-mediated	Ovary weight	Rat	52	W	Oral		ppm	No effect			
4b	EATS-mediated	Ovary weight	Rat	104	W	Oral		ppm	No effect			
9	EATS-mediated	Prostate histopathology (+ seminal vesicles and coagulating glands)	Mouse	90	D	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
10	EATS-mediated	Prostate histopathology (+ seminal vesicles and coagulating glands)	Mouse	18	M	Oral		ppm	No effect			
12	EATS-mediated	Prostate histopathology (+ seminal vesicles	Dog	90	D	Oral		ppm	No effect			

study ID	Effect		Specie	Dura-tion #	unit	Administra-tion route	Dose		Effect direction	Observed effect (+ and -)	Assessment	
	Classifica-tion	target					Lowest	unit			Each LoE	Integrated LoE
		and coagulating glands)										
5	EATS-mediated	Prostate weight	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
11	EATS-mediated	Prostate weight	Dog	4	W	Oral		ppm	No effect			
10	EATS-mediated	Seminal vesicles histopathology	Mouse	18	M	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
5	EATS-mediated	Seminal vesicles weight	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
5	EATS-mediated	Sperm morphology	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
5	EATS-mediated	Sperm motility	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
5	EATS-mediated	Sperm numbers	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
2	EATS-mediated	Testis histopathology	Rat	13	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
9	EATS-mediated	Testis histopathology	Mouse	90	D	Oral		ppm	No effect			
10	EATS-mediated	Testis histopathology	Mouse	18	M	Oral	7000	ppm	No effect			
12	EATS-mediated	Testis histopathology	Dog	90	D	Oral		ppm	No effect			
1	EATS-mediated	Testis weight	Rat	4	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
2	EATS-mediated	Testis weight	Rat	13	W	Oral		ppm	No effect			
5	EATS-mediated	Testis weight	Rat	17	W	Oral		ppm	No effect			
9	EATS-mediated	Testis weight	Mouse	90	D	Oral		ppm	No effect			

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
10	EATS-mediated	Testis weight	Mouse	18	M	Oral		ppm	No effect			
11	EATS-mediated	Testis weight	Dog	4	W	Oral		ppm	No effect			
12	EATS-mediated	Testis weight	Dog	90	D	Oral		ppm	No effect			
4a	EATS-mediated	Testis weight	Rat	52	W	Oral		ppm	No effect			
4b	EATS-mediated	Testis weight	Rat	104	W	Oral		ppm	No effect			
2	EATS-mediated	Uterus (+ cervix) histopathology	Rat	13	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
9	EATS-mediated	Uterus (+ cervix) histopathology	Mouse	90	D	Oral		ppm	No effect			
10	EATS-mediated	Uterus (+ cervix) histopathology	Mouse	18	M	Oral		ppm	No effect			
12	EATS-mediated	Uterus (+ cervix) histopathology	Dog	90	D	Oral		ppm	No effect			
4a	EATS-mediated	Uterus (+ cervix) histopathology	Rat	52	W	Oral		ppm	No effect			
1	EATS-mediated	Uterus weight (+ cervix)	Rat	4	W	Oral		ppm	No effect			
2	EATS-mediated	Uterus weight (+ cervix)	Rat	13	W	Oral	500	ppm	Change	At 500 ppm ↑37% (r), at 50000 ppm ↑13% (r), not changed at 5000 ppm→ no dose-response, NNS	EAS unlikely as inconsistent	Not EAS
51	EATS-mediated	Uterus weight (+ cervix)	Rat	17	W	Oral	50000	ppm	Decrease	↓uterine weights (a. 22%, r. 18%) on d28 *post partum*. (notifier: possible correlation between the terminal oestrus cycle stage and uterine weight at termination?).	EAS unlikely as inconsistent	Not EAS
5m	EATS-mediated	Uterus weight (+ cervix)	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
8	EATS-mediated	Uterus weight (+ cervix)	Rat	19	D	Oral		mkd	No effect			
11	EATS-mediated	Uterus weight (+ cervix)	Dog	4	W	Oral		ppm	No effect			
12	EATS-mediated	Uterus weight (+ cervix)	Dog	90	D	Oral		ppm	No effect			
4a	EATS-mediated	Uterus weight (+ cervix)	Rat	52	W	Oral		ppm	No effect			
4b	EATS-mediated	Uterus weight (+ cervix)	Rat	104	W	Oral		ppm	No effect			
5	EATS-mediated	Vagina histopathology	Rat	17	W	Oral		ppm	Change	Acute inflammatory infiltration of vaginal epithelium at 50000 ppm: F_0: 0/25 *vs.* **2**/27 (7%) F_1: 1/21 (5%) *vs.* **6**/24 (25%) however unclear if immunotoxic etiology	EAS unlikely as inconsistent F_0/F_1;	Inconclusive for EAS
9	EATS-mediated	Vagina histopathology	Mouse	90	D	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
10	EATS-mediated	Vagina histopathology	Mouse	18	M	Oral		ppm	No effect			
2	STBNDO	Adrenals histopathology	Rat	13	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
9	STBNDO	Adrenals histopathology	Mouse	90	D	Oral		ppm	No effect			
10	STBNDO	Adrenals histopathology	Mouse	18	M	Oral		ppm	No effect			
12	STBNDO	Adrenals histopathology	Dog	90	D	Oral		ppm	No effect			
4a	STBNDO	Adrenals histopathology	Rat	52	W	Oral		ppm	No effect			
4b	STBNDO	Adrenals histopathology	Rat	104	W	Oral		ppm	No effect			

study ID	Effect		Specie	Dura-tion #	unit	Administra-tion route	Dose		Effect direction	Observed effect (+ and -)	Assessment	
	Classifica-tion	target					Lowest	unit			Each LoE	Integrated LoE
1	STBNDO	Adrenals weight	Rat	4	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
2	STBNDO	Adrenals weight	Rat	13	W	Oral		ppm	No effect			
5	STBNDO	Adrenals weight	Rat	17	W	Oral		ppm	No effect			
9	STBNDO	Adrenals weight	Mouse	90	D	Oral		ppm	No effect			
10	STBNDO	Adrenals weight	Mouse	18	M	Oral		ppm	No effect			
11	STBNDO	Adrenals weight	Dog	4	W	Oral		ppm	No effect			
12	STBNDO	Adrenals weight	Dog	90	D	Oral	1000	ppm	Increase	a ↑ at 1000 ppm (13%), at 5000 ppm (14%), at 25000 ppm (21%), NSS, no histological correlate	No clear support for EAS	Inconclusive for EAS
4a	STBNDO	Adrenals weight	Rat	52	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
4b	STBNDO	Adrenals weight	Rat	104	W	Oral	2500	ppm	Increase	(♂) at 25000 ppm ↑147% (a) & 116% (r) NSS.; (♀) at 2500 ppm ↑ 10% (r) & SS., at 25000 ppm ↑ 16% (a) and 30% (r) & NSS	No clear support for EAS	Not EAS
9	STBNDO	Brain histopathology	Mouse	90	D	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
10	STBNDO	Brain histopathology	Mouse	18	M	Oral		ppm	No effect			
12	STBNDO	Brain histopathology	Dog	90	D	Oral		ppm	No effect			
4b	STBNDO	Brain histopathology	Rat	104	W	Oral		ppm	No effect			
1	STBNDO	Brain weight	Rat	4	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
5	STBNDO	Brain weight	Rat	17	W	Oral		ppm	No effect			
9	STBNDO	Brain weight	Mouse	90	D	Oral		ppm	No effect			
10	STBNDO	Brain weight	Mouse	18	M	Oral		ppm	No effect			
11	STBNDO	Brain weight	Dog	4	W	Oral		ppm	No effect			
12	STBNDO	Brain weight	Dog	90	D	Oral		ppm	No effect			

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
5	STBNDO	Fertility	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
5	STBNDO	Gestation length	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
5	STBNDO	Litter size	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
4a	STBNDO	Motor activity	Rat	50	W	Oral	2500	ppm	Decrease	↓low-beam breaks at wk 50 in 2500 & 25000 ppm ♂during 2nd & 4th intervals in the 60' assessment. ↓in the 3rd interval at 25000 ppm. Yielded lower total motor activity. No other indications of ↓motor activity. NSS ↓ in high beam breaks, no changes in adjacent intervals (1st & 5th). No clinical signs or histopathological findings to suggest a neurotoxic effect.	No clear support for EAS	Not EAS
6	STBNDO	# implantations, corpora lutea	Rat	10	D	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
8	STBNDO	# implantations, corpora lutea	Rat	19	D	Oral		mkd	No effect			
5	STBNDO	# live births	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
6	STBNDO	# embryonic or foetal deaths and viable foetuses	Rat	10	D	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
8	STBNDO	# embryonic or foetal deaths and viable foetuses	Rat	19	D	Oral		mkd	No effect			
9	STBNDO	Pituitary histopathology	Mouse	90	D	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
10	STBNDO	Pituitary histopathology	Mouse	18	M	Oral	7000	ppm	Increase	↑in cysts observed - 6/66 *vs.* controls 2/70, likely Rathke's pouch, a spontaneous finding	No clear support for EAS; cysts not likely related	Not EAS

study ID	Effect: Classification	Effect: target	Specie	Duration #	unit	Administration route	Dose: Lowest	Dose: unit	Effect direction	Observed effect (+ and -)	Assessment: Each LoE	Assessment: Integrated LoE
											endocrine tissue	
12	STBNDO	Pituitary histopathology	Dog	90	D	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
5f	STBNDO	Pituitary weight	Rat	17	W	Oral	10000	ppm	Increase	Unaltered in F_0, ↑27 & 40% r in F_1 ♂ at 10000 & 50000 ppm. (explained by notifier to excessively low pituitary wt in several F_1 controls which may skew the data)	Inconsistent results for F_0 & F_1; No clear support for EAS	Inconclusive for EAS
5g	STBNDO	Pituitary weight	Rat	17	W	Oral		ppm	No effect	Negative	No support for EAS	Not EAS
11	STBNDO	Pituitary weight	Dog	4	W	Oral		ppm	No effect			
8	STBNDO	Post implantation loss	Rat	19	D	Oral		mkd	No effect	Negative	No support for EAS	Not EAS
8	STBNDO	Pre implantation loss	Rat	19	D	Oral		mkd	No effect			
6	STBNDO	Presence of anomalies (external, visceral, skeletal	Rat	10	D	Oral		ppm	No effect	-	No support for EAS	Not EAS
7[1]	STBNDO	Presence of anomalies (external, visceral, skeletal	Rat	19	D	Oral	300	mkd	Increase	thickened rib, top-dose: incomplete ossification of cervical and sacrocaudal vertebrae		
13	STBNDO	Presence of anomalies (external, visceral, skeletal	Rabbit	17	D	Oral		mkd	No effect	-		
13	STBNDO	Reproduction	Rabbit	17	D	Oral		mkd	No effect	Negative	No support for EAS	Not EAS

Abbreviations: H= hours; D=days, W= weeks, M= months; ppm= parts per million; mkd= mg/kg b.w./d; organ weights: a=absolute, r=relative (to body weight); (N)SS= (not) statistically significant

STBNDO= Sensitive to, but not diagnostic of, EATS

[1]: structural defects not considered typical endocrine, but added by RMS for the sake of completeness, in the presence of reported no-effects in the older (but less adequate) developmental rat study (1978)

B.6.8.3.4.1.2 Thyroid

Thyroid/parathyroid gland

In some lenacil repeated dose rat studies a gross observation of dark thyroid was observed, corroborated by increased pigmentation upon histological examination, mainly at the top-dose. The Schmorl's stain was used to further characterise these changes. The ♂ controls in the 13- week rat study had a high frequency of Schmorl's staining; but with lenacil treatment at top-dose the intensity (severity) of staining increased. In ♀ there was a very low incidence of Schmorl's positive staining in controls and both the frequency and the intensity of staining increased. There was possibly also an increase of incidence at 5000 ppm in ♀. After a 4-week recovery period, Schmorl's staining in control ♂ mostly resolved to control levels but not in ♀.

Schmorl's stain is known to stain melanin and lipofuscin, and the association with a specific endocrine effect, including a potential thyroid hormone changes is unclear. Notifier stated that these staining changes may be *"related to the increased thyroid concretions and cellular debris that was also increased with dose"*, likely referred to as "altered colloid" in a recent publication of pathology nomenclature (Brändli-Baiocco *et al,* 2018). However, **RMS** reminds that such thyroid colloidal changes are also identified potential endocrine effects in the ED guidance.

Thyroid weight was increased in high dose rats (90d, 2G, 2yr) and in high-dose dogs (90d). While most cases of high thyroid weights (supported by visually enlarged thyroids) in the long-term rat study (+20-49%) was observed at doses >MTD, it is of note that a weak trend toward higher thyroid weights were also observed in the 2 yr-study at 118 mg/kg b.w./d (+14-17%).

The histopathological correlates in the rat at (mainly) top-dose was as reported black thyroids, follicular cellular debris, hypertrophy and isolated cases of follicular haemorrage/pigmented epithelium (2G) and luminal concretions (2-yr). It was noted that no marked increase in follicular cell hypertrophy in the 2-year rat study was reported.

No adverse thyroidal effects (neither weight, gross and micropathology) were observed in the mouse studies, including the long-term study. Corroborative histopathological findings supporting heavy thyroids lacked in the dog studies either.

In addition, follicular cell hypertrophy was noted at ≥25000 ppm in F_0 parental rats in the 2G rat study and at a low incidence in F_1 parental animals at top-dose in both sexes.

The incidence of follicular cell adenoma was significantly increased in high-dose ♀ of the 2yr-study, but remained within the historical control data (HCD) for the laboratory. The incidence of carcinomas was not elevated at any dose when compared to the controls. The incidence of combined adenomas and carcinomas was within the HCD for adenomas only and ECHA concluded that was no evidence that lenacil induced follicular cell tumours. In addition, the new mechanistic studies clearly demonstrated that lenacil was a strong inducer of CYP1B metabolic enzyme, and a moderate inducer of UDPGT, suggesting that any marginal elevation of thyroid follicular cell tumours would be of poor human relevance.

There was a marginal increase in C-cell tumours in ♀rats, which was considered to be age- and sex-related, and unlikely related to lenacil treatment for the notifier. These tumours are typically linked to changes in calcitonin, which modulates calcium and phosphorus, and in the rat study database for lenacil, there were no consistent changes in calcium or phosphorus to suggest modulation by calcitonin.

Finally, examination of the thyroid tissues on week 52 of the rat carcinogenicity study showed no treatment-related findings suggestive of precursor C-cell tumors. Finally, C-cell tumours are not mediated by classical thyroid hormones, and RMS agrees that there would be no relationship between them and T_3, T_4 or TSH.

RMS concludes that the findings are for now not essential for the evaluation of the T-modality covered by the current ED GD.

RMS considers that the increased absolute and/or relative thyroid weights, in combination with histopathological evidence of thyroid toxicity (especially in the ♀) in both F_0 and F_1 adults, appearing mostly at 817 mg/kg b.w./d and above, indicated that, while reproductive parameters remained unaltered, the generational studies revealed specific thyroid adverse findings. It remains unclear if the findings can be ascribed to systemic toxicity only, but it is acknowledged that meaningful findings appeared only at doses nearby or even higher than the generally accepted limit dose of 1000 mg/kg b.w./d.

Mechanistic studies show that lenacil does not inhibit the deiodinase that converts T_4 to T_3. The data also show that lenacil did not impair the uptake of iodine into the thyroid or the iodinification of organic compounds in the thyroid via thyroid peroxidases. Measurements of the thyroid hormones provided an inconsistent picture (see **table B.6.8.3.4.1.2-1** below). In the 20-week study with lenacil in ♀ Wistar rats, there was a decrease in T_4 at both 10 and 19 weeks. While these decreases were sometimes statistically significant, they occurred without an increase in TSH or a decrease in T_3, and the dose-response in the wk-19 phase was not evident.
At the 52-week timepoint in the 2-year study in Han Wistar rats there were no marked changes in ♂ or ♀for T3, T4 or TSH. In a recent 2-week study in ♂Sprague Dawley rats, while there was a dose-dependent induction of UDPGT, there was no typical pattern of T_3, T_4 or TSH changes. In all three mechanistic studies, rT_3 was examined and also showed no consistent pattern of change. These data demonstrate that there were no consistent or adverse changes in *"thyroid hormone economy"*. According to the notifier, *"the minimal changes may have represented biological variation or subtle adaptive changes in hormonal regulation.*"

RMS is of the opinion that a comparison between the 3 mechanistic studies (at least for the thyroid hormone measurement) remains problematic, since 2 different rat strains were used, that the top-dose of 50000 ppm was not tested in the most recent assay, and that the observed variation and consistency of hormonal levels (which only show a weak modification overall) is therefore difficult to interpret.

No MoA could unequivocally be identified for the finding of the thyroids blackening, but the existing mechanistic study *suggests* that a direct thyrotropic effect is unlikely. In a WoE evaluation, **RMS** agrees that there was no consistent pattern suggestive of a clear impact on the T modality. However, there is no DNT study, thus it remains unclear what the impact could be of subtle thyroid hormone changes on the behavioural development in young rats, and **RMS** suggests to discuss it more in detail in the peer review process.

A more extended table with the LoE is reproduced in table **B.6.8.3.4.1.2-2.** The latter table contains the effects which are considered T-mediated.
The basis of this table is the tab *"THYROID LOE"* of Appendix E (commented and amended by **RMS** where appropriate).

Finally, table **B.6.8.3.4.1.2-3** contains the effects which pertain to STOT or general systemic toxicity. Effects related to EAS and T can therefore be matched with possible systemic toxicity.

Cited reference:
Brändli-Baiocco A, Balme E, Bruder M, Chandra S, Hellmann J, Mark J, Hoenerhoff MJ, Kambara T, Landes C, Lenz B, Mense M, Rittinghausen S, Satoh H, Schorsch F, Seeliger F, Tanaka T, Tsuchitani T, Wojcinski Z and Rosol TJ Nonproliferative and Proliferative Lesions of the Rat and Mouse Endocrine System, J Toxicol. Pathol. 31 (3 Suppl): 1S–95S, 2018.

Table B.6.8.3.4.1.2-1 Lenacil: thyroid mechanistic studies in rats - comparison thyroid hormone levels in feeding studies

Report/Design	Treatment time (wk)	Dose (ppm)	T_3	T_4	TSH	rT_3
[redacted] 2019 Crl:CD(SD), n=13-15, serum			*ng/dL*	*µg/dL*	*ng/mL*	*ng/mL*
	2	0	63.110 ± 9.383	4.174 ± 0.505	8.237 ± 3.963	0.057 ± 0.013
		2500	62.744 ± 14.045	4.075 ± 0.606	7.719 ± 2.677 (↓6%)	**0.066 ± 0.013 (↑16%)**
		12500	**58.950 ± 13.425 (↓7%)**	4.032 ± 0.428	**7.243 ± 3.487 (↓12%)**	**0.069* ± 0.010 (↑22%)**
		25000	**49.075* ± 6.914 (↓22%)**	4.188 ± 0.792	**6.215* ± 4.223 (↓25%)**	**0.070* ± 0.013 (↑23%)**
[redacted] 2004, [redacted] 2007, Wistar ♀; n=6, plasma	**10**		*nmol/L (total)*	*nmol/L (total)*	*nmol/mL*	*ng/L*
		0	1.03 ± 0.155	32 ± 6.7	5.2 ± 0.46	0.17 ± 0.011
		250	0.97 ± 0.131	**21* ± 5.6 (↓34%)**	5.0 ± 0.23	0.16 ± 0.010
		50000	1.04 ± 0.158	**20* ± 3.9 (↓38%)**	**5.8 ± 0.75 (↑12%)**	0.16± 0.008
	19	0	-a	28 ± 7.9	6.6 ± 0.62	0.23 ± 0.0037
		250	-a	**23 ± 6.5 (↓18%)**	**5.7 ± 1.75 (↓14%)**	**0.19* ± 0.022 (↓17%)**
		50000	-a	**25 ± 7.1 (↓11%)**	6.0 ± 0.89 (↓9%)	**0.19* ± 0.012 (↓17%)**
			pmol/L (free)	*pmol/L (free)*	*nmol/mL*	*ng/mL*
[redacted] 2003 Wistar ♂; n=20, plasma	**52**	0	1.1 ± 0.26	12.4 ± 2.20	6.3 ± 1.12	n.d.
		25000	1.2 ± 0.22	13.0 ±2.50	**8.4 ± 3.58 (↑33%)**	n.d.
[redacted] 2003 Wistar ♀; n=19-20, plasma		0	1.4 ± 0.42	8.9 ± 2.94	5.4 ± 0.72	n.d.
		25000	1.5 ± 0.34	**8.0 ± 2.29 (↓10%)**	**6.9 ± 3.37 (↑28%)**	n.d.

Data expressed as mean ± s.d.; *: statistically significant from the control (see original evaluations for degree of significance) ; n.d.: not determined;
a: According to the report, this portion of the assay was unreliable;

Table **B.6.8.3.4.1.2-2:** Lines of evidence on T-mediated parameters

Study ID	Effect		Specie	Duration		Dose		Effect direction	Observed effect (+ and -)	Assessment	
	Classification	target		#	unit	lowest	unit			Each LoE	Integrated LoE
19	*In vivo* mechanistic	Sodium-iodide symporter (NIS)	Rat	20	W		ppm	No effect	Negative	No support for T	Not T
19a	*In vivo* mechanistic	T_3 and T_4 level	Rat	10 & 19	W	250	ppm	Decrease	Mean rT_3 values: not changed at W10, at W19 ↓17% at 250 & 50000 ppm, SS.	Inconsistent data	Inconclusive for T
19b	*In vivo* mechanistic	T_3 and T_4 level	Rat	10 & 19	W	250	ppm	Decrease	Mean T_4 values wk 10: ↓at 250 ppm (34%) & 50000 ppm (38%) compared to control, SS., no dose response, no corroborating TSH change & not reproducible on W19	Inconsistent data	Inconclusive for T
19c	*In vivo* mechanistic	T_3 and T_4 level	Rat	10	W		ppm	No effect	Mean T_3 values similar to controls on W10, assay failed W19	No support for T	Not T
4a	*In vivo* mechanistic	T_3 and T_4 level	Rat	52	W		ppm	No effect	No effect	No support for T	Not T
19	*In vivo* mechanistic	Thyroid-stimulating hormone level (TSH)	Rat	10 & 19	W		ppm	No effect	No effect	No support for T	Not T
4a	*In vivo* mechanistic	Thyroid-stimulating hormone level (TSH)	Rat	52	W	25000	ppm	Increase	Very mild change (♂): ↑33% (♀): ↑28%, neither value SS., no change in T_4	Inconsistent data	Inconclusive for T
2	EATS-mediated	Thyroid histopathology	Rat	13	W		ppm	No effect	No effect	No support for T	Not T
3	EATS-mediated	Thyroid histopathology	Rat	13	W	50000	ppm	Increase	Schmorl's $^+$ staining: (♂): control 9/10, at 50000 ppm 8/10; (♀): control 1/10, at 50000 ppm 7/10; (♂) intensity of staining, control 7 minimal & 2 slight, at 50000 ppm 2 minimal, 5 slight & **3 moderate↑**	~~No~~ support for T at 50000 ppm (°)	~~Not~~ T ([£])
5h	EATS-mediated	Thyroid histopathology	Rat	17	W	10000	ppm	Increase	Follicular cell hypertrophy: F_0 (♂): 1/20, 0/20, **3**/20 & **4**/20 at 0, 1000, 10000 & 50000 ppm. NSS. F_0 (♀): 0/25 in control, at 50,000 ppm **9**/27, SS F1 (♂♀) at 50000 ppm (2/20 both M&F), 0/20 in control (both M&F)	~~Only~~ support for T at ≥10000 ppm (°)	~~Not~~ T ([£])

Study ID	Effect		Specie	Duration		Dose		Effect direction	Observed effect (+ and -)	Assessment	
	Classification	target		#	unit	lowest	unit			Each LoE	Integrated LoE
5i	EATS-mediated	Thyroid histopathology	Rat	17	W	10000	ppm	Increase	F0 (M) ↑ intensity of Schmorl's + staining at ≥10000 ppm (1/20 with moderate staining in control & 1000 ppm; **3**/20 & **10**/20 at 10000 & 50000 ppm); ↑ follicular cell debris (0/20 in control & 1000 ppm, **6**/20 & **15**/20 at 10000 & 50000 ppm); F0 (♀) ≥10000 ppm ↑ incidence & degree of Schmorl's + staining (incidence: 6/25, 9/28, **20**/27 & **24**/27 at 0, 1000, 10000 & 50000 ppm), ↑ follicular cell debris (0/25, 0/28, **5**/27 & **25**/27 at 0, 1000, 10000 & 50000 ppm)	~~No~~ support for T ≥10000 ppm (°)	~~Not~~ T (£)
5j	EATS-mediated	Thyroid histopathology	Rat	17	W	50000	ppm	Change	follicular cell adenoma: 1/23 ♂	Inconclusive for T at >4000 mg/kg/d	Inconclusive for T
5k	EATS-mediated	Thyroid histopathology	Rat	17	W	50000	ppm	Increase	Pigmented follicular epithelium: (♂) 1/20 in control versus **4**/23 at 50000 ppm; (♀) 0/20 control versus **2**/22 at 50000 ppm	~~No~~ support for T at 50000 ppm (°)	~~Not~~ T (£)
9	EATS-mediated	Thyroid histopathology	Mouse	90	D		ppm	No effect	Negative	No support for T	Not T
10	EATS-mediated	Thyroid histopathology	Mouse	18	M		ppm	No effect	Negative	No support for T	Not T
12	EATS-mediated	Thyroid histopathology	Dog	90	D		ppm	No effect	Negative	No support for T	Not T
19	EATS-mediated	Thyroid histopathology	Rat	20	W	50000	ppm	Increase	Darkened thyroids: 0/6 controls, 0/6 250 ppm, **6**/6 at 50000 ppm	~~No~~ support for T at 50000 ppm (°)	~~Not~~ T (£)
4a	EATS-mediated	Thyroid macropathology	Rat	52	W	25000	ppm	Increase	No findings in controls of either sex (0/20/sex), at 25000 ppm darkening in (♂) **5**/20 (25%) & (♀) **10**/19 (52%)	~~No~~ support for T at 25000 ppm (°)	~~Not~~ T (£)
4b1	EATS-mediated	Thyroid macropathology	Rat	104	W	25000	ppm	Increase	Darkening: incidence in ♂1/50 (2%) and ♀**12**/50 (24%); versus 0/50 in controls (♂♀).	~~No~~ support for T at 25000 ppm (°)	~~Not~~ T (£)
4	EATS-mediated	Thyroid histopathology	Rat	52	W	25000	ppm	Increase	Thyroid, increased luminal concretions at 25000 ppm in (♂) **6/9** vs. 2/14 (ctrl) & (♀) **8**/14 vs. 1/9 (ctrl)	support for T at 25000 ppm (°)	T (£)
4b4	EATS-mediated	Thyroid histopathology	Rat	104	W	25000	ppm	Increase	Thyroid, increased luminal concretions at 25000 ppm in (♂) **33/50** (66%) vs. 11/50, 22% (ctrl) & (♀) **32/49** (65%) vs. 5/50, 10% (ctrl), SS.	~~Finding not linked to T~~	T

