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Draft guidance on aneugenicity assessment

EFSA Scientific Committee (SC)

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Abstract

The EFSA Scientific Committee was asked to provide guidance on the most appropriate *in vivo* tests to follow up on positive *in vitro* results for aneugenicity, and on the approach to risk assessment for substances that exhibit aneugenicity but do not induce gene mutation or clastogenicity. The Scientific Committee confirmed that the preferred approach is to perform an *in vivo* bone marrow micronucleus test with a relevant route of administration. If this is positive, it demonstrates that the substance is aneugenic *in vivo*. A negative result with evidence that the bone marrow is exposed to the test substance supports a conclusion that aneugenic activity is not expressed *in vivo*. If there is no evidence of exposure to the bone marrow, a negative result is viewed as inconclusive and further studies are required. The liver micronucleus assay, even though not yet fully validated, can provide supporting information for substances that are aneugenic following metabolic activation. The gastrointestinal micronucleus test, conversely, needs to be validated to assess aneugenic potential at the initial site of contact for substances that are aneugenic *in vitro* without metabolic activation. Based on the evidence in relation to mechanisms of aneugenicity, the Scientific Committee concluded that, in principle, health-based guidance values can be drafted for substances that are aneugenic but do not induce gene mutations or clastogenicity, provided that a comprehensive toxicological database is available. For situations in which the toxicological database is not sufficient to set up health-based guidance values, some approaches to risk assessment are proposed such as calculating the margin of exposure between a relevant reference point and the estimated human dietary exposure. The Scientific Committee recommends further development of the gastrointestinal micronucleus test, and research to improve the understanding of aneugenicity to support risk assessment.

Keywords

aneugenicity, micronucleus test, genotoxicity *in vivo* and *in vitro*

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1. Background and Terms of Reference as provided by EFSA

The genotoxicity testing strategy[1] indicated in the EFSA Scientific Committee opinion is designed to investigate the genotoxic potential of substances through the detection of three genotoxic endpoints: gene mutations, structural chromosomal aberrations (i.e. clastogenicity) and numerical chromosomal aberrations (i.e. aneuploidy).

The testing strategy is developed as a step-wise approach, beginning with a basic battery of *in vitro* tests, comprising:

- a bacterial reverse mutation assay [Organisation for Economic Co-operation and Development (OECD) TG 471, end-point: gene mutations]; *and*
- an *in vitro* mammalian cell micronucleus (MN) test (OECD TG 487, endpoints: clastogenicity and aneugenicity).

The ability of the compound to induce clastogenicity and/or aneugenicity can be discriminated *in vitro* through the *in vitro* MN test with centromere labelling [i.e. fluorescence *in situ* hybridisation (FISH) technique]. Clarification of the mechanism of action would make it possible to identify the most appropriate follow-up *in vivo* study, but this is not a mandatory requirement of the current testing strategy.

If positive results are observed *in vitro*, the substance should be tested in an appropriate *in vivo* test depending on the relevant end-point to be followed up.

The currently recommended *in vivo* tests are:

- the transgenic rodent assay (OECD TG 488, end-point: gene mutations);
- the *in vivo* mammalian alkaline comet assay (OECD TG 489, endpoints: DNA strand breaks as follow-up of compounds inducing gene mutations and/or clastogenicity);
- the mammalian erythrocyte MN test (OECD TG 474, endpoints: clastogenicity and/or aneugenicity).

Clastogenic substances induce structural chromosomal aberrations through breaks in DNA. Aneugenic substances induce numerical chromosomal aberrations through interactions with cellular targets other than DNA, such as proteins involved in the segregations of chromosomes during mitosis or meiosis. This difference in molecular targets results in features that are typical of aneugenic substances:

- a critical number of target sites must be affected before the aneugenic effect occurs;
- the onset of numerical chromosomal aberrations generally occur with steep dose–response curves in a narrow range of concentrations/doses;
- aneuploidy-inducing agents exhibit non-linear dose–response curves and a threshold is usually estimated.

According to the EFSA Scientific Committee (SC) Opinion on genotoxicity testing strategies (EFSA J, 2011) (Scenario IIa) if the available data show an aneugenic effect *in vitro*, an *in vivo* rodent MN test (in bone marrow or peripheral blood) would typically be considered appropriate. If an adequately conducted *in vivo* MN test (with evidence for significant exposure of the target tissue) is negative, it will be possible to conclude that the test substance is not aneugenic *in vivo*. However, if the *in vivo* MN test is negative and there is no demonstration that the target tissue was exposed, it cannot be concluded that there is no concern with respect to the aneugenic potential. Lack of demonstration of target tissue exposure could be due to several reasons including:

- the tested substance could be poorly absorbed or metabolised and eliminated before reaching the

[1] EFSA Scientific Committee, 2011. Scientific Opinion on Genotoxicity Testing Strategies Applicable to Food and Feed Safety Assessment. EFSA Journal 2011;9(9):2379, 69 pp. doi:10.2903/j.efsa.2011.2379

bone marrow;

- a clear demonstration that the aneugenic substance reaches the bone marrow in a sufficient amount could be difficult.

At present there are no internationally validated methods to investigate the potential aneugenic effect in tissues other than bone marrow, including the site of first contact. For food chemicals, the gastrointestinal tract (GIT) may represent a particularly relevant target because of the potential higher exposure, especially for substances that are poorly absorbed and scarcely bioavailable to systemic circulation.

Terms of Reference

Considering the current limitations in the evaluation of aneugenic substances, the SC is requested to develop guidance in relation to:

- what is the most appropriate *in vivo* follow-up for substances that are aneugenic *in vitro*;
- how should risk to human health be assessed for a substance exhibiting aneugenicity.

1.1. Audience and degree of obligation

This guidance is unconditional (i.e. required, see EFSA J, 2015) for the EFSA panels and units evaluating the genotoxicity of chemical substances in the food and feed safety area. It should be supported by sectoral guidance, if available. It also provides guidance to applicants submitting dossiers to EFSA.

2. Introduction

Mutational events can be subdivided into three categories: gene mutations, structural chromosomal aberrations (clastogenicity), and numerical chromosomal aberrations (aneugenicity). Aneugenicity designates chemically induced aneuploidy, which means a change in the chromosome number from the normal diploid or haploid number of chromosomes that is not a multiple of the haploid number. By contrast, polyploidy means an exact multiple of the haploid number of chromosomes. The two main processes leading to aneuploidy are non-disjunction at anaphase or lagging of chromosomes during cell division, both during mitosis and meiosis, resulting in loss or gain of individual chromosomes in the daughter cells (Parry et al., 2002a).

Chemicals inducing aneuploidy are called aneugens. They may interact with a number of different targets in the cell, including centromeres and telomeres, kinetochores and chromatid glue proteins involved in chromatid attachment and separation, tubulin, microtubule-associated proteins (MAPs), centrioles and other components of the spindle apparatus, the anaphase promoting complex, proteins involved in cell cycle control such as cyclins, cyclin-dependent kinases (CDKs), and p53, or targets indirectly involved in the cell cycle such as calmodulin, and the cellular or nuclear membrane (Kirsch-Volders et al., 2002). So, even though aneugenicity is a genotoxic end-point, the target of aneugens is not DNA itself, but rather proteins.

The consequence of aneuploidy is a difference in the number of copies of genes that are located on the missing or additional chromosome, and so a difference in the relative dosage of these gene products (Kirsch-Volders et al., 2019). If the genes concerned are transcriptional regulators, the dosage of other genes, located on the normally segregated chromosomes, may also be affected. Aneuploidy is recognised as a potential risk factor for cancer when impacting somatic cells, and teratogenicity, embryotoxicity/spontaneous abortions and impaired male fertility when impacting germ cells (Kirsch-Volders et al., 2002; Parry et al., 2002b).

