The core *in vitro* test battery – 2 or 3 tests?

David Kirkland

Kirkland Consulting

Background

- The *in vitro* tests recommended in the EFSA 2011 guidance are an Ames test + an *in vitro* micronucleus (MNvit) test
- This is consistent with a paper we (Kirkland et al.) published in 2011, with the recommendations of the UK Committee on Mutagenicity (2011) and the GUM (Pfuhler et al., 2007)
- Ames + MNvit detects all 3 key modes of action (gene mutations, clastogenicity and aneugenicity)
- However, for our 2011 paper, we questioned whether a mammalian cell gene mutation test (MCGM) is also needed to detect rodent carcinogens and *in vivo* genotoxins?
- We therefore analysed whether 2 or 3 tests were needed to detect rodent carcinogens and *in vivo* genotoxins.

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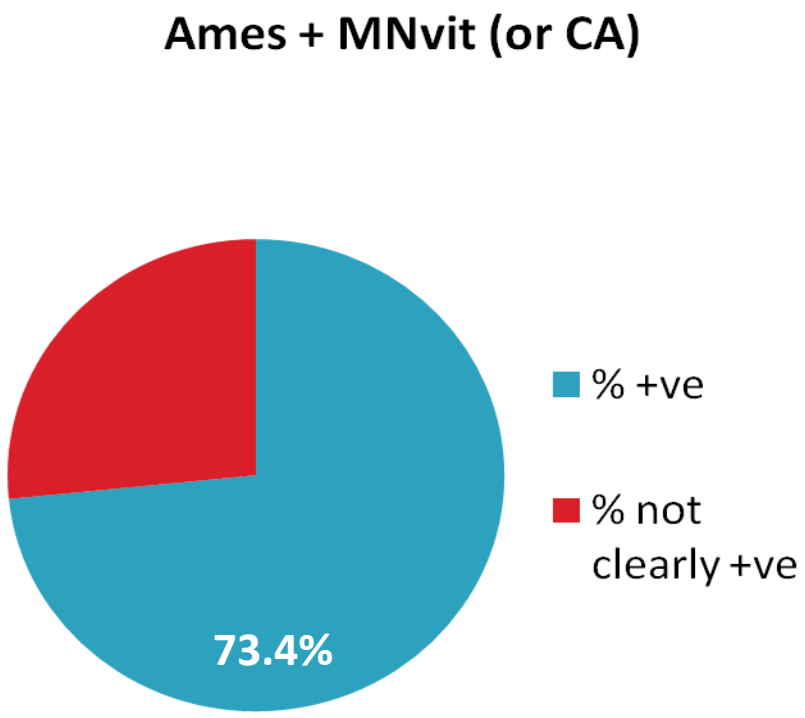
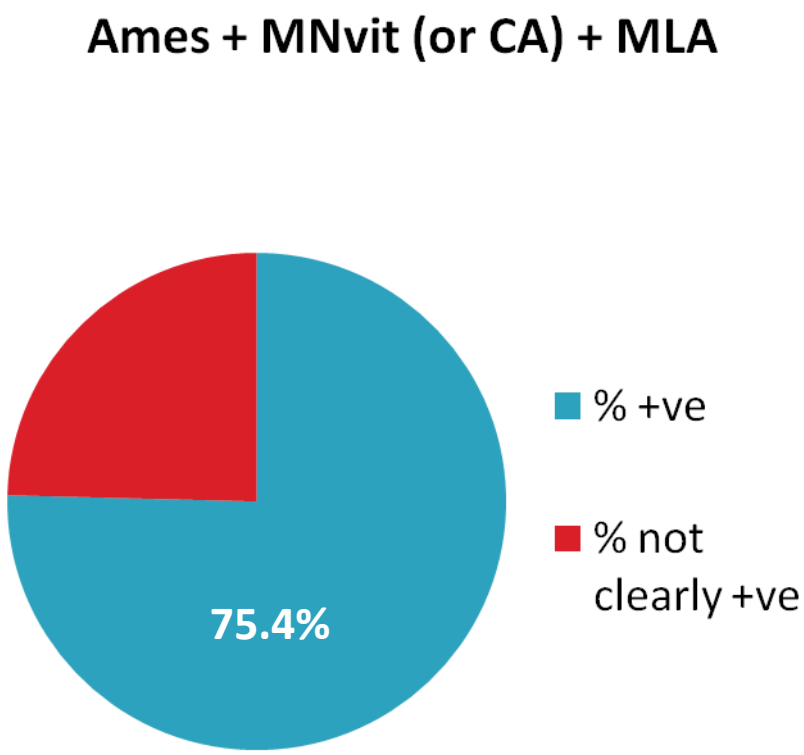
2 or 3 tests to detect rodent carcinogens?