Study ID	Effect		Specie	Duration		Dose		Effect direction	Observed effect (+ and -)	Assessment	
	Classification	target		#	unit	lowest	unit			Each LoE	Integrated LoE
										support for T at 25000 ppm (°)	
4b2	EATS-mediated	Thyroid histopathology	Rat	104	W	2500	ppm	Increase	C-cell adenoma rate: 4, 4, 16 & 14% at 0, 250, 2500 & 25000 ppm; 2 carcinomas (4%) at 25000 ppm. Marginally >HCD. No treatment-related ↑ in C-cell hyperplasia; ECHA considered equivocal-age related	No support for T	Not T
4b3	EATS-mediated	Thyroid histopathology	Rat	104	W	2500	ppm	Increase	Follicular cell adenoma (♂): 3/50, 2/50, 2/49, & 5/50 (10%) & (♀): 1/50, 0/50, 1/50, & 4/49 (8%) at 0, 250, 2500 & 25000 ppm. Follicular cell carcinoma (♂): 0/50, 0/50, 1/50 & 1/50 & (♀): 1/50, 0/50, 1/50 & 0/50 (8%) at 0, 250, 2500 & 25000 ppm; adenomas within HCD.	Equivocal response	Inconclusive for T
1	EATS-mediated	Thyroid weight	Rat	4	W		ppm	No effect	Negative	No support for T	Not T
2	EATS-mediated	Thyroid weight	Rat	13	W	50000	ppm	Increase	Thyroid+parathyroid weight together; r at 50000 ppm (♂) ↑21% & (♀) 13%, NSS	~~No~~ support for T at 50000 ppm (°)	~~Not~~ T (£)
5	EATS-mediated	Thyroid weight	Rat	17	W	50000	ppm	Increase	Thyroid+parathyroid weight r: ↑12-19% in F_0 and F_1 (♂♀) at 50000 ppm, SS	~~No~~ support for T at 50000 ppm (°)	~~Not~~ T (£)
11	EATS-mediated	Thyroid weight	Dog	4	W		ppm	No effect	Negative	No support for T	Not T
12	EATS-mediated	Thyroid weight	Dog	13	W	5000	ppm	Increase	Thyroid+parathyroid weight together; a. & adjusted means (♂) at 5000 ppm a ↑11%, adj ↑15%; at 25000 ppm a ↑20%, adj ↑23%-SS; (♀) a at 5000 ppm ↑11%, at 25000 ppm ↑24%; no histologic correlate	~~No~~ support for T at ≥5000 ppm;	Inconclusive for T (no histological correlate)
19	EATS-mediated	Thyroid weight	Rat	20	W	50000	ppm	Increase	Adjusted wt ↑23% at 50000 ppm, SS However, no effect on deiodinase/peroxidase→ no direct thyrotoxic effect	~~Inconclusive for T at >4000 mg/kg/d~~ support for T at 50000 ppm (°)	~~Not~~ T (£)
4a	EATS-mediated	Thyroid weight	Rat	52	W	25000	ppm	Increase	thyroid+parathyroid together, r: (♂)↑23%, (♀)↑20%, SS, histological correlate	~~Inconclusive for T at >4000 mg/kg/d~~ support	~~Not~~ T (£)

Study ID	Effect		Specie	Duration		Dose		Effect direction	Observed effect (+ and -)	Assessment	
	Classification	target		#	unit	lowest	unit			Each LoE	Integrated LoE
										for T at 25000 ppm (°)	
4b	EATS-mediated	Thyroid weight	Rat	104	W	25000	ppm	Increase	thyroid+parathyroid weight together, r: at 2500 ppm ↑17 & 8% (♂♀) at 104 weeks, NSS. at 25000 ppm ↑40 & 49% (♂♀) NSS, histological correlate	~~Inconclusive for T at >4000 mg/kg/d~~ support for T at 25000 ppm (°)	~~Not~~ T (£)
22*	EATS-mediated	Thyroid weight	Rat	14	D		ppm	No effect	Negative	No support for T	Not T
22*	EATS-mediated	Thyroid histopathology	Rat	14	D		ppm	No effect	Negative	No support for T	Not T
22*	EATS-mediated	Thyroid weight	Rat	14	D		ppm	No effect	Negative	No support for T	Not T
22*	EATS-mediated	Thyroid weight	Rat	14	D	≥ 12500	ppm	Increase and decrease	compared to control: rT_3 values: weak ↑at 2500 ppm (16%) NSS, 12500 ppm (22% SS) and 25000 ppm (23%) SS., no effect on T_4, ↓TSH 12500 ppm (12% NSS) and 25000 ppm (25% SS); ↓T_3 25000 ppm (22% SS) However, hormone modifications not fully reproducible in other assays	Inconsistent data	Inconclusive for T
22*	EATS-mediated	Thyroid peroxidase	Rat	14	D		ppm	No effect	Negative	No support for T	Not T
22*	EATS-mediated	Thyroid UDPGT and CYP B induction	Rat	14	D	<2500	ppm	Increase	Moderate UDPGT induction (×1.26-1.38) Stong induction of CYP2B1 (×9-15)	No support for T	Not T[$]

Abbreviations: D=days, W= weeks; ppm= parts per million; organ weights: a=absolute, r=relative (to body weight); (N)SS= (not) statistically significant
*: added by RMS (not yet in Appendix E)
(°): findings at 13W, 17W, 52W not linked to thyroid hormone change (=observed in mechanistic study #19, and in LT rat feeding study #4)
(£): while notifier considers the thyroid weights and/or histopathological evidence of thyroid toxicity not relevant ("*Not T*"), **RMS** considered that treatment-relationship for thyroid findings cannot be ignored. However, it should be recognised that the thyroidal effects occur mostly at doses associated with systemic toxicity/limit dose (~1000 mg/kg b.w./d), possibly confounding a specific endocrine disrupting effect. Therefore, the final evaluation should take into account a WoE, also considering lack of thyroid hormone level modifications, and indication of no specific inhibition of thyoid-related enzymes (deiodinase, peroxidase) and potential metabolic enzyme induction - see discussion in **B.6.8.3.4.2.**
[$]: induction effects irrelevant for positive endocrine disruptive effects

Table **B.6.8.3.4.1.2-3.:** Lines of evidence on systemic toxicity and STOT parameters

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
2	STOT	Heart histopathology	Rat	13	W	Oral		ppm	No effect	Negative	No indication of toxicity	No findings
9	STOT	Heart histopathology	Mouse	90	D	Oral		ppm	No effect			
10	STOT	Heart histopathology	Mouse	18	M	Oral		ppm	No effect			
12	STOT	Heart histopathology	Dog	90	D	Oral		ppm	No effect			
1	STOT	Heart weight	Rat	4	W	Oral		ppm	No effect	Negative		
2	STOT	Heart weight	Rat	13	W	Oral		ppm	No effect			
9	STOT	Heart weight	Mouse	90	D	Oral		ppm	No effect			
11	STOT	Heart weight	Dog	4	W	Oral		ppm	No effect			
12	STOT	Heart weight	Dog	90	D	Oral		ppm	No effect			
2	STOT	Kidney histopathology	Rat	13	W	Oral		ppm	No effect	Negative	Rodent kidney wt changes inconsistent; ↑wt in rats adaptive or related to kidney function; no histological changes in any species	target organ/ systemic toxicity
9	STOT	Kidney histopathology	Mouse	90	D	Oral		ppm	No effect			
10	STOT	Kidney histopathology	Mouse	18	M	Oral		ppm	No effect			
12	STOT	Kidney histopathology	Dog	90	D	Oral		ppm	No effect			
4a	STOT	Kidney histopathology	Rat	52	W	Oral		ppm	No effect			
1	STOT	Kidney weight	Rat	4	W	Oral		ppm	No effect	In mice (18-month) wt (r) ↓12, 13 & 16% at 100, 2500 & 7000 ppm, no histological correlate, NSS.		
2	STOT	Kidney weight	Rat	13	W	Oral		ppm	No effect			
5	STOT	Kidney weight	Rat	17	W	Oral		ppm	No effect			

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
9	STOT	Kidney weight	Mouse	90	D	Oral		ppm	No effect	In 52 wk rats at 25000 ppm r ↑8% & SS in ♀ & at 2 years slight ↑ *vs.* controls, r (♂) ↑9%, (♀) ↑12%, both SS		
10	STOT	Kidney weight	Mouse	18	M	Oral		ppm	Change			
11	STOT	Kidney weight	Dog	4	W	Oral		ppm	Decrease			
12	STOT	Kidney weight	Dog	90	D	Oral		ppm	No effect			
4a	STOT	Kidney weight	Rat	52	W	Oral	25000	ppm	Increase			
4b	STOT	Kidney weight	Rat	104	W	Oral	25000	ppm	Increase			
2	STOT	Liver histopathology	Rat	13	W	Oral	50000	ppm	Increase	Centrilobular hepatocellular hypertrophy: 5/9 ♂, 4/10 ♀ at 50000 ppm at week 13 versus 0/10 in both (♂♀) controls; not observed after recovery	↑liver wt in rat, mouse & dog, related to ↑ hepatocellular hypertrophy; equivocal ↑ in tumours in mice; ↑enzymes in rats vacuolation in rats at 2-yr at >4000 mkd	target organ/ systemic toxicity
5	STOT	Liver histopathology	Rat	17	W	Oral	50000	ppm	Increase	Centrilobular hepatocellular hypertrophy in ♂: Controls F_0: 0/28, F_1: 0/24; 50000 ppm F_0: 1/28, F_1: 3/24; Not examined in F_0♀, examined in F_1♀ & no cases of hypertrophy observed.		
9	STOT	Liver histopathology	Mouse	90	D	Oral		ppm	No effect	Negative		
10a	STOT	Liver histopathology	Mouse	18	M	Oral	7000	ppm	Increase	Centrilobular hepatocellular hypertrophy: minimal in 7/80 animals		
10b	STOT	Liver histopathology	Mouse	18	M	Oral	7000	ppm	Increase	♂Incidence of single hepatocellular adenoma not ↑, hepatocellular single or multiple incidence: 11/80, 15/79, 14/80 & **24**/80 (30%), hepatocellular carcinoma: 5/80, 3/79, 3/80 & 2/80, hepatocellular tumour any type: 14/80, 16/79, 15/80 & **25**/80. Adenoma multiple, combined, tumour any type, SS at 7000 ppm		
12	STOT	Liver histopathology	Dog	90	D	Oral	5000	ppm	Increase	Centrilob & midzonal hepatocyte hypertrophy: ♂ at 5000 ppm 2/4, at 25000 ppm 3/4; ♀ at 25000 1/4		
4a	STOT	Liver histopathology	Rat	52	W	Oral	25000	ppm	Increase	Slight centrilob hepat hypertrophy in 15/20 ♂at week 52 versus 2/20 in controls. ♀unaffected.		
4b	STOT	Liver histopathology	Rat	104	W	Oral	25000	ppm	Increase	Centrilobular hepatocellular vacuolisation: 16/50 in control, 28/50 at 25000 ppm in ♂		

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
1	STOT	Liver weight	Rat	4	W	Oral	20000/50000	ppm	Increase	a (↑28%) & r (↑13%) at top-dose SS		
2	STOT	Liver weight	Rat	13	W	Oral	5000	ppm	Increase	♀a wt: ↑22% at 50000 ppm; r ↑21% at 50000 ppm at W13, both SS.; not observed after recovery. At 5000 ppm r ↑22%, NSS. ♂ 50000 ppm ↑10% r. NSS.		
5	STOT	Liver weight	Rat	17	W	Oral	10000	ppm	Increase	r ↑ in F_0 & F_1 parental rats at 10000 ppm (5-6%) and 50000 ppm (12-16%), generally SS.		
9	STOT	Liver weight	Mouse	90	D	Oral	5000	ppm	Increase	♂a: ↑13% at 10000 ppm; ♀a: ↑21% at 5000 ppm, ↑22% at 10000 ppm; r: ↑14% at 5000 ppm, ↑17% at 10000 ppm.		
10	STOT	Liver weight	Mouse	18	M	Oral	7000	ppm	Increase	at 7000 ppm: ♂ r ↑ 16%, ♀ ↑7%, SS. in♀		
11	STOT	Liver weight	Dog	4	W	Oral		ppm	Increase	r at 20000 ppm (↑18%), at 50000 ppm (↑21%)		
12	STOT	Liver weight	Dog	90	D	Oral	25000	ppm	Increase	a ↑13% and adjusted ↑15%, NSS		
19	STOT	Liver weight	Rat	20	W	Oral		ppm	Increase	NSS ↑ in mean liver wt. reported, but adjusted liver weight ↑11% at 50000 ppm		
4a	STOT	Liver weight	Rat	52	W	Oral	25000	ppm	Increase	r ↑9%, SS		
4b	STOT	Liver weight	Rat	104	W	Oral	25000	ppm	Increase	r ♂↑13%, ♀↑11%, both SS		
2	STOT	Lung histopathology	Rat	13	W	Oral		ppm	No effect	Negative	Equivocal ↑ in lung tumours in ♂mice (977 mg/kg/d); no changes in ♀mice, rats or dogs	target organ/ systemic toxicity
9	STOT	Lung histopathology	Mouse	90	D	Oral		ppm	No effect			
10	STOT	Lung histopathology	Mouse	18	M	Oral	7000	ppm	Increase	SS. ↑ combined incidence of alveolar adenomas & adenocarcinomas (26/80, 32%) at 7000 ppm ♂ *vs.* controls (18/80, 22.5%). Literature evidence of higher incidence of bronchoalvealoar tumours in CD-1 male mice, up to 61.1% (Manenti, 2003), 43% (Fox, 2007) & 33.4% (Maita, 1988)		
12	STOT	Lung histopathology	Dog	90	D	Oral		ppm	No effect	Negative		

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
4a	STOT	Lung histopathology	Rat	52	W	Oral		ppm	No effect			
4b	STOT	Lung histopathology	Rat	104	W	Oral		ppm	No effect			
9	STOT	Pancreas histopathology	Mouse	90	D	Oral		ppm	No effect	Negative	No indication of toxicity	No findings
10	STOT	Pancreas histopathology	Mouse	18	M	Oral		ppm	No effect			
2	STOT	Spleen histopathology	Rat	13	W	Oral		ppm	No effect	Negative	Changes in 90-day mouse spleen wt potentially related to haemato-poiesis, see below; changes in rat & dog wt but no histologic change	target organ/ systemic toxicity
9	STOT	Spleen histopathology	Mouse	90	D	Oral		ppm	No effect			
10	STOT	Spleen histopathology	Mouse	18	M	Oral		ppm	No effect			
2	STOT	Spleen weight	Rat	13	W	Oral		ppm	No effect			
5	STOT	Spleen weight	Rat	17	W	Oral		ppm	No effect			
9	STOT	Spleen weight	Mouse	90	D	Oral	10000	ppm	Increase	a wt:↑43%, r: ↑36%, NSS.		
10	STOT	Spleen weight	Mouse	18	M	Oral	100	ppm	No effect	Negative		
11	STOT	Spleen weight	Dog	4	W	Oral		ppm	No effect			
12	STOT	Spleen weight	Dog	90	D	Oral	1000	ppm	Decrease	Unadjusted a. & adjusted mean wt: (♂) at 25000 ppm unadj ↓15%, adj ↓14%; (♀) only unadjusted wt calculated. At 1000 ppm ↓12%, at 5000 ppm ↓34%, at 25000 ppm ↓29%		
4a	STOT	Spleen weight	Rat	52	W	Oral		ppm	No effect	Negative		
4b	STOT	Spleen weight	Rat	104	W	Oral	25000	ppm	Increase	a: ↑11%, r: ↑23%; NSS		
2	STOT	Thymus histopathology	Rat	13	W	Oral		ppm	No effect	Negative	No correlative indications of toxicity	No clear findings
9	STOT	Thymus histopathology	Mouse	90	D	Oral		ppm	No effect			

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
10	STOT	Thymus histopathology	Mouse	18	M	Oral		ppm	No effect			
12	STOT	Thymus histopathology	Dog	90	D	Oral		ppm	Change	♂: 0/4, 1/4 minimal, 1/4 slight, 2/4 thymic involution/atrophy; ♀: 1/4 minimal, 1/4 slight, 1/4 minimal, 0/4 at 0, 1000, 5000, 25000 ppm, resp.		
1	STOT	Thymus weight	Rat	4	W	Oral		ppm	No effect	Negative		
2	STOT	Thymus weight	Rat	13	W	Oral		ppm	No effect			
12	STOT	Thymus weight	Dog	90	D	Oral		ppm	No effect			
1	Systemic toxicity	Body weight	Rat	4	W	Oral		ppm	No effect	Negative	No indications of an effect	No findings
2	Systemic toxicity	Body weight	Rat	13	W	Oral		ppm	Decrease	Possible test substance related ↓ at 5000 & 50000 ppm, but effect may be due, at least in part, to unusual weight gain in controls	No clear indication of an effect	No clear findings
5a	Systemic toxicity	Body weight	Rat	17	W	Oral	10000	ppm	Decrease	Slight to no effect in F_0 & F_1 (♂); F_0 & F_1 (♀) slightly ↓gain prior to & during mating at ≥10000 ppm. Gestation: F_0 at ≥10000 ppm & F_1 at 50000 ppm minimal ↓gain (↓7 - 9% SS.); F_0 dams: Lactation bw & gain similar to controls except D 7-14, gain ↓38% at 50000 ppm, recovered afterward. F_1 dams: at start of lactation, mean bw slightly < than controls at ≥10000 ppm (↓6 & 7% at 10000 & 50000 ppm, NSS.). Bw gain for treated F_1 dams ≥controls except at ≥10000 ppm during D 4-7 interval where gains were ↓29% at both doses & D 7-14 in which they were ↓12-18% (NSS) but afterward recovered.	Minimal body wt gain ↓	target organ/ systemic toxicity
5b	Systemic toxicity	Body weight	Rat	17	W	Oral	10000	ppm	Decrease	Overall BW gain over lactation (d1-21): at 10000 ppm only minimal; at 50000 ppm F_1 (↓6-7%) & F_2 (↓10%)	Minimal body wt gain ↓	target organ/ systemic toxicity
5c	Systemic toxicity	Body weight	Rat	17	W	Oral		ppm	No effect	Overall unaffected by treatment		No findings

study ID	Effect: Classification	Effect: target	Specie	Duration #	Duration unit	Administration route	Dose: Lowest	Dose: unit	Effect direction	Observed effect (+ and -)	Assessment: Each LoE	Assessment: Integrated LoE
6	Systemic toxicity	Body weight	Rat	10	D	Oral		ppm	No effect		No indications of an effect	
6	Systemic toxicity	Body weight	Rat	10	D	Oral		ppm	No effect			
8	Systemic toxicity	Body weight	Rat	19	D	Oral		mkd	No effect			
8	Systemic toxicity	Body weight	Rat	19	D	Oral		mkd	No effect			
9	Systemic toxicity	Body weight	Mouse	90	D	Oral		ppm	No effect			
10	Systemic toxicity	Body weight	Mouse	18	M	Oral		ppm	No effect			
11	Systemic toxicity	Body weight	Dog	4	W	Oral		ppm	Decrease	♂>10% bw change: at 20000 ppm (↓23%), at 50000 ppm (↓38%); ♀ at 50000 ppm (↓22%)	Body wt ↓	target organ/ systemic toxicity
12	Systemic toxicity	Body weight	Dog	90	D	Oral		ppm	No effect	Negative	No indications of an effect	No findings
19	Systemic toxicity	Body weight	Rat	20	W	Oral	50000	ppm	Decrease	marginal ↓ overall bw gain, wks 0 - 19 (8%), NSS	Marginal ↓ BW gain	Marginal finding
4b	Systemic toxicity	Body weight	Rat	104	W	Oral	25000	ppm	Decrease	♀overall bw ↓9% & SS., bw gain ↓13%, NSS	Body wt ↓ & BW gain	Marginal finding
2a	Systemic toxicity	Clinical chemistry and haematology	Rat	13	W	Oral	5000	ppm	Decrease	phosphorous in ♀ at 5000 ppm ↓21% & at 50000 ppm ↓18%, both SS; reversed at recovery	Clinical chemistry findings related to kidney function	target organ/ systemic toxicity
2b	Systemic toxicity	Clinical chemistry and haematology	Rat	13	W	Oral	50000	ppm	Decrease	phosphorous in ♂at 50000 ppm ↓6%, SS reversed at recovery		
2c	Systemic toxicity	Clinical chemistry and haematology	Rat	13	W	Oral	50000	ppm	Increase	creatinine ↑10% in ♀& SS; ↑7% at recovery, NSS		
2c	Systemic toxicity	Clinical chemistry and haematology	Rat	13	W	Oral	500	ppm	Decrease	SS WBC ↓19% & LYM ↓26% in ♂ at 50000 ppm. ♀at 5000 & 50000 ppm WBC ↓26/26%, lymphocyte ↓31/27%, monocytes ↓36/45% & leucocyte ↓33/33%, and at 50000 ppm eosinophils ↓25%	Haematology changes adverse at ≥5000 ppm (468	target organ/ systemic toxicity

study ID	Effect: Classification	Effect: target	Specie	Duration #	Duration unit	Administration route	Dose: Lowest	Dose: unit	Effect direction	Observed effect (+ and -)	Assessment: Each LoE	Assessment: Integrated LoE
											mg/kg) in ♀	
9a	Systemic toxicity	Clinical chemistry and haematology	Mouse	90	D	Oral	1000	ppm	Decrease	D45: WBC: ↓27% at 1000 ppm, ↓31% at 5000 ppm, ↓29% at 10000 ppm; D90: ↓30% at 1000 ppm, ↓38% at 5000 ppm, ↓34% at 10000 ppm; NEU: ↓51% at 1000 ppm, ↓40% at 5000 ppm, ↓39% at 10000 ppm; D90: WBC: ↓30% at 1000 ppm, ↓38% at 5000 ppm, ↓34% at 10000 ppm; NEU: ↓40% at 1000 ppm, ↓37% at 5000 ppm, ↓64% at 10000 ppm; LYM: ↓29% at 1000 ppm, ↓37% at 5000 ppm, ↓26% at 10000 ppm	Haematology changes; adverse at ≥5000 ppm (787 mg/kg) in (M)	target organ/systemic toxicity
9b	Systemic toxicity	Clinical chemistry and haematology	Mouse	90	D	Oral	1000	ppm	Increase	Red cell mass parameters, D45: RBC ↑13% at 1000 ppm, ↑10% at 5000 ppm, ↑12% at 10000 ppm, HB ↑11% at 1000 ppm, no change at 5000 ppm, ↑11% at 10000 ppm, HCT ↑11% at 1000 ppm, ↑7% at 5000 ppm, ↑11% at 10000 ppm, most changes were SS; no effect at 90 D	Haematology changes	target organ/systemic toxicity
11a	Systemic toxicity	Clinical chemistry and haematology	Dog	4	W	Oral		ppm	Increase	↑ urea values: high *vs.* low dose (M) (↑91%), (F) (↑14%)	Clinical chemistry changes	target organ/systemic toxicity
11b	Systemic toxicity	Clinical chemistry and haematology	Dog	4	W	Oral		ppm	Increase	Creatinine values at 50000 ppm *vs.* predose (↑24%) *vs.* low-dose (↑22%)	Clinical chemistry changes	target organ/systemic toxicity
11c	Systemic toxicity	Clinical chemistry and haematology	Dog	4	W	Oral		ppm	Increase	ALP: 50000 ppm *vs.* predose (↑29%) *vs.* low-dose (↑61%) at 20000 ppm, (↑59%) at 50000 ppm		
11d	Systemic toxicity	Clinical chemistry and haematology	Dog	4	W	Oral		ppm	Change	Haematology - ↑ and ↓	Haematology changes	No clear toxicity
12a	Systemic toxicity	Clinical chemistry and haematology	Dog	90	D	Oral	25000	ppm	Increase	ALP ♂wk 6 ↑29%, wk 13 ↑54%, ♀wk 6 ↑22%, wk 13 ↑31%	Clinical chemistry changes	No clear toxicity
12b	Systemic toxicity	Clinical chemistry and haematology	Dog	90	D	Oral	5000	ppm	Decrease	Reticulocytes: at 5000 ppm wk 6 (↓46%) and 13 (↓34%); at 25000 wk 6 (↓30%) and 13 (↓41%); within lab HCD	Haematology changes	No clear toxicity
12c	Systemic toxicity	Clinical chemistry and haematology	Dog	90	D	Oral	25000	ppm	Increase	↑ APTT: wk 13 (22%) SS, but not different from pre-dosing values	Haematology changes	No clear toxicity

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
4a	Systemic toxicity	Clinical chemistry and haematology	Rat	52	W	Oral	25000	ppm	Increase	Urinary protein levels: ♂wk 12 ↑32% & wk 51 ↑50%, ♀wk 51 ↑68%. Transient increases (NSS at other sample times). No kidney histopath.	Clinical chemistry changes	target organ/ systemic toxicity
5	Systemic toxicity	Clinical signs	Rat	17	W	Oral	10000	ppm	Increase	↑ alopecia dorsal surface in F_0 & F_1 at 50000 ppm (♂♀), F_0 generation: 2/28, 1/28, 3/28, 5/28 in ♂, 7/28, 3/28, 6/28, 10/28 in ♀, at 0, 1000, 10000 & 50000 ppm. Similar pattern in F_1 animals. ↑alopecia at 10000 ppm in ♂ (1/24 at 0 ppm vs 5/24 at 10000 ppm).	Possible clinical signs of toxicity	target organ/ systemic toxicity
6	Systemic toxicity	Clinical signs	Rat	10	D	Oral		ppm	No effect	Negative	No indications of an effect	No findings
8	Systemic toxicity	Clinical signs	Rat	19	D	Oral	1000	mkd	Change	At 1000 mg/kg forepaw alopecia in 5/22 dams (23%). In control & lower doses the incidence of alopecia was 1-2/22 dams.	Possible clinical signs of toxicity	target organ/ systemic toxicity
8	Systemic toxicity	Clinical signs	Rat	19	D	Oral	1000	mkd	Change	Brown staining of forelimbs slightly ↑at 1000 mg/kg (5/22 cases *vs* 1 to 3 cases in 21 to 22 dams in all other groups).		
13	Systemic toxicity	Clinical signs	Rabbit	17	D	Oral	4000	mkd	Increase	Alopecia in the controls was higher than in the treated groups, *e.g.* GD 20-29 7/20 in control *vs.* 3/19 at top dose		
13	Systemic toxicity	Clinical signs	Rabbit	17	D	Oral	4000	mkd	Increase	↑stained tail: GD 7-19, 6/20 in control & 11/20 at top dose; GD 20-29, 5/10 in control & 12/19 at top dose		
4b	Systemic toxicity	Clinical signs	Rat	104	W	Oral	25000	ppm	No effect	Negative	No indications of an effect	No findings
1	Systemic toxicity	Food consumption	Rat	4	W	Oral		ppm	No effect	Negative	No indications of an effect	
2	Systemic toxicity	Food consumption	Rat	13	W	Oral		ppm	No effect			
5	Systemic toxicity	Food consumption	Rat	17	W	Oral		ppm	No effect			

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
5d	Systemic toxicity	Food consumption	Rat	17	W	Oral		ppm	No effect			
5e	Systemic toxicity	Food consumption	Rat	17	W	Oral		ppm	No effect			
6	Systemic toxicity	Food consumption	Rat	10	D	Oral		ppm	No effect			
8	Systemic toxicity	Food consumption	Rat	19	D	Oral		mkd	No effect			
9	Systemic toxicity	Food consumption	Mouse	90	D	Oral		ppm	No effect			
10	Systemic toxicity	Food consumption	Mouse	18	M	Oral		ppm	No effect			
11	Systemic toxicity	Food consumption	Dog	4	W	Oral		ppm	Decrease			
12	Systemic toxicity	Food consumption	Dog	90	D	Oral		ppm	No effect			
19	Systemic toxicity	Food consumption	Rat	20	W	Oral		ppm	No effect			
4a	Systemic toxicity	Food consumption	Rat	52	W	Oral		ppm	No effect			
4b	Systemic toxicity	Food consumption	Rat	104	W	Oral		ppm	No effect			
1	Systemic toxicity	Mortality	Rat	4	W	Oral		ppm	No effect	Negative	No indications of an effect	
8	Systemic toxicity	Mortality	Rat	19	D	Oral		mkd	No effect			
9	Systemic toxicity	Mortality	Mouse	90	D	Oral		ppm	No effect			
10	Systemic toxicity	Mortality	Mouse	18	M	Oral		ppm	No effect			

study	Effect		Specie	Dura-tion		Administra-tion	Dose		Effect	Observed effect	Assessment	
ID	Classifica-tion	target		#	unit	route	Lowest	unit	direction	(+ and -)	Each LoE	Integrated LoE
11	Systemic toxicity	Mortality	Dog	4	W	Oral		ppm	No effect			
12	Systemic toxicity	Mortality	Dog	90	D	Oral		ppm	No effect			
4a	Systemic toxicity	Mortality	Rat	52	W	Oral		ppm	No effect			
4b	Systemic toxicity	Mortality	Rat	104	W	Oral		ppm	No effect			
1	[Not in list]	[Not in list]	Rat	4	W	Oral		ppm	No effect	Negative	No indications of an effect	No findings
2	[Not in list]	[Not in list]	Rat	13	W	Oral		ppm	No effect			
9c	[Not in list]	[Not in list]	Mouse	90	D	Oral	10000	ppm	Increase	Extramedullary haematopoiesis-liver & spleen; Liver: 4/10; spleen: 5/10	Likely compensatory changes due to hematology findings	target organ/ systemic toxicity
10	[Not in list]	[Not in list]	Mouse	18	M	Oral	7000	ppm	Increase	↑Harderian gland adenoma: 6/80, 2/23, 2/16 & 9/80 at 0, 100, 2500 & 7000 ppm. NSS.	No clear indication of an effect	No clear toxicity
7	No relevant effect observed	No relevant effect observed	Rat	22	D	Oral	1000	mkd	Increase	Rat Developmental tox: Alopecia in 4/11 (36%) dams at 4000 mg/kg. Lower incidences (2/11, 18%) noted at 500 & 1000 & one animal in control (1/11, 9%) - Laboratory HCD, 25 studies from 1991 to 2001, mean=14%, range 0-28%	Possible clinical signs of toxicity	target organ/ systemic toxicity
8	No relevant effect observed	No relevant effect observed	Rat	19	D	Oral		mkd	No effect	Negative	No indications of an effect	No findings
18	No relevant effect observed	No relevant effect observed	Rat	5	D	Oral		mkd	No effect			

Abbreviations: H= hours; D=days, W= weeks, M= months; ppm= parts per million; mkd= mg/kg b.w./d; organ weights: a=absolute, r=relative (to body weight); (N)SS= (not) statistically significant

B.6.8.3.4.1.3 Liver

Notifier clarified that, although not necessarily related to endocrine changes, increases in liver weights and hepatocellular hypertrophy could be associated with endocrine effects since the liver is the primary site of biotransformation of steroid and thyroid hormones. Increased hepatic metabolism may lead to alterations in circulating levels of hormones, which may in turn affect the target organs of these hormones (WHO, 2002; 2012). In this regard, increases in liver weight that usually correlated with hepatocellular hypertrophy were observed at the highest dose tested in all species (*e.g.* 90-day rat, 90-day dog, 2-year rat and 18-month mouse study).
In addition, a recently conducted 2-week study in rats has shown increased liver weight and minimal hepatocellular hypertrophy at dietary concentrations of 12500 and 25000 ppm lenacil. Consistent with an adaptive response, hepatic UDPGT activity and hepatic microsomal CYP2B1 gene expression were increased in a test substance-related manner.
Notifier re-iterated that, although hepatic UDPGT activity was induced, there was no difference observed in serum T_4 concentration in either treatment group. Furthermore, there were no effects on thyroid organ weights, no evidence of thyroid follicular cell hypertrophy and TSH was not elevated.