The impact of aneuploidy during gametogenesis and embryogenesis is a significant cause of reproduction failure. Heritable aneugenic hazard has been characterised for a few chemicals in experimental models. Limited evidence suggests that male human germ cells might be more sensitive than mouse spermatocytes to chemically induced aneuploidy (Baumgartner et al., 2001, Adler et al., 2002). Although aneuploidy in

germ cells is a significant cause of infertility and pregnancy loss in humans, there is currently limited evidence that aneugens induce hereditary diseases in human populations because the great majority of aneuploid conceptuses die *in utero* (Pacchierotti et al., 2019).

Aneuploidy was observed at many stages of cancer progression in humans, although the evidence that it is a primary driver of carcinogenesis is limited. Human constitutive aneuploidies show increased risks of cancer early in life. As an example, individuals with Down's syndrome have an increased risk of acute megakaryoblastic leukaemia (AMKL) in childhood and constitutive trisomy 21 is viewed as an early event in carcinogenesis. In the available rodent carcinogenicity studies of known chemical aneugens it is difficult to characterise the role of aneuploidy in tumour induction and/or progression, as carcinogenic aneugens often show, in addition, other properties including gene mutation, structural chromosomal damage, toxicity, immunosuppression, epigenetic and hormonal effects (Tweats et al., 2019). However, growing scientific evidence indicates that aneuploidy in somatic cells is involved in the development of cancer. Chromosome loss (included in micronuclei) can be the result of chromothripsis (chromosome breakage) followed by a random recombination of the fragments in any position on chromosomes, leading to increased chromosomal instability (Guo et al., 2019). In addition, recognition of cytosolic self DNA contained in micronuclei (MN) by cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP–AMP) or cGAMP synthase (cGAS) may activate a proinflammatory pathway that promotes the metastatic phenotype (Bakhoum et al., 2018).

In its Opinion on genotoxicity testing for risk assessment of substances in food and feed, the SC of the European Food Safety Authority (EFSA) recommended an *in vitro* test battery consisting of a bacterial reverse mutation test (OECD TG 471), and an *in vitro* mammalian cell MN test (OECD TG 487) to cover all three genotoxic endpoints (EFSA J, 2011). The bacterial reverse mutation assay covers gene mutations and the *in vitro* MN test covers both structural and numerical chromosome aberrations. Aneugens are detectable in the MN test if a staining technique (FISH or CREST, see Annex A) is used to distinguish whole chromosomes from acentric chromosome fragments.

For a positive result *in vitro*, an *in vivo* follow-up test should cover the same end-point that was found to be positive *in vitro* (EFSA J, 2011). The only validated *in vivo* MN test is carried out in mammalian erythrocytes, sampled either from the bone marrow or peripheral blood cells of rodents (OECD TG 474). However, the usefulness of this test in the follow-up of *in vitro* aneugens may be limited when the test substance in its active form does not reach the bone marrow in sufficient amounts and, therefore, effects at other potentially relevant targets, such as the site of first contact, need to be evaluated. This is particularly the case when the test substance itself, rather than a metabolite, is the aneugenic substance, i.e. positive results are seen in the absence of metabolic activation. Because of these limitations, and the lack of an internationally agreed strategy for the *in vivo* assessment of aneugenic hazards, in this document the SC provides guidance on the *in vivo* follow-up of *in vitro* aneugens, and on the risk characterisation of substances evaluated as aneugenic *in vivo*. The current Opinion addresses purely aneugenic substances, yet if a substance is both clastogenic and aneugenic *in vivo*, the clastogenicity would already trigger a concern, and aneugenicity is not investigated further.

3. *In vivo* follow-up of *in vitro* aneugens

The mammalian erythrocyte MN Test (OECD TG 474), covering the endpoints of structural and numerical chromosomal aberrations of erythroblasts, is the only validated assay for the *in vivo* follow-up of *in vitro* aneugenic compounds. The experimental protocol was standardised to evaluate MN formation in young erythrocytes (polychromatic) sampled in bone marrow and/or reticulocytes of peripheral blood cells of rodents. Newly formed micronucleated erythrocytes are identified and quantitated by staining followed by visual scoring using a microscope. Automated analysis of micronuclei on cell suspensions using flow cytometry allows scoring of a large number of cells, reducing the scorer subjectivity and increasing the statistical power. The application of kinetochore staining or FISH with pan-centromeric probe provides the frequency of centromere-positive micronuclei, possibly confirming the aneugenic mechanism.

The erythrocyte MN test is one of the most widely used *in vivo* genotoxicity tests and it has been shown to be very sensitive in detecting aneugenic compounds that are systemically available. However, organ-specific compounds and unstable compounds or metabolites might not be detected with this assay. Negative results obtained in immature erythrocytes need to be supported by a proof of bone marrow exposure. The EFSA opinion (EFSA J, 2017b) specifically addressed the lines of evidence to demonstrate bone marrow exposure and to decide on the validity of the assay. Toxicity at the bone marrow level, detected as a reduction in the per cent of immature erythrocytes in total erythrocytes was considered as direct evidence of bone marrow exposure. However, evidence of systemic bioavailability can also be obtained from independent absorption, distribution, metabolism and excretion (ADME) and/or toxicity studies using the same route and same species.

Based on these considerations, the interpretation of the outcome from the *in vivo* erythrocyte MN assay needs to take into account the results from the *in vitro* studies (metabolic activation, condition of exposure) and all the available data on the substance, such as the chemical reactivity (which might predispose to site of contact effects), bioavailability, metabolism, toxicokinetics and any target organ specificity.

The scheme in Figure 1 describes the possible outcomes of the oral *in vivo* erythrocyte MN test with substances that are aneugenic *in vitro*. Positive results could be detected with systemically available compounds. These should be viewed as reliable and risk assessment should be conducted as described in Section 5.

Negative results obtained together with evidence of bone marrow exposure allow the conclusion that the substance is not aneugenic *in vivo* and therefore aneugenicity is not a safety concern.

