



Which mammalian cell tests best complement the Ames test in terms of detecting rodent carcinogens and *in vivo* genotoxins

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In vitro mammalian cell tests (1)

- ▶ Most guidelines recommend:
 - a test for induction of gene mutations in bacteria
 - a test for induction of gene mutations in mammalian cells (preferably the mouse lymphoma *tk* assay with colony sizing)
 - an *in vitro* chromosomal aberration or micronucleus test
- ▶ There is no dispute that
 - bacterial and mammalian cell tests need to be included,
 - the endpoints of gene mutation, chromosomal damage and aneuploidy need to be investigated
- ▶ However, is it necessary to include 2 mammalian cell tests in order to achieve this?

In vitro mammalian cell tests (2)

- ▶ The principles behind the tests in most guidelines are to detect gene mutation, chromosomal damage and aneuploidy.
- ▶ The Ames test 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, 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 chromosomal aberrations and aneuploidy.
- ▶ **Is a battery with Ames + MNvit sufficient?**

Rodent carcinogens

- ▶ We first analysed the published carcinogenicity data in order to address 2 questions:
 - Q1. “Are there Ames-negative rodent carcinogens that are positive in the mouse lymphoma assay (MLA) that are not detected in the MNvit or CA assays?”
 - Q2. “Are there MNvit-negative rodent carcinogens that are positive in the mouse lymphoma assay (MLA) that are not detected in the Ames assay?”

Question 1

- ▶ “Are there Ames-negative rodent carcinogens that are positive in the mouse lymphoma assay (MLA) that are not detected in the MNvit or CA assays?”
- ▶ Out of 757 rodent carcinogens in Kirkland *et al* (2005), 562 had published Ames results
 - Most were +ve
- ▶ Of 215 that were Ames –ve, 65 were +ve in MLA
- ▶ Of these 65, 16 were also tested in MNvit, and 14/16 were +ve
 - Cadmium sulphate was E for MNvit but +ve for CA
 - Toluene was –ve in both MNvit and CA (see later)

Question 1 – additional analysis

- ▶ Of the 65 Ames –ve carcinogens that were +ve in MLA:
 - 22 were +ve in CA but not tested in MNvit. Given the concordance between these 2 tests for clastogens, +ve MNvit results would be expected
 - 19 were –ve in CA but not tested in MNvit.
 - Most of these were from NTP studies using short treatments and early sampling times, so may be +ve for CA (and therefore also for MN) if using a modern protocol
 - The reliability (acceptability according to current criteria) of the MLA +ve results for these chemicals is being reassessed (Moore and Gollapudi) and the +ve results may not be upheld
 - The remainder were either equivocal, inadequate (technically compromised, TC), inconclusive or not tested.

Question 2

- ▶ “Are there MNvit-negative rodent carcinogens that are positive in the mouse lymphoma assay (MLA) that are not detected in the Ames assay?”
- ▶ Analysis of the database published by Kirkland *et al* (2005) has identified 87 rodent carcinogens for which MNvit studies have given clear +ve (70 carcinogens) or clear -ve (17 carcinogens) results.
- ▶ The 17 carcinogens –ve in MNvit are:

17 Carcinogens –ve in MNvit

Chemical	CAS No.	Ames result	MLA result	MNvit result
Benzyl acetate	140-11-4	-	TC	-
(4-chloro-6-(2,3-xylylidino)-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	-	-	-
Di(2-ethylhexyl)phthalate	117-81-7	-	-	-
Hexachloroethane	67-72-1	-		-
Nafenopin	3771-19-5	-		-
N-nitrosodiphenylamine	86-30-6	-	-	-
Phenacetin	62-44-2	+	E	-
Phenobarbital	50-06-6	+	+	-
Tertachloroethylene	127-18-4	-	E	-
12-O-tetradecanoylphorbol 13-acetate	16561-29-8	-		-
Titanium dioxide	13463-67-7	-	-	-
Toluene	108-88-3	-	+	-
Urethane	51-79-6	+	-	-

In vitro mammalian cell tests (4)

- ▶ Many of the 17 carcs -ve in MNvit were also -ve in Ames and MLA
- ▶ Many of these are accepted non-genotoxic carcs, e.g.
 - clofibrate
 - di(2-ethylhexyl)adipate
 - di(2-ethylhexyl)phthalate
 - 12-*O*-tetradecanoylphorbol 13-acetate]
- ▶ There were 3 carcs -ve in MNvit but +ve in MLA
 - 2 of these (dichloroacetic acid, phenobarbital) were +ve in Ames
- ▶ **Thus, toluene is the only rodent carc that was –ve in both Ames and MNvit, but +ve in the MLA.**

MLA with toluene

- ▶ In McGregor *et al* (1988) the lowest conc giving a +ve result (induced MF exceeds GEF, Moore *et al*, 2006) was 250 µg/ml (2.7 mM)
 - 4 hr treatment –S9
- ▶ A new MLA test on toluene has recently been performed at Covance UK
 - Protocol compliant with the latest recommendations
 - L5178Y cells treated for 3 hr – and + S9, and 24 hr -S9.
 - Positive controls were MMS -S9 and B[a]P +S9, at 2 concs 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.
- ▶ Negative control mutant frequencies fell in acceptable ranges
- ▶ Positive control chemicals induced significant responses at modest levels of toxicity
- ▶ Top concentrations of toluene reduced RTG by at least 80%

