Table 5
Summary of *in vivo* genotoxicity and carcinogenicity (in terms of the IARC classification) data on the 15 different chemicals that were positive in the Ames test.

JEC ID	Chemical name	CAS No.	Ames	*in vivo* MN	Carcinogenicity[a]	Ref.
5	*N*-(Aminoethyl)ethanolamine	111-41-1	+	–		[47,49]
7	2-Amino-1-naphthalenesulfonic acid	81-16-3	+			[47]
10	Azodicarbonamide	123-77-3	+	–		[47,49]
13	1,3-Bis(aminomethyl)cyclohexane (mixtures of cis-, trans-)	2579-20-6	+			[47]
17	1-Bromo-3-chloropropane	109-70-6	+			[47]
51	2-(Dimethylamino)ethyl methacrylate	2867-47-2	+	–		[44,47]
52	2,3-Dimethylaniline (2,3-Xylidine)	87-59-2	+			[47]
53	2,6-Dimethylaniline (2,6-Xylidine)	87-62-7	+	–	2B	[47,49,50]
54	3,5-Dimethylaniline (3,5-Xylidine)	108-69-0	+			[47]
71	Hydrazine monohydrate	7803-57-8	+	+[b]	2B[b]	[47,50]
82	3-Methoxybenzeneamine	536-90-3	+	+		[47]
101	4,4'-Oxybis(benzenesulfonylhydrazide)	80-51-3	+	–		[47]
112	Thiourea dioxide	4189-44-0	+			[47]
114	Tolylene diisocyanate (Toluene diisocyanate)	26471-62-5	+	–	2B	[47,50]
121	2,4,6-Trinitrophenol (Picric acid)	88-89-1	+	–		[47,49]

+, positive; –, negative; MN, micronucleus.
[a] In terms of the IARC classification.
[b] As hydrazine (CAS No. 302-01-2).

JEC ID 76. 3-Hydroxy-2-naphthalenecarboxylic acid (CAS No. 92-70-6): 3-Hydroxy-2-naphthalenecarboxylic acid induced CAs in Chinese hamster V79 cells after a 6-h or 18-h treatment with the highest test concentration (0.75 mg/mL, *i.e.*, 4.0 mM) without S9. With S9, no CAs were observed [49]. No information about the frequency of CAs or cytotoxicity was available. The Ames test was negative with or without S9 [49,51]. In an *in vivo* CA test in bone-marrow cells of hamsters, no clastogenic activity and no toxicity were observed at the maximum recommended dose of 2000 mg/kg. However, the test had severe limitations (only 50 metaphases were examined per animal and there was no indication that the target tissue was reached by the chemical). Still, recent *in vivo* mouse bone-marrow MN tests were negative after oral administration of up to 500 mg/kg/day for 2 days. One animal died at 700 mg/kg/day in a dose-range finding study [47]. The weight-of-evidence suggests that the level of concern is negligible.

JEC ID 117. 2,4,6-Trimercapto-S-triazine (CAS No. 638-16-4): 2,4,6-Trimercapto-S-triazine induced CAs at the highest concentration of 0.8 mg/mL (4.5 mM) after 6-h treatment with or without S9 (19.0% or 5.5%, respectively); the relative cell growth, as measured by monolayer confluence, was 73% or 55%, respectively. The pH of the medium at 1.2 mg/mL or more was approximately 6.0 or less. The pH at 0.8 mg/mL was not measured. In a confirmatory test in pH-adjusted medium with S9, the chemical induced CAs (31.5%) at the highest concentration of 1.2 mg/mL, and precipitation was observed at the beginning of the treatment; the relative cell growth was 77%. No CAs were observed up to 0.31 mg/mL after 24-h treatment without the S9 mix [47]. The Ames test was negative with or without S9 [47]. An *in vivo* mouse bone-marrow MN test was negative after oral administration of up to 1000 mg/kg/day for 2 days. One animal died at 2000 mg/kg/day in a dose-range finding study [47]. The weight-of-evidence suggests that the level of concern is negligible.

