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Discussion paper on the Stage 1 (core *in vitro* tests) in the new COM strategy

A proposal for a 2-test core battery consisting of Ames + *in vitro* micronucleus

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Introduction

Whilst there is no dispute that bacterial and mammalian cell tests need to be included at the *in vitro* stage of testing, and that the endpoints of gene mutation, chromosomal damage and aneuploidy need to be investigated, it may not be necessary to include 2 mammalian cell tests in order to achieve this. The Ames test, of course, measures gene mutations, and it is often reasoned that a mammalian cell test for gene mutations should also be included, but such reasoning does not seem to be based on analysis of data. In addition, if both bacterial and mammalian cell tests for gene mutation are included, there is no guidance as to which result takes precedence in the event of one test being positive and the other negative. If the *in vitro* micronucleus test (MNvit) is included, it detects both additional endpoints, namely chromosomal aberrations and aneuploidy. It should be noted that this is preferred to the alternative chromosomal aberration assay, which does not adequately detect aneugens.

It is not advisable for an *in vitro* battery to contain more tests than necessary. The more *in vitro* (particularly p53-defective mammalian cell) tests that are performed, the greater the chance of “misleading” positive results (Kirkland *et al*, 2005, 2007) that would have to be followed up *in vivo*, leading to unnecessary use of animals. In 2007, a working group of the German Speaking section of the European Environmental Mutagen Society (GUM) recommended (Pfuhrer *et al*, 2007) that Stage 1 *in vitro* genotoxicity testing should consist of an Ames test plus MNvit, since these tests cover all of the essential mutagenic endpoints (gene mutations, structural chromosome damage, and aneuploidy), and moreover cover testing in both prokaryotic and eukaryotic systems. Therefore, we decided to investigate whether an *in vitro* battery consisting of Ames plus MNvit would be sufficient to detect genotoxic potential, and this was accomplished by analysis of published data in order to address 2 key questions:

“Are there rodent carcinogens that are positive in the mouse lymphoma assay (MLA) that are not detected in either Ames or MNvit?”

“Are there *in vivo* genotoxins that are positive in the mouse lymphoma assay (MLA) that are not detected in either Ames or MNvit?”

(i) Analysis of rodent carcinogens

In the database published by Kirkland *et al* (2005), out of 756 rodent carcinogens, 542 had published Ames results. Most of these were positive, but the compounds that gave negative results in the Ames test provide the greatest focus in terms of expecting the mammalian cell test(s) to give positive results. Thus, from the 215 rodent carcinogens that were negative in

Ames, 65 were apparently positive in the MLA. However, after the re-evaluation by Gollapudi *et al* (2010) of NTP MLA studies, and (by the authors) of non-NTP MLA studies using the same criteria, many were found to be equivocal or uninterpretable. Only 26 remained clear or weak positives. Of these 26:

- 9 were tested in MNvit, and 8 of these were positive, 1 was equivocal
- 6 were positive in CA (one induced polyploidy) but not tested in MNvit. Given the concordance between these 2 tests for clastogens, positive MNvit results would be expected
- 6 were negative in CA but not tested in MNvit.
 - Most of these were from NTP studies using short treatments and early sampling times, so may be positive for CA (and therefore also for MN) if using a modern protocol
- The remaining 5 were either equivocal, inadequate (technically compromised, TC), inconclusive or not tested.

Confidence in the conclusions presented later would be improved if the 11 MLA-positive chemicals that were either negative, equivocal, TC or inconclusive in CA, and not tested in MNvit, were to be retested according to current recommendations. They are:

- 1,4-benzoquinone
- Benzyl acetate
- Butylated hydroxytoluene
- N,N'-diethyl-2-thiourea
- FD&C Red 1 (Ponceau 3R)
- Malonaldehyde sodium salt
- Methyl tert-butyl ether
- Piperonyl butoxide
- Procarbazine HCl (Natulan)
- Trimethylthiourea
- Vinylidene chloride

Toluene was also thought to be positive in MLA yet was negative in both CA and MNvit tests, but the MLA result has not been confirmed in recent re-testing. Toluene and benzyl acetate are discussed in more detail below.