RMS agrees that the observed findings in the liver are well consistent with a potential CYP450 induction process. As discussed above, all top-dose thyroidal effects may however not totally be explained by liver enzyme induction only, but they appear on the other hand also at doses >>MTD.

B.6.8.3.4.1.4 Summary
The mechanistic studies aiming to clarify possible mechanisms for EATS-mediated effects did not clearly point to an endocrine disrupting effects by lenacil. In **table B.6.8.3.4.4-1**, a summary of endocrine-related mechanistic studies (or special measurements in guideline studies) for EATS modalities is provided. *In-vitro* assays include 7 studies, where oestrogenic (2), androgenic (2), steroidogenic (2) and thyroidotropic (1) effects were studied. None of them indicated evidence of endocrine effects after treatment with lenacil.

The *in-vivo* studies included an uterotrophic assay, where no adverse findings were observed after treatment with lenacil up to and including the limit dose.
Further *in-vivo* mechanistic studies were limited to either *ad-hoc* studies (stand-alone or incorporated in existing guideline studies) in order to investigate thyroid hormone levels after dietary treatment with lenacil (2wk, 10-20wk, 52 wk). The outcome was a disparate picture regarding circulating levels of T_3, T_4 or TSH, which were difficult to interprete, in view of the different rat strains used, sexes evaluated and top-doses included. The final evaluation would be that lenacil caused an equivocal effect on the thyroid hormones, possibly associated but not completely explained by liver metabolic enzyme induction. On the other hand, existing assays would indicate that lenacil is devoid of a primary thyrotropic effect on deiodinase and peroxidase.

It could thus be proposed by **RMS** that lenacil was devoid of any oestrogenic, anti-oestrogenic, androgenic or anti-androgenic activity. Inconsistent effects were noted on the thyroid hormone-levels, but from the guideline studies is became clear that lenacil may be thyrotoxic, however at doses associated with relatively high systemic toxicity, and mainly at doses exceeding the accepted limit dose of 1000 mg/kg b.w./d.

While no reprotoxic effects were reported, it could be discussed whether a DNT study would be desirable, in the case that developing young animals would possibly be more sensitive for neurodevelopmental endpoints.

Table B.6.8.3.4.4-1 Lenacil : summary of endocrine-related mechanistic studies (or special measurements in guideline studies) –EATS modalities

Type of assay test system - duration *(in-vivo)*, test method (TM) used - ref. in DAR B.6	Modality [a]	Concentrations/Doses tested, route of administration *(in-vivo)*	Lenacil, B.n°, purity	Result	Reference
IN-VITRO ASSAYS					
Oestrogen receptor binding assay (ER-RUC) OPPTS 890.1250 - (**B.6.8.3.1.1**)	**E**	10^{-10} – 10^{-3} M	B.n° 047303003, purity 99.33%	negative	Nabb, 2018a
Oestrogenic receptor agonism/antagonism (hERα-HeLa-9903 cells), OECD TG 455 - (**B.6.8.3.1.2**)	**E**	*Agonism*: 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM; *Antagonism*: 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, 100 pM	B.n° 047303003, purity 99.33%	negative	Rijk, 2018a
Androgen receptor binding assay (AR-RPC) OPPTS 890.1150 -(**B.6.8.3.1.3**)	**A**	10^{-10} – 10^{-3} M	B.n° 047303003, purity 99.33%	negative	Nabb, 2018b
Androgenic agonism/antagonism, (AR EcoScreen™, OECD TG 458 - (**B.6.8.3.1.4**)	**A**	*Agonism*: 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, 1 pM; *Antagonism*: 1 µM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM	B.n° 047303003, purity 99.33%	negative	Rijk, 2018b
In vitro aromatase inhibition assay, human recombinant microsomes, OPPTS 890.1200 - (**B.6.8.3.1.6**)	**S**	0.00001 µM – 31.6 µM (10^{-11} – 3.16×10^{-5} M)	B.n° 047303003, purity 99.33%	negative	Rijk, 2019
In vitro steroidogenesis assay, H295R cells EC TM B.57 - OECD TG 456 - (**B.6.8.3.1.7**)	**S**	0.00001 µM – 3.16 µM (10^{-11} – 3.16×10^{-6} M)	B.n° 047303003, purity 99.33%	negative	Verkaart, 2019
Thyroid mechanistic assay, peroxidase inhibition assay in isolated porcine thyrocytes, no TM - **(B.6.8.3.1.8)**	**T**	0.5-500 µM	B.n° 151/152, purity 98.8%	negative	[redacted] 2019
IN-VIVO ASSAYS					
Uterotrophic assay 15♀ SD rats/dose, 5d, EC TM B.54 - OECD TG 440 - (**B.6.8.3.1.5**)	**E**	0, 500, 1000 mg/kg b.w./d (gavage)	B.n°, 141712003 purity 98.6%	negative	[redacted] 2018
Repeated toxicity, 20♂♀ Wistar rats/dose, 52wk, thyroid hormone measurements, EC TM B.30 - OECD TG 453 - (**B.6.5.1**)	**T**	0, 1446/1894 mg/kg b.w./d (dietary)	B.n°, 141712003 purity 98.6%	negative	[redacted] 2003
Thyroid mechanistic assay 15♂ SD rats/dose, 14d, thyroid hormone measurements, no TM, - **(B.6.8.3.1.8)**	**T**	0, 189, 923, 1841 mg/kg b.w./d (dietary)	B.n° 151/152, purity 98.8%	equivocal	[redacted] 2019
Thyroid mechanistic assay 18♀ Wistar rats/dose, 10-20 wk, perchlorate discharge test, deiodinase inhibition, thyroid hormone measurements, no TM - **(B.6.8.2)**	**T**	0, 21, 4421 mg/kg b.w./d (dietary)	B.n°, 141712003 purity 98.6%	equivocal	[redacted] 2004 – [redacted] 2007

[a]: according to OECD GD 150

B.6.8.3.4.1.5 Sensitive to, but not diagnostic of EATS

B.6.8.3.4.1.5.1 Pituitary

-Minimal changes in the pituitary gland (pituitary cysts and weight). This included a slight increase in the incidence of cysts in high-dose ♂in the 13-week dog study and 18-month mouse study, and weight changes in rats. Notifier noted that the incidence of cysts in high dose ♂dogs was the same as the incidence in control ♀(2/4 animals), and the incidence in the high dose ♀dogs was the same as the ♂control (0/4 animals). Given this pattern, notifier considered it doubtful that these findings are test substance-related. **RMS** considers the effect relevant (comparing study controls across sexes is not considered adequate), but given the magnitude of the top-dose (>1000 mg/kg b.w./d) of insufficient concern in the absence of other ED-related findings.

-In top-dose ♂mice from the 18-month carcinogenicity study there were 6 cases of pituitary cysts *vs.* 2 cases in controls. Pituitary cysts in rodents are non-proliferative lesions and typically represent either the remnants of the craniophyrangeal (Rathke's) pouch or cysts or pseudocysts. Given the low numbers in this study and their lack of statistical significance, notifier considered this finding incidental (as supported by the interpretation of the study pathologist). **RMS** retained the pituitary cyst conservatively as part of the top-dose-effects (HCD awaited), but noted the absence of other pituitary findings, and highlighted the absence of concerning endocrine effects in the thyroids, adrenals, ovaries, and testes, all primarily regulated by the pituitary, whose dysfunction would have caused pleiotropic effects none of which were observed, indicating no meaningful endocrine impact.

-Pituitary weights were statistically significantly increased in ♂parental F_1 rats at 817 mg/kg b.w./d and above in the 2G reproduction study. While notifier considered the pituitary weights elevations merely be a spurious finding, **RMS** agreed that the pituitary weight was unaltered in F_0 but showed an increase in the F_1♂ at 817 mg/kg b.w./d and above. It was still questioned whether there could be an association with the increased thyroid weight in this cohort at the two highest doses.

B.6.8.3.4.1.5.2 Kidney

There was no indication of a test substance related effect on kidneys in the 13-week dietary study in dogs. In rats at dose levels of ~4000 mg/kg bw/d (50000 ppm) in the 13-week study changes in kidney function were suggested based on statistically significant decreases in blood electrolytes and increases in creatinine. Suggestive changes in urinary parameters were also observed but there was no correlation with dose. However, there were no effects on kidney weight or histopathology. In the chronic cancer rat study at 25000 ppm (~1200-1900 mg/kg/d), ♂ at 2-years and ♀at ≥52 weeks had minimal increases in relative kidney weight (8-12%); however, with no histological correlates. In the 18-month mouse study, relative (bw) kidney weight was minimally reduced in ♀but they were not statistically significant, and there was no histological correlate. Given the available results, there is no indication that these changes were related to modulation of any endocrine-related parameters, for which **RMS** agrees.

Ecotoxicology (see **Vol. 3, B.9**)

B.6.8.3.4.2 General conclusions on the ED properties of lenacil (mammalian toxicology)

Table B.6.8.3.4.2-1 Selection of relevant scenario for EATS-mediated effects/adversity for mammalian toxicology

Adversity based on EATS-mediated parameters	Positive mechanistic OECD CF	Scenario	Next step of the assessment	Scenario selected
No (sufficiently investigated)	Yes/No	1a	Conclude: ED criteria not met because there is no "EATS-mediated" adversity	
Yes (sufficiently investigated)	Yes/No	1b	Perform MoA analysis	**X**
No (not sufficiently investigated)	Yes	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no EATS-mediated endocrine activity observed	
No (not sufficiently investigated)	No (not sufficiently investigated)	2a (iii)	Generate missing level 2 and 3 information. Alternatively, generate missing "EATS-mediated" parameters. Depending on the outcome move to corresponding scenario	
Yes (not sufficiently investigated)	Yes/No	2b	Perform MoA analysis	

Notifier considered that lenacil fulfilled all conditions to propose a scenario "1a" (*i.e.* sufficiently investigated, no adversity indicated by "EATS-mediated" parameters. Taking into account all data of the dossier, **RMS** is of the opinion that lenacil fulfilled the condition of scenario **"1b"** (EATS-mediated adversity observed, postulate MoA).

Rationale:
The potential of lenacil to induce adverse effects on components of the endocrine system has initially been assessed in published *in vitro* assays. Based on the available receptor-based *in vitro* studies it was suggested that lenacil has no PXR, mPPARα, mPPARγ or AhR agonistic activities at concentrations of $\leq 10^{-5}$M, *in vitro*. Further OECD Level-2 studies were submitted at the occasion of the renewal in 2018-2019.
Potential endocrine-related adverse findings were also identified in in the guideline short-term and long-term feeding studies, and in the multi-generation reproduction study. The adverse effect pertained to (i) thyroid findings, but also some indications of (ii) mammary tumours and (iii) uterus adversity, with a possible underlying endocrine MoA.

No effects on fertility, reproductive performance and/or development and sexual maturation of the offspring was seen even when lenacil was orally administered at dose levels exceeding the limit dose level of 1000 mg/kg b.w./d.

Regarding the observed potential endocrine adverse findings:

(i) In both the sub-chronic and the chronic feeding studies with rats (**B.6.3.2.1.1 -**ACD 002/013903 and **B.6.5.1.1-** ACD 045/024288-), adverse effects on the thyroid were observed, without any evidence of organ atrophy, but with evidence of histopathologically diagnosed blackening, possibly indicating lipofuscin deposition. Follicular tumours were weakly induced, however without being eligible for carcinogenicity classification, as the incidence was low, comprised into HCD and because the likely MoA was UDPGT induction, with relevance for rodents but not for the human.

The effect of the substance on thyroid pathways was not entirely elucidated. A perchlorate discharge study with lenacil showed no direct toxicity to the thyroid which could be explained via an inhibition of deiodinase

or peroxidase. The key findings from this test was that lenacil at doses up to >4000 mg/kg b.w./d did not affect the ability of the thyroid to take-up and organify iodide. It was demonstrated that lenacil did not act as an inhibitor of the deiodinase and/or peroxidase which converts T_4 to T_3. A separate peroxidase inhibition assay on porcine thyrocytes demonstrated that lenacil did not act via this pathway. Inconsistent effects on thyroid hormone levels were reported, but overall, the thyroid hormone modifications were not indicating a clear effect of the substance.

No MoA could unequivocally be identified for the finding of the thyroids blackening, but the existing mechanistic study *suggests* that a direct thyrotropic effect is unlikely. In a WoE evaluation, **RMS** agrees that there was no consistent pattern suggestive of a clear impact on the T modality.

However, there is no DNT study, thus it remains unclear what the impact could be of subtle thyroid hormone changes (if relevant) on the behavioural development in young rats, and **RMS** suggests to discuss it more in detail in the peer review process.

(ii) Based on an increased incidence of malignant mammary adenocarcinomas in the rat carcinogenicity study, which was considered to be *possibly* relevant for humans in the EFSA conclusion on the peer review of lenacil (EFSA, 2009) and more recently by the ECHA Risk Assessment Committee (RAC) lenacil was classified as a category 2 carcinogen (Carc. 2; H351). This harmonised classification proposal has meanwhile been adopted.

On the other hand, the mode of action (MoA) for the induction of mammary tumours observed in the rat carcinogenicity study with lenacil is still unknown. The absence of a genotoxic potential of lenacil does, however, not suggest that mammary gland tumours are caused be a genotoxic MoA and for this reason an epigenetic, endocrine-mediated MOA could be the cause for the induction of these tumours.

However, as the mechanistic *in-vitro* studies aiming to investigate effects on EAS-modalities demonstrated no interaction, the emergence of mammary tumours was considered most likely not endocrine-mediated. The mammary tumours may be either of equivocal relevance, or an epigenetic MoA could be at the basis of the tumourigenicity.

(iii) Uterus changes occur in several studies in the present DRAR which could be considered possibly treatment-related. The data with effect on the uterus pertain to findings observed in the Wistar rat, and increased uterus weight, along with fluid distention and/or luminal dilatation, or hyperplasia were observed. It is observed that the effects occur at relatively high dose (mostly ≥ 4000 mg/kg b.w./d), and that a dose-response was observed at times but not consistently. In the key study (2G), top-dose findings are poorly dose-responsive and/or increased histopathological findings are only borderline when compared to controls. No effects were observed in the dog, and in mice it was not investigated.

In addition, a the newly submitted uterotrophic assay was conducted up to a dose of 1000 mg/kg b.w./d (guideline limit dose) but also with another rat strain (Sprague-Dawley derived), and the assay turned out negative for uterus findings. As mentioned in (ii), lower-tier *in-vitro* studies do not indicate interference with EAS pathways. **RMS** concludes that the observed uterus findings are unlikely caused by an endocrine-dependent pathway. The overall WoE would suggest that the effect of lenacil on uterus was limited.

In conclusion, adverse endocrine findings possibly involving EAS-pathways were identified and further investigated. Both *in-vitro* and *in-vivo* mechanistic studies were performed in order to investigate an EATS-mediated MoA, and the latter could on the basis of these studies be excluded.

Thyroid adverse effects were also identified, and existing and new *in-vivo* mechanistic studies indicated that lenacil is not a primary thyrotoxicant. It was demonstrated that lenacil induced CYP4501B metabolic enzymes, as well as UDPGT, possibly explaining a number of adverse findings. Overall, the data showed that the adverse findings were mainly associated to systemic toxicity at doses nearby or >MTD, exceeding the limit dose of 1000 mg/kg b.w./d.

It is therefore plausible that no primary endocrine MoA for the EATS-modalities would be at the basis of all observations. For the thyroid findings, no other explanation than top-dose toxicity and UDPGT induction could be formulated. Therefore, **RMS** would be inclined to consider lenacil **not meeting the criteria for endocrine disruption**. While potentially adverse EATS findings have been detected, subsequent mechanistic studies and a WoE consideration leads to the conclusion that the ED criteria are not met.

One residual doubt subsist, for which a final discussion is seeked, namely the absence of a developmental neurotoxicity study. It is not excluded that any thyroidal effect could have more impact on young animals. Therefore, the question whether or not a DNT is necessary needs further discussion.

B.6.8.4 Studies on immunotoxicity

No immunotoxicity assay was submitted. **RMS** considers this unfortunate.

Whereas no overt signs of immunotoxic action could be identified in any repeated toxicity assay, there is still some uncertainty, since the lymphoid blood line and related organs such as spleen and/or thymus are consistently altered, albeit not always in a non-dose-dependent way. However, it seems quite obvious that high-dose administrations were consistently freight with leukopenia and some uncertainty remains as regards the interpretation of the observed findings throughout the repeated toxicity studies.

Therefore, **RMS** requests the conduct of a guideline GLP-immunotoxicity study, guideline according to *e.g.* OPPTS 870.7800 Immunotoxicity (EPA) or equivalent.

B.6.9 (CA 5.9) Medical data

B.6.9.1 (CA 5.9.1) Medical surveillance on manufacturing plant personnel and monitoring studies

The manufacturing sites of lenacil technical and the representative formulation are considered confidential information and are reported in the Lenacil EU Renewal Dossier, Document J, Part 1, DuPont-43886 EU. During the past 23 years there have been no incidents or accidents involving lenacil that have been reported at the formulations sites of the finished product.

Staff involved in the production of lenacil have been supervised since 1993 and examined clinically at regular intervals (Klotzbach, K. 2016). The medical care at the production site includes medical history, eye test, listening test, functional check of the lungs, blood and urine tests, ECGs, measurement of blood pressure, neurological status, clinical checks as well as consultation on the use of personal protective equipment. There were no findings attributable to the involvement in lenacil production. No consequential damages of the staff involved in the production of lenacil are known.

B.6.9.2 (CA 5.9.2) Data collected on humans

No cases of accidental poisoning were identified in the literature search that was conducted in conjunction with this EU Renewal submission (see Section 9).

The literature search indicated no available reports on incidents related to the agricultural use of lenacil.

B.6.9.3 (CA 5.9.3) Direct observations

Lenacil-based products have been commercially available in Europe since 1993. No reports of adverse health effects have been reported.

B.6.9.4 (CA 5.9.4) Epidemiological studies

There is no relevant exposure of the general population which would allow epidemiological investigations to be conducted. Hence no epidemiological studies have been conducted with lenacil. This product has been used commercially in Europe since 1993 and there have been no reports of adverse health effects associated with the manufacture or labelled uses of lenacil.

B.6.9.5 (CA 5.9.5) Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

There was no accidental poisoning resulting from production, formulation and agricultural use of lenacil.

With respect to acute oral toxicity, testing in rats showed that the active substance is well tolerated at 5000 mg/kg b.w./d, *i.e.,* it is of very low acute oral toxicity. Only non-specific clinical signs of hunched posture, lethargy and diarrhoea were seen at this dose level in an earlier study, however in a recently performed acute oral toxicity study, no such signs were observed at any dose up to 5000 mg/kg. Whilst these signs are non-specific it is possible that symptoms of exposure in humans may be different.

Similar non-specific clinical signs were seen in rats after acute inhalation exposure. Test rats exhibited exaggerated breathing during exposure and this persisted in a proportion of rats until 2h post exposure. No deaths occurred and no treatment-related findings at necropsy in either macroscopic abnormalities or lung weights were evident. The substance proved to be not irritating to the skin and eye. A transient diffuse, crimson colouration of the conjunctivae with or without above normal swelling was seen in all animals from approximately 1h after dosing, resolving completely by one or two days after instillation. The degree of findings did not trigger EU-classification. Testing for sensitising properties by the method of Magnusson & Kligman did not show an allergenic potential. Given the low acute oral, dermal and respiratory toxicity of lenacil, it would not be expected that accidental overexposure would lead to serious illness or mortality.

In rats, after long term exposure, the incidences of malignant mammary adenocarcinoma was found to be above the historical background range of the laboratory and was, therefore, considered *potentially* relevant for humans by the RAC Committee in the framework of the assessment of the CLH Report on the harmonised classification and labelling of lenacil. Lenacil is classified as a Category 2 carcinogen under the CLP (Carc. 2; H351). Therefore, after long-term exposure lenacil is *suspected* of causing cancer in humans.

B.6.9.6 (CA 5.9.6) Proposed treatment: first aid measures, antidotes, medical treatment

Lenacil is a uracil herbicide of very low acute toxicity. Therefore, it is most unlikely to produce any signs of toxicity. Consequently, the risk of poisoning when used under recommended use patterns is extremely low.

First aid measures

General

Measures:	In case of unconsciousness lay and transport person in stable position. If consulting a physician, show safety data sheet
After skin contact:	Remove contaminated clothing. Wash off immediately with soap and plenty of water. In case of persistent irritation consult a physician.
After eye contact:	Immediately rinse with running water for at least 15 min with spreaded eyelids (protect unharmed eye, remove contact lenses). In case of persistent irritation consult an ophthalmologist
After inhalation:	Move to fresh air. If necessary, artificial respiration. Rest. In case of persistent trouble consult a physician
After ingestion:	Rinse mouth and drink 1 or 2 glasses of water. Induce vomiting (only if person is fully conscious). If feeling sick, consult a physician
Advice for physician:	Decontamination. Symptomatic treatment

(No symptoms have been identified in humans to date)

Medical treatment and antidotes:

Local contamination:	Symptomatic after decontamination. In cases of skin or eye contamination, treat as documented above under FIRST AID MEASURES.
Systemic poisoning:	In the event of a very large amount being ingested the following measures should be considered: gastric lavage followed by charcoal and sodium sulphate. There is no specific antidote. Hence only symptomatic treatment is recommended after gastric lavage and treatment with charcoal.

First aid measures in the case of a suicidal intake of lenacil should consist of standard hygienic measures, i.e. decontamination and symptomatic treatment of non-specific symptoms.

B.6.9.7 (CA 5.9.7) Expected effects of poisoning

Expected effects and duration of poisoning as a function of the type, level and duration of exposure or ingestion

Findings in animal experiments indicated that unspecific symptoms of poisoning may appear after acute oral ingestion of high doses. However there have been no reports of clinical symptoms by exposed humans. Clinical signs caused by acute inhalation may be seen only on the day of exposure and may also be non-specific. Whilst dermal contact is not expected to cause any clinical symptoms in exposed humans, some mild temporary irritancy could occur following ocular exposure.

Since no cases of poisoning have been reported so far, no information on the nature and duration of effects as a function of the time between exposure and treatment exists. Based on the findings in animal tests, only symptomatic treatment of non-specific symptoms is recommended.

Expected effects and duration of poisoning as a function of varying time periods between exposure or ingestion and commencement of treatment

No specific human symptoms of lenacil toxicity are known. Effects of human exposure to lenacil should be transitory and resolved by 24h after exposure. The time between overexposure and commencement of treatment should be as short as possible but is not expected to be crucial for the final health status.

REFERENCE LIST, CA, SECTION 5, MAMMALIAN TOXICOLOGY - DOCUMENTS SUBMITTED.

Unless otherwise specified data submitted with this dossier are necessary for the renewal of the approval of lenacil because they address standard data requirements or reflect changes in scientific and/or technical knowledge or changes in uses since the first inclusion of the active substance. The reasons why individual studies are necessary are specified in a separate column below. The corresponding studies were conducted according to GLP or GEP standards and did not benefit from a previous period of protection.

In line with Article 60(1) of Regulation (EC) No. 1107/2009, the Rapporteur Member State shall prepare a list of the test and study reports necessary for the renewal of the approval of lenacil and the reference list below can be used as a basis.

FMC will make final claims of data protection for these necessary active substance and plant protection product data at application for authorisation or renewal of authorisation of our plant protection products after the approval renewal of lenacil in line with the provisions set in Articles 33.4 and 59 of Regulation (EC) No. 1107/2009.

The search strategy, analysis , overview and notifier opinion on the open scientific literature on lenacil is put in Appendix 1 of this DRAR.

B.6.10 Studies relied upon

REFERENCE LIST, CA, SECTION 5, MAMMALIAN TOXICOLOGY - DOCUMENTS SUBMITTED.