The results of the evaluation of the level of concern are summarized in Table 6. Of the 53 different chemicals, four chemicals were of 'real concern', 16 were of 'some concern', eight were of 'minimal concern', and the remaining 25 chemicals were of 'negligible concern'. Importantly, the 'of some concern' category in some cases was due to the absence of relevant additional data and not based available data suggesting real concern [9]. In this analysis, 15 Ames-positive chemicals were included in the 53 different (*i.e.*, missed by the application of the ICH TG) chemicals (Table 5). All of the Ames-positives were classified as of 'some concern' or of 'real concern' (Table 6). If the Ames-positive chemicals were excluded from the analysis due to detection by the test-battery system, 38 chemicals would be missed. Among the 38 chemicals, five were of 'some concern'; eight were of 'minimal concern'; and the remaining 25 chemicals were of 'negligible concern' (Table 6).

3.4. Distribution of chemical MWs

The distribution of the MWs of the 267 CA-positives from the CGX database or 124 CA-positives from the JEC database is presented in Table 7. The MWs of the majority of chemicals (71.9% in CGX, 84.7% in JEC) were between 100 and 300. Approximately half (141/267) of the 267 CA-positives from the CGX database had a MW below 200. Similar distributions in MWs have been shown in carcinogens and non-carcinogens. Approximately 70% (85/124) of the 124 CA-positives from the JEC data set, based on CSCL for industrial chemicals, had a MW of less than 200. These distributions indicate that 10 mM can be considered equivalent to 2 mg/mL for industrial chemicals.

4. Discussion

The present reduction in the top-concentration limit in the *in vitro* CA test is expected to reduce the number of false or misleading positives, and hopefully, it will not greatly affect the assay's sensitivity or specificity for rodent carcinogenicity. We investigated the effects of this reduction by means of two chemical data sets from the CGX and JEC databases, by applying three test guidelines, *i.e.*, the 1997-OECD [1], r-OECD [12] and ICH [11] TGs. The chemical dataset from the CGX [16] or JEC [47] databases consisted of a variety of chemical categories, including natural products, pharmaceuticals and pesticides or industrial chemicals. The sensitivity and specificity analysis of the 435 chemicals from the CGX database revealed that application of the r-OECD TG (10 mM or 2 mg/mL) did not affect the sensitivity (63.1%) or specificity (59.3%) against carcinogenicity compared with those (sensitivity 63.1%, specificity 59.3%) seen with the 1997-OECD TG (10 mM or 5 mg/mL). However, the ICH TG (1 mM or 0.5 mg/mL) showed a different outcome, *i.e.*, approximately a 18% decrease in sensitivity (45.4%) and a 14% increase in specificity (72.9%) (Table 3). These results indicate that the r-OECD TG demonstrated the same ability to detect rodent carcinogens as the 1997-OECD TG for chemicals in the CGX database. However, the ICH TG showed a low sensitivity (less than 50%) and was not useful for its detection. Analysis of the changes in the number of 124 CA-positives from the JEC database revealed a small reduction in the number induced under the r-OECD TG, and a remarkable reduction (about half) under the ICH TG (Table 4). These data indicate that application of ICH TG did not lead to an effective detection of rodent carcinogens among non-pharmaceuticals (*e.g.*, general

Table 6
Evaluation of level of concern for human health-risk assessment on the 53 different chemicals.