It is also important to analyse the published data in terms of those carcinogens not detected as positive in MNvit. For such chemicals, are they detected by the Ames test, or is the MLA needed in order to provide a positive result somewhere in the *in vitro* battery? Analysis of the Kirkland *et al* (2005) database identified 87 rodent carcinogens for which MNvit results were available. Of these studies, 70 carcinogens gave clear positive results and 17 carcinogens gave clear negative results. The 17 carcinogens that were negative in MNvit are listed in the table below, together with their respective Ames and MLA results. The MLA calls as presented in Kirkland *et al* (2005) are given alongside the re-evaluation calls from Gollapudi *et al* (2010).

Chemical	CAS No.	Ames result	MLA result Kirkland 2005/Re-evaluation	MNvit result
Benzyl acetate	140-11-4	-	TC/+	-
(4-chloro-6-(2,3-xylidino)-2-pyrimidinyl(thio)acetic acid (Wyeth 14,643)	50892-23-4			-
Clofibrate	637-07-0	-		-
Coumarin	91-64-5	+		-
Dichloroacetic acid	79-43-6	+	+/+	-
Di(2-ethylhexyl)adipate	103-23-1	-	-/U	-
Di(2-ethylhexyl)phthalate	117-81-7	-	-/U	-
Hexachloroethane	67-72-1	-		-
Nafenopin	3771-19-5	-		-
N-nitrosodiphenylamine	86-30-6	-	-/E	-
Phenacetin	62-44-2	+	E/U	-
Phenobarbital	50-06-6	+	+/U	-
Tertachloroethylene	127-18-4	-	E/U	-
12-O-tetradecanoylphorbol 13-acetate	16561-29-8	-		-
Titanium dioxide	13463-67-7	-	-/U	-
Toluene	108-88-3	-	+/E	-
Urethane	51-79-6	+	-/U	-

TC = technically compromised

E = equivocal

U = uninterpretable

See Kirkland *et al* (2005) for references to these results.

It can be seen that many of these 17 chemicals negative in MNvit were also negative in both the Ames test and in the MLA. This is perhaps not surprising since many of these are accepted as inducing tumours by a non-genotoxic mechanism [e.g. clofibrate, di(2-ethylhexyl)adipate, di(2-ethylhexyl)phthalate, 12-O-tetradecanoylphorbol 13-acetate]. There were 3 carcinogens negative in the MNvit that were positive in the MLA, either in the Kirkland *et al* (2005) analysis or after re-evaluation. Two of these (dichloroacetic acid, phenobarbital) were positive in the Ames test. Although dichloroacetic acid is a clear mutagen in the MLA (even after re-evaluation), phenobarbital produced very weak responses that would not be considered positive by current criteria (i.e. where mutant frequency exceeded the concurrent control by more than the Global Evaluation Factor - GEF, Moore *et al*, 2006) at concentrations inducing 70-80% toxicity, and was considered uninterpretable in the Gollapudi *et al* (2010) re-evaluation. Thus, benzyl acetate and toluene are the only rodent carcinogens (from the 87 carcinogens giving results in the MNvit) that were negative in both Ames and MNvit, but apparently positive in the MLA.

Benzyl acetate was called “technically compromised” in Kirkland *et al* (2005) on the basis of the review of Mitchell *et al* (1997), which noted that benzyl acetate reacts with the plastic of culture dishes, and therefore the expert panel chaired by Mitchell could not reach a conclusion, even though clear increases in mutant frequency were seen in the presence of S9. Although the re-evaluation by Gollapudi *et al* (2010) gave a positive call, the result may be an artefact. In addition, it is not clear whether benzyl acetate is a genotoxic carcinogen. It does not induce tumours in rats or mice when administered by diet. It did induce pancreatic tumours in rats, and forestomach and liver tumours in mice, when administered in corn oil, but the irritation effects of both benzyl acetate and corn oil may be confounding factors in the aetiology of the tumours. If benzyl acetate is a non-genotoxic carcinogen, then the negative findings in the Ames and MNvit are a true reflection of its mode of action.