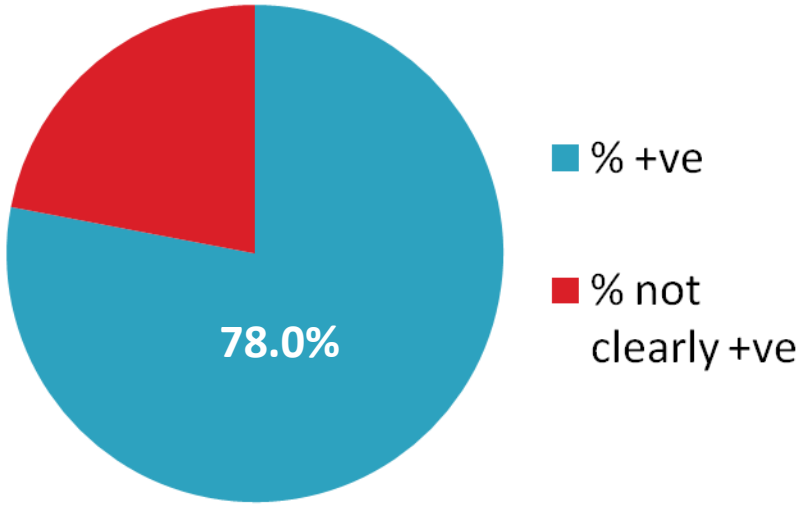




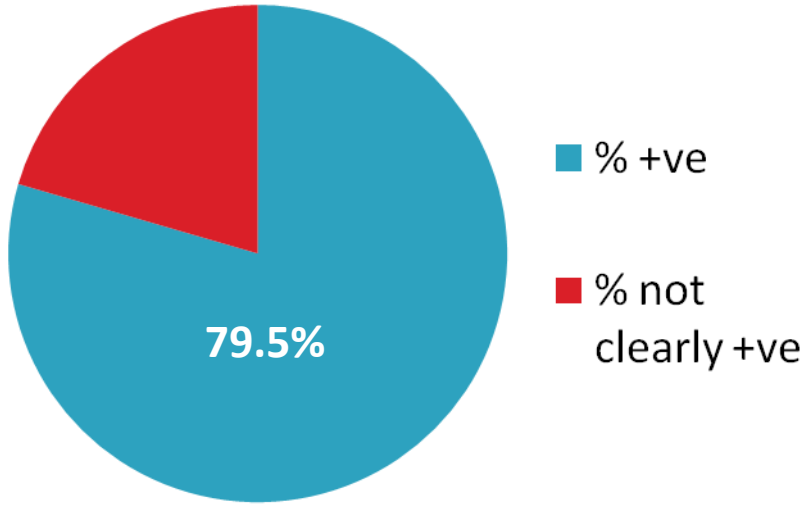
557 rodent carcinogens had available *in vitro* data.
Most carcinogens that were –ve in Ames + MNvit were inadequately tested, weak, equivocal or inconclusive/insufficient detail.
An additional 11 substances were detected by MLA but all were questionable or not tested for chromosome damage *in vitro*.

2 or 3 tests to detect *in vivo* genotoxins?