Data Requirement No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	Vertebrate study Y/N	Data Protection Y/N	Justification if data protection is claimed	Owner
KCA, 5.1.1/01	Pineiro Costas, N	2016	Interspecies comparison of *in vitro* metabolism of [pyrimidine-2-^{14}C]Lenacil in mouse, rat, dog and human hepatocytes WIL Research Europe B.V,'s-Hertogenbosch 512721 GLP: Yes Published: No	N	Y	Study necessary for the regulatory decision, and not previously submitted.	FMC
KCA, 5.2.7/01	Westerink, W.M.A.	2016	Evaluation of *in vitro* phototoxicity of lenacil TGAI in 3T3 fibroblasts using the neutral red uptake assay WIL Research Europe B.V. 511052 GLP: Yes Published: No	N	Y	Study necessary for the regulatory decision, and not previously submitted.	FMC
KCA, 5.8.1/01	Kurubaran, S.	2016	Expert statement: Assessment of the toxicological relevance of the groundwater metabolites of lenacil and proposal with a view to a human health-based risk assessment Dr Knoell Consult Limited DuPont-47276 GLP: No Published: No	N	N		FMC
KCA, 5.8.1/02	Tier, G.T	2014	Position paper title: A non-testing approach to evaluate the relevance of specific groundwater metabolites of lenacil E.I. du Pont de Nemours and Company DuPont-39162 GLP: No Published: No	N	N		FMC

Data Requirement No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	Vertebrate study Y/N	Data Protection Y/N	Justification if data protection is claimed	Owner
KCA, 5.8.3	Nabb, D.L.	2018a	Lenacil (DPX-B0634) technical: Estrogen receptor binding assay using rat uterine cytosol (ER-RUC) DuPont Report No.: 49349 GLP: Yes Published: No	N	Y	Study necessary for the regulatory decision, and not previously submitted.	FMC
KCA, 5.8.3	Nabb, D.L.	2018b	Lenacil (DPX-B0634) technical: Androgen receptor binding assay using rat prostate cytosol (AR-RPC) DuPont Report No.: 49367 GLP: Yes Published: No	N	Y	Study necessary for the regulatory decision, and not previously submitted.	FMC
KCA, 5.8.3	Rijk J.C.W.	2018a	Evaluation of the oestrogenic agonist and antagonist activity of lenacil (DPX-B0634) technical using the stably transfected human oestrogen receptor-α transactivation assay (hERα-HeLa-9903 cell line) DuPont Report No.: 49351 GLP: Yes Published: No	N	Y	Study necessary for the regulatory decision, and not previously submitted.	FMC
KCA, 5.8.3	Rijk J.C.W.	2018b	Evaluation of the androgenic agonist and antagonist activity of lenacil (DPX-B0634) technical using the stably transfected human androgen receptor transcriptional activation assay (AR EcoScreenTM) DuPont Report No.: 50113 GLP: Yes Published: No	N	Y	Study necessary for the regulatory decision, and not previously submitted.	FMC
KCA, 5.8.3	[redacted]	2018	Lenacil (DPX-B0634) technical: 5-day uterotrophic assay for detecting estrogenic activity in ovariectomised rats DuPont Report No.: 49350 GLP: Yes Published: No	Y	Y	Study necessary for the regulatory decision, and not previously submitted.	FMC

Data Requirement No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	Vertebrate study Y/N	Data Protection Y/N	Justification if data protection is claimed	Owner
KCA, 5.8.3	Rijk J.C.	2019	Lenacil (DPX-B0634) technical : *in vitro* aromatase inhibition using human recombinant microsomes. DuPont Report No.: 51364 GLP: Yes Published: No	N	Y	Study necessary for the regulatory decision, and not previously submitted.	FMC
KCA, 5.8.3	[redacted]	2019	Lenacil (DPX-B0634) technical: thyroid mechanistic 14-day feeding study in rats DuPont Report No.: 49352 GLP: Yes Published: No	Y	Y	Study necessary for the regulatory decision, and not previously submitted.	FMC
KCA, 5.8.3	Verkaart S	2019	Screening Lenacil (DPX-B0634) technical for modulation of steroidogenesis using the human H295R adeno-carcinoma cell line Report No. FMC-51365 GLP: Yes Published: No	N	Y	Study necessary for the regulatory decision, and not previously submitted.	FMC
KCA, 5.9.1	Klotzbach, K	2016	Medical expertise for the lenacil production E.I. du Pont de Nemours and Company 101581-5-9-1-01 GLP: No Published: No	N	N		FMC

REFERENCE LIST, CA, SECTION 5, MAMMALIAN TOXICOLOGY - DOCUMENTS NOT SUBMITTED, PREVIOUSLY SUBMITTED AND RELIED UPON

Data Requirement No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	Vertebrate study Y/N	Data Protection Y/N	Justification if data protection is claimed	Owner
CA, 5.1.1	[redacted]	1996	Absorption, distribution, metabolism and excretion of [2- 14C]-lenacil ([2-14C]-DPX-B634) in the rat [redacted] HLR 62-94 GLP: Yes Published: No	Y	Y		FMC
CA, 5.2.1	[redacted]	2001	Lenacil technical acute oral toxicity to the rat (acute toxic class method) [redacted] ACD 004/013224/AC GLP: Yes Published: No	Y	Y		FMC
CA, 5.2.2	[redacted]	2001	Lenacil technical acute dermal toxicity to the rat [redacted] ACD 005/013220/AC GLP: Yes Published: No	Y	Y		FMC
CA, 5.2.3	[redacted]	2001	Lenacil technical acute (four-hour) inhalation study in rats [redacted] ACD 021/013229 GLP: Yes Published: No	Y	Y		FMC

Data Requirement No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	Vertebrate study Y/N	Data Protection Y/N	Justification if data protection is claimed	Owner
CA, 5.2.4	[redacted]	2001	Lenacil technical skin irritation to the rabbit [redacted] ACD 006/013201/SE GLP: Yes Published: No	Y	Y		FMC
CA, 5.2.5	[redacted]	2001	Lenacil technical eye irritation to the rabbit [redacted] ACD 007/013273/SE GLP: Yes Published: No	Y	Y		FMC
CA, 5.2.6	[redacted]	1992	Closed-patch repeated insult dermal sensitization study (maximization method) with DPX-B634-91 in guinea pigs [redacted] HLO 34-92 GLP: Yes Published: No	Y	Y		FMC
CA, 5.3.1	[redacted]	2001	Lenacil technical preliminary study by dietary administration to Han Wistar rats for 4 weeks [redacted] ACD 001/010098 GLP: Yes Published: No	Y	Y		FMC
CA, 5.3.1	[redacted]	2001	Lenacil technical preliminary study by dietary administration to beagle dogs for 4 weeks [redacted] ACD 003/013230 GLP: Yes Published: No	Y	Y		FMC
CA 5.3.2	[redacted]	2002	Lenacil technical toxicity study by dietary administration to HAN Wistar rats for 13 weeks followed by a 4 week recovery period [redacted]	Y	Y		FMC

Data Requirement No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	Vertebrate study Y/N	Data Protection Y/N	Justification if data protection is claimed	Owner
			ACD 002/013903 GLP: Yes Published: No				
CA, 5.3.2	[redacted]	2004	Lenacil technical additional histopathological investigations to a toxicity study by dietary administration to han wistar rats for 13 weeks followed by a 4-week recovery period [redacted] ACD 055/024499 GLP: Yes Published: No	Y	Y		FMC
CA, 5.3.2	[redacted]	1991	Subchronic oral toxicity: 90-day study with DPX-B634- 91 (lenacil) feeding study in mice [redacted] HLR 293-91 GLP: Yes Published: No	Y	Y		FMC
CA, 5.3.2	[redacted]	2002	Lenacil technical toxicity study by dietary administration to beagle dogs for 13 weeks [redacted] ACD 022/014297 GLP: Yes Published: No	Y	Y		FMC
CA, 5.4.1	May, K.	2001	Lenacil technical bacterial mutation assay Huntingdon Life Sciences Ltd ACD 016/013217 GLP: Yes Published: No	N	Y		FMC
CA, 5.4.1	Russel, J.F.	1977	Mutagenic activity of uracil, 3-cyclohexyl-5,6,-trimethylene in the Salmonella/microsome assay DuPont Haskell Laboratory HLR 601-77 GLP: Yes	N	N		FMC

Data Requirement No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	Vertebrate study Y/N	Data Protection Y/N	Justification if data protection is claimed	Owner
			Published: No				
CA, 5.4.1	D'Amico, S.W.	1994	Mutagenicity testing of DPX-B634-107 (lenacil) in the Salmonella typhimurium plate incorporation assay DuPont Haskell Laboratory HLR 413-94 GLP: Yes Published: No	N	N		FMC
CA, 5.4.1	Grancharov, K., Gorneva, G., Mladenova, J., Norpoth, K., Golovinsky, E.	1986	Lack of genotoxic and cytotoxic effects of the herbicide lenacil on mouse tumor cells and on some *Salmonella typhimurium* strains Arzneimittelforschung 369110, 1660-1663 GLP: No Published: Yes	N	N		Authors
CA, 5.4.1	De Marco A, De Salvia R, Polani S, Ricordy R, Sorrenti F, Perticone P, Cozzi R, D'Ambrosio C, De Simone C, Guidotti M, Albanesi T, Duranti G, Festa F, Gensabella G, Owczarek M.	2000	Evaluation of genotoxic and cytotoxic properties of pesticides employed in Italian agricultural practices. Environ Res. 2000 Jul;83(3):311-21. GLP: No Published: Yes	N	N		Authors
CA, 5.4.1	Allais, L	2001	Lenacil technical *in vitro* mammalian chromosome aberration test in human lymphocytes Huntingdon Life Sciences Ltd ACD 017/013707 GLP: Yes Published: No	N	Y		FMC
CA, 5.4.1	Clare, G	2003	Lenacil technical *in vitro* mammalian cell gene mutation test Huntingdon Life Sciences Ltd ACD 053/023530	Y	Y		FMC

Data Requirement No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	Vertebrate study Y/N	Data Protection Y/N	Justification if data protection is claimed	Owner
			GLP: Yes Published: No				
CA, 5.4.1	Riach, C.G., Mohammed, R.	1990	Lenacil: assessment of genotoxicity in an unscheduled DNA synthesis assay using adult rat hepatocyte primarycultures Inveresk Research International IRI 6135 GLP: Yes Published: No	Y	Y		FMC
CA, 5.4.2	[redacted]	2001	Lenacil technical mouse micronucleus test [redacted] ACD 018/013472 GLP: Yes Published: No	Y	Y		FMC
CA.5.5	[redacted]	2002	Lenacil technical combined chronic toxicity and carcinogenicity study by dietary administration to HAN Wistar rats over 104 weeks interim report: 0-52 weeks [redacted] ACD 045/024288 GLP: Yes Published: No	Y	Y		FMC
CA.5.5	[redacted]	2004	Lenacil technical combined chronic toxicity and carcinogenicity study by dietary administration to han HAN Wistar rats over 104 weeks [redacted] ACD 045/042214 GLP: Yes Published: No	Y	Y		FMC
CA.5.5	[redacted]	1994	Oncogenicity study with DPX-B634-91 (lenacil) eighteen-month feeding study in mice [redacted] HLR 336-93	Y	Y		FMC

Data Requirement No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	Vertebrate study Y/N	Data Protection Y/N	Justification if data protection is claimed	Owner
			GLP: Yes Published: No				
CA 5.6.1	[redacted]	2002	Lenacil technical preliminary study of effects on reproductive performance in Han Wistar rats by dietary administration [redacted] ACD 019/010186 GLP: Yes Published: No	Y	Y		FMC
CA, 5.6.1	[redacted]	2003	Lenacil technical study of effects on reproductive performance in han wistar rats treated continuously through two successive generations by dietary administration [redacted] ACD 020/023865 GLP: Yes Published: No	Y	Y		FMC
CA, 5.6.2	[redacted]	1978	Embryotoxic and teratogenic study in rats with lenacil (INB-634) [redacted] HLR 405-78 GLP: Yes Published: No	Y	N		FMC
CA, 5.6.2	[redacted]	1996	DPX-B634-91 (lenacil): Pilot developmental toxicity study in rats [redacted] HLR 996-96 GLP: Yes Published: No	Y	N		FMC
CA, 5.6.2	[redacted]	2003	Lenacil technical preliminary study of effects on embryo- fetal development in CD rats treated by oral gavage administration	Y	Y		FMC

Data Requirement No., Reference No.	Author(s)	Year	Title Source Company Report No. GLP or GEP Status (where relevant) Published or not	Vertebrate study Y/N	Data Protection Y/N	Justification if data protection is claimed	Owner
			[redacted] ACD 057/030001 GLP: Yes Published: No				
CA, 5.6.2	[redacted]	2003	Lenacil technical study on effects on embryo-fetal development in CD rats treated by oral gavage administration [redacted] ACD 058/032316 GLP: Yes Published: No	Y	Y		FMC
CA, 5.6.2	[redacted]	1991	Teratogenicity study of DPX-B634-91 in rabbits [redacted] HLR 626-91 GLP: Yes Published: No	Y	Y		FMC
CA, 5.8.2	[redacted]	2004 2007	Lenacil technical Investigation into potential effects on thyroid function after 20 weeks of treatment in female HAN Wistar rats using the "perchlorate discharge test" and amendment (2007) [redacted] ACD 060/033946 GLP: Yes Published: No	Y	Y		FMC

B.6.11 Appendices

ANNEX B

Lenacil

Appendices

Following appendices are inserted in order to support the RMS evaluation for section B.6 (AS)

APPENDIX 1:
Litterature review as submitted by the notifier (discussed in the appropriate sections of the renewal DRAR)

A1a Notifier evaluation of the retained scientific publications
A1b Notifier search strategy and paper selection

APPENDIX 2:
Key characteristics of the six polar metabolites of lenacil

APPENDIX 3:
Key characteristics of lenacil, IN-KE121 and IN-KF313

APPENDIX 4:
OECD QSAR Toolbox (v3.3.5) analysis of carcinogenicity alerts for lenacil and its groundwater metabolites

APPENDIX 5 :
OECD QSAR Toolbox (v3.3.5) analysis of endocrine disrupting alerts for lenacil and its groundwater metabolites

Appendix 1: Litterature review as submitted by the notifier (discussed in the appropriate sections of the renewal DRAR)

A1a Notifier evaluation of the retained scientific publications

CA 5.8.2

Report: Kojima, H., Sata, F., Takeuchi, S., Sueyoshi, T., and Nagai, T. (2011); Comparative study of human and mouse pregnane X receptor activity in 200 pesticides using in vitro gene assays.

Source: Toxicology, 280:77-87

Abstract: The nuclear receptor, pregnane X receptor (PXR), is a ligand-dependent transcription factor that regulates genes involved in xenobiotic metabolism. Recent studies have shown that PXR activation may affect energy metabolism as well as the endocrine and immune systems. In this study, we characterized and compared the agonistic activities of a variety of pesticides against human PXR (hPXR) and mouse PXR (mPXR). We tested the hPXR and mPXR agonistic activity of 200 pesticides (29 organochlorines,11 diphenyl ethers, 56 organophosphorus pesticides, 12 pyrethroids, 22 carbamates, 12 acid amides, 7 triazines, 7 ureas, and 44 others) by reporter gene assays using COS-7 simian kidney cells. Of the 200 pesticides tested, 106 and 93 activated hPXR and mPXR, respectively, and a total of 111 had hPXR and/or mPXR agonistic activity with greater or lesser inter-species differences. Although all of the pyrethroids and most of the organochlorines and acid amides acted as PXR agonists, a wide range of pesticides with diverse structures also showed hPXR and/or mPXR agonistic activity. Among the 200 pesticides, pyributicarb, pretilachlor, piperophos and butamifos for hPXR, and phosalone, prochloraz, pendimethalin, and butamifos for mPXR, acted as particularly potent activators at low concentrations in the order of 10−8–10−7 M. In addition, we found that several organophosphorus oxon- and pyributicarb oxon-metabolites decreased PXR activation potency compared to their parent compounds. These results suggest that a large number of structurally diverse pesticides and their metabolites possess PXR-mediated transcriptional activity, and their ability to do so vary in a species-dependent manner in humans and mice.

The publication on *in vitro* human and mouse pregnane x receptor agonistic activity of pesticides in Toxicology, 280:77-87 is being submitted for the first time in this submission and has been conducted with lenacil. A review of this publication indicates that even though it doesn't follow a defined guideline, the study is well documented and is scientifically sound. Therefore, the study is reliable and is relevant for risk assessment.

MATERIALS AND METHODS

A.	MATERIALS	
1.	Test material:	Lenacil
	Purity:	97-100%
	CAS #:	2164-08-1
2.	Control materials	
	Vehicle control:	Dimethyl sulphoxide (DMSO).
	Positive control:	Rifampicin (RIF, >97% pure) and Pregnenolone 16-α-carbonitrile (PCN, >98% pure).

3. Cell line and cell culture materials

COS-7 simian kidney cells were obtained from the American Type Culture Collection. Fetal bovine serum (FBS) and charcoal-dextran treated FBS (CS-FBS) were obtained from Hyclone (Logan, UT, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from GIBCO-BRL (Invitrogen, Rockville, MD, USA). Glutamine solution and penicillin-streptomycin (antibiotics) solution were obtained from Dainippon Pharmaceutical Co.Ltd. (Osaka, Japan), and 0.25% trypsin/0.02% ethylenediamine tetra-acetic acid (EDTA) disodium salt solution was obtained from Life Technologies (Paisley, UK).

4. Plasmids

The expression plasmids of pSG5-hPXR and pSG5-mPXR encoding the full-length receptor protein were provided by Steven Kliewer (Department of Molecular Biology, University of Texas, Southwestern Medical Centre, Dallas, TX, USA), and the reporter plasmid pXREM-3A4-Luc was provided by Bryan Goodwin (High Throughput Biology, Discovery Research, GlaxoSmithKline,

Research Triangle Park, NC, USA). The internal control plasmid, pCMVβ-Gal, was purchased from Clontech (Palo Alto, CA, USA).

B. STUDY DESIGN AND METHODS

1. Transfection of plasmids to cells and luciferase activity assay

The host COS-7 cells were plated in 96-well microtiter plates (Nalge, Nunc, Denmark) at a density of 8400 cells per well in DMEM containing 10% CD-FBS (complete medium) 1 day before transfection. For detection of hPXR or mPXR activity, cells were transfected with 12 ng pSG5-hPXR or pSG5-mPXR, 48 ng pXREM-3A4-Luc, and 12 ng pCMVβ-Gal per well using FuGENE®6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN, USA). After a 3-h transfection period, cells were dosed with various concentrations of the test compounds or with 0.1% DMSO (vehicle control) in complete medium. To avoid any cytotoxic effects associated with the test compounds, screening assays were performed for test compounds at concentrations from $1\times10-7$ to 1×10^{-5} M, except for chlorothalonil and fluazinam, which were screened at concentrations from $1\times10-8$ to 1×10^{-6} M. After an incubation period of 24 h, cells were rinsed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (50µl/well; Promega, Madison, WI, USA). In addition, to confirm that the luciferase inductions of test chemicals are PXR-dependent, another assay using COS-7 cells transfected with 48 ng pXREM-3A4-Luc, and 12 ng pCMVβ-Gal per well (without the PXR expression plasmid) was performed. The firefly luciferase activity was measured in a 5-µl aliquot of the cell lysate in one reaction tube with a MiniLumat LB 9506 luminometer (Berthold, Wildbad,Germany) using the Luciferase Assay System (Promega). The luciferase activity was normalized against the β-galactosidase activity for each treatment.

2. β-Galactosidase activity assay

Bovine serum albumin (BSA) and 4-methylumbeliferyl-β-d-galactoside (4-MUG) were purchased from Sigma-Aldrich. β-galactosidase activity was measured to check the cytotoxicity of the test materials against transcriptional activity using a fluorescence method.

3. Evaluation of PXR agonistic activities

To estimate the potency of the receptor agonistic activity of the test compounds, the luminescence intensity of the assay as a dose-response curve was determined. The concentration of the compound equal to 20% of the maximal response of positive control (RIF or PCN) from the dose-response curve of the luminescence intensity was determined and expressed as an REC_{20} value (20% relative effective concentration). When the agonistic activity of the test compound was higher than the REC_{20} value for the concentration tested ($\leq1x10^{-5}$ M), then the test compound was concluded to be positive for agonistic activity against hPXR or mPXR. Each REC_{20} value is the mean of three independent experiments.

4. Data analysis

Results are expressed as mean±SD from at least three independent experiments performed in triplicate.

II. RESULTS AND DISCUSSION

Species-specific activity of typical PXR ligands, RIF and PCN, in the hPXR and mPXR assays

hPXR was preferentially activated at very low concentrations of RIF, but not activated even at 1×10^{-5} M of PCN. The maximal hPXR activity of RIF was 8.5 fold that of the vehicle control at 1×10^{-5} M. In contrast, mPXR was preferentially activated at very low contractions of PCN, but not activated even at 1×10^{-5} M of RIF. Again, the maximal hPXR activity of PCN was 8.5 fold that of the vehicle control at 1×10^{-5} M. From the dose-response curves, the REC_{20} values of RIF for hPXR and PCN for mPXR were estimated to be 4.3×10^{-7} M and 5.7×10^{-8} M, respectively.

Transcriptional activity of lenacil in the hPXR and mPXR assays

Lenacil did not show either hPXR or mPXR agonistic activity at concentrations of $\leq1x10^{-5}$ M. The outcome of the β-galactosidase activity assay was that lenacil is not cytotoxic at the screening doses tested.

III. CONCLUSION

Lenacil did not activate the human or the mouse pregnane x receptor (PXR) at concentrations of $\leq1\times10^{-5}$ M and therefore lenacil is concluded to have no PXR agonistic activities, *in vitro*.

(Kojima, H., Sata, F., Takeuchi, S., Sueyoshi, T., and Nagai, T., 2011)

CA 5.8.2

Report: Takeuchi, S., Matsuda, T., Kobayashi, S., Takahasi, T and Kojima, H. (2006); In vitro screening of 200 pesticides for agonistic activity via mouse peroxisome proliferator-activated receptor (PPAR)α and PPARγ and quantitative analysis of in vivo induction pathway.

Source: Toxicology and Applied Pharmacology, 217: 235-244

Abstract: Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors and key regulators of lipid metabolism and cell differentiation. However, there have been few studies reporting on a variety of environmental chemicals, which may interact with these receptors. In the present study, we characterized mouse PPARα and PPARγ agonistic activities of 200 pesticides (29 organochlorines, 11 diphenyl ethers, 56 organophosphorus pesticides, 12 pyrethroids, 22 carbamates, 11 acid amides, 7 triazines, 8 ureas and 44 others) by in vitro reporter gene assays using CV-1 monkey kidney cells. Three of the 200 pesticides, diclofop-methyl, pyrethrins and imazalil, which have different chemical structures, showed PPARα-mediated transcriptional activities in a dose-dependent manner. On the other hand, none of the 200 pesticides showed PPARγ agonistic activity at concentrations ≤10^{-}5 M. To investigate the in vivo effects of diclofop-methyl, pyrethrins and imazalil, we examined the gene expression of PPARα-inducible cytochrome P450 4As (CYP4As) in the liver of female mice intraperitoneally injected with these compounds (≤300 mg/kg). RT-PCR revealed significantly high induction levels of CYP4A10 and CYP4A14 mRNAs in diclofop-methyl- and pyrethrinstreatedr mice, whereas imazalil induced almost no gene expressions of CYP4As. In particular, diclofop-methyl induced as high levels of CYP4A mRNAs as WY-14643, a potent PPARα agonist. Thus, most of the 200 pesticides tested do not activate PPARα or PPARγ in in vitro assays, but only diclofop-methyl and pyrethrins induce PPARα agonistic activity in vivo as well as in vitro.

The publication on *in vitro* mouse peroxisome proliferator receptor agonistic activity of pesticides in Toxicology and Applied Pharmacology, 217: 235-244 is being submitted for the first time in this submission and has been conducted with lenacil. A review of this publication indicates that even though it doesn't follow a defined guideline, the study is well documented and is scientifically sound. Therefore, the study is reliable and is relevant for risk assessment.

MATERIALS AND METHODS

A. MATERIALS

1. Test material: Lenacil
   Purity: 95-100%
   CAS #: 2164-08-1

2. Control materials
   Vehicle control: Dimethyl sulphoxide (DMSO).
   Positive control: WY-14643 and pioglitazone.

3. Cell line and cell culture materials

CV-1 monkey kidney cells were obtained from Dainippon Pharmaceutical Co. Ltd. The cells were routinely cultured in MEM supplemented with 10% FBS and antibiotics at 37 °C in an atmosphere of 5% CO2/95% air under saturating humidity, and passaged every week by trypsinization with 0.25% trypsin/0.02% EDTA.

4. Plasmids

Expression plasmids, pCMX-mPPARα, pCMX-mPPARγ andvpCMX-mRXRα, and the PPRE-containing reporter plasmid, PPRE3-TK-LUC, were gifts from Dr. R. M. Evans (Salk Institute, La Jolla, CA, USA). The internal control plasmid pCMVβ-Gal was purchased from Clontech (Palo Alto, CA, USA).

B. STUDY DESIGN AND METHODS

1. Reporter gene assay for PPARα and PPARγ

Host CV-1 cells were plated in 48-well microtiter plates (Corning Costar Corporation, NY, USA) at a density of 50,000 cells per well in phenol red-free MEM supplemented with 10% CD-FBS (complete medium) 1 day before transfection. To detect mPPARα or mPPARγ activity, host cells were transfected with 24 ng of either pCMX-mPPARα or pCMX-mPPARγ, 96 ng PPRE3-TK-LUC and 20 ng pCMVβ-

Gal per well using the FuGene6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN, USA) following the manufacturer's instructions. After a 3-h period of transfection, various concentrations of pesticides, positive control compounds or 0.1% DMSO (vehicle control) in complete medium were administered to the cells. To avoid cell toxicity by the pesticides, assays were performed for pesticides at concentrations of less than 10−5 M. After an incubation period of 24 h, cells were rinsed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (100 µL/well; Promega, Madison, WI, USA). The firefly luciferase activity was measured with a MiniLumat LB 9506 luminometer (Berthold, Wildbad, Germany) in a reaction tube with a 10 µL aliquot of cell lysate using the Luciferase Assay System (Promega) following the manufacturer's instructions. The β-galactosidase activity was also measured in the cell lysate using the fluorescence method previously reported (Takeuchi et al., 2005). The luciferase activity was normalized based on the β-galactosidase activity for each treatment. Results are expressed as means±SD from at least three independent experiments. To estimate the potency of receptor-agonistic activity of the compounds tested, the concentration of the compound exhibiting the response equal to 20% of the maximal response of 1×10−5 M WY-14643 or 1×10−5 M pioglitazone for PPARα or PPARγ, respectively, was evaluated from a dose–response curve of the luminescence intensity, and expressed as the 20% relative effective concentration (REC_{20}).

2. Reporter gene assay for RXR

The mRXRα assay was performed by the same procedure used for the PPARα and PPARγ assays. Namely, CV-1 cells were transfected with 24 ng of pCMX-mRXRα, 96 ng PPRE3-TK-LUC and 20 ng pCMVβ-Gal per well using the FuGene6 transfection reagent. 9-cis retinoic acid (9-cis RA), an RXR ligand, was utilized as a positive control in this RXR assay.

3. Data analysis

An analysis of variance (ANOVA) followed by Bonferroni correction was used to evaluate the differences in CYP4A10 and CYP4A14 mRNA levels by TaqMan between the control group and each of the chemical groups. The level of significance was $p<0.05$. Data were presented as the mean±SD of five mice per group.

II. RESULTS AND DISCUSSION

Responses of WY-14643 in the mPPARα assay and of pioglitazone in the mPPARγ assay

CV-1 cells were transiently transfected with the expression plasmid for mouse PPARα or PPARγ, the PPAR-responsive luciferase reporter plasmid and control plasmid. After transfection, cells were treated with WY-14643 for PPARα and with pioglitazone for PPARγ. A dose-dependent transactivation of PPARα by WY-14643 was observed, indicating that the receptor is activated at very low concentrations of the chemical. The PPARα agonistic activity of WY-14643 exhibited approximately 17.8-fold activity over the vehicle control at the concentrations of 1×10−5 M. The maximal pioglitazone-induced PPARγ activity was 15.7-fold greater than that of the vehicle control at the concentrations of 1×10−5M and above. From the dose–response curves, the REC_{20} values of WY-14643 for PPARα and of pioglitazone for PPARγ were deduced to be 4.9×10−7 M and 1.6×10−7 M, respectively.