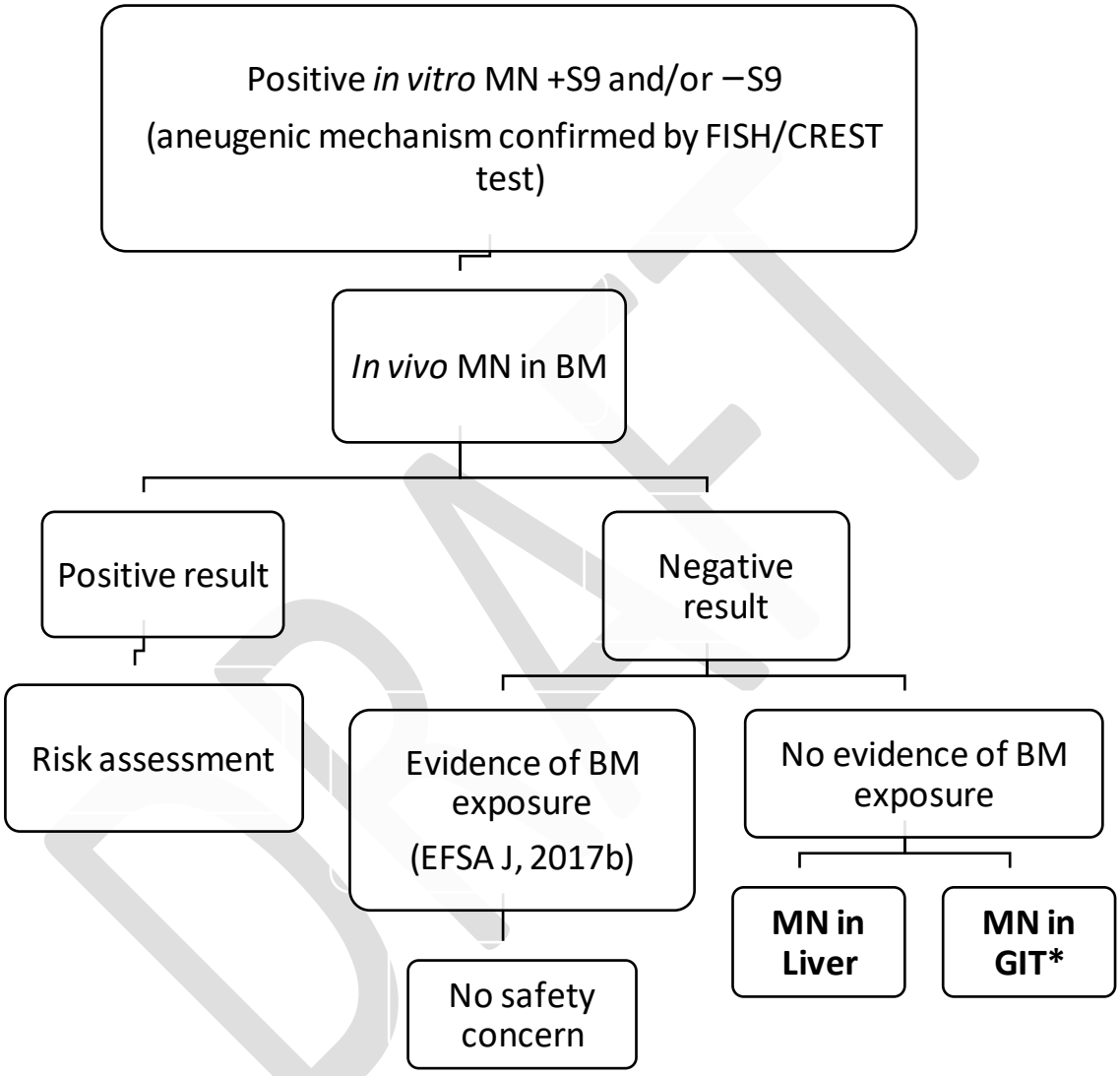
Negative results observed without evidence of bone marrow exposure may be unreliable for hazard assessment and further *in vivo* tests are needed to rule out the concern for *in vivo* aneugenic effects. In this case, different target organs must be considered for directly and indirectly acting genotoxic chemicals, as indicated in the scheme. Aneugenic compounds detected *in vitro* exclusively or predominantly in the presence of liver supernatant S9 fraction, suggesting the involvement of liver-specific metabolites, could be detected in liver. Direct aneugenic substances inducing *in vitro* increased micronuclei frequency in the absence of S9 mix could be detected at the site of first contact (e.g. GIT). However, although micronuclei induction has been studied in the liver and GIT for many years (see Annex A) validated standard methods have not yet been set up. The MN assay in these alternative tissues was applied to detect aneugenic compounds and genotoxic carcinogens with target organ specificity.

The state of the standardisation and validation of the MN assays in tissues other than bone marrow and peripheral blood were discussed at different International Workshops on Genotoxicity Testing (IWGT) during the meetings of the International Association of Environmental Mutagen Societies (IAEMS) (Hayashi et al., 2000; Hayashi et al., 2007; Uno et al., 2013; Uno et al., 2015a; Uno et al., 2015b). The IWGT Working Group in 2017 concluded that the liver MN assay is sufficiently validated for the development of an OECD guideline. The GIT MN assay appeared to be useful for the site-specific analysis of micronuclei induction and some evaluation of the sensitivity and specificity was possible, based on a very limited number of substances. However, many more substances would need to be tested to draw firm conclusions and the IWGT Working Group concluded that more work is required for an OECD guideline (Kirkland et al., 2019).

MN analysis in different target organs such as bone marrow, liver and GIT has the potential to be integrated into routine *in vivo* toxicity studies. Young rats (6 weeks old) are used for liver MN assays; periodical blood samples could be obtained from the same animals to also assess erythrocyte micronuclei. The major advantages of incorporating the MN analysis into conventional repeat dose toxicity assays are the reduction in the number of animals, saving time and the possibility to use kinetics data and general toxicology observations for the interpretation of genotoxicity results.

When following up on a positive *in vitro* result for aneugenicity in the presence of metabolic activation, there is potential for evaluating micronuclei in the liver and erythrocytes of the same animals to reduce the animal usage, cost and time required for consecutive testing.

When following up on a positive *in vitro* result for aneugenicity in the absence of metabolic activation, the application of the MN test also in the GIT should be considered. However, further development of micronuclei assessment in the GIT is required before the SC can make recommendations on its applicability as follow-up of substances that are aneugenic *in vitro*.



BM: bone marrow; GIT: gastrointestinal tract; MN: micronucleus test.
*: For a positive *in vitro* MN in the absence of S9, a GIT micronucleus assay would be appropriate, but is not yet sufficiently developed for validation.

Figure 1: Proposed aneugenicity testing scheme

4. Evidence for thresholds in aneugenicity

4.1. Theoretical considerations

The analysis of the dose–effect relationship (hazard characterisation) is a key component in chemical risk assessment, in which usually a reference point (also known as a point of departure) is identified. This is commonly the no observed adverse effect level (NOAEL) or the lower confidence limit of the benchmark dose (BMD) termed as the benchmark dose level (BMDL) associated with a specific benchmark response (BMR), identified from the dose–effect relationship for the critical effect (i.e. the relevant effect occurring at the lowest doses). Both approaches have limitations, however the BMD approach aims at estimating the dose that corresponds to a low, but measurable change in an adverse response. Health-based guidance values are derived from the reference point. This approach is currently not applied to substances that induce gene mutation or clastogenicity, for which a linear dose–effect relationship is assumed for the reaction with DNA. The hypothesis of linearity was first proposed for ionising radiations, based on the one-hit model of action of radiation, and subsequently extended to DNA reactive chemicals, in consideration of the prevailing mechanisms of mutagenicity that rely on single molecular interactions between the agent and its target (DNA).

Aneugens have non-DNA targets and induce abnormal chromosome segregation, interacting with a variety of molecular and structural targets of the mitotic/meiotic machinery within the cell. The induction of chromosome mis-segregation, leading to aneuploidy, requires multiple molecular interactions due to the multiplicity of copies of critical targets present in the cell, and the need to affect a critical number of these to elicit a functional effect. As a consequence, the shape of the dose–effect relationship will not be linear but will have a variable slope, depending on the number of involved events. Aneugenicity is typically observed in a narrow dose range. As the dose of aneugen increases, the cell division apparatus becomes more compromised resulting in cells with chromosome imbalances, and the potential for the effects of aneuploidy to be detected. Doses causing moderate cell cycle alterations may allow cells with imbalanced chromosome number to survive mitosis and aneuploidy, while higher doses inducing strong cell cycle alterations may not allow cell survival and/or completion of mitosis (Lynch et al., 2019). It is therefore widely accepted that genotoxicants with an aneugenic mode of action exhibit non-linear dose–response relationships with a dose below which no effects are observed and defined as the 'threshold dose'.

A threshold dose was defined by the World Health Organization (WHO, 2009) as 'the dose at which an effect just begins to occur', i.e. at a dose immediately below the threshold dose, the effect will not occur, and immediately above the threshold dose, the effect will occur. For a given chemical, there can be multiple threshold doses, in essence one for each definable effect. For a given effect, there may be different threshold doses in different individuals. Furthermore, the same individual may vary from time to time as to his or her threshold dose for any effect.'

Therefore, a threshold cannot be observed experimentally, but investigations of dose–response relationships can provide evidence for the existence of a threshold for specific effects such as aneugenicity.

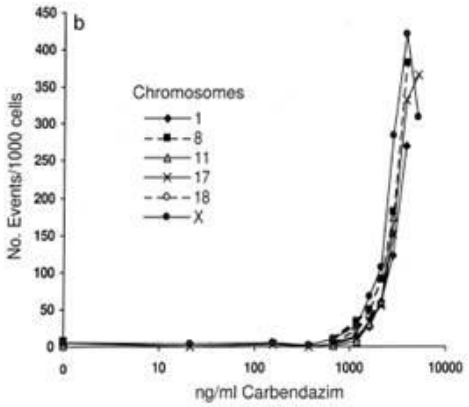
4.2. Threshold in aneuploidy – empirical evidence

Some aneugens have been shown to have well characterised threshold responses *in vitro* and *in vivo* using the induction of micronuclei as a surrogate end-point to measure chromosome loss, or the induction of non-disjunction using chromosome-specific probes.