In the original publication of the MLA result on toluene by McGregor *et al*, 1988 (which was part of the NTP program and summarised by Mitchell *et al*, 1997), the lowest concentration of toluene giving a positive result by current criteria (i.e. induced mutant frequency exceeded the GEF) was 225 µg/ml (2.4 mM). This was in the absence of S9, but only a 4 hr treatment was used, whereas modern protocols would include a prolonged (e.g. 24 hr) treatment in the absence of S9. This response was not reproducible in 2 other experiments conducted by McGregor *et al* where increases in mutant frequency did not exceed the GEF at similar concentrations. A re-evaluation of NTP MLA studies by Gollapudi *et al* (2010) categorised this result as equivocal. A new MLA test on toluene has therefore recently been performed at Covance Laboratories (Harrogate, UK), using a protocol compliant with the latest recommendations. L5178Y cells were treated for 3 hr in the absence or presence of S9, or for 24 hr in the absence of S9. Positive controls were methyl methanesulphonate (MMS) in the absence of S9 and benzo(a)pyrene (B[a]P) in the presence of S9, at 2 concentrations each. After a 2-day expression period in which cell density was controlled, cells were counted and plated in 96-well plates in the presence of the TFT selective agent. Colonies were counted 12-14 days later. The results are summarised in the table below.

It can be seen that:

- Negative control mutant frequencies fell in acceptable ranges
- Positive control chemicals induced significant responses at modest levels of toxicity
- Concentrations of toluene tested exceeded those found positive in McGregor *et al* (1988)
- Concentrations of toluene were tested that reduced RTG by at least 80%

and yet there were no increases in mutant frequency following toluene treatment that reached the Global Evaluation Factor increase of 126 mutants per 10⁶ viable cells (for the microwell method; Moore *et al*, 2006). Thus, toluene is not confirmed as a genotoxin *in vitro* in the MLA, and thus the published negative MNvit result actually is consistent with the negative Ames and (new) negative MLA results.

Treatment period (hr)	S9	Concentration of toluene (or positive control) in µg/ml	Mutant frequency per 10 ⁶ viable cells	% Relative total Growth
3	-	0	69.01	100
		36.85	60.52	89
		73.70	60.31	81
		110.6	62.56	68
		147.4	53.75	56
		184.3	48.80	47
		221.1	70.23	36
		258.0	78.51	10
		15 (MMS)	437.44	48
20 (MMS)	501.46	38		
3	+	0	79.37	100
		36.85	45.76	118
		73.70	57.40	82
		110.6	69.01	70
		147.4	75.61	48
		184.3	77.86	38
		221.1	100.28	23
		258.0	90.57	7
		2 (B[a]P)	550.65	79
3 (B[a]P)	679.68	45		
24	-	0	63.38	100
		36.85	40.04	95
		73.70	38.53	95
		110.6	36.33	85
		147.4	38.71	75
		184.3	38.36	64
		221.1	37.55	44
		258.0	46.18	29
		294.8	50.33	16
		5 (MMS)	805.69	51
		7.5 (MMS)	1339.47	32

Thus, benzyl acetate and toluene, the only carcinogens possibly positive in the MLA that are negative in CA and MNvit tests, are not robust positives in the MLA. They therefore do not make a strong case for inclusion of the MLA in the test battery.

Thus, from the available data, no genotoxic rodent carcinogens would be “missed” by using an *in vitro* battery consisting of Ames and *in vitro* micronucleus tests.

Examination of the ability of a 2-test battery (Ames + MNvit) to clearly detect rodent carcinogens gives the following:

- Ames + MNvit (or CA in the absence of MNvit data, or where MNvit data are inadequate) detects 409/557 (73.4%) rodent carcinogens with available *in vitro* data

- The remainder are negative, negative but inadequate (technically compromised, TC) according to current testing standards, weak, equivocal or inconclusive/insufficient detail
- By adding MLA to this battery of 2 tests an additional 29 carcinogens were initially found (i.e. from Kirkland *et al*, 2005) with reported positive results in the MLA. However, on re-evaluation of the results according to current (IWGT) criteria, most of these are equivocal or uninterpretable (Gollapudi *et al*, 2010). Thus, after re-evaluation, only an additional 11 carcinogens that are weak or clearly positive in the MLA would be picked up by adding MLA to the proposed 2-test battery (detection rate increases to 420/557 = 75.4%). However, of these 11 only one (benzyl acetate) was tested in MNvit, and the positive MLA with this chemical may be an artefact.

Thus, a 2-test battery consisting of Ames + MNvit is comparable to a 3-test battery consisting of Ames + MLA + MNvit in terms of detecting genotoxic rodent carcinogens as positive. There is no notable advantage achieved by adding MLA to a battery consisting of Ames + MNvit.