Transcriptional activity of 200 pesticides in the mPPARα and mPPARγ assays

Lenacil did not show any agonistic effects in the mPPARα and the mPPARγ transactivation assays at concentrations of ≤10⁻5 M.

III. CONCLUSION

Lenacil did not activate transcription of the reporter gene in mPPARα and mPPARγ assays at concentrations of ≤10⁻5 M and therefore lenacil is concluded to have no mPPARα and mPPARγ agonistic activities, *in vitro.*

(Takeuchi, S., Matsuda, T., Kobayashi, S., Takahasi, T and Kojima, H., 2006)

CA 5.8.2

Report: Takeuchi, S., Iida, M., Yabushita, H, Matsuda, T and Kojima, H. (2008); In vitro screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, DR-EcoScreen cells, and in vivo mouse liver cytochrome P450-1A induction by propanil, diuron and linuron.

Source: Chemosphere, 74:155-165

Abstract: The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that regulates genes involved in xenobiotic metabolism, cellular proliferation and differentiation. In this study, we have developed a

highly sensitive AhR-mediated reporter cell line, DR-EcoScreen cells, which are mouse hepatoma Hepa1c1c7 cells stably transfected with a reporter plasmid containing seven copies of dioxin-responsive element. Using these DR-EcoScreen cells, we performed the reporter gene assay and characterized the AhR agonistic activities of 200 pesticides (29 organochlorines, 11 diphenyl ethers, 56 organophosphorus pesticides, 12 pyrethroids, 22 carbamates, 12 acid amides, 7 triazines, 6 ureas, and 45 others). Eleven of the 200 pesticides (acifluorfen-methyl, bifenox, chlorpyrifos, isoxathion, quinalphos, chlorpropham, diethofencarb, propanil, diuron, linuron, and prochloraz) showed AhR-mediated transcriptional activity. In particular, three herbicides (propanil, diuron, and linuron) have a common chemical structure and showed more potent agonistic activity than other pesticides. To investigate the in vivo effects, we examined the gene expression of AhR-inducible cytochrome P450 1As (CYP1As) in the liver of female C57BL/6 mice intraperitoneally injected with these three herbicides (≤ 300 mg $kg^{-}1$) by quantitative RT-PCR, resulting in induction of significant high levels of CYP1A1 and CYP1A2 mRNAs. This indicates that propanil, diuron and linuron possess AhR-mediated transactivation effect in vivo as well as in vitro. Through the present study, we demonstrated that DR-EcoScreen cells are useful for sensitive, rapid and simple identification of AhR agonists among a large number of environmental chemicals.

The publication on *in vitro* aryl hydrocarbon receptor agonistic activity of pesticides in Chemosphere, 74:155-165 is being submitted for the first time in this submission and has been conducted with lenacil. A review of this publication indicates that even though it doesn't follow a defined guideline, the study is well documented and is scientifically sound. Therefore, the study is reliable and is relevant for risk assessment.

I. MATERIALS AND METHODS

A.		MATERIALS	
	1.	Test material:	Lenacil
		Purity:	95-100%
		CAS #:	2164-08-1
	2.	Control materials	
		Vehicle control:	Dimethyl sulphoxide (DMSO).
		Positive control:	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[α]pyrene, β-naphtoflavone, 3,3',4,4',5-Pentachlorobiphenyl (PCB # 126) and 3-methylcholanthrene (3-MC)

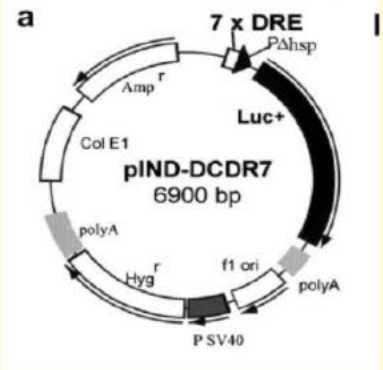
3. Cell line and cell culture materials

Mouse hepatoma Hepa1c1c7 cells were obtained from the American Type Culture Collection. Fetal bovine serum (FBS) was obtained from JRH Bioscience, Inc. (Lenexa, KS, USA). Charcoal– Dextran treated FBS (CD-FBS) was obtained from Hyclone (Logan, UT, USA). Alpha-modified Eagle's minimum essential medium a-MEM) was obtained from MP Biomedicals (Germany). Hygromycin was obtained from Invitrogen (San Diego, CA, USA). Glutamine solution and penicillin– streptomycin (antibiotics) solution were obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). 0.25% Trypsin/0.02% ethylenediamine tetra-acetic acid (EDTA) disodium salt solution was obtained from Life Technologies (Paisley, UK). The luciferase substrate, Steady-Glo™ reagent and luciferin, were purchased from Promega (Madison, WI, USA). FuGENE®6 Transfection Reagent was purchased from Roche Diagnostics Corp. (Indianapolis, IN, USA).

4. Construction of reporter plasmid, pIND-GCDR7

The regulatory element containing DREs of the CYP1A1 gene from the genome of C57BL/6 strain mouse was cloned and amplified PCR (Pyrobest TAKARA, Japan) using three following primers. Small letters represent franking site sequences of the primers, and italics represent sequences of restriction enzymes. 50-attggtaccttatggatccTTCAGGGCCAGAGAGCACCTG-30: DR-s1, 50-aatagatctGACAAGGTGCCCCGGAGTTGCGTGAG-30: DR-a1, 50-aatcagatctACGCGAGACAGCAGGAGGGGGT-30: DR-a2.
DR-s1 is located from _1334 to _1314 upstream of the transcription start site for mouse CYP1A1 gene (Fisher et al., 1990) and flanked with two restriction sites (KpnI and BamHI) at the 50 end. DR-a1 and DR-a2 are antisense primers and located from 989 to 962 and 844 to 822, respectively, and both of them are flanked with a restriction site (BglII) at 50 end. The regulatory fragments amplified with DR-

s1 and DR-a1, and with DR-s1 and DR-a2, were designated as DRE1 and DRE2, respectively. DRE1 has three Ah receptor recognition sequences. DRE2 has four Ah receptor recognition sequences and a GC box for signal enhancer (Fisher et al., 1990). DRE1 and DRE2 were digested with KpnI and BglII. DRE2 was cloned into KpnI-BamHI site of pUC-18 (TAKARA) to create pUC-18 DRE2. Digested DRE1 was ligated into KpnI and BamHI sites of pUC-18 DRE2 to create pUC-18 DRE1/2, and then digested with BamHI and SalI to obtain the fragment of about 950 bp containing DRE1 and DRE2. A new reporter plasmid for the AhR-mediated transcriptional assay was constructed using pIND-MCS-LUC, based on the ecdysoninducible mammalian expression vector pIND/Hygro harbouring hygromycin resistant gene (Invitrogen), the construction method of pIND-MCS-LUC being previously described (Kojima et al., 2003). First, pIND-MCS-LUC was digested with BanII followed by blunting treatment with T4 DNA polymerase, and digested with HindIII. Next, the fragment containing luciferase gene (luc+) and SV40 late poly(A) signal was obtained from pGL3-Basic Luciferase Reporter Vector (Promega) by digesting with SalI, blunting with Klenow enzyme, and digesting with HindIII. Then, this fragment was ligated into the pIND-MCS-LUC already digested as mentioned above to create pIND-MCS-LUC2. Finally, the pUC-18 DRE1/2 fragment (about 950 bp) obtained as mentioned above was inserted into BglII and XhoI sites of the pIND-MCS-LUC2, and designated as pIND-GCDR7 (Fig. a), which contained seven DREs sequences and one GC box. After each recombinant DNA treatment, the arrangement of the insert was verified by DNA sequencing.



B. STUDY DESIGN AND METHODS

1. Establishment of DR-EcoScreen cells and cell culture condition

About 16 h prior to the transfection, Hepa1c1c7 cells were seeded at a confluency of 50% in a 6-well plate in 2 mL of culture medium (a-MEM with 5%FBS) per well. Transfection was carried out with FuGENE 6 Transfection Reagent according to the instruction of the manufacturer. Briefly, 1 µg of pIND-GCDR7 and 3 µL of FuGENE®6 Transfection Reagent were added to each well. After 24 h incubation, the cells in each well were trypsinized, re-suspended in the culture medium containing 150 µg mL-1 of hygromycin, and divided equally to plate in two 100 mm petri dishes. The culture medium was replaced every four d until colonies became available for isolation (for about two weeks). After cells were exposed to 20 pM of TCDD for 24 h, luciferin (final concentration: 20 nM) was added into a 100 mm petri dish. After further incubation for 5 min, luminescence image was scanned through a photon detecting charge-coupled device (CCD) camera (Night OWL, Perkin–Elmer), which took 10 min per dish. Each luciferase positive clone was isolated using a cloning ring and individually cultured in a well of a 24-well plate. After proliferation, each clone was trypsinized, re-suspended, seeded a 1/10 volume into a 100 mm petri dish, and further cultured. With repeated these steps, the clone having the highest luciferase activity and the greatest fold of induction ratio to background luciferase activity was selectednd named as DR-EcoScreen.

2. Luciferase reporter gene assay using DR-EcoScreen cells

The DR-EcoScreen cell line was maintained in a-MEM supplemented with 5% FBS, antibiotics and glutamine at 37°C in an atmosphere of 5% CO2/95% air under saturating humidity, and passaged twice every week by trypsinization with 0.25% trypsin/0.02% EDTA. For screening assay, cells were trypsinized and suspended at a density of 1.0 x 105 cells mL-1 in a-MEM containing 5% of CD-FBS. Ninety µL of the cell suspension was seeded in a well of a 96-well white bottom plate (#136102 NunclonTM, Nalge Nunc, Denmark) (final density: 9000 cells well-1). After 24 h cultivation at 37°C, 10 µL of various concentrations of chemicals that were dissolved in 1% DMSO was added to each well (final concentration of DMSO was 0.1%). Following further 24 h of cultivation, 100 µL of Steady-

Glo™ reagent was added to each well. After the plate was shaken at room temperature for five min, the luminescence was measured with a microplate-luminometer (Wallac 1420 ARVOTM SX, Perkin–Elmer).

1. Cytotoxicity test and evaluation of AhR agonistic activity

DR-EcoScreen cells were plated and cultured in the same way as the luciferase reporter gene assay, and added alamar blue dye instead of Steady-Glo™ reagent. The cytotoxicity was measured in accordance with the manufacturer's protocol of alamarBlueTM (AbD Serotec, Kidlington, UK). This is a colorimetric assay to measure the metabolic activity of living cells based on the conversion of alamar blue dye (resorufin, blue) into resorufin (red). Absorbance was read at a test wavelength of 575 nm and a reference wavelength of 610 nm with a microplate-spectrophotometer, MPRA4i (TOSOH Co., Ltd., Osaka, Japan). To estimate the potency of receptor–agonistic activity of the pesticide tested, its concentrations exhibiting the response equal to 50% and 20% of the maximal response of $10^{-}10$ M TCDD for AhR were evaluated from a dose–response curve of the luminescence intensity, and expressed as 50% and 20% relative effective concentration, REC_{50} and REC_{20}, respectively. When the activity of the test compound was higher than REC_{20} within the concentration tested (~$10^{-}7$–$10^{-}5$ M), the pesticide was concluded to be positive for activity

3.1Data analysis

An analysis of variance (ANOVA) followed by Bonferroni correction was used to evaluate the differences in CYP1A1 and CYP1A2 mRNA levels determined by TaqMan® (Applied Biosystems) assay between the control group and each of the chemical groups. Statistical significance was set at $p < 0.05$.

II. RESULTS AND DISCUSSION

A. RESPONSES OF AHR LIGANDS IN THE DR-ECOSCREEN ASSAY

The DR-EcoScreen cells were treated with well-known AhR ligands such as TCDD, PCB # 126, 3-MC, β-naphthoflavone, and benzo[a]pyrene. These chemicals dose-dependently transactivated the receptor even at very low concentrations. From the dose–response curves, both REC50 and REC20 values of the five AhR ligands for AhR were derived. The relative potency calculated from the REC20 values indicates that the AhR agonistic activity of PCB # 126, 3 MC, b-naphthoflavone, and benzo[a]pyrene were about 21, 1250, 5900 and 10000-fold smaller than that of TCDD, respectively. Thus, a rapid and simple assay system using DR-EcoScreen cells is highly sensitive and reasonable to detect TCDD and related AhR ligands.

B. TRANSCRIPTIONAL ACTIVITY OF LENACIL IN DR-ECOSCREEN ASSAY

The AhR agonistic activity of lenacil at concentrations from $1x\ 10^{-7}$ to $1x10^{-5}$ M was examined by the *in vitro* reporter gene assay using DR-EcoScreen cells. Lenacil did not show any AhR-mediated transcriptional activity in this study.

III. CONCLUSION

Lenacil did not result in AhR-mediated transcriptional activity at concentrations of 1×10^{-7} to 1×10^{-5} M and therefore lenacil is concluded to have no AhR agonistic activity, *in vitro*.

(Takeuchi, S., Iida, M., Yabushita, H, Matsuda, T and Kojima, H., 2008);

CA 5.8.3

Report: Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K., and Kobayashi, K. (2004); Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells.

Source: Environmental health perspectives, 112:524-531

Abstract: We tested 200 pesticides, including some of their isomers and metabolites, for agonism and antagonism to two human estrogen receptor (hER) subtypes, hERα and hERβ, and a human androgen receptor (hAR) by highly sensitive transactivation assays using Chinese hamster ovary cells. The test compounds were classified into nine

groups: organochlorines, diphenyl ethers, organophosphorus pesticides, pyrethroids, carbamates, acid amides, triazines, ureas, and others. These pesticides were tested at concentrations < 10^{-5} M. Of the 200 pesticides tested, 47 and 33 showed hERα- and hERβ-mediated estrogenic activities, respectively. Among them, 29 pesticides had both hERα and hERβ agonistic activities, and the effects of the organochlorine insecticides β-benzene hexachloride (BHC) and δ-BHC and the carbamate insecticide methiocarb were predominantly hERβ rather than hERα agonistic. Weak antagonistic effects toward hERα and hERβ were shown in five and two pesticides, respectively. On the other hand, none of tested pesticides showed hAR-mediated androgenic activity, but 66 of 200 pesticides exhibited inhibitory activity against the transcriptional activity induced by 5α-dihydrotestosterone. In particular, the antiandrogenic activities of two diphenyl ether herbicides, chlornitrofen and chlomethoxyfen, were higher than those of vinclozolin and *p,p′*-dichlorodiphenyl dichloroethylene, known AR antagonists. The results of our ER and AR assays show that 34 pesticides possessed both estrogenic and antiandrogenic activities, indicating pleiotropic effects on hER and hAR. We also discussed chemical structures related to these activities.

Taken together, our findings suggest that a variety of pesticides have estrogenic and/or antiandrogenic potential via ER and/or AR, and that numerous other manmade chemicals may also possess such estrogenic and antiandrogenic activities. *Key words:* antiandrogenic activity, Chinese hamster ovary cells, estrogenic activity, human androgen receptor, human estrogen receptor α, human estrogen receptor β, pesticide, reporter gene assay.

The publication on *in vitro* estrogen and androgen receptor agonistic activity of pesticides in Environmental health perspectives, 112:524-531 is being submitted for the first time in this submission and has been conducted with lenacil. A review of this publication indicates that even though it doesn't follow a defined guideline, the study is well documented and is scientifically sound. Therefore, the study is reliable and is relevant for risk assessment.

MATERIALS AND METHODS

A.		MATERIALS	
	1.	Test material: Purity: CAS #:	Lenacil 95-100% 2164-08-1
	2.	Control materials Vehicle control: Positive control:	 Dimethyl sulphoxide (DMSO). 17β-Estradiol (E2; > 87% pure) and 5α-dihydrotestosterone (DHT; 95% pure).

3. Cell line and cell culture conditions

CHO-KI cells were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). For routine maintaince, cells were grown in DMEM/F-12 supplemented with 10% FBS and antibotics at 37°C in an atmosphere of 5% CO2/95% air under saturating humidity and passaged every week by trypsinisation with 0.25% trypsin/0.02% ethylenediamine tetraacetic acid (EDTA) disodium salt solution (Life Technologies, Paisley, UK)

4. Construction of plasmid

The human ERα (hERα) and AR (hAR) expression vectors (pcDNAERα and pZeoSV2AR) were constructed as previously described in Kojlima et al. 2003. The hERβ expression vector was newly constructed as follows: The ERβ cDNA was cloned by reverse transcriptase-polymerase chain reaction from human placental RNA (Clontech, Palo Alto, CA, USA). The sequence of the cloned hERβ cDNAwas verified and inserted into the mammalian expression vector pcDNA3.1Zeo(-) (Invitrogen, San Diego, CA, USA), to create pcDNAERβ. The estrogen-resposive element (ERE)-containing reportor plasmid pGL3-tkERE and the androgen-responsive element (ARE)-containing reporter plasmid pIND-ARE was constructed as described previously described in Kojlima et al. 200. pRL-SV40 containing the *Renilla* luciferase gene was purchased from Promega (Madison, WI, USA) and used as an internal control for transfection efficiency.

B. STUDY DESIGN AND METHODS

1. Reporter gene assays for hERα, hERβ and hAR.

The host CHO-K1 cells were plated in 96-well microtiter plates (Nalge Nunc, Rochester, NY, USA) at a density of 8,400 cells/well in phenol red–free DMEM/F-12 containing 5% CD–FBS (complete medium) 1 day before transfection. For detection of hERα or hERβ activity, cells were transfected with 5 ng pcDNAERα or 5 ng pcDNAERβ, 50 ng pGL3-tkERE, and 5 ng pRL-SV40 per well using the transfection reagent FuGene6 (Roche Diagnostics Corp., Indianapolis, IN, USA). For detection of hAR activity, cells were transfected with 2.5 ng pZeoSV2AR, 50 ng pIND-ARE, and 5 ng pRL-SV40 per well. After a 3-hr transfection period, cells were dosed with various concentrations of test compounds or with 0.1% DMSO (vehicle control) in complete medium. For measurement of the antagonistic activity to hERα, hERβ, and hAR, either 10–11 M E2, 10–10 M E2, or 10–10 M DHT was added to the cell cultures along with the test compound, respectively. After an incubation period of 24 hr, cells were rinsed with phosphate-buffered saline (pH 7.4) and lysed with passive lysis buffer (50 µL/well) provided with the Dual-Luciferase Reporter Assay kit (Promega). The firefly luciferase activity with a MiniLumat LB 9506 luminometer (Berthold, Wildbad, Germany) was measured before measuring*Renilla* luciferase activity in one reaction tube with 5-µL aliquots of cell lysates using the Dual-Luciferase Reporter Assay kit, following the manufacturer's instructions. The firefly luciferase activity was normalized based on the *Renilla* luciferase activity of the cotransfected pRL-SV40.

2. Data analysis

Statistical significance was evaluated using the Student's *t*-test (two-tailed, equal variance) calculated by software (Excel; Microsoft, Redmond, WA, USA). The level of significance was $p < 0.05$. Data are presented as the mean and, where shown, the SD of at least three separate experiments with duplicate wells.

II. RESULTS AND DISCUSSION

Estrogenic or antiestrogenic effects of lenacil

Lenacil did not show any ERα or ERβ agonistic or antagonistic activity at concentrations ≤10-5 M.

Androgenic effects of lenacil

Lenacil did not show androgenic or antiandrogenic activity at concentrations $\leq 10^{-5}$ M.

Response of 17β-E2 in ERα and ERβ assays, and of 5α-DHT in AR assay

A dose-dependent transactivation of ERα and ERβ by 17β-E2 was observed at very low hormone concentrations ($\leq 10^{-12}$ M). This indicates that both receptors can be activated at very low hormone concentrations. The maximal ERα activity was achieved at 10^{-10} M E2 or more, exhibiting approximately 10-fold that of the control solvent. The maximal ERβ activity induced was 8.5-fold that of the solvent control at 10–9 M E2 or more. Thus, E2 was more potent for ERα than for ERβ. From these dose–response curves, REC20 values of E2 for ERα and ERβ were deduced to be 2.5 x 10–12 M and 5.3 x 10–12 M, respectively. Furthermore, a dose-dependent transactivation of AR by 5α-DHT was observed. Its activity was detectable from 10–11 M DHT and reached a plateau at 10–9 M DHT. The maximum induction was 21-fold that of the control solvent. The REC20 value of DHT for AR was 3.1 × 10–11 M.

III. CONCLUSION

Lenacil did not show any estrogenic or androgenic transcriptional activity at concentrations $\leq 10^{-5}$ M via the two human estrogen receptor (hER) subtypes, hERα and hERβ, and via the human androgen receptor (hAR). Therefore, lenacil is conlcluded to have no ERα, ERβ or AR agonistic activities, *in vitro*.

(Kojima, H., Katsura, E., Takeuchi, S., Niiyama, K., and Kobayashi, K., 2004)

A1b Notifier search strategy and paper selection

*Note: formally curated by **RMS** for reasons of efficiency (insertion of text into this DRAR) and readability; please also refer to: **Document Number – DuPont-43896 EU***

"LENACIL Active substance, document M-CA, section 9: Literature data - Supplemental submission in support of renewal - Applicant DuPont De Nemours (Deutschland) GMBH
Author: Rosa Criollo, 16.06.2016"

CA 9.0 Literature Data

Introduction: literature data of the a.s. lenacil

Lenacil
IUPAC name: 3-cyclohexyl-1,5,6,7-tetrahydrocyclopentapyrimidine-2,4(3H)-dione
CAS Number: 2164-08-1
It is the active substance in over 5 products used in 20 countries worldwide for DuPont registered products.

Article 8(5) of Regulation (EC) No 1107/2009 requires applicants submitting dossiers for approval of active substances to provide relevant scientific peer reviewed open literature. This summary of scientific peer reviewed open literature conforms to EFSA guidance "Submission of scientific peer-reviewed open literature under Regulation (EC) No 1107/2009, EFSA Journal 2011; 9(2):2092".
Peer reviewed open literature containing data and analysis dealing with the side effects on health, environment, and non-target species for lenacil, and its metabolites. The data published within the last ten years before the date of the submission of lenacil (renewal) dossier were reviewed for this document. The most recent scientific literature published from 2005 to 2016 is included.
The document contains the search criteria and results of those searches of "scientific peer-reviewed open literature" performed under Regulation (EC) No 1107/2009 for lenacil.

CA 9.1 Relevance criteria

Peer reviewed open literature relevant to the dossier may satisfy or partially satisfy data requirements as set out in Regulation (EC) No 1107/2009. The relevance criteria chosen for the selection of peer reviewed scientific open literature is consistent with the OECD guidance and does not restrict the selection of literature (**Table A1a-1**). The relevance criteria guide the selection of literature dealing with the side effects on health, environment and non-target species for lenacil, and its relevant metabolites when used according to the legally registered label. Non-GLP studies in open literature may be considered relevant if the design and execution of the study is consistent with generally accepted scientific practice and guidelines. Clearly non-relevant studies are excluded.

Table A1a-1 lists the selection criteria applied to the results of the search for peer reviewed open literature relevant to lenacil and its metabolites.

Table A1a-1 Relevance criteria

Data requirement(s)*	Criteria for relevance
All Data Points	1. The dose levels or application rates reflect the proposed GAP.
	2. The test system, target crop, or species are prescribed by Regulation (EC) No 1107/2009 or the relevance is explained if not standard.
	3. Well identified test material, including its purity and impurity profile, is described.
	4. Study design and/or execution are consistent with relevant study guidelines.
	5. The endpoint is relevant to an EU data point as prescribed by Regulations (EU) No 283/2013 and 284/2013
Toxicological and toxicokinetic studies	**6. Description of the observations, examinations, analysis performed, or necropsy are well described.**
	7. The conditions of exposure should be from a legally registered use of the product.
Residues in or on treated products, food and feed (metabolism and residues data)	8. The application method(s) complies with Good Agriculture Practice (GAP)
	9. Appropriate in-life/processing conditions are used and/or are well described
Fate and behaviour in the environment	10. The model is appropriate for European regulatory requirements.
	11. The input parameter selection is appropriate based on European regulatory requirements.
	12. The pedoclimatic conditions are appropriate.
Ecotoxicological studies	13. A relevant route of exposure is presented.