Classical spindle poisons, such as colchicine, carbendazim, mebendazole and nocodazole, were chosen as model molecules to evaluate the possible threshold, because their mechanism of action is well known, consisting of direct interaction with the spindle fibres by inhibiting the polymerisation of tubulin. No increase in the frequencies of centromere-positive micronuclei, using FISH with a pancentromeric probe, in human peripheral lymphocytes was observed over a range of low concentrations. While a steep increase to highly statistically significant values of centromere-positive micronuclei was observed over a range of higher

concentrations (Elhajouji et al., 1995). As shown in Figure 2, similarly shaped dose–response curves have been obtained from another study using similar *in vitro* experimental conditions – binucleated human lymphocytes coupled with FISH – in which a thresholded mechanism of action for two spindle inhibitors, including carbendazim, has been reported (Bentley et al., 2000). The shape of this dose–response relationship is strongly indicative of a threshold.

Studies (Elhajouji et al., 1995; Marshall et al., 1996; Bentley et al., 2000; Tweats et al., 2016) on benomyl and carbendazim, frequently used as model aneugenic compounds, indicate that threshold values may differ depending on the end-point and scoring method applied, e.g. polyploidy or aneuploidy in metaphase preparations, non-disjunction or frequency of chromosome loss with the MN assay.



Amended from Bentley et al. (2000).

Figure 2: Aneugenicity occurrence in a narrow dose range of aneugens

The possibility of an aneuploidy threshold for these compounds was also investigated based on non-disjunction using chromosome-specific probes on binucleated cells in the cytokinesis-blocked MN assay. The results indicated that non-disjunction occurred at lower concentrations than chromosome loss (Zijno et al., 1996; Elhajouji et al., 1997). Results described in the published literature on the sensitivity of the two main endpoints and analysed to evaluate an aneugenicity threshold, the chromosome loss and non-disjunction, varied among the aneugens and the cell systems used, the chromosome chosen and the interpretation of results for non-disjunction and the method applied to analyse chromosome loss (Elloway et al., 2017).

Evidence for the existence of thresholds for aneuploidy induced by spindle poisons was also obtained *in vivo* using the flow cytometry peripheral blood MN test in mice. Dose–response curves observed for vinblastine, vincristine and colchicine, based on chromosome loss, were not linear and the results were consistent with *in vitro* findings (Cammerer et al., 2010).

5. Risk assessment of substances that are aneugenic but not clastogenic

5.1. Setting up reference points for aneugenicity

Although there is a general consensus that genotoxic agents acting using non-DNA-reactive mechanisms exhibit a thresholded, non-linear dose–response, an absolute no-effect threshold level cannot definitively be set up due to the impossibility of discerning experimentally no effect from small effects within the normal background range. Therefore, in the quantitative risk assessment of genotoxic agents, emphasis is placed on determination of reference points, rather than thresholds. From reference points, acceptable exposure levels can be determined by extrapolation using available mechanistic information and appropriate uncertainty factors, or be used in a margin of exposure approach.

There are currently no internationally accepted guidelines for the quantitative analyses of genetic toxicity.

Protocols to evaluate the dose–response curves for aneugenic compounds have been reported using the MN assay *in vitro* and *in vivo* as described below (Elhajouji et al., 1995; Cammerer et al., 2010; Tweats et al., 2016).

Dose–response analysis of data from the *in vitro* MN test using flow cytometry sorting and FISH with pancentromeric probe in human peripheral lymphocytes treated with colchicine, carbendazim, mebendazole and nocodazole was carried out by Elhajouji et al. (1995) applying a piecewise linear regression analysis. The piecewise linear regression analysis (or breakpoint regression) is a discontinuous regression model that reflects a 'jump' in the regression line. The equation used is the sum of a constant function (if the concentration is lower than the breakpoint) and a linear function (if the concentration is higher than the breakpoint). Determination of the breakpoint was carried out based on the results of the chi-squared test *P*-values. The first concentration that induced a statistically significant increase in the MNCen+ frequency compared with the control was chosen as the breakpoint. During the calculations of regressions, the program calculates the expected values (values of the regression curve at the given concentrations) and the correlation coefficients based on the data and the fixed breakpoints. Analysis comparing the observed and predicted values showed a high correlation for the tested substances (Elhajouji et al., 1995).

Evaluation of the threshold dose has been carried out *in vivo* using the flow cytometry peripheral blood MN test in mice (Cammerer et al., 2010). Vinblastine, vincristine and colchicine were tested. A specific study design was applied based on small size groups of animals, high range of doses (10 to 12) and the automated scoring of a high number of cells (around 20,000) to increase the statistical power. A specific statistical analysis of the dose–response curves was performed to determine the no observed-effect level (NOEL). The intersection point of piecewise regression lines was determined as the breakpoint of the dose–response curve (Cammerer et al., 2010).

An example of application of the BMD approach to evaluate *in vitro* and *in vivo* dose–responses and to derive a reference point was reported for the aneugenic anti-parasitic benzimidazole flubendazole, which has been used for many years to treat intestinal infections in humans and animals. The compound was shown to be aneugenic *in vitro* with the MN test in cultured human peripheral lymphocytes testing nine concentrations with the standardised protocol. A first *in vivo* experiment using the mouse bone marrow MN assay was negative without demonstration of bone marrow exposure. In a further experiment using a new oral formulation with improved bioavailability of flubendazole, micronuclei induction was observed. Analysis of the plasma from the treated animals showed that there was exposure to flubendazole. Analysis of the *in vivo* data allowed establishment of a reference point for aneugenicity, which could be compared with therapeutic exposures of flubendazole (Tweats et al., 2016). The BMR of one standard deviation above the spontaneous value was used in calculating the BMDL as the reference point for the *in vivo* MN dose–response data, following the recommendations of the International Workgroup on Genotoxicity Testing (MacGregor et al., 2015). The EPA's Benchmark Dose Software was applied and the polynomial model was selected as the most suitable for these data.

The SC considers that reference points for aneugenicity can be set up using dose–response analysis. The preferred approach is BMD modelling following the EFSA guidance (EFSA J, 2019b) and selection of the BMR needs to be justified. If BMD modelling is not possible, a breakpoint (NOEL) may be identified as the reference point using expert judgement.

5.2. Hazard characterisation for substances that are aneugenic but not clastogenic

As described above, a thresholded mode of action is plausible for aneugenic substances and, therefore, in principle a health-based guidance value (HBGV) can be set up based on the most sensitive end-point. So, the first step is to look at the entire toxicological database. Several scenarios can be envisaged, depending on the completeness of the database for the substance under consideration and its toxicological properties.

5.2.1. Data-rich substances, *in vivo* dose–response data are available for aneugenicity

If it has been possible to identify a reference point for aneugenicity, generally from an *in vivo* MN test using an appropriate study protocol, then this can be compared with the reference points for other effects. The reference point would preferably be identified applying the benchmark dose approach. However, even if it has not been possible to calculate a reliable BMDL, due to insufficient dose–response data, then comparison of the breakpoint (NOEL – see Section 4.1) for *in vivo* induction of micronuclei with the reference points for other toxicity endpoints can be informative about whether aneugenicity should be viewed as the most sensitive effect, i.e. that occurring at lowest dose levels.

If the reference point for aneugenicity *in vivo* is higher than that for another effect, HBGVs (acute and chronic) can be set up using the well-established principles applied for chemicals in food, as is seen in the examples in Table 1 (Annex B).