Ames + MNvit (or CA)



Ames + MNvit (or CA) + MLA



405 *in vivo* genotoxins had available *in vitro* data.
Most *in vivo* genotoxins that were –ve in Ames + MNvit were inadequately tested, weak, equivocal or inconclusive/insufficient detail.
An additional 6 substances were detected by MLA but were either equivocal in MNvit or had not been tested.

However …

- Since we analysed the available data for our 2011 paper, new publications have appeared and there are more data on *in vivo* gene mutations detected in the transgenic rodent mutation (TGR) assay and the Pig-a assay
- Since substances inducing gene mutations are a particular concern (representing stable genetic change and a key step in the carcinogenic process) a new question was raised:

Are there any substances that are +ve in TGR or Pig-a tests, that are negative in Ames, but are positive in MCGM?

A new analysis – source material (1)

- Five database review publications containing tables of results (including Ames and TGR assays) were analysed for Ames -ve/TGR +ve substances. The 5 publications were:
  - **Lambert et al. (2005)** which contained the data submitted to OECD as part of the detailed review paper for the development of TG488.
  - **Kirkland et al. (2011)** referred to earlier.
  - **Morita et al. (2016)** which recommended the *in vivo* micronucleus (MNviv) and TGR as an *in vivo* battery to detect rodent carcinogens.
  - **Madia et al. (2020)** which reported on the compilation of a database of Ames-negative substances.
  - **Luijten et al. (2024)** which analysed various combinations of *in vitro* tests to predict in vivo genotoxicity (2022 IWGT working group report).

Source material (2)

- All 5 review publications contained Ames and TGR data.
  - 4 of these publications also reported results for MCGM tests.
  - Morita et al. (2016) did not report MCGM data so the literature was searched (Google Scholar, PubMed) for any published data on the Ames -ve/TGR +ve substances found only in that database.
  - The IWGT database (Luijten et al., 2024) also contained data from Japan, ECHA and the Pig-a detailed review paper for OECD.
- It should be noted that **procarbazine** was the only substance that was +ve in Pig-a and -ve in Ames, but this was also +ve in TGR.
- The “calls” for the different tests as listed in the reviews were considered first but were not always accompanied with references.
  - The “calls” may also not have been checked against the original publications.
  - It was therefore important to check whether the reported “calls” were accurate.
  - This required extensive searching in Google Scholar and PubMed.
- Moreover, for some endpoints it was reported that no data were available.

How many substances?

- Out of several hundred substances with TGR/Pig-a and Ames data, 26 were identified that were reported as being Ames -ve/TGR (or Pig-a) +ve in the 5 database publications mentioned before.

Acetic acid	α-Chaconine
Acrylamide	Comfrey (symphitine oxide)
Benzene	Dicyclanil
CC-1065 (Rachelmycin)	Ferric nitrilotriacetate
Crocidolite asbestos	Leucomalachite green
Cyproterone acetate	Solanidine
Oxazepam	α-Solanine
Phenobarbital	Hexachlorobutadiene
Sucrose	Procarbazine HCl (Natulan)
Tamoxifen	Aroclor 1254
Uracil	Methyleugenol
Wyeth 14,643	Nickel (II) sulfate hexahydrate
Amosite asbestos	Sodium arsenite

The process of reviewing the “calls”

- Where possible, the original publications or data sources from which the “calls” were made in the 5 database publications, were identified, checked and any additional or new data added.
  - Also data from other relevant endpoints – *in vitro* chromosomal aberration (CAvit), MNvit, MCGM, *in vivo* chromosomal aberration (CAviv) and MNviv – were searched and checked.
  - In total, 134 published references, reports and data sources have been checked (included results on the NTP website, JECFA report, MAK commission report)
- In some cases the “calls” made in the 5 database publications were not confirmed, or were questionable, when the original publications and reports were checked. Some results were conflicting, or new results were found.
- The patterns of results are grouped in the following slides.
  - Note some substances appear in more than one list

No MCGM data could be found (11 substances)

- Acetic acid
- Tamoxifen
- Uracil
- Wyeth 14,643
- Amosite asbestos
- α-Chaconine
- Comfrey
- Solanidine
- α-Solanine
- Hexachlorobutadiene
- Aroclor 1254