*: (indicated by the correspondent data point number(s) as identified in Commission Regulation (EU) 283/2013)

CA 9.2 Search criteria

Reasonable effort was taken to locate all sources of relevant peer reviewed open literature concentrated on comprehensive databases containing worldwide coverage of biology, chemistry, biomedical, agricultural and environmental fields. The search ranged up **to 10 years and within 6 months of the submission date**. The initial search is a single concept search capturing all data points using search terms and synonyms for the active substance. If a large number of search results are returned from the single concept search making assessment for relevance impractical, a separate, focussed search is conducted for grouped data points. The search by discipline was conducted for **lenacil and its metabolites** (metabolites submitted separately from initial CA9 document)

Table A1a-2 lists the literature search details: search statements and search strategy. Following databases were listed:

- AGRICOLA,
- BIOSIS,
- CABA,
- CAPLUS,
- CSNB,
- DDFU,
- EMBASE,
- ESBIOBASE,
- FSTA,
- IPA,
- MEDLINE,
- NTIS,
- PASCAL,
- PQSCITECH,
- SCISEARCH,
- TOXCENTER,
- CAS Registry and
- HSDB

Table A1a-2 Details of the literature search for lenacil and its metabolites (AGRICOLA, BIOSIS, CABA, HCAPLUS) Mammalian toxicology, Metabolism, Residues, Environmental fate, Ecotoxicology

Data requirement(s) captured in the search	Details of the searches			
	AGRICOLA	*BIOSIS*	*CABA*	*CAPLUS*
Justification for choosing the source:	Agriculture Online Access is a bibliographic database containing selected worldwide literature of agriculture and related fields. More than 5.2 million records (01/2016)	The largest and most comprehensive life science database in the world, BIOSIS covers original research reports, reviews, and selected U.S. patents in biological and biomedical areas, with subject coverage ranging from aerospace biology to zoology. More than 24.8 million records (02/2016)	The CAB Abstracts database covers worldwide literature from all areas of agriculture and related sciences including biotechnology, forestry, and veterinary medicine. More than 8.0 million records (01/2016)	Chemical Abstracts Plus covers worldwide literature from all areas of chemistry, biochemistry, chemical engineering, and related sciences. More than 42.9 million records (03/2016)
Date of the search	11 FEB 2016	11 FEB 2016	11 Feb 2016	11 FEB 2016
Date span of the search:	2005 to 2016	2005 to 2016	2005 to 2016	2005 to 2016
Date of the latest database update included in the search:	02 Feb 2016	10 Feb 2016	10 Feb 2016	10 FEB 2016
Language limit:	No	No	No	No
Other limit set:	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT
Search strategy:	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**
Total number of records retrieve	**4**	**19**	**65**	**255**

Table A1a-2 (continued) **Details of the literature search for lenacil and its metabolites (CSNB, DDFU, EMBASE, ESBIOBASE)**
Mammalian toxicology, Metabolism, Residues, Environmental fate, Ecotoxicology

Data requirement(s) captured in the search	Details of the searches			
	CSNB	***DDFU***	***EMBASE***	***ESBIOBASE***
Justification for choosing the source:	The Chemical Safety NewsBase provides access to chemical information related to fire and explosions, storage and transport, toxic substances, studies on laboratory animals, waste removal, and other subjects related to chemistry, health and safety. More than 108,102 records (02/2016)	The Derwent Drug File provides information from worldwide pharmaceutical literature on all aspects of drugs, from drug design to post marketing surveillance. More than 1.5 million records in the literature segment; more than 200.447 records in the registry segment (01/2016)	The Excerpta Medica database, covers worldwide literature in the biomedical and pharmaceutical fields, including biological science, biochemistry, human medicine, forensic science, pediatrics, pharmacy, pharmacology and drug therapy, pharmacoeconomics, psychiatry, public health, biomedical engineering and instrumentation, and environmental science. More than 31.2 million records (01/2016)	Elsevier BIOBASE is a bibliographic current awareness database providing comprehensive coverage of the entire spectrum of biological research worldwide. Coverage includes the following areas: applied microbiology, biotechnology, cancer research, cell & developmental biology, clinical chemistry, ecological & environmental sciences, endocrinology, genetics, immunology, infectious diseases, metabolism, molecular biology, neuroscience, plant and crop science, protein biochemistry, and toxicology. More than 6.6 million records (01/2016)
Date of the search	11 FEB 2016	11 FEB 2016	11 Feb 2016	11 FEB 2016
Date span of the search:	2005 to 2016	2005 to 2016	2005 to 2016	2005 to 2016
Date of the latest database update included in the search:	08 Dec 2015	08 Feb 2016	10 Feb 2016	11 FEB 2016
Language limit:	No	No	No	No
Other limit set:	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT
Search strategy:	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**
Total number of summary records retrieved	**0**	**0**	**16**	**9**

Table A1a-2 (continued) **Details of the literature search for lenacil and its metabolites (FSTA, IPA, MEDLINE, NTIS)**
Mammalian toxicology, Metabolism, Residues, Environmental fate, Ecotoxicology

Data requirement(s) captured in the search	Details of the searches			
	FSTA	***IPA***	***MEDLINE***	***NTIS***
Justification for choosing the source:	The Food Science and Technology Abstracts database provides worldwide coverage of all scientific and technological aspects of the processing and manufacture of human food products. Coverage includes basic food sciences, biotechnology, hygiene and toxicology, engineering, packaging, and all individual foods and food products. More than 1.2 million records (02/2016)	The International Pharmaceutical Abstracts (IPA) database, contains international coverage of pharmacy and health-related literature, including drug therapy, pharmacy practice and management, and the legal aspects of pharmacy and drugs. More than 616,800 records (02/2016)	MEDLINE contains information on every area of medicine. More than 25.3 million records (01/2016)	The National Technical Information Service database contains abstracts on government-sponsored research, which corresponds to Government Reports Announcements & Index. NTIS also includes a significant number of German research reports. The file contains records for all areas of science, engineering, technology, and environmental protection. 2.566.763 records (static file)
Date of the search	11 FEB 2016	11 FEB 2016	11 FEB 2016	11 FEB 2016
Date span of the search:	2005 to 2016	2005 to 2016	2005 to 2016	2005 to 2016
Date of the latest database update included in the search:	08 Feb 2016	03 FEB 2016	11 FEB 2016	03 NOV 2014
Language limit:	No	No	No	No
Other limit set:	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT
Search strategy:	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**
Total number of summary records retrieved	**5**	**0**	**10**	**0**

Table A1a-2 (continued) **Details of the literature search for lenacil and its metabolites (PASCAL, PQSCITECH, SCISEARCH, TOXCENTER) Mammalian toxicology, Metabolism, Residues, Environmental fate, Ecotoxicology**

Data requirement(s) captured in the search	Details of the searches			
	PASCAL	***PQSCITECH***	***SCISEARCH***	***TOXCENTER***
Justification for choosing the source:	The PASCAL database provides access to the world's scientific and technical literature including physics and chemistry, life sciences (biology, medicine, and psychology), applied sciences and technology, earth sciences, and information sciences. 18.5 million records (static file)	PQSCITECH (ProQuest Science and Technology) is a valuable and huge resource of over 27 Mio documents in all areas of science and technology from engineering to lifescience. More than 30.4 million records (02/2016)	Science Citation Index, one of the largest multidisciplinary scientific databases, is an international index to the literature covering virtually every subject area within the broad fields of science, technology, and biomedicine. More than 40.1 million records (02/2016)	Toxicology Center covers the pharmacological, biochemical, physiological, and toxicological effects of drugs and other chemicals. More than 12.2 million records (01/2016)
Date of the search	11 FEB 2016	11 FEB 2016	11 FEB 2016	11 FEB 2016
Date span of the search:	2005 to 2016	2005 to 2016	2005 to 2016	2005 to 2016
Date of the latest database update included in the search:	22 DEC 2014	21 JAN 2016	08 Feb 2016	09 Feb 2016
Language limit:	No	No	No	No
Other limit set:	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT	Document types excluded that are not "scientific peer-reviewed open literature": PATENT
Search strategy:	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**	Details are listed in **Table A1a-4**
Total number of summary records retrieved	**8**	**13**	**12**	**150**

Total number of summary records retrieved after removing duplicates N= 312

Total number of summary records retrieved after applying search filters (tox, ecotox, e-fate, residues) N= 311

Table A1a-3 Summary of search results (non-bibliographic databases CAS Registry and HSDB)

Data requirement(s) captured in the search	Details of the searches	
	CAS REGISTRY	**HSDB**
Justification for choosing the source:	The CAS REGISTRY File is a chemical structure and dictionary database that contains unique substance records identified by CAS. Records contain CAS Registry Numbers, CA index names, commonly used synonyms, polymer class terms, structure diagrams (many with stereo-chemical information), molecular formulas, and calculated physical properties, all of which are searchable. More than 107.1 million organic and inorganic substances (02/2016)	HSDB is a toxicology data file on the National Library of Medicine's (NLM) Toxicology Data Network (TOXNET®). It focuses on the toxicology of potentially hazardous chemicals. It is enhanced with information on human exposure, industrial hygiene, emergency handling procedures, environmental fate, regulatory requirements, nanomaterials, and related areas. All data are referenced and derived from a core set of books, government documents, technical reports and selected primary journal literature. HSDB is peer-reviewed by the Scientific Review Panel (SRP), a committee of experts in the major subject areas within the data bank's scope. HSDB is organised into individual chemical records, and contains over 5000 such records.
Date of the search	11 FEB 2016	11 FEB 2016
Date span of the search:	not applicable	not applicable
Date of the latest database update included in the search:	10 FEB 2016	MAY 2015
Language limit:	No	No
Other limit set:	None	None
Search strategy:	Search by CAS Registry No.: L1 1 S **2164-08-1** L1-Ln = Single step(s) of the search S = search operator	Details are listed in A1a-2
Total number of summary	**Total number of CAS Registry records retrieved: 1**	**Total number of HSDB records retrieved: 0**

Table A1a-4: Details of the List of search statements and Search strategy

List of search statements and Search strategy	No. of Hits / Comments
STN Search	
FILE 'MEDLINE'	Database Medline
L1 12 S 2164-08-1	Lenacil substance search
L2 0 S 50642-91-6 OR 66113-95-9 OR 91867-45-7	
L3 26 S LENACIL	
L4 0 S LENACILE	
L5 0 S 1H CYCLOPENTAPYRIMIDINE 2 4 3H 5H DIONE 3 CYCLOHEXYL 6 7 DIHYDRO	
L6 0 S 6 7 DIHYDRO 3 CYCLOHEXYL 1H CYCLOPENTAPYRIMIDINE 2 4 3H 5H DIONE	
L7 0 S 3 CYCLOHEXYL 1 5 6 7 TETRAHYDROCYCLOPENTAPYRIMIDINE 2 4 3H DIONE	
L8 1 S 3 CYCLOHEXYL 6 7 DIHYDRO 1H CYCLOPENTAPYRIMIDINE 2 4 3H 5H DIONE	
L9 0 S 5 CYCLOHEXYL 5 7 DIAZA 2 3 4 5 6 7 HEXAHYDROINDENE 4 6 DIONE	
L10 2 S 3 CYCLOHEXYL 5 6 TRIMETHYLENEURACIL	
L11 0 S 3 CYCLOHEXYL 5 6 TRIMETHYLENURACIL	
L12 1 S 3 CYCLOHEXYL 5 6 TRIMETHYLURACIL	
L13 0 S ADOL PESTICIDE	
L14 0 S ADOL 80WP	
L15 0 S BURACYL	
L16 0 S DU PONT 634	
L17 0 S ELBATAN	
L18 0 S HERBICIDE 634	
L19 0 S HEXILURE	
L20 1 S HEXYLURE	
L21 0 S URACIL 634	
L22 8 S VENZAR	31 substance related hits
L23 31 S L1-L22	
	Excluding patents
L24 31 S L23 NOT PATENT/DT	
	Date span 2005-2016 =>
L25 10 S L24 AND 2005-2016/PY	10 Hits database Medline
FILE 'AGRICOLA'	4 Hits database Agricola
L26 4 S L25	
FILE 'BIOSIS'	19 Hits database Biosis
L27 19 S L25	
FILE 'CABA'	65 Hits database Caba
L28 65 S L25	
FILE 'DDFU'	0 Hits database Ddfu
L29 0 S L25	
FILE 'FSTA'	5 Hits database Fsta
L30 5 S L25	
FILE 'PASCAL'	8 Hits database Pascal
L31 8 S L25	
FILE 'TOXCENTER'	150 Hits database Toxcenter
L32 150 S L25	
	0 Hits database Ntis
FILE 'NTIS'	
L33 0 S L25	
FILE 'IPA'	0 Hits database Ipa
L34 0 S L25	
FILE 'PQSCITECH'	13 Hits database Pqscitech
L35 13 S L25	
FILE 'EMBASE'	16 Hits database Embase
L36 16 S L25	
FILE 'ESBIOBASE'	9 Hits database Esbiobase

List of search statements and Search strategy	No. of Hits / Comments
L37 9 S L25	
FILE 'CSNB'	0 Hits database Csnb
L38 0 S L25	
FILE 'HCAPLUS'	255 Hits database HCAplus
L39 255 S L25	
FILE 'SCISEARCH'	12 Hits database Scisearch
L40 12 S L25	
	Entering file cluster (multifile environment)
FILE 'MEDLINE, AGRICOLA, BIOSIS, CABA, DDFU, FSTA, PASCAL, TOXCENTER, NTIS, IPA, PQSCITECH, EMBASE, ESBIOBASE, CSNB, HCAPLUS, SCISEARCH'	312 Hits after duplicate removal
L41 312 DUP REM L25-L28 L30-L32 L35-L37 L39 L40 (254 DUPLICATES REMOVED)	Applying search filter Toxicology
L42 156 S L41 AND (TOXI? OR HAZARD OR ADVERSE OR HEALTH OR NOAEL OR NOEL OR LOAEL OR LOEL OR BMD# OR ACUTE OR SUBACUTE OR SUBCHRONIC? OR CHRONIC? OR REPEATED DOSE OR ORAL OR DERMAL OR GAVAGE OR DIET? OR INHAL? OR SKIN OR EYE? OR IRRIT? OR DERMATITIS OR ECZEM?)	
L43 93 S L41 AND (SENSI? OR HYPERSENSI? OR ALLERG? OR ANAPHYLA? OR ASTHMA? OR MAMMAL? OR RAT OR RATS OR DOG# OR RABBIT# OR HARE OR GUINEA PIG# OR MOUSE OR MICE OR METABOLI? OR DISTRIBUTION OR ADSORPTION OR EXCRETION)	
L44 22 S L41 AND (ELIMINATION OR KINETIC OR PBPK OR TOXICOKIN? OR CYP OR CYTOCHROME OR ENZYM? OR GENE# OR GENETIC? OR GENOME OR MUTA? OR CHROMOSOM? OR CLASTOGEN? OR DNA OR GENOTOXI? OR CARCINO? OR CANCER? OR TUMOR? OR TUMOUR? OR NEOPLAS? OR IN VIVO OR IN VITRO)	
L45 68 S L41 AND (MECHANIS? OR IMMUN? OR NEURO? OR BEHAV? OR ENDOCRIN? OR HORMON? OR XENOESTROGEN? OR ESTROGENIC? OR ANTIANDROGEN? OR REPRODUCT? OR DEVELOPMENT? OR MALFORMATION? OR ANOMAL? OR FERTIL? OR FOET? OR FETUS? OR FETOTOX? OR MATERN? OR PREGNAN?)	
L46 26 S L41 AND (EMBRYO? OR EPIDEM? OR MEDICAL? OR POISON? OR INTOXICA? OR EXPOSURE OR OPERATOR? OR BYSTANDER? OR RESIDENT? OR WORKER? OR OCCUPAT?)	229 Hits Toxicology
L47 229 S L42-L46	Applying search filter Ecotoxicology
L48 124 S L41 AND (PHYTOTOXI? OR ECOTOXI? OR HAZARD OR ADVERSE OR ENDOCRINE DISRUPT? OR BIOACCUMULATION OR BIOMAGNIFICATION OR BIOCONCENTRATION OR EFFECT? OR BIRD# OR MALLARD OR DUCK OR QUAIL OR BOBWHITE OR ANAS OR COLINUS?)	
L49 14 S L41 AND (AQUATIC OR FISH OR DAPHNI? OR ALGA? OR CHIRON? OR SEDIMENT DWELL? OR LEMNA OR MARIN# OR ESTUARINE OR FRESHWATER OR CRUSTAC? OR GASTROPOD? OR MOLLUSC OR REPTILE OR AMPHIB? OR CERIODAPHN? OR GAMMARUS OR HYALELLA OR WATERFLEA OR WATER FLEA)	
L50 106 S L41 AND (BEE# OR APIS OR BUMBLEBEE# OR HONEYBEE# OR VERTEBRAT? OR INVERTEBRAT? OR ARTHROPOD? OR BENEFICIALS OR TYPHLODROMUS OR APHIDIUS OR INSECT# OR WORM# OR ?WORM OR EISENIA OR COLLEMBOL? OR MACRO ORGANISM OR FOLSOMIA OR SPRINGTAIL)	
L51 94 S L41 AND (MICRO ORGANISMS OR MICROBIAL OR PLANT# OR VEGETATIVE VIGO? OR SEEDLING OR GERMINATION OR MONOCOT? OR DICOT? OR SEWAGE OR ACTIVATED SLUDGE)	208 Hits Ecotoxicology
L52 208 S L48-L51	Applying search filter Environmental fate
L53 133 S L41 AND (SOIL OR DEGRADATION OR METABOLITE# OR	

List of search statements and Search strategy	No. of Hits / Comments
PHOTOLYSIS OR SOIL RESIDUE# OR SOIL ACCUMULATION OR CONTAMINATION OR MOBILITY OR ADSORPTION OR DESORPTION OR LYSIMETER OR MODELING OR PEC OR FOCUS MODELING OR GROUNDWATER OR LEACHING OR SURFACEWATER) L54 98 S L41 AND (WATER? OR SEDIMENT OR DISSIPATION OR SATURATED ZONE OR HYDROLYSIS OR PHOTOTRANSFORMATION OR BIODEGRAD? OR BIODETERIO? OR DRIFT OR RUN OFF OR DRAINAGE OR AIR OR VOLAT? OR ATMOSPHERE OR LONG RANGE TRANSPORT OR SHORT RANGE TRANSPORT) L55 183 S L53 OR L54 L56 239 S L41 AND (RESIDUE? OR MULTIRESIDUE? OR STORAGE STABILITY OR METABOLIC OR METABOLISM OR DEGRADATION OR BREAKDOWN OR PLANT# OR CROP? OR FEED OR ANIMAL# OR LIVESTOCK# OR HEN OR CATTLE OR RUMINANT# OR GOAT? OR COW# OR PIG? OR FISH OR MILK OR HONEY) L57 126 S L41 AND (PROCESS? OR HYDROLY? OR ROTATION? OR SUCCEED? OR RISK OR ASSESSMENT OR RISK ASSESSMENT OR CONSUME? OR EXPOSURE OR CROSS CONTAMINATION OR BIOMONITORING OR MONITORING OR ENVIRONMENTAL CONTAMINA?) L58 264 S L56 OR L57 L59 311 S L47 OR L52 OR L55 OR L58 STN command language: File Medline = Entering database Medline L1 – L59 = Single steps of the online search ("search statements") s = search operator AND / OR / NOT = Boolean search operators /dt = search term in field "document type" Dup rem = remove duplicate citations	183 Hits Environmental fate Applying search filter Residues 264 Hits Residues 311 Hits combined for all 4 search filters
HSDB search: 2164-08-1 Search results: 0 hits	 Lenacil 0 Hits database HSDB

CA 9.3 Relevant study selection-results of the selection process

As first step, the rapid assessment was performed by expert reviewers based on summary records (title/abstracts). Summary records clearly related to one of the following topics were classified as **obviously irrelevant:**

- Efficacy
- Resistance of targets
- Analytical method development, calibration
- New ways of synthesis
- Studies on a molecular level, which cannot be related to environmental risk assessment
- Non-EU monitoring studies, non-EU field studies
- Publications in non-EU language without English abstract
- Abstract refers to a conference contribution and does not contain data, full text not available
- Not relevant due to missing information: Studies with target organisms

Obviously non-relevant studies in open literature search were excluded by applying the relevance criteria previously defined in **Error! Reference source not found.** of this document. A total of **311 summary records** were reviewed; of these **305 were not relevant**. When the summary records did not contain sufficient information to assess relevance, full text documents were reviewed in detail for relevance according to the previously defined criteria. After reviewing full text documents of potentially relevant studies, 40 were excluded from further consideration. **Relevant studies (6) have been selected for inclusion in the dossier.**

Table A1a-5 summarise the results of the selection process including the number of summary records and full text documents assessed for the section of **toxicology**.

Table A1a-5: Literature search results: Toxicology

Data requirement(s) captured in the search	Number
Total number of summary records retrieved after all searches of peer-reviewed literature (excluding duplicates)	14
Number of summary records excluded from the search results after rapid assessment for relevance	9
Total number of full-text documents assessed in detail	5
Number of studies excluded from further consideration after detailed assessment for relevance	1
Number of studies not excluded for relevance after detailed assessment (*i.e.*, relevant studies and studies of unclear relevance)	4

CA 9.4 Literature included in the dossier after detailed assessment

Literature included in the dossier are classified and summarised as an Appendix to the applicable data point in the summary (renewal) dossier for lenacil, Document M-CA. Studies are classified in the dossier in the following categories:

- Studies that provide data for establishing or refining risk assessment parameters. These studies are summarised in detail following the OECD guidance and can be found in an Appendix to the applicable data point.
- Studies that are relevant to the data requirement but only supply supplementary information for the risk assessment parameters. A justification for this decision is given in an Appendix to the applicable data point.
- Studies that remain of unclear relevance. An explanation as to why relevance of such studies could not be definitively determined is in an Appendix to the applicable data point.

Reliability assessments for each relevant study and that of unclear relevance are included in Document M-CA of the dossier.

Copies of the full text documents, abiding applicable copyright laws, have been attached in

of document M-CA section 9. (**RMS**: not inserted into this DRAR) The list of literature excluded by rapid assessment is presented in **table A1a-8.**

Table A1a-6 contains the bibliographic references (List by author) for all relevant mammalian toxicological and toxicokinetic studies included in the dossier after detailed assessment by data point and author, respectively.

Table A1a-6 Literature to be included after detailed assessment: Mammalian Toxicology.

Author(s)	Data Requirement No., Reference No.	Year	Title	Source
Kojima, H., Sata, F., Takeuchi, S., Sueyoshi, T., and Nagai, T	MCA 5.8.2/02	2011	In vitro screening of 200 pesticides for agonistic activity via mouse peroxisome proliferator-activated receptor (PPAR)α and PPARγ and quantitative analysis of in vivo induction pathway	Toxicology and Applied Pharmacology, 217: 235-244
Takeuchi, S., Iida, M., Yabushita, H, Matsuda, T and Kojima, H.	MCA 5.8.3/01	2004	Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells	Environmental health perspectives, 112:524-531
Takeuchi, S., Iida, M., Yabushita, H, Matsuda, T and Kojima, H.	MCA 5.8.2/04	2008	In vitro screening for aryl hydrocarbon receptor agonistic activity in 200 pesticides using a highly sensitive reporter cell line, DR-EcoScreen cells, and in vivo mouse liver cytochrome P450-1A induction by propanil, diuron and linuron	Chemosphere, 74:155-165
Takeuchi, S., Matsuda, T., Kobayashi, S., Takahasi, T and Kojima, H.	MCA 5.8.2/03	2006	In vitro screening of 200 pesticides for agonistic activity via mouse peroxisome proliferator-activated receptor (PPAR)α and PPARγ and quantitative analysis of in vivo induction pathway	Toxicology and Applied Pharmacology, 217: 235-244

CA 9.5 Literature excluded after detailed assessment

Technical experts, after detailed assessment of the full text document, determined certain literature not relevant to the risk assessment parameters. A list of this non-relevant literature is given below with an explanation for non-inclusion. **Table A1a**-7 contains the bibliographic references for all potentially relevant and unclear relevance for mammalian toxicological and toxicokinetic studies determined to be not relevant.

Table A1a-7 Literature to be excluded after detailed assessment: Mammalian Toxicology

Author(s)	Year	Title	Source	Reason (s) for non-inclusion
Nougadere, A.; Reninger, J.-C.; Volatier, J.-L.; Leblanc, J.-C.	2011	Chronic dietary risk characterization for pesticide residues: A ranking and scoring method integrating agricultural uses and food contamination data	Food and Chemical Toxicology, (2011) Vol. 49, No. 7, pp. 1484-1510	Exposure cannot be related to representative uses. The study presents a discussion on the revision of chronic dietary intake indicators developed and adapted nationally for French population. The paper is not relevant for the risk assessment as it does not include information on new endpoints for lenacil. The paper does not contain any experimental data for mammalian toxicological purposes. Based on surveys, this paper proposes a method for ranking and scoring pesticide residues according to the chronic risk. The paper is not relevant for the risk assessment.

LITERATURE EXCLUDED BY RAPID ASSESSMENT

Obviously non-relevant studies in open literature search were excluded by applying the relevance criteria previously defined in **table A1a-1** of this document. A list of this non-relevant literature is given below.

Table A1a-8 Literature excluded by rapid assessment: Mammalian Toxicology

Author(s)	Year	Title	Source	Reason(s) for non-inclusion
Fantke, Peter; Friedrich, Rainer; Jolliet, Olivier	2012	Health impact and damage cost assessment of pesticides in Europe	Environment International, (2012) Vol. 49, pp. 9-17.	The evaluation of mechanistic aspects not assessed for lenacil. The article was not relevant for exposure considerations since no endpoint is altered and no influence on risk assessment is concluded
Hayat, Khizar; Ashfaq, Muhammad; Ashfaq, Umair; Saleem, Mushtaq Ahmad	2010	Determination of pesticide residues in blood samples of villagers involved in pesticide application at District Vehari (Punjab), Pakistan	African Journal of Environmental Science and Technology, (2010) Vol. 4, No. 10, pp. 666-684.	Analytical method development, calibration.
Hu, Le-Le; Chen, Chen; Huang, Tao; Cai, Yu-Dong; Chou, Kuo-Chen	2011	Predicting biological functions of compounds based on chemical-chemical interactions	PLoS One, (2011) Vol. 6, No. 12, pp. e29491.	As no information is available on relevance of the results for humans or comparison to human exposure levels is made, the test results are not further considered for risk assessment.
Hug, Christine; Krauss, Martin; Nuesser, Leonie; Hollert, Henner; Brack, Werner	2015	Metabolic transformation as a diagnostic tool for the selection of candidate promutagens in effect-directed analysis	Environmental Pollution (Oxford, United Kingdom), (2015) Vol. 196, pp. 114-124.	As no information is available on relevance of the results for humans or comparison to human exposure levels is made, the test results are not further considered for risk assessment.
Kim, J.; Kim, S.; Schaumann, G. E.	2013	Development of QSAR-based two-stage prediction model for estimating mixture toxicity	SAR and QSAR in Environmental Research, (2013) Vol. 24, No. 10, pp. 841-861.	As no information is available on relevance of the results for humans or comparison to human exposure levels is made, the test results are not further considered for risk assessment.
Li, Jiazhong; Gramatica, Paola	2010	Classification and Virtual Screening of Androgen Receptor Antagonists	Journal of Chemical Information and Modeling, (2010) Vol. 50, No. 5, pp. 861-874.	As no information is available on relevance of the results for humans or comparison to human exposure levels is made, the test results are not further considered for risk assessment. Note **RMS**: reference is made to Lenacil (under the form of its CAS RN 1264-08-1), but data in this article refer back to the Kojima 2004 publication, already assessed elsewhere.
Lozowicka, B.; Kaczynski, P.; Paritova, A. E.; Kuzembekova, G. B.; Abzhalieva, A. B.; Sarsembayeva, N. B.; Alihan, K.	2014	Pesticide residues in grain from Kazakhstan and potential health risks associated with exposure to detected pesticides	Food and Chemical Toxicology, (2014) Vol. 64, pp. 238-248.	Analytical method development, calibration. Not subject to relevant for residues analyzed and/or residues occurrence from lenacil application on crops and side effects on health

Table A1a-8 Literature excluded by rapid assessment: Mammalian Toxicology

Author(s)	Year	Title	Source	Reason(s) for non-inclusion
Nougadere, Alexandre; Reninger, Jean-Cedric; Volatier, Jean-Luc; Leblanc, Jean-Charles	2011	Chronic dietary risk characterization for pesticide residues: A ranking and scoring method integrating agricultural uses and food contamination data	Food and Chemical Toxicology, (2011) Vol. 49, No. 7, pp. 1484-1510.	Analytical method development, calibration. Not subject to relevant for residues analyzed and/or residues occurrence from lenacil application on crops and side effects on health
Schummer, Claude; Salquebre, Guillaume; Briand, Olivier; Millet, Maurice; Appenzeller, Brice M. R.	2012	Determination of farm workers' exposure to pesticides by hair analysis	Toxicology Letters, (2012) Vol. 210, No. 2, pp. 203-210.	As no information is available on relevance of the results for humans or comparison to human exposure levels is made, the test results are not further considered for risk assessment.