(ii) *In vivo* genotoxins

It is acknowledged that not all hazardous genotoxins have been tested for carcinogenicity, or have been shown to induce tumours. There are numerous *in vivo* genotoxins for which carcinogenicity data do not exist or are inadequate, that should be detected by an *in vitro* battery of tests. We have therefore compiled a database of published *in vivo* genotoxins that have not been tested for carcinogenicity, or are not carcinogenic, or are carcinogenic but were not included in the Kirkland *et al* (2005) database discussed above, which was based on the Carcinogenic Potency DataBase (CPDB) of Gold (2004).

A total of 461 *in vivo* genotoxins were identified in the published literature (where 2 similar forms of the same chemical had been tested separately we grouped them together in a single category, e.g. chlordiazepoxide and chlordiazepoxide hydrochloride). Data were obtained from the following *in vivo* endpoints - micronuclei, chromosomal aberrations, unscheduled DNA synthesis (UDS), transgenic mutations or Comets. Although in some cases there may be reason to question the validity or relevance of an *in vivo* positive result, these papers all appeared in peer-reviewed journals and therefore we accepted the author's conclusions of a positive outcome without further question.

As for the rodent carcinogens analysis, we searched the literature (mainly using Toxline, CCRIS, PubMed, IARC and NTP websites) for *in vitro* genotoxicity data from Ames, MLA, MNvit and CA tests. We accepted positive results for HPRT mutation as being indicative of positive results in the MLA (had the test been performed), but because the HPRT test does not readily detect clastogens, we did not accept a negative HPRT result as being indicative of a negative MLA. We were particularly careful to evaluate published negative results in terms of current testing methods. For example if an Ames test did not include TA102 or an *E. coli* strain, or if an MLA or CA test did not include a continuous (20-24 hr) treatment in the absence of S9, a negative result was considered to be inadequate (technically compromised, TC).

As before, the analysis was designed to answer whether Ames + MNvit would be sufficient to detect genotoxic potential.

Of the 461 chemicals in this database, no *in vitro* data were found for 55 substances. Thus some *in vitro* data were available for 406 chemicals that had not been analysed as part of the rodent carcinogens database. Of the 406 chemicals with *in vitro* data, 367 had been tested in the Ames test

- 202 gave clear positive results.
- 39 were “weak”, equivocal, inconclusive, or with insufficient detail
- 61 were negative, but did not meet current standards (TC)
- 65 were convincing negatives.

If we group together the 165 chemicals in the last 3 categories as being convincingly negative or not clearly detected in the Ames test, and in fact those being equivocal, inconclusive or inadequate (TC) could also be clearly negative in the Ames test if rigorously tested to current standards, then the mammalian cell test(s) become critical in detecting their potential to induce genotoxicity *in vivo*. Of these 165 chemicals, 34 were clearly positive in MLA (after recent re-evaluation by Gollapudi *et al* and the authors) or HPRT tests:

- 19 were tested in MNvit of which 17 were clearly positive, one (morphine/morphine sulphate) was negative, and one (thiabendazole) was equivocal (see later)
- 11 were positive for CA but not tested in MNvit. Given the high concordance between clastogenic activity in CA and induction of MN in MNvit, these 11 chemicals would be expected to be positive.
- 3 were not tested either in MNvit or CA
- 1 was negative for CA but not tested in MNvit (*o*-dichlorobenzene)

As before, it is also important to analyse the database in terms of the MNvit results. Of the 406 *in vivo* genotoxins with *in vitro* results, 128 were tested in the MNvit test:

- 103 were clearly positive
- 6 were equivocal
- 5 were negative but inadequate (TC)
- 14 were clearly negative.

If we group together the 25 chemicals in the last 3 categories as being convincingly negative or not clearly detected in the MNvit, and in fact those which are equivocal or inadequate (TC) could also be clearly negative in the MNvit if rigorously tested to current standards, then the Ames test becomes critical in detecting their potential to induce genotoxicity *in vivo*:

- 6 were not tested in Ames
- 11 were clearly positive in Ames
- 2 were equivocal in Ames
- 2 were negative but inadequate by current standards (TC)
- 4 were clearly negative in Ames.