Level of concern	Number of chemicals with different result based on the different top-concentration limit between r-OECD and ICH TGs (chemical JEC ID)[a]	
Negligible	25	(JEC IDs 3, 4, 6, 19, 28, 33, 36, 39, 44, 45, 47, 57, 66, 69, 74, 75, 76*, 79, 88, 97, 100, 107, 108, 117*, 122)
Minimal	8	(JEC IDs 1*, 16, 55, 62, 64, 78, 85, 119)
Some	16	(JEC IDs 5*, 7*, 10*, 13*, 17*, 35, 51*, 52*, 54*, 73*, 77, 86, 101*, 112*, 118, 121*)
Real	4	(JEC IDs 53*, 71*, 82*, 114*)

[a] Positive by the revised OECD test guideline (r-OECD), but negative by the ICH S2(R1) guideline (ICH).
* Evaluated in this paper. Other chemicals without asterisk were evaluated by Morita et al. [9].
Underlined: Ames-positive chemicals.

Table 7
Distribution of the molecular weights of the 267 or 124 CA-positives from the CGX or JEC database, respectively.

Database	Dataset		Number of chemicals (%) in various ranges of molecular weight					
			<100	100–<200	200–<300	300–<400	400–<500	≥500
CGX	267 CA-positives	210 C	22(10.5)	92(43.8)	60(28.6)	30(14.3)	2(1.0)	4(1.7)
		57 NC	3(5.3)	25(43.9)	15(26.3)	6(10.5)	5(8.8)	3(5.3)
		Total	25(9.4)	117(43.8)	75(28.1)	36(13.5)	7(2.6)	7(2.6)
JEC	124 CA-positives		6(4.8)	79(63.7)	26(21.0)	7(5.6)	2(1.6)	4(3.2)

C, carcinogen; NC, non-carcinogen.

industrial chemicals). These data were supported by a relevance analysis of the *in vitro* CA results (Tables 5 and 6). Fifty-three chemicals, including 15 Ames-positives, were detected as CA-positive with the r-OECD TG, but not with the ICH TG. Twenty-five chemicals were considered to be of negligible concern; thus, a negative call upon the application of the ICH TG was not an issue in such cases. However, the remaining 28 chemicals, of which four chemicals were of real concern (*i.e.*, possible human carcinogens or *in vivo* genotoxins), were not detected as CA-positive under the ICH TG. These results indicate that the ICH TG will miss critical potential carcinogens. Importantly, 15 (*i.e.*, 11 of 15 chemicals of some concern and all four chemicals of real concern) of 28 chemicals of various concern levels were positive in the Ames test, and could be detected with the test-battery system, such as the ICH TG to detect genotoxic carcinogens. No or small changes in the sensitivity/specificity for carcinogenicity or alterations in the number of CA-positives with the r-OECD TG may be explained with the MW analysis of the chemical data set from the CGX and JEC databases. More than half (68.5%) of the CA-positive industrial chemicals had a MW of less than 200, and 90.3% had less than MW 300 in the JEC database (Table 7). Similar results (53.2% < MW 200, 81.3% < MW 300) were shown in the CA-positive data set from the CGX database, which included several pharmaceuticals. Because the MWs of the majority (84.7%) of industrial chemicals are between 100 and 300, 10 mM is considered to be equivalent to 2 mg/mL. Thus, the r-OECD TG showed effects similar to those of the 1997-OECD TG. The top-concentration limit in the ICH TG is 1 mM or 0.5 mg/mL, whichever is lower, although higher test concentrations should be considered for pharmaceuticals with unusually low MWs (*e.g.*, less than 200) [12]. However, no clear recommendation is provided in the ICH TG to determine exactly which 'higher concentrations' should be considered. In the CGX database, 142 chemicals (114 carcinogens and 28 non-carcinogens) had an MW < 200 (Table 7). Of the 142 chemicals, 65 compounds (50 carcinogens and 15 non-carcinogens) were CA-negative upon application of the ICH TG (Table 1). If r-OECD TG were applied to the 65 CA-negatives with MW < 200 (*i.e.*, application of modified ICH TG), 40 of 50 carcinogens and 6 of 15 non-carcinogens would be positive (Table 1). The sensitivity was increased to 58.0% from 45.4%, and the specificity was decreased to 67.8% from 72.9% (Table 3). These values were similar to those after the application of the r-OECD TG. In the JEC database, 85 chemicals were less than MW 200 (Table 7). Forty-seven of the 85 chemicals were negative in the CA test upon application of the ICH TG (Table 2). If r-OECD TG were applied to the 47 CA-negatives with MW < 200, 41 chemicals would be positive (Table 2). The number of CA-positives increased to 101 from 60 upon application of the modified ICH TG (Table 4). The number was similar to that found upon application of the r-OECD TG. This approach suggests the usefulness of applying the r-OECD TG for pharmaceutical substances with MW < 200. Recently, a simulation study performed by Brookmire et al. [10] suggested that lowering the highest concentration on the mg/mL scale to a value close to 2 mg/mL would result in an assay sensitivity close to the 10-mM limit; thus testing up to 5 mg/mL did not increase the sensitivity of the assay. The simulation study suggested also that lowering the current high concentration limit from 10 mM would dramatically impact the sensitivity of the assay. Our analysis with real data was consistent with this simulation study. We also revealed that the top concentration of 2 mg/mL did not decrease the specificity of the assay, although the simulation study did not dictate what the highest concentration should be, or address the specificity. In addition, the lack of significant changes in the sensitivity and specificity after the application of the r-OECD TG suggests that the new top-concentration limit proposed by the r-OECD TG would not affect the evaluation of chromosome damage in in-silico models.