MCGM tests were –ve for 6 substances

- Benzene
  - Overall conclusion from collaborative trial
- Cyproterone acetate
  - *Hprt* in V79 cells
- Oxazepam
  - MLA/*Tk* assay
- Dicyclanil
  - Probably *Hprt* in V79 cells
- Leucomalachite green
  - *Hprt* in CHO cells
- Methyleugenol
  - *Hprt* in V79 cells

MCGM uninterpretable, questionable or inconsistent results (4 substances)

- Acrylamide
  - +ve, equivocal and -ve results reported
- Sucrose
  - Uninterpretable by Schisler et al. (2018) in re-evaluation of NTP MLA data
- Nickel (II) sulfate hexahydrate
  - MLA/*Tk* only clearly +ve at >80% reduction in RTG;
  - +ve at *gpt* locus of G12 Chinese hamster cells only seen with 6-hr treatment. It was -ve at equitoxic doses with 16-hr treatment
- Sodium arsenite
  - Mutant frequency in MLA/*Tk* did not exceed GEF until >80% toxicity. Almost entirely small colony mutants i.e. clastogenic (it was +ve in MNvit and CAvit);
  - <2-fold increase in *gpt* mutations in CHO-AS52 cells at 15% survival

Ames not clearly -ve (e.g. weak +ve responses or both +ve and -ve results found) – 9 substances

-ve Ames results found for the following but …

- Acrylamide
  - Weak +ve in 1 or 2 labs, -ve in 3rd lab
- Benzene
  - +ve in TA100 by microsuspension fluctuation method
- CC-1065
  - Weak +ve, but only TA98 and TA100 tested
- Oxazepam
  - 1 of 3 studies reported +ve in TA98 and TA100
- Phenobarbital
  - Weak +ve in TA1535, but neither TA102 nor *E. coli* included
- α-Chaconine
  - Weak +ve in TA98, but only TA98 and TA100 tested
- Comfrey
  - +ve with acetone extract
- Hexachlorobutadiene
  - +ve with S9 containing higher protein content
- Sodium arsenite
  - Reported +ve in a “Masters” thesis but unable to check data

-ve Ames results not robust (insufficient strains tested or data cannot be checked) – 13 substances

- Cyproterone acetate
  - Neither TA102 nor *E. coli* included
- Oxazepam
  - Neither TA102 nor *E. coli* included in 1 lab reporting -ve results
- Tamoxifen
  - Neither TA102 nor *E. coli* included
- Uracil
  - Unable to check which strains were tested
- Wyeth 14,643
  - Unable to check which strains were tested
- Comfrey
  - Neither TA102 nor *E. coli* included in 2 labs reporting -ve results
- Dicylanil
  - Unable to check which strains were tested
- Ferric nitrilotriacetate
  - Unable to check which strains were tested
- Solanidine
  - Only TA98 and TA100 tested
- α-Solanine
  - Only TA98 and TA100 tested
- Hexachorobutadiene
  - Neither TA102 nor *E. coli* included in 1 lab reporting -ve results
- Methyleugenol
  - Neither TA102 nor *E. coli* included in 1 of 2 labs reporting -ve results
- Sodium arsenite
  - Only *E. coli* tested in 1 lab reporting -ve results

TGR result questionable (unusual dosing period, non-physiological route of administration or not actually a transgenic mutation study) - 19 substances (p1)

- Acetic acid
  - Induced 2-fold increase in *LacZ* mutations only at a dose causing skin ulcers. Dose may have exceeded MTD. Inflammation could lead to clonal expansion of pre-existing mutations.
- Acrylamide
  - Weak +ve, <2-fold
- Benzene
  - <2-fold increases in *LacI* mutations in lung and spleen, but not liver, after 12 weeks inhalation exposure
- Crocidolite asbestos
  - *LacI* mutations induced 4 weeks after 5 daily inhalation exposures, but mutant spectrum same as in controls so could be clonal expansion due to inflammation