Again we can group together the 8 compounds in the last 4 categories that were tested in Ames but not clearly positive and the data are summarised in the following table:

Chemical	CAS No.	Ames result	MLA result	MNvit result	<i>In vivo</i> result
Bromobenzene	108-86-1	-TC		-	MN
Cesium chloride	7647-17-8	E		-	CA
Dimethyl terephthallate	120-61-6	-	- TC (U after re-evaluation*)	-TC	MN
Dursban (chlorpyrifos)	2921-88-2	-TC		-	CA/Comet
Imipramine	50-49-7	-		-TC	MN
Morphine/ Morphine sulphate	57-27-2 64-31-3	-	+ (HPRT)	-TC	MN
Sucrose	57-50-1	-	- (U after re-evaluation*)	-	Transgenic
Thiabendazole	148-79-8	E	+	E	MN/Comet

* Re-evaluation of NTP MLA studies by Gollapudi *et al* (2010); other re-evaluation by authors.

Thus, only morphine and thiabendazole are positive in the MLA (or HPRT) test, yet are not clearly detected in either MNvit or Ames tests. These are discussed below.

Morphine (grouped together with morphine sulphate) was clearly positive for induction of HPRT mutants (Shafer *et al*, 1994), and therefore would be expected to be positive in the MLA, but was not clearly detected in either MNvit (negative result) or CA (equivocal result) tests. However, the negative MNvit test was unusual in that it was conducted in mouse splenic lymphocytes and only included a 21 hr continuous treatment in the absence of S9 (Sawant and Couch, 1995), so it is not clear whether a rigorous test in commonly-used cells would produce a positive response. An equivocal CA result was reported by Snyder (2009) in a review of marketed pharmaceuticals contained in the Physicians' Desk Reference, but no details are given. It is therefore not known whether the equivocal CA result was from a robust study to current standards or not. However, morphine does induce DNA strand breaks (comets) in human cells (Shafer *et al*, 1994) and may be expected to induce CA and MN if rigorously tested.

Thiabendazole was in fact positive for MNvit in several papers (Von Der Hude *et al*, 2000; Antoccia *et al*, 1991; Lynch & Parry, 1993), was equivocal in Natarajan *et al* (1993), negative in Van Hummelen *et al* (1995) and the SFTG trial (Lorge *et al*, 2006) where it was only tested in human lymphocytes, and inconclusive in the GUM evaluation (Miller *et al*, 1998) of the MNvit (because of the mix of published positive and negative results). It appears that, like many aneugens, thiabendazole has a very steep toxicity profile and positive MNvit results can only be detected in a very narrow concentration range. Careful testing is therefore required to detect its MNvit activity.

Based on the above analysis there are no examples of *in vivo* genotoxins for which it is essential to include the MLA in addition to Ames plus MNvit in order to detect genotoxic potential.

Examination of the ability of a 2-test battery (Ames + MNvit) to clearly detect *in vivo* genotoxins gives the following:

- A combination of Ames + MNvit (or CA where MNvit data not obtained, or inadequate) clearly detects 317/406 (78.1%) *in vivo* genotoxins with available *in vitro* data
 - The remainder are negative, negative but inadequate (TC), weak, equivocal or inconclusive/insufficient detail
 - By adding the MLA to this battery of 2 tests, only an additional 6 *in vivo* genotoxins are detected (323/406 = 79.6%); 4 of these 6 have not been tested in either MNvit or CA

One of those tested in MNvit and CA was morphine, discussed above. The other, where the CA test was considered inadequate by current standards, was *o*-dichlorobenzene. Confidence in the conclusions reached would be improved if all 6 of these chemicals were to be re-tested according to current recommendations. They are:

- Morphine/morphine sulphate
- 5-Bromo-2-deoxyuridine
- Cycloheximide
- *o*-Dichlorobenzene
- RSU-1069
- Tyramine.

Thus, a 2-test battery consisting of Ames + MNvit is comparable to a 3-test battery consisting of Ames + MLA + MNvit in terms of detecting *in vivo* genotoxins as positive. There is no notable advantage achieved by adding MLA to a battery consisting of Ames + MNvit.

Conclusions

From this analysis of 963 rodent carcinogens and *in vivo* genotoxins with *in vitro* data, **there is no convincing evidence that any genotoxic rodent carcinogens or *in vivo* genotoxins would be “missed” by using an *in vitro* battery consisting of Ames + MNvit.** At worst there are 4 “questionable” compounds – benzyl acetate, toluene, morphine and thiabendazole – where the MLA may be more sensitive than MNvit, but the data are not convincing. Therefore it is concluded that a core battery of Ames + MNvit provides acceptable sensitivity.

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