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

Toluene – toxic but negative

	Treatment/recovery (hrs)		
MLA	3 hr -S9	3 hr +S9	24 hr -S9
	-ve up to 2.8 mM (10% RTG)	-ve up to 2.8 mM (7% RTG)	-ve up to 3.2 mM (16% RTG)

No mutant frequency (MF) values in treated cultures exceeded control MF by $>126 \times 10^{-6}$ (Global Evaluation Factor [GEF] for microwell method, recommended by Moore *et al*, 2006).

Thus the +ve published MLA has not been confirmed in a robust modern study to $>80\%$ toxicity

Overall conclusions on rodent carcinogens

- ▶ Ames + MNvit (or CA in the absence of MNvit data) detects 410/557 (73.6%) rodent carcinogens with available *in vitro* data
 - Remainder are –ve, -ve but TC, weak, equivocal or inconclusive/insufficient detail
- ▶ By adding MLA to this battery of 2 tests an additional 24 carcinogens are detected (434/557 = 77.9%)
 - Only 1 was tested in MNvit – toluene, already shown not to be reproducible MLA +ve
 - 4 were not tested either in MNvit or CA
 - 17 were –ve but TC in CA (including toluene)
 - 3 were apparently real –ves in CA (malonaldehyde, methyl tertbutyl ether, sodium o-phenylphenol) but not tested in MNvit
 - Note that re-analysis of the NTP MLA database by Moore and Gollapudi has questioned many of the +ve results

In vivo genotoxins

- ▶ Since not all hazardous chemicals are carcinogens, or have been tested for carcinogenicity, the analysis was extended to include 464 *in vivo* genotoxins not present in the Kirkland *et al* (2005) carcinogens database
- ▶ Searched literature for positive results for *in vivo* MN, CA, UDS, transgenic mutation and comet assays
 - Accepted authors conclusions of a +ve finding as being accurate, since these papers all appeared in peer-reviewed journals
- ▶ Searched for the same *in vitro* data (Ames, MLA, MNvit and CA) as for the carcinogens

Questions for *in vivo* genotoxins database

- ▶ Q1. “Are there Ames-negative *in vivo* genotoxins that are positive in the mouse lymphoma assay (MLA) that are not detected in the MNvit or CA assays?”
- ▶ Q2. “Are there MNvit-negative *in vivo* genotoxins that are positive in the mouse lymphoma assay (MLA) that are not detected in the Ames assay?”

Question 1

- ▶ “Are there Ames-negative *in vivo* genotoxins that are positive in the mouse lymphoma assay (MLA) that are not detected in the MNvit or CA assays?”
- ▶ We looked carefully at any –ve results to ensure study design met current criteria (if not then Technically Compromised, TC)
 - Insufficient Ames strains
 - No continuous treatments in MLA or CA tests
 - Sampling times in CA too early
- ▶ We accepted an HPRT +ve as indicative of an MLA +ve, but did not include any HPRT –ve results

In vitro data for *in vivo* genotoxins

- ▶ Out of 464 *in vivo* genotoxins:
 - 55 had not been tested for *in vitro* genotoxicity
 - Therefore 409 chemicals had results in at least 1 *in vitro* assay
 - Most of these were in the Ames test (369 results)
- ▶ Of the 369 Ames results:
 - 202 (55%) were clearly +ve
 - in a few cases with modified conditions e.g. Prival modification for azo dyes
 - 38 were “weak”, equivocal, inconclusive, or with insufficient detail
 - 63 were –ve, but did not meet current standards (TC)
 - 66 were convincing -ves

Ames –ve *in vivo* genotoxins

- ▶ Of the 66 Ames –ve *in vivo* genotoxins, 14 were clearly +ve in the MLA (or HPRT)
 - 4 of these were +ve both in MNvit and CA
 - 1 was +ve in MNvit but –ve in CA (ferric nitrilotriacetate)
 - 1 was +ve in MNvit but not tested in CA
 - 7 were +ve in CA but not tested in MNvit
 - We would expect these to be +ve in MNvit based on concordance between the 2 tests for clastogens
- ▶ Only morphine/morphine sulphate was +ve in MLA and not clearly detected in MNvit or CA
 - MNvit was –ve but not a robust study (splenic lymphocytes only treated –S9)
 - CA was equivocal according to Snyder 2009
 - However, morphine does induce Comets *in vitro*

Ames –ve (but TC) *in vivo* genotoxins

- ▶ 63 *in vivo* genotoxins were Ames –ve (but TC). Let's assume these would be clearly –ve. 13 were clearly +ve in the MLA (or HPRT)
 - 3 were not tested either in MNvit or CA
 - 2 were +ve both in MNvit and CA
 - 1 was +ve in MNvit but inconclusive in CA (lead nitrate)
 - 2 were +ve in MNvit but not tested in CA
 - 1 was –ve but TC for CA and not tested in MNvit (*o*-dichlorobenzene)
 - 4 were +ve in CA but not tested in MNvit
 - We would expect these to be +ve in MNvit based on concordance between the 2 tests for clastogens
- ▶ Thus no chemicals in this subset were +ve in MLA and clearly not detected in MNvit or CA