If the reference point for aneugenicity *in vivo* is lower than or close to those for other effects, an acute reference dose (ARfD) can be set up from the reference point, applying the 100-fold default uncertainty factor (i.e. 10 for interspecies and 10 for intraspecies differences). This approach is appropriate because aneugenicity is an acute effect and exhibits a steep dose–response relationship at doses higher than the breakpoint. In this situation, setting up an acceptable daily intake (ADI) is not considered necessary as the reference point for the ARfD would be the lowest from all the reference points in the database and so any ADI based on another effect would be higher.

5.2.2. Data-rich substances, *in vivo* data are not sufficient to set up a dose–response relationship for aneugenicity

If it has not been possible to identify doses associated with aneugenicity *in vivo*, for example if the MN assay is negative with insufficient evidence of exposure to the bone marrow, then the first approach is to seek additional data to follow up on the *in vitro* data. If that is not an option, or *in vivo* dose–response data for aneugenicity cannot be obtained, then it is not possible to set up an HBGV and a margin of exposure approach (MOE) for risk characterisation should be applied using the well-established principles applied for chemicals in food as described in Section 5.3.

5.2.3. Data-poor substances, i.e. gaps in the toxicological database

In vivo aneugenicity might or might not be available but overall the data are insufficient for establishment of an HBGV and one of the approaches described in Section 5.3 could be applied.

5.3. Risk characterisation for substances that are aneugenic but not clastogenic

If an HBGV (e.g. ADI/TDI, ARfD) has been set up following the approaches described in Section 4.2, then the ADI or ARfD is compared with the estimated chronic or acute dietary exposure, in line with the commonly accepted risk assessment paradigm.

If the data are insufficient to set up an HBGV, the MOE approach should be used to assess human risk. Similarly to setting an HBGV, the entire toxicological database and its limitations should be taken into account. The MOE should be calculated based on the lowest reference point from the available *in vivo* toxicological studies and interpreted taking into account the overall uncertainties in the toxicological database. For data-poor chemical substances, the reference point might be from a subchronic study. Currently, read-across from a structurally related chemical substance is not applicable due to the lack of knowledge on structure–activity relationships for aneugens. In each of these circumstances, the concentration or dose range for which aneugenicity was observed needs to be considered and the overall

uncertainties should be assessed in determining the size of MOE that is not expected to be a safety concern, using expert judgement. This would normally be larger than the default uncertainty factor (UF) of 100 applied to allow for interspecies and intraspecies differences because of the additional uncertainties related to gaps in the database, such as dosing duration, absence of NOAEL, absence of key toxicological studies. As described in the EFSA guidance (EFSA J, 2012), the preferred option is to request additional data but, if this is not feasible, then 'the use of an additional UF to take account of the deficiency of a database should be considered on a case-by-case basis and justified. It is not possible to propose a default value for this UF, as it will be directly dependent on the dataset available'.

In the event that there is no concern for gene mutations and clastogenicity and aneugenicity is observed but no other toxicological data are available, in principle, application of the threshold of toxicological concern (TTC) could be considered. If a reference point for aneugenicity can be identified, then the TTC approach can be applied to allow for the absence of toxicological data. Provided that the substance does not belong to one of the exclusion categories, the TTC scheme can be applied starting at step 4 (EFSA J, 2019b). The relevant TTC value can be applied provided that it is at least 100-fold lower than the reference point for aneugenicity. Otherwise the reference point for aneugenicity may be used in a MOE approach. The SC stressed that the TTC approach should not be used when toxicological data are available, or for regulated products requiring toxicological data.

For aneugens for which a reference point cannot be identified, the SC noted that the TTC approach cannot be currently recommended. This is because there is insufficient information on chemical structures leading to aneugenicity and whether aneugens are adequately represented in the TTC databases. Therefore, further investigations are needed and no conclusions on the risk to human health can be made.

A further possibility for substances that are aneugenic without metabolic activation (i.e. most likely to have effects at the site of contact) is to compare the concentrations resulting in aneugenicity *in vitro* with the estimated concentration of the substance in the GIT following ingestion of food or beverage. Estimation of the concentration of the substance in the GIT needs to take into account:

- possible dilution by the presence of other foods/beverages at the time of consumption, and by gastric juices;
- gastric/small intestine/large intestine passage time;
- whether the substance is likely to be altered by gastric juices or to react with other constituents in the GIT;
- information on absorption, which could decrease the concentration further along the GIT.

Taking these factors into account, if the concentration in the GIT is likely to be of a similar order of magnitude to concentrations shown to be aneugenic *in vitro*, there is concern for aneugenicity.

5.4. Risk assessment for combined exposure to multiple substances that are aneugenic but not clastogenic

For simultaneous exposure to multiple aneugenic agents, the default assumption of dose additivity (EFSA J, 2019a) should be used. The possibility of combination effects of aneugenic compounds with the same mechanism of action has been demonstrated *in vitro* by analysis of micronuclei induced by a mixture of seven benzimidazoles acting by binding to the colchicine-binding site on tubulin monomers and disrupting microtubule polymerisation. Additive effects were observed with the mixture of seven compounds at their estimated threshold concentrations as predicted according to the principles of concentration addition (Ermler et al., 2013). Studies on aneugens acting by different modes of action have not been found, but in line with the EFSA guidance (EFSA J, 2019a), the default assumption is dose additivity.

6. Conclusions

The SC stresses that this guidance applies specifically to substances that are aneugenic, but do not induce clastogenicity or gene mutations, for which other considerations are required. Based on the information on mechanisms and testing methods on aneugenicity, the current limitations of *in vivo* genotoxicity assays (EFSA J, 2017b) and recent developments in genotoxicity risk characterisation, the SC considered that the questions presented in the Terms of Reference (ToR) of this opinion can be addressed as follows:

6.1. Question 1: What is the most appropriate *in vivo* follow-up for substances that are aneugenic *in vitro*?

The preferred approach commences with an *in vivo* bone marrow MN test, conducted with a relevant route of administration and in accordance with OECD test guideline 474 (OECD, 2016). Evidence that the bone marrow is exposed to the test substance should be obtained following the EFSA opinion (EFSA J, 2017b). There are three possible outcomes to this test:

1) **A positive result**, which confirms that the substance is aneugenic *in vivo* and risk assessment is required.
2) **A negative result in a study testing an acceptable maximum dose with evidence of exposure to the bone marrow.** This supports the conclusion that the aneugenic activity observed *in vitro* is not expressed *in vivo*, and aneugenicity is not a safety concern (see Section 2.1).
3) **A negative result in a study testing an acceptable maximum dose without evidence of exposure to the bone marrow.** This is viewed as an inconclusive result and it is considered prudent to regard the substance as potentially active also under *in vivo* conditions, unless further follow-up studies are performed. Appropriate follow-up studies could include a liver MN assay, particularly for substances that produce positive results in the presence of metabolic activation *in vitro*. The SC notes that there is as yet no OECD guideline for the liver MN assay, but that it is sufficiently validated for development of a guideline (see also Section 2.1).

The SC also notes that aneugenic substances are frequently active without requiring metabolic activation, and therefore are likely to act at the first site of contact. There is no validated *in vivo* test that detects aneugenicity at the first site of contact.

Progress has been made in the development of the MN assay in the GIT. However, further development is required before the SC can make recommendations on its applicability for follow-up of substances that are aneugenic *in vitro*.

The SC presents its guidance in a step-wise approach, but suggests that consideration be given to combining the bone marrow and liver MN assays, to minimise use of experimental animals, cost and time. This would require a protocol based on the liver MN assay, with periodical removal of blood samples to assess micronudei in the reticulocytes.

For a positive aneugenic response *in vivo*, or if a conclusion on *in vivo* aneugenic potential cannot be drawn based on the available data, the evaluation should continue with risk characterisation (Question 2).

6.2. Question 2: How should risk to human health be assessed for a substance exhibiting aneugenicity?

There is a general consensus that genotoxic agents acting by non-DNA-reactive mechanisms exhibit a thresholded, non-linear dose–response. However, as for chemical substances that exhibit other types of toxicity, it is not possible to identify a threshold level. Therefore, the first step in risk assessment for an aneugenic substance is to identify a suitable reference point, to be used in risk characterisation.