CA 9.7 Original search query – complete STN search to be included - Raw data

```
    (FILE 'MEDLINE' ENTERED AT 14:45:14 ON 11 FEB 2016)
L1          12 SEA SPE=ON  ABB=ON  PLU=ON  2164-08-1
L2           0 SEA SPE=ON  ABB=ON  PLU=ON  50642-91-6 OR 66113-95-9 OR
             91867-45-7
L3          26 SEA SPE=ON  ABB=ON  PLU=ON  LENACIL
L4           0 SEA SPE=ON  ABB=ON  PLU=ON  LENACILE
L5           0 SEA SPE=ON  ABB=ON  PLU=ON  1H CYCLOPENTAPYRIMIDINE 2 4 3H 5H
             DIONE 3 CYCLOHEXYL 6 7 DIHYDRO
L6           0 SEA SPE=ON  ABB=ON  PLU=ON  6 7 DIHYDRO 3 CYCLOHEXYL 1H
             CYCLOPENTAPYRIMIDINE 2 4 3H 5H DIONE
L7           0 SEA SPE=ON  ABB=ON  PLU=ON  3 CYCLOHEXYL 1 5 6 7 TETRAHYDROCYCL
             OPENTAPYRIMIDINE 2 4 3H DIONE
L8           1 SEA SPE=ON  ABB=ON  PLU=ON  3 CYCLOHEXYL 6 7 DIHYDRO 1H
             CYCLOPENTAPYRIMIDINE 2 4 3H 5H DIONE
L9           0 SEA SPE=ON  ABB=ON  PLU=ON  5 CYCLOHEXYL 5 7 DIAZA 2 3 4 5 6 7
             HEXAHYDROINDENE 4 6 DIONE
L10          2 SEA SPE=ON  ABB=ON  PLU=ON  3 CYCLOHEXYL 5 6 TRIMETHYLENEURACIL
L11          0 SEA SPE=ON  ABB=ON  PLU=ON  3 CYCLOHEXYL 5 6 TRIMETHYLENURACIL
L12          1 SEA SPE=ON  ABB=ON  PLU=ON  3 CYCLOHEXYL 5 6 TRIMETHYLURACIL
L13          0 SEA SPE=ON  ABB=ON  PLU=ON  ADOL PESTICIDE
L14          0 SEA SPE=ON  ABB=ON  PLU=ON  ADOL 80WP
L15          0 SEA SPE=ON  ABB=ON  PLU=ON  BURACYL
L16          0 SEA SPE=ON  ABB=ON  PLU=ON  DU PONT 634
L17          0 SEA SPE=ON  ABB=ON  PLU=ON  ELBATAN
L18          0 SEA SPE=ON  ABB=ON  PLU=ON  HERBICIDE 634
L19          0 SEA SPE=ON  ABB=ON  PLU=ON  HEXILURE
L20          1 SEA SPE=ON  ABB=ON  PLU=ON  HEXYLURE
L21          0 SEA SPE=ON  ABB=ON  PLU=ON  URACIL 634
L22          8 SEA SPE=ON  ABB=ON  PLU=ON  VENZAR
L23         31 SEA SPE=ON  ABB=ON  PLU=ON  (L1 OR L2 OR L3 OR L4 OR L5 OR L6
             OR L7 OR L8 OR L9 OR L10 OR L11 OR L12 OR L13 OR L14 OR L15 OR
             L16 OR L17 OR L18 OR L19 OR L20 OR L21 OR L22)
L24         31 SEA SPE=ON  ABB=ON  PLU=ON  L23 NOT PATENT/DT
L25         10 SEA SPE=ON  ABB=ON  PLU=ON  L24 AND 2005-2016/PY
            SAVE L25 LENAMED/A

    FILE 'AGRICOLA' ENTERED AT 14:53:20 ON 11 FEB 2016
CHARGED TO COST=101581
L26          4 SEA SPE=ON  ABB=ON  PLU=ON  L24 AND 2005-2016/PY
            SAVE L26 LENAAGRI/A

    FILE 'BIOSIS' ENTERED AT 14:56:31 ON 11 FEB 2016
CHARGED TO COST=101581
L27         19 SEA SPE=ON  ABB=ON  PLU=ON  L24 AND 2005-2016/PY
            SAVE L27 LENABIOSI/A

    FILE 'CABA' ENTERED AT 14:58:22 ON 11 FEB 2016
CHARGED TO COST=101581
L28         65 SEA SPE=ON  ABB=ON  PLU=ON  L24 AND 2005-2016/PY
            SAVE L28 LENACAB/A

    FILE 'DDFU' ENTERED AT 14:59:52 ON 11 FEB 2016
CHARGED TO COST=101581
L29          0 SEA SPE=ON  ABB=ON  PLU=ON  L24 AND 2005-2016/PY

    FILE 'FSTA' ENTERED AT 15:01:01 ON 11 FEB 2016
CHARGED TO COST=101581
L30          5 SEA SPE=ON  ABB=ON  PLU=ON  L24 AND 2005-2016/PY
            SAVE L30 LENAFST/A

    FILE 'PASCAL' ENTERED AT 15:02:50 ON 11 FEB 2016
CHARGED TO COST=101581
L31          8 SEA SPE=ON  ABB=ON  PLU=ON  L24 AND 2005-2016/PY
            SAVE L31 LENAPAS/A

    FILE 'TOXCENTER' ENTERED AT 15:04:20 ON 11 FEB 2016
CHARGED TO COST=101581
L32        150 SEA SPE=ON  ABB=ON  PLU=ON  L24 AND 2005-2016/PY
            SAVE L32 LENATOX/A

    FILE 'NTIS' ENTERED AT 15:06:36 ON 11 FEB 2016
```

CHARGED TO COST=101581
L33 0 SEA SPE=ON ABB=ON PLU=ON L24 AND 2005-2016/PY

FILE 'IPA' ENTERED AT 15:07:33 ON 11 FEB 2016
CHARGED TO COST=101581
L34 0 SEA SPE=ON ABB=ON PLU=ON L24 AND 2005-2016/PY

FILE 'PQSCITECH' ENTERED AT 15:09:00 ON 11 FEB 2016
CHARGED TO COST=101581
L35 13 SEA SPE=ON ABB=ON PLU=ON L24 AND 2005-2016/PY
SAVE L35 LENAPQSC/A

FILE 'EMBASE' ENTERED AT 15:11:40 ON 11 FEB 2016
CHARGED TO COST=101581
L36 16 SEA SPE=ON ABB=ON PLU=ON L24 AND 2005-2016/PY
SAVE L36 LENAEMB/A

FILE 'ESBIOBASE' ENTERED AT 15:14:20 ON 11 FEB 2016
CHARGED TO COST=101581
L37 9 SEA SPE=ON ABB=ON PLU=ON L24 AND 2005-2016/PY
SAVE L37 LENAESBI/A

FILE 'CSNB' ENTERED AT 15:15:36 ON 11 FEB 2016
CHARGED TO COST=101581
L38 0 SEA SPE=ON ABB=ON PLU=ON L24 AND 2005-2016/PY

FILE 'HCAPLUS' ENTERED AT 15:20:12 ON 11 FEB 2016
CHARGED TO COST=101581
L39 255 SEA SPE=ON ABB=ON PLU=ON L24 AND 2005-2016/PY
SAVE L39 LENAHCAP/A

FILE 'SCISEARCH' ENTERED AT 15:29:12 ON 11 FEB 2016
CHARGED TO COST=101581
L40 12 SEA SPE=ON ABB=ON PLU=ON L24 AND 2005-2016/PY
SAVE L40 LENASCI/A

FILE 'MEDLINE, AGRICOLA, BIOSIS, CABA, FSTA, PASCAL, TOXCENTER, PQSCITECH, EMBASE, ESBIOBASE, HCAPLUS, SCISEARCH' ENTERED AT 15:38:07 ON 11 FEB 2016
CHARGED TO COST=101581
L41 312 DUP REM L25-L28 L30-L32 L35-L37 L39 L40 (254 DUPLICATES REMOVED
ANSWERS '1-10' FROM FILE MEDLINE
ANSWER '11' FROM FILE AGRICOLA
ANSWERS '12-20' FROM FILE BIOSIS
ANSWERS '21-72' FROM FILE CABA
ANSWERS '73-76' FROM FILE FSTA
ANSWER '77' FROM FILE PASCAL
ANSWERS '78-208' FROM FILE TOXCENTER
ANSWER '209' FROM FILE PQSCITECH
ANSWERS '210-312' FROM FILE HCAPLUS
SAVE L41 LENASUBST/A

L42 156 SEA SPE=ON ABB=ON PLU=ON L41 AND (TOXI? OR HAZARD OR ADVERSE OR HEALTH OR NOAEL OR NOEL OR LOAEL OR LOEL OR BMD# OR ACUTE OR SUBACUTE OR SUBCHRONIC? OR CHRONIC? OR REPEATED DOSE OR ORAL OR DERMAL OR GAVAGE OR DIET? OR INHAL? OR SKIN OR EYE? OR IRRIT? OR DERMATITIS OR ECZEM?)

L43 93 SEA SPE=ON ABB=ON PLU=ON L41 AND (SENSI? OR HYPERSENSI? OR ALLERG? OR ANAPHYLA? OR ASTHMA? OR MAMMAL? OR RAT OR RATS OR DOG# OR RABBIT# OR HARE OR GUINEA PIG# OR MOUSE OR MICE OR METABOLI? OR DISTRIBUTION OR ADSORPTION OR EXCRETION)

L44 22 SEA SPE=ON ABB=ON PLU=ON L41 AND (ELIMINATION OR KINETIC OR PBPK OR TOXICOKIN? OR CYP OR CYTOCHROME OR ENZYM? OR GENE# OR GENETIC? OR GENOME OR MUTA? OR CHROMOSOM? OR CLASTOGEN? OR DNA OR GENOTOXI? OR CARCINO? OR CANCER? OR TUMOR? OR TUMOUR? OR NEOPLAS? OR IN VIVO OR IN VITRO)

L45 68 SEA SPE=ON ABB=ON PLU=ON L41 AND (MECHANIS? OR IMMUN? OR NEURO? OR BEHAV? OR ENDOCRIN? OR HORMON? OR XENOESTROGEN? OR ESTROGENIC? OR ANTIANDROGEN? OR REPRODUCT? OR DEVELOPMENT? OR MALFORMATION? OR ANOMAL? OR FERTIL? OR FOET? OR FETUS? OR

FETOTOX? OR MATERN? OR PREGNAN?)

L46 26 SEA SPE=ON ABB=ON PLU=ON L41 AND (EMBRYO? OR EPIDEM? OR MEDICAL? OR POISON? OR INTOXICA? OR EXPOSURE OR OPERATOR? OR BYSTANDER? OR RESIDENT? OR WORKER? OR OCCUPAT?)

L47 229 SEA SPE=ON ABB=ON PLU=ON (L42 OR L43 OR L44 OR L45 OR L46)
SAVE L47 LENATO/A

L48 124 SEA SPE=ON ABB=ON PLU=ON L41 AND (PHYTOTOXI? OR ECOTOXI? OR HAZARD OR ADVERSE OR ENDOCRINE DISRUPT? OR BIOACCUMULATION OR BIOMAGNIFICATION OR BIOCONCENTRATION OR EFFECT? OR BIRD# OR MALLARD OR DUCK OR QUAIL OR BOBWHITE OR ANAS OR COLINUS?)

L49 14 SEA SPE=ON ABB=ON PLU=ON L41 AND (AQUATIC OR FISH OR DAPHNI? OR ALGA? OR CHIRON? OR SEDIMENT DWELL? OR LEMNA OR MARIN# OR ESTUARINE OR FRESHWATER OR CRUSTAC? OR GASTROPOD? OR MOLLUSC OR REPTILE OR AMPHIB? OR CERIODAPHN? OR GAMMARUS OR HYALELLA OR WATERFLEA OR WATER FLEA)

L50 106 SEA SPE=ON ABB=ON PLU=ON L41 AND (BEE# OR APIS OR BUMBLEBEE# OR HONEYBEE# OR VERTEBRAT? OR INVERTEBRAT? OR ARTHROPOD? OR BENEFICIALS OR TYPHLODROMUS OR APHIDIUS OR INSECT# OR WORM# OR ?WORM OR EISENIA OR COLLEMBOL? OR MACRO ORGANISM OR FOLSOMIA OR SPRINGTAIL)

L51 94 SEA SPE=ON ABB=ON PLU=ON L41 AND (MICRO ORGANISMS OR MICROBIAL OR PLANT# OR VEGETATIVE VIGO? OR SEEDLING OR GERMINATION OR MONOCOT? OR DICOT? OR SEWAGE OR ACTIVATED SLUDGE)

L52 208 SEA SPE=ON ABB=ON PLU=ON (L48 OR L49 OR L50 OR L51)
SAVE L52 LENAET/A

L53 133 SEA SPE=ON ABB=ON PLU=ON L41 AND (SOIL OR DEGRADATION OR METABOLITE# OR PHOTOLYSIS OR SOIL RESIDUE# OR SOIL ACCUMULATION OR CONTAMINATION OR MOBILITY OR ADSORPTION OR DESORPTION OR LYSIMETER OR MODELING OR PEC OR FOCUS MODELING OR GROUNDWATER OR LEACHING OR SURFACEWATER)

L54 98 SEA SPE=ON ABB=ON PLU=ON L41 AND (WATER? OR SEDIMENT OR DISSIPATION OR SATURATED ZONE OR HYDROLYSIS OR PHOTOTRANSFORMATION OR BIODEGRAD? OR BIODETERIO? OR DRIFT OR RUN OFF OR DRAINAGE OR AIR OR VOLAT? OR ATMOSPHERE OR LONG RANGE TRANSPORT OR SHORT RANGE TRANSPORT)

L55 183 SEA SPE=ON ABB=ON PLU=ON L53 OR L54
SAVE L55 LENAEF/A

L56 239 SEA SPE=ON ABB=ON PLU=ON L41 AND (RESIDUE? OR MULTIRESIDUE? OR STORAGE STABILITY OR METABOLIC OR METABOLISM OR DEGRADATION OR BREAKDOWN OR PLANT# OR CROP? OR FEED OR ANIMAL# OR LIVESTOCK # OR HEN OR CATTLE OR RUMINANT# OR GOAT? OR COW# OR PIG? OR FISH OR MILK OR HONEY)

L57 126 SEA SPE=ON ABB=ON PLU=ON L41 AND (PROCESS? OR HYDROLY? OR ROTATION? OR SUCCEED? OR RISK OR ASSESSMENT OR RISK ASSESSMENT OR CONSUME? OR EXPOSURE OR CROSS CONTAMINATION OR BIOMONITORING OR MONITORING OR ENVIRONMENTAL CONTAMINA?)

L58 264 SEA SPE=ON ABB=ON PLU=ON L56 OR L57
SAVE L58 LENARE/A

L59 311 SEA SPE=ON ABB=ON PLU=ON L47 OR L52 OR L55 OR L58
SAVE L59 LENAFILT/A

CA 9.8 Literature data concerning the metabolites of lenacil

Note **RMS** : after the first renewal submission, where notifier conducted a literature search on the active substance lenacil, RMS requested the notifier to extend its search for the metabolites IN-KE121, IN-KF313, IN-KC943, IN-KQ961, IN-KD304 and IN-KQ957.

Notifier submitted during the evaluation phase a report of this search, which was named:

"Literature Review Report - Scientific peer-reviewed of open literature for the approval of pesticide active substance lenacil (focus on its metabolites) as under Article 8(5) of Regulation (EC) No 1107/2009 (Ref. EFSA Journal 2011; 9(2) 2092)
Report number 106052-CA9-1, Author Anonymus, 2018
Sponsor Cheminova Deutschland GmbH & Co. KG FMC Agricultural Solutions, Office Frankfurt am Main Westhafenplatz 1 - 60327 Frankfurt am Main Germany; Reporting Date 03 September, 2018.

This report was evaluated and reproduced in the DRAR under this point CA 9.8.

The Scientific peer-reviewed open literature was carried out as requested by the **RMS** Belgium (July-August 2018) for the metabolites of lenacil. The review itself is in accordance with the EFSA Guidance document as published in EFSA Journal 2011; 9(2):20921. The peer-reviewed open literature evaluation dealing with side- effects on health, the environment and non-target species and published within the last 10 years before the date of submission of the dossier of lenacil was performed.
All steps in the literature review report (LRR) are based on the EFSA Guidance Document mentioned above. The evaluation and identification of relevant public literature articles for the lenacil metabolites, in the context of side-effects on health, the environment and non-target species was prepared.

CA 9.8.1 **Search strategy**

CA 9.8.1.1 **Date of the search**: the search was carried out on August 14th, 2018.

CA 9.8.1.2 **Time window of the literature search**
According with the Article 8(5) of Regulation (EC) Nº 1107/2009 the search ranged up to 10 years before the date of submission of the dossier. As done for the LRR for the approval of the active substance lenacil in 2016, the window of literature search was 2005-2016.

CA 9.8.1.3 **Bibliographic Databases used in the literature review**
Reasonable effort was taken to locate all sources of relevant peer reviewed open literature concentrated on comprehensive databases containing worldwide coverage of biology, chemistry, biomedical, agricultural and environmental fields. Following the latest recommendations from EFSA for submission of peer-review open literature1, the following information has been gathered from the databases used in the literature search. In an analogious way as for the a.s. lenacil, the literature search was performed in the following databases (slightly different than for the a.s. (databases: SNB, DDFU, IPA, NTIS, PASCAL, CAS Registry and HSDB not included):

- AGRICOLA,
- BIOSIS,
- CABA,
- CAPLUS,
- MEDLINE,
- EMBASE,
- TOXCENTER,
- FSTA,
- PQSCITECH,
- ESBIOBASE,
- SCISEARCH,

CA 9.8.1.4 **Input parameters for literature search**
The approach used for the search was the "single concept search", for the metabolite names, and CAS RN's where available.

Table A1a-9 Key words used for the litterature search of the metabolites of lenacil

No.	Metabolite	Key words to be used for the literature search	Compound found in:
1	IN-KE121	**CAS number**: Not available **Chemical name:** 3-(4-oxocyclohexyl)-1,5,6,7-tetrahydrocyclopenta[d]pyrimidine-2,4-dione **Molecular weight:** 248.3 g/mol	Soil, water and sediment
2	IN-KF313	**CAS number**: 1270965-07-5 **Chemical name:** 3-cyclohexyl-1,6,7-trihydrocyclopenta[d]pyrimidine- 2,4,5-trione **Molecular weight:** 248.3 g/mol	Soil, water and sediment
3	IN-KC943	**CAS number**: 1018147-92-6 **Chemical name** 3-cyclohexyl-7-hydroxy-1,5,6,7-tetrahydrocyclopenta[d]pyrimidine-2,4-dione **Molecular weight:** 250.3 g/mol	Plant (sugar beet)
4	IN-KQ961	**CAS number**: Not available **Chemical name:** 3-cyclohexyl-5-hydroxy-1,5,6,7-tetrahydrocyclopenta[d]pyrimidine-2,4-dione **Molecular weight:** 250.3 g/mol	Water (aquatic photolysis at alkaline conditions, but not relevant for risk assessment)
5	IN-KD304	**CAS number**: Not available **Chemical name:** 3-(4-hydroxycyclohexyl)-1,5,6,7-tetrahydrocyclopenta[d]pyrimidine-2,4-dione **Molecular weight:** 250.3 g/mol	Residues
6	IN-KQ957	**CAS number**: Not available **Chemical name:** 3-(2-oxocyclohexyl)-1,5,6,7-tetrahydrocyclopenta[d]pyrimidine-2,4-dione **Molecular weight:** 248.3 g/mol	Residues

CA 9.8.1.5 **Original search query – complete search to be included - Raw data**

(FILE 'HOME' ENTERED AT 11:22:08 ON 14 AUG 2018) SET ACCOUNT

FILE 'MEDLINE' ENTERED AT 11:22:26 ON 14 AUG 2018 CHARGED TO COST=106052
L1 0 S 1018147-92-6
L2 0 S 1270965-07-5
L3 0 S 3 1R 4R 4 HYDROXYCYCLOHEXYL 1H 5H 6H 7H CYCLOPENTA D PYRIMIDINE 2
4 DIONE
L4 0 S 3 2 OXOCYCLOHEXYL 1 5 6 7 TETRAHYDROCYCLOPENTA D PYRIMIDINE 2 4 DIONE
L5 0 S 3 2 OXOCYCLOHEXYL 1H 5H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 DIONE
L6 0 S 3 4 HYDROXYCYCLOHEXYL 1 5 6 7 TETRAHYDROCYCLOPENTA D PYRIMIDINE
2 4 DIONE
L7 0 S 3 4 OXOCYCLOHEXYL 1 5 6 7 TETRAHYDROCYCLOPENTA D PYRIMIDINE 2 4 DIONE
L8 0 S 3 4 OXOCYCLOHEXYL 1H 5H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 DIONE
L9 0 S 3 4 OXOCYCLOHEXYL 6 7 DIHYDRO 1H CYCLOPENTA D PYRIMIDINE 2 4 3H 5H DIONE
L10 0 S 3 4 OXOCYCLOHEXYL 6 7 DIHYDRO 1H CYCLOPENTA D PYRIMIDINE 2 4 3H 5H DIONE
L11 0 S 3 CYCLOHEXYL 1H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 5 TRIONE
L12 0 S 3 CYCLOHEXYL 2 7 DIHYDROXY 3 5 6 7 TETRAHYDRO 4H CYCLOPENTA D
PYRIMIDIN 4 ONE
L13 0 S 3 CYCLOHEXYL 5 HYDROXY 1H 5H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 DIONE
L14 0 S 3 CYCLOHEXYL 6 7 DIHYDRO 1H CYCLOPENTA D PYRIMIDINE 2 4 5 3H TRIONE
L15 0 S 3 CYCLOHEXYL 6 7 DIHYDRO 1H CYCLOPENTA E PYRIMIDINE 2 4 5 TRIONE
L16 0 S 3 CYCLOHEXYL 6 7 DIHYDRO 7 1H CYCLO PENTAPYRIMIDINE 2 4 5 3H TRIONE
L17 0 S 3 CYCLOHEXYL 6 7 DIHYDRO 7 1H CYCLOPENTAPYRIMIDINE 2 4 5 3H TRIONE
L18 0 S 3 CYCLOHEXYL 7 HYDROXY 1 5 6 7 TETRAHYDROCYCLOPENTA E
PYRIMIDINE 2 4 DIONE
L19 0 S 3 CYCLOHEXYL 7 HYDROXY 1H 5H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 DIONE
L20 0 S 3 CYCLOHEXYL 7 HYDROXY 6 7 DIHYDRO 1H CYCLOPENTA D PYRIMIDINE 2 4 3H 5H
DIONE
L21 0 S 3 CYCLOHEXYL 7 HYDROXY 1 5 6 7 TETRAHYDROCYCLOPENTA D
PYRIMIDINE 2 4 DIONE
L22 0 S 3 CYCLOHEXYL 5 HYDROXY 1 5 6 7 TETRAHYDROCYCLOPENTA D
PYRIMIDINE 2 4 DIONE
L23 0 S IN KC 943
L24 0 S IN KC943
L25 0 S IN KD 304
L26 0 S IN KD304
L27 0 S IN KE 121
L28 0 S IN KE121
L29 0 S IN KF 313
L30 0 S IN KF313
L31 0 S IN KQ 957
L32 0 S IN KQ 961
L33 0 S IN KQ957
L34 0 S IN KQ961
L35 0 S L1-L34

L36 0 S L35 NOT PATENT/DT L37 0 S L36 AND 2005-2016/PY

FILE 'AGRICOLA' ENTERED AT 11:36:01 ON 14 AUG 2018 CHARGED TO COST=106052
L38 0 S L37

FILE 'BIOSIS' ENTERED AT 11:38:13 ON 14 AUG 2018 CHARGED TO COST=106052
L39 0 S L37

FILE 'CABA' ENTERED AT 11:39:35 ON 14 AUG 2018 CHARGED TO COST=106052
L40 0 S L37

FILE 'DDFU' ENTERED AT 11:42:28 ON 14 AUG 2018 CHARGED TO COST=106052
L41 0 S L37

FILE 'FSTA' ENTERED AT 11:43:45 ON 14 AUG 2018 CHARGED TO COST=106052
L42 0 S L37

FILE 'TOXCENTER' ENTERED AT 11:45:19 ON 14 AUG 2018 CHARGED TO COST=106052

L43 0 S L37

FILE 'NTIS' ENTERED AT 11:47:01 ON 14 AUG 2018 CHARGED TO COST=106052
L44 0 S L37

FILE 'IPA' ENTERED AT 11:48:22 ON 14 AUG 2018 CHARGED TO COST=106052
L45 0 S L37

FILE 'PQSCITECH' ENTERED AT 11:49:30 ON 14 AUG 2018 CHARGED TO COST=106052
L46 0 S L37

FILE 'EMBASE' ENTERED AT 11:51:06 ON 14 AUG 2018 CHARGED TO COST=106052
L47 0 S L37

FILE 'ESBIOBASE' ENTERED AT 11:54:25 ON 14 AUG 2018 CHARGED TO COST=106052
L48 0 S L37

FILE 'HCAPLUS' ENTERED AT 11:56:31 ON 14 AUG 2018 CHARGED TO COST=106052
L49 2 S L37

FILE 'SCISEARCH' ENTERED AT 12:00:37 ON 14 AUG 2018 CHARGED TO COST=106052
L50 0 S L37

FILE 'HCAPLUS' ENTERED AT 12:03:35 ON 14 AUG 2018 CHARGED TO COST=106052
L51 2 DUP REM L37-L50 (0 DUPLICATES REMOVED) SAVE L51 LRRDUPREM/A

CA 9.8.1.6 **Bibliographic Databases used in the literature review : detailed description and output**

The specification of the consulted databases and their output, regarding the search for the lecacil metabolites is found in table A1a-10, below:

Table A1a-10 Details of the literature search for the lenacil metabolites (AGRICOLA, BIOSIS, CABA, HCAPLUS) Mammalian toxicology, Metabolism, Residues, Environmental fate, Ecotoxicology

Data requirement(s) captured in the search	Details of the searches			
	AGRICOLA	*BIOSIS*	*CABA*	*CAPLUS*
Justification for choosing the source:	Agriculture Online Access is a bibliographic database containing selected worldwide literature of agriculture and related fields. More than 5.7 million records (06/2017)	The largest and most comprehensive life science database in the world, BIOSIS covers original research reports, reviews, and selected U.S. patents in biological and biomedical areas, with subject coverage ranging from aerospace biology to zoology. More than 25.7 million records (03/2017)	The CAB Abstracts database covers worldwide literature from all areas of agriculture and related sciences including biotechnology, forestry, and veterinary medicine. More than 8.6 million records (06/2017)	Chemical Abstracts Plus (CAplusSM) provides current and comprehensive worldwide cover- age of chemistry and related scientific disciplines. CAplus covers international journals, patents, patent families, technical disclosures, technical reports, books, conference proceedings, dissertations, electronic-only journals, and web pre-prints from all areas of chemistry, biochemistry, chemical engineering, and related sciences from 1907 to the present. More than 45 million records (03/2017)
Date of the search	14 AUG 2018	14 AUG 2018	14 AUG 2018	14 AUG 2018
File covers	1970-present	1926-present	1973-present	1907-present plus more than 180,000 pre- 1907 records
File last updated	2 AUG 2018	8 Aug 2018	8 AUG 2018	13 Aug 2018
Language limit	No	No	No	No
Document types excluded that are not "scientific peer- reviewed open literature"	PATENT	PATENT	PATENT	PATENT
Search strategy	Details are listed in **CA 9.8.1.5**			
Total number of records retrieved	**0**	**0**	**0**	**2**

Table A1a-10 (continued) Details of the literature search for the lenacil metabolites (MEDLINE, EMBASE, TOXCENTER) Mammalian toxicology, Metabolism, Residues, Environmental fate, Ecotoxicology

Data requirement(s) captured in the search	Details of the searches		
	MEDLINE	*EMBASE*	*TOXCENTER*
Justification for choosing the source:	MEDLINE contains information on every area of medicine. More than 27.1 million records (04/2017)	The Excerpta Medica database, covers worldwide literature in the biomedical and pharmaceutical fields, including biological science, biochemistry, human medicine, forensic science, pediatrics, pharmacy, pharmacology and drug therapy, pharmacoeconomics, psychiatry, public health, biomedical engineering and instrumentation, and environmental science. More than 32.7 million records (07/2017)	TOXCENTER (Toxicology Center) is a bibliographic database that covers the pharmacologi-cal, biochemical, physiological, and toxicological effects of drugs and other chemicals. More than 12.9 million records (04/2017)
Date of the search	14 AUG 2018	14 AUG 2018	14 AUG 2018
File covers	1946-present	1974-present	1907-present
File last updated:	13 Aug 2018	13 Aug 2018	13 Aug 2018
Language limit:	No	No	No
Document types excluded that are not "scientific peer- reviewed open literature":	PATENT	PATENT	PATENT
Search strategy:	Details are listed in Details are listed in **CA 9.8.1.5**		
Total number of summary records retrieved	**0**	**0**	**0**

Table A1a-10 (continued) Details of the literature search for the lenacil metabolites (FSTA, PQSCITECH, ESBIOBASE, SCISEARCH) Mammalian toxicology, Metabolism, Residues, Environmental fate, Ecotoxicology

Data requirement(s) captured in the search	Details of the searches			
	FSTA	*PQSCITECH*	*ESBIOBASE*	*SCISEARCH*
Justification for choosing the source:	The Food Science and Technology Abstracts database provides worldwide coverage of all scientific and technological aspects of the processing and manufacture of human food products. Coverage includes basic food sciences, biotechnology, hygiene and toxicology, engineering, packaging, and all individual foods and food products. More than 1.3 million records (06/2017)	The ProQuest Science and Technology is a valuable and huge resource of over 27 Mio documents in all areas of science and technology from engineering to lifescience. More than 32 million records (07/2017)	Elsevier BIOBASE is a bibliographic current awareness database providing comprehensive coverage of the entire spectrum of biological research worldwide. Coverage includes the following areas: applied microbiology, biotechnology, cancer research, cell & developmental biology, clinical chemistry, ecological & environmental sciences, endocrinology, genetics, immunology, infectious diseases, metabolism, molecular biology, neuroscience, plant and crop science, protein biochemistry, and toxicology. More than 7.2 million records (05/2017)	Science Citation Index, one of the largest multidisciplinary scientific databases, is an international index to the literature covering virtually every subject area within the broad fields of science, technology, and biomedicine. More than 43 million records (08/2017)
Date of the search	14 AUG 2018	14 AUG 2018	14 AUG 2018	14 AUG 2018
File covers	1969-present	1962-present	1994-present	1974-present
File last updated	10 AUG 2018	25 JUL 2018	10 AUG 2018	13 Aug 2018
Language limit	No	No	No	No
Document types excluded that are not "scientific peer- reviewed open literature"	PATENT	PATENT	PATENT	PATENT
Search strategy	Details are listed in **CA 9.8.1.5**			
Total number of records retrieved	**0**	**0**	**0**	**0**
Total number of summary records retrieved after removing duplicates	**N=2**			

CA 9.8.2 **Search results**

CA 9.8.2.1 **Rapid and detailed assessment**

A total of 2 summary records were reviewed. One article was the EFSA Conclusions on confirmatory data of lenacil and a second article which had information related to the toxicology section.