Ames weak, E or I *in vivo* genotoxins

- ▶ 38 *in vivo* genotoxins were weak, equivocal or inconclusive in the Ames test. Let's assume these are also clearly –ve. 14 were clearly +ve in the MLA (or HPRT)
 - 6 were +ve both in MNvit and CA
 - 1 induced numerical abs in CA (carbendazim)
 - 1 was E in MNvit (both +ve and –ve published results) but induced numerical abs in CA (thiabendazole)
 - 1 was +ve in MNvit but E in CA (hexamethylphosphoramide)
 - 6 were +ve in CA but not tested in MNvit
 - We would expect these to be +ve in MNvit based on concordance between the 2 tests for clastogens
- ▶ Thus no chemicals in this subset were +ve in MLA and clearly not detected in MNvit or CA

Question 1

- ▶ “Are there Ames-negative *in vivo* genotoxins that are positive in the mouse lymphoma assay (MLA) that are not detected in the MNvit or CA assays?”
- ▶ Thus, as a worst case, 167 *in vivo* genotoxins could be considered –ve in the Ames test
- ▶ None of the 41 that gave clearly +ve results in MLA (or HPRT) was uniquely +ve in this test
- ▶ All that were tested in MNvit or CA either gave clearly +ve results in 1 or both tests, or testing was inadequate
 - Only “questionable” compounds are morphine and thiabendazole (discussed later)

Question 2

- ▶ “Are there MNvit-negative *in vivo* genotoxins that are positive in the mouse lymphoma assay (MLA) that are not detected in the Ames assay?”
- ▶ Of the 464 *in vivo* genotoxins, 127 had published results in MNvit
 - 102 were clearly +ve
 - 6 were equivocal
 - 5 were –ve but inadequate (TC)
 - 14 were clearly –ve
- ▶ Assume as a worst case that the last 3 categories (25 chemicals) are all -ve

In vivo genotoxins –ve, TC or E in MNvit

- ▶ Of the 25 *in vivo* genotoxins that were clearly –ve, or –ve but inadequate (TC), or equivocal in MNvit:
 - 6 were not tested in Ames
 - 11 were clearly +ve in Ames
 - 1 was equivocal in Ames
 - 1 had insufficient detail
 - 2 were –ve but inadequate by current standards (TC)
 - 4 were clearly –ve in Ames
- ▶ The 8 compounds that were –ve, -ve TC, E or I in the Ames test are shown on the next table

In vivo genotoxins not clearly +ve in Ames + MNvit

Chemical	CAS No.	Ames	MLA	MNvit	<i>In vivo</i> results
Bromobenzene	108-86-1	-TC		-	MN
Cesium chloride	7647-17-8	I		-	CA
Dimethyl terephthalate	120-61-6	-	-TC	-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	-	-	-	Transgenic
Thiabendazole	148-79-8	E	+	E	MN/Comet

Morphine and thiabendazole

- ▶ Only morphine and thiabendazole were not clearly detected in Ames + MNvit, yet were +ve in MLA or HPRT
- ▶ The MNvit with morphine was a non-standard assay (splenic lymphocytes, only treated –S9 for 21 hr)
 - May be +ve in a standard assay
- ▶ Thiabendazole was:
 - +ve for MNvit in several papers (Von Der Hude *et al*, 2000; Antoccia *et al*, 1991; Lynch & Parry, 1993)
 - Equivocal in Natarajan *et al* (1993)
 - Inconclusive in the GUM evaluation (Miller *et al*, 1998) – some +ve and some –ve results
 - -ve in Van Hummelen *et al* (1995) and the SFTG trial (Lorge *et al*, 2006) where it was only tested in human lymphocytes

Overall conclusions on *in vivo* genotoxins

- ▶ A combination of Ames + MNvit (or CA where MNvit data not obtained) clearly detects 317/409 (77.5%) *in vivo* genotoxins with available *in vitro* data
 - The remainder are –ve, -ve but 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 (323/409 = 78.9%)
 - 4 of these 6 have not been tested in either MNvit or CA
 - Morphine and thiabendazole not convincingly –ve in MNvit

The essential *in vitro* battery

- ▶ It is not advisable for an *in vitro* battery to contain more tests than necessary.
 - The more *in vitro* tests that are performed, (particularly in p53-defective mammalian cells) 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.
- ▶ From this analysis of 971 rodent carcinogens and *in vivo* genotoxins with *in vitro* data, **there is no convincing evidence that any rodent carcinogens or *in vivo* genotoxins would be “missed” by using an *in vitro* battery consisting of Ames + MNvit.**
- ▶ At worst there are 3 “questionable” compounds – toluene, morphine and thiabendazole – where MLA may be more sensitive than MNvit, but data are not convincing.