Reference points for aneugenicity should be set up by analysis of dose–response data for induction of micronuclei from *in vivo* studies if available. The preferred approach is BMD modelling following the EFSA guidance (EFSA J, 2017a) and selection of the BMR needs to be justified. If BMD modelling is not possible, a breakpoint (NOEL) may be identified as the reference point using expert judgement.

The subsequent steps of risk assessment should be determined by the completeness of the dataset for the substance under consideration.

For substances with a comprehensive database (data-rich substances), for which a reference point for aneugenicity can be identified, this should be compared with the reference points for other toxicity endpoints, to identify the most sensitive effect. If the reference point for aneugenicity *in vivo* is higher than that for another effect, acute and chronic HBGVs, can be set up using the well-established principles applied for chemicals in food.

If the reference point for aneugenicity *in vivo* is lower than or close to those for other effects, an ARfD can be set up from the reference point, applying the default UF of 100 (i.e. 10 for interspecies and 10 for intraspecies differences). A chronic HBGV, such as an ADI, is not considered necessary as any ADI based on another effect would be higher than the ARfD, which is not meaningful. Risk characterisation then follows the commonly accepted paradigm of comparing the estimated acute and chronic exposure to the ARfD and ADI, respectively.

If it has not been possible to identify doses associated with aneugenicity *in vivo*, then the first approach is to seek additional data. If that is not an option, or *in vivo* dose–response data for aneugenicity cannot be obtained, then it is not possible to set up an HBGV, and an alternative approach should be identified (and justified) based on the available data and expert judgement. Possible approaches include:

- An MOE approach applied to the reference point for another toxicological end-point, taking into account the uncertainties in the entire toxicological database. The size of MOE that is not expected to be a safety concern, should be assessed using expert judgement, and would normally be larger than the default UF of 100 applied to allow for interspecies and intraspecies differences in setting HBGVs, because of the additional uncertainties related to gaps in the database.
- If no toxicological data other than genotoxicity data are available, and a reference point for aneugenicity can be identified, then the TTC approach can be applied to allow for the absence of toxicological data, as described in Section 5.
- For aneugens for which a reference point cannot be identified, the TTC approach cannot be currently recommended and further investigations are needed before the safety assessment can be completed.
- Comparison of the concentrations resulting in aneugenicity *in vitro*, with the concentration of substance estimated to be present in the GIT following ingestion of a food or beverage might provide additional information for site of contact exposure.

7. Recommendations

The SC recommends further development of the GIT MN assay before implementing it in the testing strategy. In addition, research to improve understanding of aneugenicity is required, including:

- establishment of structure–activity relationships for aneugens
- investigation of the applicability of the TTC approach to aneugens
- quantitative comparison of aneugenic activity of model compounds in immortalised cell lines and human primary cells
- relative sensitivity of rodent and human somatic and germ cells to aneuploidy induced by model compounds

- comparison of plasma or tissue levels associated with lowest effective doses *in vivo* with lowest effective concentrations *in vitro* (for the calibration of *in vitro* versus *in vivo* data).

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693

Annex A. Methods to detect aneuploidy

Aneuploidy is the result of malsegregation events, namely chromosome loss and chromosome non-disjunction, which give rise to changes in chromosome number. For chromosome loss, the chromosome lost during cell division may lead to the formation of an MN or may be re-incorporated in one of the two daughter nuclei randomly forming monosomic, trisomic or diploid cells. Chromosome non-disjunction results in one of the two daughter nuclei becoming trisomic and the other one monosomic. Methods are available to detect these effects in mitotic and interphase cells by microscopy analysis.

A.1. Detection of aneuploidy in mitotic cells

In mitotic cells, condensed chromosomes can be easily visualised and counted to determine whether increase (hyperploidy) or reduction (hypoploidy) of their number are induced. To allow the expression of the aneugenic effect of a treatment, the analysis needs to be restricted to metaphase cells that have divided twice in culture (Parry et al., 1995). In addition, only hyperploid cells should be recorded to avoid the inclusion of false aneuploid cells resulting from the mechanical loss of chromosomes during the preparation of the slides.

Besides chromosome counting methods, also the study of abnormalities in cell division through the investigation of mitotic spindle structure and chromosome segregation can be indicative of a potential aneugenic activity. The analysis of the spindle apparatus can be achieved by use of specific staining procedures involving a fixation technique to maintain the integrity of the spindle apparatus and differential staining to visualise proteins of the spindle and chromosomes. In particular, the application of this visualisation technique is suitable for the quantification of the so-called C-metaphases, namely cells lacking a mitotic spindle, typically resulting from the action of inhibitors of the spindle apparatus, such as colchicine (Parry and Parry, 1987). Conversely, analysis of chromosomes at anaphase makes it possible to identify lagging chromosomes. Single lagging chromosomes or chromatids can be detected in bipolar ana-telophases, while more severe damage can be detected in cells at anaphase, i.e. C-anaphases (Minissi et al., 1999).

A.2. Detection of aneuploidy in interphase cells

A.2.1. Analysis of binucleated cells

Acentric chromosome fragments or whole chromosomes not included in the daughter nuclei at the end of mitosis may give rise to micronuclei when the cells enter subsequent interphase. The analysis of micronudei in interphase cells allows faster and easier measurement of chromosome damage compared with the count of chromosomes particularly when the cytokinesis-block assay is applied. This is a method that improves the accuracy and sensitivity of the MN test, focusing the analysis on cells that have divided only once after treatment (Fenech, 2007). These cells can be detected as binucleated cells using the inhibitor of cytokinesis cytochalasin B (CytB), which prevents the separation of the cytoplasm into two daughter cells after mitosis and leads to the formation of cells with two nuclei within a cytoplasm (binucleated cells).

As the origin of micronuclei is associated with the induction of both structural and numerical aberrations, it is necessary to characterise the content of micronuclei to clarify the mechanism of their induction; here, micronuclei containing whole chromosomes are considered the result of an aneugenic event, while micronuclei containing acentric chromosome fragments are related to clastogenic activity. Several methods have been applied to discriminate aneugens from clastogens, such as the size-classified counting of micronuclei based on the assumption that increased frequency of large-size micronuclei could be an alerting index for aneugenic effects; in addition, C-banding was applied as well as measurement of DNA content in micronuclei (Parry et al., 2002a). However, the use of molecular cytogenetic methods allows a more precise and reliable classification of micronuclei.

Here, the approaches most widely used to distinguish between clastogenic and aneugenic effects are based on the detection of the centromere into the MN by immunochemical labelling of kinetochores using anti-

kinetochore antibodies obtained from the serum of patients with the autoimmune disease scleroderma, formerly called CREST (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly and telangiectasia) syndrome, (CREST analysis) and FISH with DNA probes specific for centromeric regions (FISH analysis). Occasionally CREST staining could falsely identify micronuclei with whole chromosomes as micronuclei containing acentric chromosome fragments when the test compound: (i) causes the detachment of the kinetochore; (ii) produces an alteration of the kinetochore epitope targeted by the CREST-antibody; and (iii) binds to the kinetochore epitope, interfering with antibody recognition. The interaction of substances such as caffeine and mitomycin C with the kinetochore and consequences for reliability of the CREST analysis has been known for decades (Miller et al., 1991). However, while the specific characteristics of the two techniques should be taken into account in the evaluation of the data, both CREST and FISH can be considered reliable tools to obtain information on the origin of micronuclei and the mechanisms of genotoxicity, as also indicated in OECD TG 487 (OECD, 2016a).