From the 2 summary records reviewed, the article on the EFSA Conclusions on confirmatory data of lenacil2 was not considered relevant because it does not analyse any of the metabolites. After reviewing the abstract of the article related to the toxicology3 section, the article was considered irrelevant (RMS agrees: it pertains an unrelated reference to KE-121, pleaserefer to Table A1a-11).

Table A1a-11 Literature excluded by rapid assessment: Mammalian Toxicology

Nr.	Author(s)	Year	Title	Source	Reason(s) for exclusion
1	European Food Safety Authority (EFSA)	2013	Conclusion on the peer review of the pesticide risk assessment of confirmatory data submitted for the active substance lenacil	European Food Safety Authority	Public document produced also by the Rapporteur Member State (RMS) Belgium. Since there is no new information provided; then this document is considered already available by the RMS.
2	Ginj, Mihaela; Zhang, Hanwen; Eisenwiener, Klaus-Peter; Wild, Damian; Schulz, Stefan; Rink, Hans; Cescato, Renzo; Reubi, Jean Claude; Maecke, Helmut R.	2008	New Pansomatostatin Ligands and Their Chelated Versions: Affinity Profile, Agonist Activity, Internalization, and Tumor Targeting	Clinical Cancer Research (2008), 14(7), 2019-2027 CODEN: CCREF4; ISSN: 1078-0432	The publication was retrieved on the basis of the keyword KE121 (Metabolite of Lenacil IN-KE121). In the publication KE121 refers to a (cyclo-) peptide with D-diaminobutyric acid as basis (*"The parent peptide of these is cyclo(D-Dab-Arg- Phe-Phe-D-Trp-Lys-Thr-Phe) (KE121)"*). Therefore, it is assumed there is a similar abbreviation (by chance), but evidently totally different molecular formulas.

CA 9.8.2 **Conclusion**

The extra search on the lenacil metabolites generated no scientific paper which would be helpful for the toxicological risk assessment.

Following text reflects the finbal search strategy for both lenacil and its metabolites:

Original search query – complete STN search to be included. Raw data

(FILE 'HOME' ENTERED AT 11:22:08 ON 14 AUG 2018) SET ACCOUNT

FILE 'MEDLINE' ENTERED AT 11:22:26 ON 14 AUG 2018 CHARGED TO COST=106052
L1 0 SEA SPE=ON ABB=ON PLU=ON 1018147-92-6 L2 0 SEA SPE=ON ABB=ON PLU=ON 1270965-07-5
L3 0 SEA SPE=ON ABB=ON PLU=ON 3 1R 4R 4 HYDROXYCYCLOHEXYL 1H 5H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 DIONE
L4 0 SEA SPE=ON ABB=ON PLU=ON 3 2 OXOCYCLOHEXYL 1 5 6 7 TETRAHYDR OCYCLOPENTA D PYRIMIDINE 2 4 DIONE
L5 0 SEA SPE=ON ABB=ON PLU=ON 3 2 OXOCYCLOHEXYL 1H 5H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 DIONE
L6 0 SEA SPE=ON ABB=ON PLU=ON 3 4 HYDROXYCYCLOHEXYL 1 5 6 7 TETRAHYDROCYCLOPENTA D PYRIMIDINE 2 4 DIONE
L7 0 SEA SPE=ON ABB=ON PLU=ON 3 4 OXOCYCLOHEXYL 1 5 6 7 TETRAHYDR OCYCLOPENTA D PYRIMIDINE 2 4 DIONE
L8 0 SEA SPE=ON ABB=ON PLU=ON 3 4 OXOCYCLOHEXYL 1H 5H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 DIONE
L9 0 SEA SPE=ON ABB=ON PLU=ON 3 4 OXOCYCLOHEXYL 6 7 DIHYDRO 1H CYCLOPENTA D PYRIMIDINE 2 4 3H 5H DIONE
L10 0 SEA SPE=ON ABB=ON PLU=ON 3 4 OXOCYCLOHEXYL 6 7 DIHYDRO 1H CYCLOPENTA D PYRIMIDINE 2 4 3H 5H DIONE
L11 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 1H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 5 TRIONE
L12 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 2 7 DIHYDROXY 3 5 6 7 TETRAHYDRO 4H CYCLOPENTA D PYRIMIDIN 4 ONE
L13 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 5 HYDROXY 1H 5H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 DIONE
L14 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 6 7 DIHYDRO 1H CYCLOPENTA D PYRIMIDINE 2 4 5 3H TRIONE
L15 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 6 7 DIHYDRO 1H CYCLOPENTA E PYRIMIDINE 2 4 5 TRIONE
L16 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 6 7 DIHYDRO 7 1H CYCLO PENTAPYRIMIDINE 2 4 5 3H TRIONE
L17 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 6 7 DIHYDRO 7 1H CYCLOPENTAPYRIMIDINE 2 4 5 3H TRIONE
L18 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 7 HYDROXY 1 5 6 7 TETRAHYDROCYCLOPENTA E PYRIMIDINE 2 4 DIONE
L19 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 7 HYDROXY 1H 5H 6H 7H CYCLOPENTA D PYRIMIDINE 2 4 DIONE
L20 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 7 HYDROXY 6 7 DIHYDRO 1H CYCLOPENTA D PYRIMIDINE 2 4 3H 5H DIONE
L21 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 7 HYDROXY 1 5 6 7 TETRAHYDROCYCLOPENTA D PYRIMIDINE 2 4 DIONE
L22 0 SEA SPE=ON ABB=ON PLU=ON 3 CYCLOHEXYL 5 HYDROXY 1 5 6 7 TETRAHYDROCYCLOPENTA D PYRIMIDINE 2 4 DIONE L23 0 SEA SPE=ON ABB=ON PLU=ON IN KC 943
L24 0 SEA SPE=ON ABB=ON PLU=ON IN KC943

Appendix 1. Original search query – complete STN search to be included. Raw data

L25 0 SEA SPE=ON ABB=ON PLU=ON IN KD 304 L26 0 SEA SPE=ON ABB=ON PLU=ON IN KD304 L27 0 SEA SPE=ON ABB=ON PLU=ON IN KE 121 L28 0 SEA SPE=ON ABB=ON PLU=ON IN KE121 L29 0 SEA SPE=ON ABB=ON PLU=ON IN KF 313 L30 0 SEA SPE=ON ABB=ON PLU=ON IN KF313 L31 0 SEA SPE=ON ABB=ON PLU=ON IN KQ 957 L32 0 SEA SPE=ON ABB=ON PLU=ON IN KQ 961 L33 0 SEA SPE=ON ABB=ON PLU=ON IN KQ957 L34 0 SEA SPE=ON ABB=ON PLU=ON IN KQ961
L35 0 SEA SPE=ON ABB=ON PLU=ON (L1 OR L2 OR L3 OR L4 OR L5 OR L6 OR L7 OR L8 OR L9 OR L10 OR L11 OR L12 OR L13 OR L14 OR L15 OR L16 OR L17 OR L18 OR L19 OR L20 OR L21 OR L22 OR L23 OR L24 OR L25 OR L26 OR L27 OR L28 OR L29 OR L30 OR L31 OR L32 OR L33 OR L34)
L36 0 SEA SPE=ON ABB=ON PLU=ON L35 NOT PATENT/DT L37 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

FILE 'AGRICOLA' ENTERED AT 11:36:01 ON 14 AUG 2018 CHARGED TO COST=106052
L38 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

FILE 'BIOSIS' ENTERED AT 11:38:13 ON 14 AUG 2018 CHARGED TO COST=106052
L39 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

FILE 'CABA' ENTERED AT 11:39:35 ON 14 AUG 2018 CHARGED TO COST=106052
L40 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

FILE 'DDFU' ENTERED AT 11:42:28 ON 14 AUG 2018 CHARGED TO COST=106052
L41 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

FILE 'FSTA' ENTERED AT 11:43:45 ON 14 AUG 2018 CHARGED TO COST=106052
L42 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

FILE 'TOXCENTER' ENTERED AT 11:45:19 ON 14 AUG 2018 CHARGED TO COST=106052
L43 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

FILE 'NTIS' ENTERED AT 11:47:01 ON 14 AUG 2018 CHARGED TO COST=106052
L44 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

FILE 'IPA' ENTERED AT 11:48:22 ON 14 AUG 2018 CHARGED TO COST=106052
L45 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

FILE 'PQSCITECH' ENTERED AT 11:49:30 ON 14 AUG 2018 CHARGED TO COST=106052
L46 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY FILE 'EMBASE' ENTERED AT 11:51:06 ON 14 AUG 2018

Appendix 1. Original search query – complete STN search to be included. Raw data

CHARGED TO COST=106052
L47 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

FILE 'ESBIOBASE' ENTERED AT 11:54:25 ON 14 AUG 2018 CHARGED TO COST=106052
L48 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

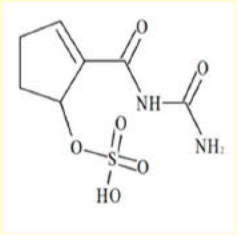
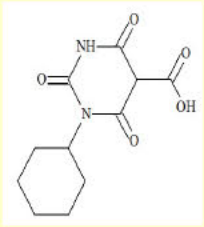
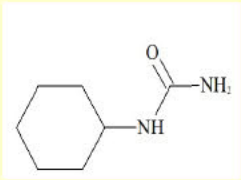
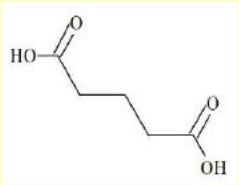
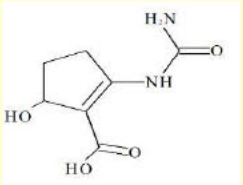
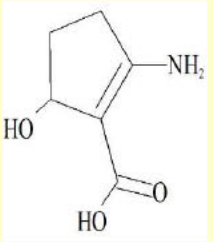
FILE 'HCAPLUS' ENTERED AT 11:56:31 ON 14 AUG 2018 CHARGED TO COST=106052
L49 2 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY D SCAN TI

FILE 'SCISEARCH' ENTERED AT 12:00:37 ON 14 AUG 2018 CHARGED TO COST=106052
L50 0 SEA SPE=ON ABB=ON PLU=ON L36 AND 2005-2016/PY

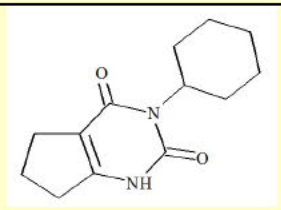
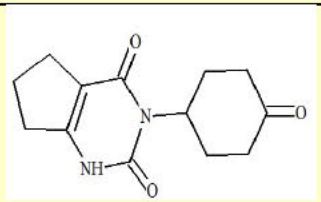
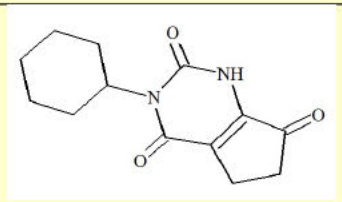
FILE 'HCAPLUS' ENTERED AT 12:03:35 ON 14 AUG 2018 CHARGED TO COST=106052
L51 2 DUP REM L37-L50 (0 DUPLICATES REMOVED) SAVE L51 LRRDUPREM/A
D TI 1-2

STN INTERNATIONAL SESSION SUSPENDED AT 12:04:56 ON 14 AUG 2018

APPENDIX 2: Key characteristics of the six polar metabolites of lenacil

NAME	IDENTIFIED POLAR	PROPOSED #1	PROPOSED #2	PROPOSED #3	PROPOSED #4	PROPOSED #5
ID	IPM1	PM1	PM2	PM3	PM4	PM5
STRUCTURE						
SMILES	NC(=O)NC(=O)C1=CCCC1OS(=O)(=O)O	OC(=O)C1C(=O)NC(=O)N(C1=O)C2CCCCC2	NC(=O)NC1CCCCC1	OC(=O)CCCC(=O)O	NC(=O)NC1=C(C(O)CC1)C(=O)O	NC1=C(C(O)CC1)C(=O)O
IUPAC	{2-[(carbamoylamino) carbonyl]cyclopent-2-en-1-yl}oxidanesulfonic acid	1-cyclohexyl-2,4,6-trioxo-1,3-diazinane-5-carboxylic acid	cyclohexylurea	pentanedioic aci		
MW	250.23	254.24	142.20	132.11	186.17	143.14
ALogP	-0.73	0.34	1.022	0.096	-1.196	-1.008
LogD @ pH 7.4	-2.97	-1.13	1.022	-2.648	-2.648	-2.407
$LogK_{ow}$ KOWWIN v1.68	-3.2	0.41	1.18	-0.26 -0.29 EXP	-1.62	-2.87

APPENDIX 3: Key characteristics of lenacil and non-polar metabolites IN-KE121 and IN-KF313

NAME	LENACIL	IN-KE121	IN-KF313
STRUCTURE			
SMILES	O=C1NC2=C(CCC2)C(=O)N1C3CCCCC3	O=C1CCC(CC1)N2C(=O)NC3= C(CCC3)C2=O	O=C1CCC2=C1NC(=O)N(C3 CCCCC3)C2=O
MW	234.29	248.28	248.28
ALogP	2.164	0.741	1.298
LogD @ pH 7.4	2.164	0.741	1.298
LogKow KOWWIN v1.68	3.09	1.04	3.111

Appendix 4: OECD QSAR Toolbox (v3.3.5) analysis of carcinogenicity alerts for lenacil and its groundwater metabolites

A4.1 Introduction

The following profilers were screened:

Carcinogenicity (genotoxic and nongenotoxic) alerts by ISS

Oncologic Primary Classification

(Carcinogenicity (genotoxic and nongenotoxic) alerts by ISS

This profiler is an expanded and updated version of the correspondent module of the software Toxtree. It works as a decision tree for estimating carcinogenicity, based on a list of 55 structural alerts (SAs). Out of them, 35 are derived from the Toxtree module and 20 are newly derived structural alerts for non-genotoxic carcinogenicity. Most of the new SAs are relative to non-genotoxic carcinogenicity whereas the SAs in the initial list mainly coded genotoxic carcinogenicity. The SAs for carcinogenicity are molecular functional groups or substructures known to be linked to the carcinogenicity activity of chemicals. As one or more SAs embedded in a molecular structure are recognised, the system flags the potential carcinogenicity of the chemical.

Oncologic Primary Classification

The oncologic primary classifier of molecular definitions developed by Laboratory of Mathematical Chemistry (LMC) and OCED to mimic the structural criteria of chemical classes of potential carcinogens covered by the U.S. Environmental Protection Agency's OncoLogic Cancer Expert System for predicting the carcinogenicity potential. In the QSAR Toolbox, the OncoLogic Primary Classifier is used solely for the purpose of categorisation based on the definition of an OncoLogic class. The profiler is introduced for categorisation purpose and not for predicting carcinogenicity.

A4.2 Summary of the results

Table A4.2-1: Results of screen for carcinogenicity structural alerts of lenacil and its metabolites

Substance	NAME	SMILES	Carcinogenicity (genotoxic and nongenotoxic) alerts by ISS	Oncologic Primary Classification
Lenacil	lenacil;3-cyclohexyl-6,7-dihydro-1h-cyclopenta[d]pyrimidine-2,4(3h,5h)-dione;3-cyclohexyl-6,7-dihydro-1h-cyclopentapyrimidine-2,4(3h,5h)-dione;3-cyclohexyl-1,5,6,7-tetrahydrocyclopentapyrimidine-2,4(3h)-dione	O=C1C2CCCC=2NC(=O)N1C1CCCCC1	No alert found, not genotoxic (tests)	Not classified
IN-KE121	3-(4-oxocyclohexyl)-1H,2H,3H,4H,5H,6H,7H-cyclopenta[d]pyrimidine-2,4-dion	O=C1CCC(N2C(=O)C3CCCC=3NC2=O)CC1	No alert found	Not classified
IN-KF313	3-cyclohexyl-1H,2H,3H,4H,5H,6H,7H-cyclopenta[d]pyrimidine-2,4,7-trione	O=C1CCC2=C1NC(=O)N(C1CCCCC1)C2=O	No alert found	Not classified
IPM1	{2-[(carbamoylamino)carbonyl]cyclopent-2-en-1-yl}oxidanesulfonic acid	NC(=O)NC(=O)C1=CCCC1OS(O)(=O)=O	alpha,beta-unsaturated carbonyls **(Genotox):Structural alert for genotoxic carcinogenicity**	Not classified
PM1	1-cyclohexyl-2,4,6-trioxo-1,3-diazinane-5-carboxylic acid	OC(=O)C1C(=O)NC(=O)N(C2CCCCC2)C1=O	No alert found	Not classified
PM2	cyclohexylurea;1-cyclohexylurea	NC(=O)NC1CCCCC1	No alert found	Not classified
PM3	carboxylic acids, c6-18 and c5-15-di-	OC(=O)CCCC(O)=O	No alert found	Not classified
PM4	2-(carbamoylamino)-5-hydroxycyclopent-1-ene-1-carboxylic acid	NC(=O)NC1CCC(O)C=1C(O)=O	No alert found	Not classified
PM5	2-amino-5-hydroxycyclopent-1-ene-1-carboxylic acid	NC1CCC(O)C=1C(O)=O	No alert found	Not classified

Appendix 5: OECD QSAR toolbox (v3.3.5) analysis of endocrine disrupting alerts for lenacil and its groundwater metabolites

A5.1 Introduction

The following profilers were screened:

A Oestrogen receptor binding

B rtER Expert System ver. 1 – USEPA

A Oestrogen receptor binding

A Estrogen receptor (ER) binding is a molecular initiating event much like protein binding. It is an endpoint where several comprehensive databases exist, which has led to the development of several approaches for using (Q)SARs to predict ER binding and possible subsequent endocrine disruption.

The incorporated Toolbox ER binding profiling scheme is based on structural and parametric rules extracted from literature sources and supported by experimental data. The ER-binding profiler classifies chemicals as non-binders or binders depending on molecular weight (MW) and structural characteristics of the chemicals:

1. Very strong binders: Chemicals with MW between 200 and 500 Da and two rings with a hydroxyl group connected to each of them.

2. Strong binders: Chemicals with at least one 5-or 6-members carbon ring with an unhindered hydroxyl or amino group and MW between 200 and 500 Da;

3. Moderate binders: Chemicals with at least one 5-or 6-members carbon ring with an unhindered hydroxyl or amino group and MW between 170 and 200 Da;

4. Weak binders: Chemicals with at least one 5-or 6-members carbon ring with an unhindered hydroxyl or amino group and MW less than 170 Da;

If the target chemical does not meet some of the structural and parametric requirements listed above it is classified as a Non-binder:

Non-binder with impaired hydroxyl or amino group;

Non-binder, MW more than 500 Da;

Non-binders without hydroxyl or amino group;

Non-binder, non-cyclic.

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The rtER Expert System ver.1 – USEPA profiler consists of molecular definitions which mimic the structural criteria of chemical classes potential estrogen receptor-binders covered by US EPA Estrogen Receptor Expert System (ERES). The ERES profiler is an effects-based automated system used to predict estrogen receptor binding affinity.

The ERES was originally developed to address a defined regulatory purpose, specifically for prioritizing chemicals from two specific inventories, food use pesticidal inerts (FI) and antimicrobial pesticides (AM) which do not include any chemicals with steroidal-type chemical structures, and thus not capable of higher affinity ER interactions. This system was built upon a training set of chemicals to cover the defined regulatory inventories using in vitro assays specifically optimized to pick up any indication of binding by testing up to chemical solubility or cytotoxicity within the assays to increase confidence that a chemical predicted negative is unlikely to bind ER. A chemical class-based approach was designed to allow extrapolating from a limited number of well-characterized TrSet chemicals to a broader inventory of chemicals by employing effects-based chemical category and read-across concepts.

The ERES is a logic rule-based decision tree that encodes the experts' mechanistic understanding with respect to both the chemical and biological aspects of the well-defined endpoint, or the ER bioassay domain. The transparency (relationship of predicted chemicals to tested chemicals) and usefulness of the system for the intended purpose (predictions provided for FI and AM chemicals) was emphasized in the approach to develop the ERES. For example, the relationship between relative binding affinity (RBA) and LogKow that was identified for the ERES chemical groups was used within each group to ensure the predicted chemical was bounded by TrSet chemicals. Chemicals falling outside the boundaries of known ability to predict (whether active or inactive) were considered to have "Unknown Binding Potential" (UnkBP). The automated version of the ERES enables users to compare the predicted chemical to TrSet chemicals within each chemical group (i.e., the decision tree node).

In the Toolbox, the rtER Expert System ver.1 – USEPA profiler is used for the purpose of categorization based on the structural definitions of the original ERES chemical classes. The rtER Expert System ver.1 – USEPA profiler is introduced for categorization purpose and not for predicting relative binding affinity (RBA).

A5.2 Summary of the results

Table 5.2-1: Results of screen for ED structural alerts for lenacil and its metabolites

Compound	SMILES	Profiler: Estrogen receptor binding	Profiler: rtER Expert System ver. 1 – USEPA
Lenacil	O=C1C2CCCC=2NC(=O)N1C1CCCCC1	Non binder, without OH or NH2 group	No alert found
IN-KE121	O=C1CCC(N2C(=O)C3CCCC=3NC2=O)CC1	Non binder, without OH or NH2 group	No alert found
IN-KF313	O=C1CCC2=C1NC(=O)N(C1CCCCC1)C2=O	Non binder, without OH or NH2 group	No alert found
IPM1	NC(=O)NC(=O)C1=CCCC1OS(O)(=O)=O	Non binder, without OH or NH2 group	No alert found
PM1	OC(=O)C1C(=O)NC(=O)N(C2CCCCC2)C1=O	Non binder, without OH or NH2 group	No alert found
PM2	NC(=O)NC1CCCCC1	Non binder, without OH or NH2 group	No alert found
PM3	OC(=O)CCCC(O)=O	Non binder, non-cyclic structure	No alert found
PM4	NC(=O)NC1CCC(O)C=1C(O)=O (MW = 186.2)	**Moderate binder, OH group**	No alert found
PM5	NC1CCC(O)C=1C(O)=O (MW = 143.1)	**Weak binder, OH group**	No alert found

A5.3 Discussion of the results

A Oestrogen receptor binding

The results obtained for 9 compounds can be divided into two groups: non-binders (7 out of 9) and weak/moderate binders (2 out of 9). In case of non-binders, either an OH (NH2) group or a ring is missing. In case of weak-moderate binders, the criterion is molecular weight: the smaller the MW, the weaker the binder; threshold between weak/moderate at 170 Da (g/mol).

Introduction for all compounds:

Oestrogen receptor (ER) binding is a molecular initiating event much like protein binding (1) that may lead to a series of adverse outcomes, which are typically linked to reproductive and development hazards. It is an endpoint where several comprehensive databases exist, which has led to the development of several approaches for using (Q)SARs to predict ER-binding and possible subsequent endocrine disruption (2). Popular among these are the "four phase" assessment that includes Comparative Molecular Field Analysis (CoMFA) (3) and the Common Reactivity Pattern Approach (COREPA) (4).

Characteristics of non-binders:

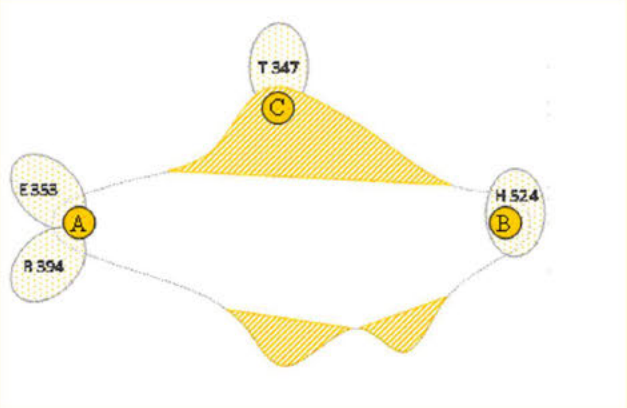
Non binder, without OH or NH2 group

Non binder, non-cyclic structure

Non-binder, without OH or NH2 group - chemicals with cycles and MW =<500 and without OH

Non-binder, non-cyclic structure – chemicals without cycles and MW =<500

Since the RE-binding is a receptor mediated event, particular organic functional groups, size and shape are critical to binding potency. A schematic representation of an ER binding pocket with its three sites of interaction (A, B, C) is shown in **figure A5.3-1** below:



Chemicals that have a molecular weight less than 500, and possess a cyclic structure but one without a hydroxyl or amino group are reported to be non-binders to the receptor (2, 3).

Chemicals that have a molecular weight of less than 500, but do not possess a cyclic structure are reported to non-binders to the receptor (2, 3).

Characteristics of binders:

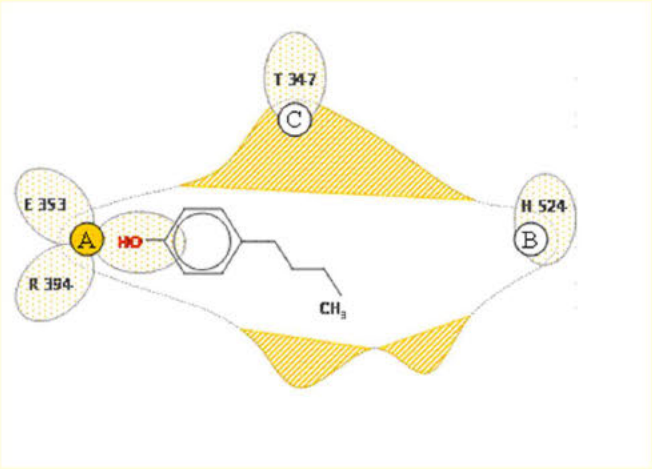
Moderate binder, OH group

Weak binder, OH group

Moderate binder, OH - MW >= 170 and MW =< 200 and with a non-impaired OH group attached to 5 or 6 C-atoms ring.

Weak binder, OH - MW < 170 and with a non-impaired OH group attached to 5 or 6 C-atoms ring.

Since the RE-binding is a receptor mediated event, particular organic functional groups, size and shape are critical to binding potency. A schematic representation of a hydroxylated ligand interacting at site A of the ER binding pocket is shown in **figure A5.3-2** below:.



Chemicals with a single 5-or 6-member carbon ring structure with an unhindered hydroxyl-group (-OH) (a hydroxyl group in the para- or meta-position on the ring and without ortho substituents to the hydroxyl group) (5) are ER binders. Binding potency is related to the size and shape of non-hydroxylated-ring aspect of the molecule, which can be grossly measured by molecular weight.

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No alert found for any of the compounds.

Available alerts are depicted schematically in **figure A5.3-3**.