In conclusion, the present analysis suggests that the application of the top-concentration limit (10 mM or 2 mg/mL, whichever is lower) proposed by the r-OECD TG will not affect the sensitivity or specificity of the detection of rodent carcinogens, indicating the validity of the guideline. Thus, the effects on the in-silico evaluation will also be small. However, the r-OECD TG has resulted in little or no reduction in the number of positive chemicals under the 1997-OECD TG, and nearly no improvements in reducing possible false positives for industrial chemicals have been made. Other approaches, *e.g.*, the consideration of the cell systems used, cytotoxicity measurements, non-physiological conditions or metabolic activation systems will be necessary to reduce the number of false positives [5].

Conflict of interests

There are no conflicts of interests.

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Can *in vitro* mammalian cell genotoxicity test results be used to complement positive results in the Ames test and help predict carcinogenic or *in vivo* genotoxic activity? I. Reports of individual databases presented at an EURL ECVAM Workshop

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ABSTRACT

Positive results in the Ames test correlate well with carcinogenic potential in rodents. This correlation is not perfect because mutations are only one of many stages in tumour development. Also, situations can be envisaged where the mutagenic response may be specific to the bacteria or the test protocol, *e.g.*, bacterial-specific metabolism, exceeding a detoxification threshold, or the induction of oxidative damage to which bacteria may be more sensitive than mammalian cells *in vitro* or tissues *in vivo*. Since most chemicals are also tested for genotoxicity in mammalian cells, the pattern of mammalian cell results may help identify whether Ames-positive results predict carcinogenic or *in vivo* mutagenic activity. A workshop was therefore organised and sponsored by the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) to investigate this further. Participants presented results from other genotoxicity tests with Ames-positive compounds. Data came from published, regulatory agency, and industry sources. The question was posed whether negative results in mammalian cell tests were associated with absence of carcinogenic or *in vivo* genotoxic activity despite a positive Ames test. In the limited time available, the