A.2.2. Simultaneous detection of chromosome loss and non-disjunction

The relative contribution of chromosome loss and non-disjunction to aneuploidy induction can be investigated following the segregation of single chromosomes in the two daughter nuclei at the completion of mitosis; this can be achieved applying the FISH technique with centromeric DNA probes for a specific chromosome in association with the cytokinesis-block MN assay (Zijno et al., 1994). By this approach it is possible to recognise the correct segregation of an autosomic chromosome when two fluorescent spots per nucleus are observed in the binucleated cells (two diploid cells), while malsegregation events are identified by an unbalanced distribution of the specific chromosome. For non-disjunction, one nucleus contains three spots (hyperploid cell) and the other nucleus one spot (hypoploid cell). Conversely, chromosome loss is identified when a nucleus contains two spots (diploid), the other nucleus one fluorescent spot (hypoploid) and the MN one spot, corresponding to the lost chromosome. With this method, technical artefacts can be excluded from the total counting as only binucleated cells with the expected number of labelled chromosome are included in the analysis independently of the distribution of the spots in the two daughter nuclei.

A.2.3. Detection of monosomy and trisomy in mononucleated cells

The *in situ* hybridisation technique, DNA probes complementary to centromeric sequences of specific chromosomes have also been applied to easily estimate the frequency of monosomic and trisomic cells (Eastmond and Pinkel, 1990). This procedure allows the measurement of aneuploidy induction by counting the number of labelled signals, representing the chromosome of interest. Simultaneous hybridisation with adjacent double-labelled pericentromeric probes (Eastmond et al., 1993) has been proposed to partially overcome any technical limitations that may reduce the reliability of this approach. Indeed, elevated frequency of apparently trisomic cells has been reported and associated with non-specific hybridisation or breakage within the hybridisation region (Eastmond and Pinkel, 1990). In addition, the original method could be relatively insensitive for detection of hypoploidy, like most of the classical cytogenetic techniques, because overlap of two spots may occur resulting in visualisation of a single signal. Another limitation is related to the application of this approach to cell culture in the absence of cytB. The inability to distinguish subpopulations of cells that have divided once from the others, in fact, may introduce a bias in the assessment of aneugenic effects.

A.2.4. Battery of tests for the identification of aneugens

An additional approach to identify an aneugenic effect could rely on the comparison, in the same experimental system, of results obtained in the MN test and those obtained in assays that measure only DNA strand breaks (chromosome aberrations and comet assays). Positive results obtained in both the MN and DNA strand breaks assays would be indicative of clastogenic activity. In contrast, negative results in the DNA strand break assays associated with a positive MN test are indicative that MN formation is due to the loss of chromosomes. The subsequent characterisation of the content of MN by FISH analysis will confirm the induction of aneuploidy and elucidate the mechanism of malsegregation underneath.

A.2.5. Detection of aneuploidy in mononucleated cells

It is generally recommended to restrict the analysis of micronuclei to binucleate cells, but recently some studies have reported that aneugens increase the frequency of micronuclei in mononucleate and binucleate cells, while clastogens induce the formation of micronuclei only in binucleated cells (Elhajouji et al., 1995; Kirsch-Volders and Fenech, 2001; Dutta et al., 2007; Kirkland, 2010). The unknown origin of micronuclei in mononucleated cells, however, represents a strong limitation for the reliability of this approach.

A.3. Micronucleus assay techniques in tissues other than bone marrow and peripheral blood

The mammalian erythrocyte MN test (OECD, 2016b), which is performed on bone marrow or peripheral blood may not detect substances or their metabolites that do not reach the target cells in sufficiently high concentrations or which are too short lived to reach the target cells. Therefore, several attempts have been made in the past to perform the MN assay in liver and other tissues.

Amendments to the MN assay have already been developed several years ago (see e.g. Barbason et al., 1975; Tates et al., 1980; Braithwaite and Ashby, 1988; Das and Roy, 1988; Cliet et al., 1989; Uryvaeva and Delone, 1995; Parton and Garriott, 1997; Igarashi and Shimada, 1997; etc.). Most studies were carried out using MN assays in liver (reviewed by Morita et al., 2011; Uno et al., 2015a) but there are also studies on several other organs including spleen (Krishna et al., 1990; Benning et al., 1992; Benning et al., 1994), oesophagus (Mehta et al., 1987), stomach and colon (reviewed by Uno et al., 2015b).

As proliferating cells are required for the MN assay and hepatocyte turnover is slow, several methods have been developed for stimulation of hepatocyte proliferation (Uno et al., 2013; Uno et al., 2015a). Partial hepatectomy was initially used to stimulate hepatocyte proliferation, e.g. by Barbason et al. (1975) and Tates et al. (1980). Chemical mitogens were later used by Braithwaite and Ashby (1988) and Uryvaeva and Delone (1995). However, this method seems to be no longer in use, as the mitogens might interfere with the genotoxic response (Uno et al., 2013; Martus et al., 2015; Uno et al., 2015a). Parton and Garriott (1997) developed a MN assay using juvenile rats without partial hepatectomy or chemical mitogens considering that the percentage of hepatocytes in S-phase is about 5% in 4-week-old rats, while it is much less in adult rats. More recently, a MN assay using young (6-week-old) adult rats and repeated-dose administration (for 14 or 28 days) have been developed based on the observation that micronuclei could accumulate in the liver of adult rats (Narumi et al., 2012; Takasawa et al., 2013; Narumi et al., 2013).

A Collaborative Study Group of the Micronucleus Test (CSGMT), a subgroup of the Mammalian Mutagenicity Study Group (MMS), of the Japanese Environmental Mutagen Society (JEMS), organised several collaborative studies to evaluate MN methods detecting chromosomal aberrations in the liver and the GIT (Ohyama et al., 2002; Suzuki et al., 2005; Suzuki et al., 2009; Takasawa et al., 2010a; Takasawa et al., 2010b; Hamada et al., 2015).

Micronucleus assays in tissues other than bone marrow and peripheral blood were discussed at the 2nd, 4th, 6th and 7th International Workshop on Genotoxicity Test Procedures (IWGTP) under the umbrella of the IAEMS in 1999, 2005, 2013 and 2017, respectively (Hayashi et al., 2000; Hayashi et al., 2007; Uno et al., 2013; Uno et al., 2015a; Uno et al., 2015b; Kirkland et al., 2019). The strengths and limitations of these methods and the need for further data were described in the report of the 6th IWGTP (Uno et al., 2015a; Uno et al., 2015b). Since then, further studies have been performed on specific aspects of these methods addressing the issues identified at the 6th IWGTP (e.g. Itoh et al., 2015; Shimada et al., 2015c; Shigano et al., 2016; Avlasevich et al., 2018; Hori et al., 2019; Itoh and Hattori, 2019).

In 2013, the IWGT working group recommended protocols for the liver MN test (LMNT), agreed on the most likely situations in which this method would be applicable and 'reached consensus that the LMNT is a useful and promising assay for regulatory use' (Uno et al., 2015a). At that time, the Working Group, however, also pointed out that further actions for development will be needed for the liver MN assay (Uno et al., 2015a). Based on additional data, the IWGT Working Group concluded in 2017 that the liver MN

assay is sufficiently validated for the development of an OECD guideline and that for the GIT MN assay, some evaluation of the sensitivity and specificity is possible. However, the Working Group also noted that more work is required for an OECD guideline for the GIT MN assay (Kirkland et al., 2019).

Overall, among the MN assays in tissues other than bone marrow and peripheral blood, the repeated-dose liver MN assay in young (6-week-old) adult rats reached the highest level of development and validation. It belongs to the assays for which internationally agreed protocols have already been set up and it can be combined with repeated-dose general toxicity studies and with other repeated-dose genotoxicity assays such as the bone marrow/peripheral blood MN assay (OECD, 2016b). In addition, there is some evidence that it can also be combined with a gene mutation assay (Hori et al., 2019). The available data suggest that the GIT MN assay could be integrated into general toxicity studies. However, additional data are required for the evaluation of its sensitivity and specificity.

Annex A. References

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Annex B. Examples of active substances (possibly) causing aneugenic effects

Table 1: Examples of active substances (possibly) causing aneugenic effects that have previously been evaluated for the maximum residue limit (MRL) classification for use in food producing animals. Note that these are published examples and have not been re-evaluated by EFSA for this guidance document.