TGR result questionable (unusual dosing period, non-physiological route of administration or not actually a transgenic mutation study) - 19 substances (p2)

- Oxazepam
  - 180 days dosing required for +ve response in *cII* and *LacI* genes of TGR mice. Mutational spectra not different from controls so could be clonal expansion of spontaneous mutations. Primary mechanism likely to be oxidative damage due to induction of CYP2B isozymes. Non-genotoxic carcinogen.
- Phenobarbital
  - 180 days dosing required to for +ve response in TGR rats and mice. Primary mechanism likely to be oxidative damage due to induction of CYP2B isozymes. Concluded not mutagenic in TGR after clonal correction. Non-genotoxic carcinogen.
- Uracil
  - *LacI* mutations probably due to irritation of bladder after long (10, 20 & 51 weeks) treatment. Predominant mutations in treated and control animals were G to A transitions at CpG sites indicating clonal expansion of spontaneous mutations.

TGR result questionable (unusual dosing period, non-physiological route of administration or not actually a transgenic mutation study) - 19 substances (p3)

- Wyeth 14,643
  - 180 days dosing required for +ve response in *cII* and *LacI* genes of TGR. Mutational spectra not different from controls so could be clonal expansion of spontaneous mutations. Non-genotoxic carcinogen.
- Comfrey
  - 3 months dietary dosing of ground comfrey roots to obtain +ve TGR in Big Blue rat liver.
- Dicyclanil
  - TGR +ve only in female *gpt*-delta mice due to oxidative damage leading to cell proliferation in liver.
- Ferric nitrilotriacetate
  - Weak induction of *LacZ* mutations after IP dosing 2 or 3x per week, not significant and within control range. Increases probably due to oxidative damage since 8-OHdG levels were increased.

TGR result questionable (unusual dosing period, non-physiological route of administration or not actually a transgenic mutation study) - 19 substances (p4)

- Leucomalachite green
  - +ve TGR in female Big Blue mice only after 16 weeks oral dosing.
  - TGR was -ve in female Big Blue rats also dosed for 16 weeks.
- Solanidine
  - Dosing was IP (for 3 days)
- α-Solanine
  - Dosing was IP (for 3 days)
- Hexachlorobutadiene
  - +ve TGR according to Lambert et al. (2005) but unpublished data so cannot be checked
- Aroclor 1254
  - 7-week dosing period in TGR and increase in MF was only 1.4-fold. Liver weight increased significantly and hypertrophy of hepatocytes in the centrilobular region was seen.

TGR result questionable (unusual dosing period, non-physiological route of administration or not actually a transgenic mutation study) - 19 substances (p5)

- Methyleugenol
  - +ve TGR after 13 weeks dosing
- Nickel (II) sulfate hexahydrate
  - Not a TGR study. This "*in vivo* mutation" result was actually a dominant lethal assay, and dominant lethal mutations result form chromosomal aberrations in germ cells. Full paper is in Russian. English abstract does not give much detail.
- Sodium arsenite
  - TGR response probably due to oxidative stress since mutations induced in *gpt*-delta mice associated with significant induction of 8-OHdG.

- Would any of the above 19 substances be +ve in a standard (OECD 488) TGR assay using a physiologically relevant route of administration?
  - With mutant sequencing included to check for clonal expansion?

That leaves procarbazine HCl …

- -ve Ames results seem robust
- +ve in TGR after both oral and IP dosing
- Also +ve in Pig-a
- +ve MCGM results (*Tk* and *Hprt*) also seem robust
- However, metabolic activation conditions may be critical to detecting +ve results *in vitro*.
  - Metabolism is complex and not fully understood.
  - It is also a stronger clastogen than a gene mutagen.
- Is this a case where the -ve results are not due to the test system (bacterial mutation) *per se*, but are due to non-optimal metabolism, which is complex and not fully understood.
- **Is this 1 example sufficient to justify adding an MCGM test to the core *in vitro* battery?**

Thank You for Your Attention

Questions?