Abbreviations: API, active pharmaceutical ingredients; Carc, carcinogenicity; CAvit, *in vitro* chromosomal aberration test; CAviv, *in vivo* chromosomal aberration test; CCRIS, Chemical Carcinogenesis Research Information System; CGX, Carcinogenicity and Genotoxicity eXperience; CMR, carcinogen/mutagen/reproductive toxicant; CSCL, Japanese Chemical Substances Control Law; CTA, cell transformation assay; DG SANCO, Directorate General Health & Consumers; ECHA, European Chemicals Agency; EURL ECVAM, EU Reference Laboratory for Alternatives to Animal Testing; GHS, Globally Harmonized System of Classification and Labeling of Chemicals; GLP, Good Laboratory Practices; GSK, GlaxoSmithKline; *Hprt*, hypoxanthine-guanine phosphoribosyl transferase locus; IARC, International Agency for Research on Cancer; ISHL, Japanese Industrial Safety and Health Law; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-b]quinoxaline; MLA, mouse lymphoma $Tk^{+/-}$ gene mutation assay; MNvit, *in vitro* micronucleus test; MNviv, *in vivo* micronucleus test; NTP, National Toxicology Program; OECD, Organisation for Economic Cooperation and Development; QSAR, Quantitative Structural Activity Relationship; SCE, sister chromatid exchange; SIDs, screening information data set; SSCS, Scientific Committee on Consumer Safety; TTC, threshold of toxicological concern; UDSviv, *in vivo* unscheduled DNA synthesis test.

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presented data were combined and an initial analysis suggested that the association of negative *in vitro* mammalian cell test results with lack of *in vivo* genotoxic or carcinogenic activity could have some significance. Possible reasons why a positive Ames test may not be associated with *in vivo* activity and what additional investigations/tests might contribute to a more robust evaluation were discussed. Because a considerable overlap was identified among the different databases presented, it was recommended that a consolidated database be built, with overlapping chemicals removed, so that a more robust analysis of the predictive capacity for potential carcinogenic and *in vivo* genotoxic activity could be derived from the patterns of mammalian cell test results obtained for Ames-positive compounds.

1. Introduction

During an evaluation of the genotoxicity of new and existing chemical substances, some regulatory and advisory bodies (*e.g.*, European Chemicals Agency; European Food Safety Authority; UK Committee on Mutagenicity; the US Environmental Protection Agency) operate a tiered approach to testing which means that positive results obtained *in vitro* usually lead to follow-up tests *in vivo*. Some agencies no longer allow follow-up *in vivo* testing (*e.g.*, for cosmetics in the EU [1]) and a substance may be banned based on positive *in vitro* data alone. Alternatively, the development of a new substance may be stopped rather than incur the time and costs of *in vivo* testing. Therefore, knowing whether positive *in vitro* results are an accurate indicator of carcinogenic or *in vivo* mutagenic potential is important in determining whether and when (during the process of development) follow-up *in vivo* tests are needed, or whether substances should be dropped from development. This is not only important for industry and the regulatory agencies, but there are potential savings in animal usage and cost if unnecessary follow-up *in vivo* testing is avoided.

With regard to mammalian cell genotoxicity tests, we now understand much better when a positive result may be misleading, *i.e.* not predictive of carcinogenic or *in vivo* mutagenic activity, and a number of protocol-driven artefacts leading to positive responses have been identified, *e.g.*, high osmolality, high ionic strength, and high toxicity. As a consequence, test protocols have been modified to avoid or minimise these potential artefacts.

In 2006, a workshop was organised by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) to look at other issues that might contribute to the high frequency of "misleading" positive results in mammalian cells [2]. As a result of that workshop, and through detailed follow-up experimental work, we now know that we can reduce the occurrence of "misleading" positive results in mammalian cells by choosing measures of cytotoxicity based on cell proliferation [3], and carefully checking the source and characterisation of the cells [4,5]. Although non-carcinogenic Ames-positive chemicals have previously been identified and discussed [6], an analysis of such Ames-positive results as "misleading" indicators of carcinogenic or *in vivo* mutagenic potential in the context of *in vitro* mammalian cell results, has not been previously performed.

It is widely accepted that positive results in the Ames test correlate well with carcinogenic potential, at least in rodents [7,8]. However, situations can be envisaged where the mutagenic response may be specific to the bacteria or the test protocol. Such situations might involve bacterial-specific metabolism, exceeding a detoxification threshold, the induction of oxidative damage to which bacteria may be more sensitive than mammalian cells *in vitro* or tissues *in vivo*, or an *in vitro* metabolic activation preparation that does not mimic the *in vivo* situation.