Active substance	Genotoxicity	Experi-mental evidence of aneu-genicity	Carcinogenicity	Risk assessment	CR (EU) 37/2010 entry*	Reference**
Colchicine	Positive results *in vivo* and *in vitro* for gene and chromosome mutation. Aneugenic potential *in vivo* (1 mg/kg) and *in vitro* (0.006 µg/ml)	Yes	No data available	No ADI could be set up based on genotoxicity, teratogenicity, effects on fertility, no data on absorption after local treatment of cattle and horses)	Table 2	EMEA/MRL/044/95-FINAL
Benzimidazole derivatives						
Albendazole	*In vivo* aneugens	Yes	No evidence of neoplasia in either rats or mice in suitable carcinogenicity bioassays	ADI of 0–0.005 mg/kg bw per day based on NOEL for teratogenicity of 5 mg/kg per day and a safety factor of 1000	Table 1	MRL Summary Report (1) (no reference number available) and EMEA/MRL/865/03-FINAL
Albendazole oxide		Yes	No studies with albendazole oxide	ADI of 0–0.005 mg/kg bw per day based on albendazole data	Table 1	EMEA/MRL/555/99-FINAL February 1999
Netobimin		Yes	No studies with netobimin	ADI of 0–0.005 mg/kg bw per day based on albendazole data	Table 1	EMEA/MRL/556/99-FINAL April 1999

Mebendazole	Not a direct acting mutagen or clastogenic. Aneugenic in mammalian somatic cells. Not possible to identify an *in vivo* NOEL for aneugenicity, but a no-effect concentration of 85 ng/ml was identified from *in vitro* FISH studies	Yes	No evidence of carcinogenicity in rats and mice but studies were considered inadequate	ADI of 0.0125 mg/kg bw per day based on NOEL of 2.5 mg/kg bw per day in a 13-w study in dogs and in developmental toxicity studies in rats and mice using a safety factor of 200	Table 1	EMEA/MRL/625/99-FINAL July 1999 and EMEA/MRL/781/01-FINAL March 2001
Thiabendazole	No gene mutation or structural chromosomal damage. Consistent evidence of aneugenicity *in vitro*. Negative in validated oral *in vivo* mutagenicity assays. Some reports of aneuploidy in bone marrow cells *in vivo* following intraperitoneal administration	Yes, but conclusion that negative *in vivo* with the oral route	No excess incidence of any type of tumour in mice. In rats, no increases in tumours except thyroid follicular adenomas, which was considered to be due to by a nongenotoxic mechanism related to liver enlargement	ADI of 0.1 mg/kg bw based on an overall toxicological NOEL of 10 mg/kg bw/day for a range of toxicological endpoints including effects on the liver, thyroid, and bone marrow, spleen, effects on reproductive performance, teratogenicity in mice and fetotoxicity in rats. A safety factor of 100 was applied	Table 1	EMEA/MRL/868/03-FINAL June 2004
Oxfendazole	Benzimidazole compound, which are known to be mitotic spindle poisons. The mutagenicity data available for febantel, fenbendazole and oxfendazole show no clear evidence of genotoxicity and although no specific tests for aneugenicity have been conducted, the clastogenicity studies that have been conducted are generally reassuring'	No	No evidence of carcinogenicity in rats or mice	ADI of 7 µg/kg bw per day based on the NOEL of 0.65 mg/kg bw per day for hepatic vacuolation in a carcinogenicity study in rats with a safety factor of 100 to febantel, fenbendazole and oxfendazole share the same metabolism with oxfendazole being most toxic	Table 1	EMEA/MRL/888/03-FINAL June 2004
Fenbendazole	Benzimidazole compound, which are known to be mitotic spindle poisons. The mutagenicity data available for febantel, fenbendazole and oxfendazole show no clear evidence of genotoxicity	No	No evidence of carcinogenicity in rats or mice	ADI of 7 µg/kg bw per day, based on toxicity data for oxfendazole	Table 1	EMA/CVMP/914694/2011

	and although no specific tests for aneugenicity have been conducted, the clastogenicity studies that have been conducted are generally reassuring'					
Febantel	Benzimidazole compound, which is known to be a mitotic spindle poison. The mutagenicity data available for febantel, fenbendazole and oxfendazole show no clear evidence of genotoxicity and although no specific tests for aneugenicity have been conducted, the clastogenicity studies that have been conducted are generally reassuring	No	No evidence of carcinogenicity in rats and mice	ADI of 7 µg/kg bw per day, based on toxicity data for oxfendazole	Table 1	EMEA/MRL/192/97-FINAL June 1997
Benzimidazole derivatives without indication of aneugenicity in the MRL summary reports						
Flubendazole	Negative for gene mutation, DNA damage, a sex-linked recessive lethal assay in Drosophila melanogaster, a dominant lethal assay in mice and *in vivo* micronucleus tests in rats and mice	No	No evidence of carcinogenicity in rats and mice although both studies were marred by poor survival	ADI of 0–12 µg/kg bw per day based on the NOEL of 2.5 mg/kg bw per day in a 3-month study in dogs, with a safety factor of 200	Table 1	EMEA/CVMP/33128/2006-FINAL
Triclabendazole	Negative in a range of genotoxicity studies, including MN in hamster bone marrow	No	No evidence of carcinogenicity in rats and mice	ADI of 0.0015 mg/kg bw, based on a NOEL of 0.15 mg/kg bw/day for increased post-partum mortality of the F_2 generation in a two-generation rat reproduction study and an UF of 100	Table 1	No reference number of CVMP's MRL Summary Report (1) available ***

*CR (EU) 37/2010: Use of active substances in veterinary medicinal products for food producing animals; Table 1 substances with numerical MRLs or 'No MRL required' classification, made it possible to be used in veterinary medicines; Table 2 – prohibited substances

**MRL list available: https://www.ema.europa.eu/en/find-medicine/veterinary-medicines/maximum-residue-limit-assessment-reports/2

*** https://www.ema.europa.eu/en/documents/mrl-report/triclabendazole-summary-report-1-committee-veterinary-medicinal-products_en.pdf

Annex C. List of Abbreviations

ADI	Acceptable Daily Intake
ADME	Absorption, Distribution, Metabolism, Excretion
AMKL	Acute Megakaryoblastic Leukaemia
ARfD	Acute Reference Dose
BMD	Benchmark Dose
BMDL	Benchmark Dose Level
BMR	Benchmark Response
CDKs	Cyclin-dependent kinases
cGAMP or cyclic GMP–AMP	Cyclic guanosine monophosphate–adenosine monophosphate
cGAS	Cyclic guanosine monophosphate–adenosine monophosphate synthase
CREST	Calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly and elangiectasia
CSGMT	Collaborative Study Group of the Micronucleus Test
CytB	Cytochalasin B
EFSA	European Food Safety Authority
FISH	Fluorescence *in situ* hybridisation
GIT	Gastro-Intestinal Tract
GIT MN assay	Gastro-Intestinal Tract Micronucleus assay
HBGV	Health-based Guidance Value
IWGT	International Workshop on Genotoxicity Test
IWGTP	International Workshop on Genotoxicity Test Procedures
JEMS	Japanese Environmental Mutagen Society
LMNT	Liver Micronucleus test
MAPs	Microtubule-Associated proteins
MMS	Mammalian Mutagenicity Study
MN	Micronucleus/micronuclei
MOE	Margin of Exposure
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level

MNCen+	Micronuclei centromere-positive
OECD	Organisation for Economic Co-operation and Development
OECD TG 474	The mammalian erythrocyte MN test
OECD TG 488	Transgenic rodent assay
OECD TG 489	*In vivo* mammalian alkaline comet assay
SC	Scientific Committee
TDI	Tolerable Daily Intake
ToR	Terms of Reference
TTC	Threshold of Toxicological Concern
UF	Uncertainty Factor
WHO	World Health Organization