Since most chemicals are tested for genotoxicity in *in vitro* mammalian cell assays in addition to the Ames test, the pattern of mammalian cell results may be informative. For example, the proportions of chemicals that are carcinogens or *in vivo* mutagens may be different if some or all mammalian cell tests are positive, compared to those situations where all mammalian cell tests are negative. In order to identify whether an Ames-positive chemical is truly predicting the *in vivo* positive response of the chemical, it might therefore be important to know whether the chemical is genotoxic *in vitro* in mammalian cells (and for what endpoints), whether it has structural alerts (and the type of alerts), and whether data can be obtained from mechanistic *in vitro* studies that more clearly define the risk. If such data indicate a lower possibility of carcinogenic or *in vivo* mutagenic potential, it may indicate that *in vivo* testing can be avoided or minimised.

A workshop to address this issue was therefore hosted and sponsored by EURL ECVAM, in Ispra, Italy from 23 to 25 January 2013. Fifteen genotoxicity experts from academia, government and industry were invited to participate. Data from public databases, regulatory agencies, and company *in-house* archives were reviewed. The primary effort was to look at the patterns of results in the *in vitro* mammalian cell genotoxicity assays for:

- compounds positive in the Ames test but NEGATIVE in carcinogenicity studies and,
- compounds positive in the Ames test but NEGATIVE in *in vivo* genotoxicity assays.

For comparison, the participants also looked at data from *in vitro* mammalian cell assays for:

- compounds positive in the Ames test and POSITIVE in carcinogenicity studies and,
- compounds positive in the Ames test and POSITIVE in *in vivo* genotoxicity assays.

If the patterns of results in the *in vitro* mammalian cell genotoxicity assays for Ames-positive chemicals were different for non-carcinogens and chemicals that were not genotoxic *in vivo* compared with carcinogens and *in vivo* genotoxins, how informative was this? Could additional data be obtained that would help further in identifying whether the chemical was or was not likely to be positive *in vivo*? The participants therefore also discussed what types of additional tests and investigations might be useful, *e.g.*, metabolism studies, identification of potential detoxification thresholds, presence of oxidative damage, and the use of other *in vitro* test systems such as cell transformation.

2. Summaries of presented material

Relevant data and analyses from the presentations given by the various participants that are pertinent to the objectives of the workshop, are summarised below. Initially, the different sources of data were reviewed intact, *i.e.*, knowing that there was likely to be overlap of chemicals among the various data sets, for example by using data from the same source (*e.g.*, the National Toxicology Program [NTP] database; International Agency for Research on Cancer [IARC] publications; Chemical Carcinogenesis Research

Information System [CCRIS] database) rather than independent study data from different sources. It was also clear that some chemicals that appeared in more than one database were associated with different response patterns in the same tests. Some participants also analysed their data after omitting chemicals that were known to overlap with other databases. Interestingly, while all analyses reviewed *in vitro* mammalian cell assay data obtained with Ames-positive compounds, some of the analyses considered mostly data from compounds found positive in carcinogenicity studies or *in vivo* genotoxicity assays, while others focused on the analysis of data obtained with non-carcinogenic compounds. Each presenter used his or her own approach to the analysis of their databases.

2.1. Review of CGX and in vivo genotoxin databases

D. Kirkland presented a review of the results on *in vitro* mammalian cell assays available in the Carcinogenicity and Genotoxicity eXperience (CGX) database of rodent carcinogens [9], where 318 rodent carcinogens were found that had positive results in the Ames test. More than one third of these Ames-positive rodent carcinogens had been tested in 2 mammalian cell tests with different endpoints (gene mutation and either chromosomal aberrations or micronuclei). Moreover, from the database of *in vivo* genotoxins published by Kirkland et al. [10], 202 *in vivo* genotoxins were found that gave positive results in the Ames test. Almost 75% of these had been tested in 2 mammalian cell tests with different endpoints. The patterns of results in the *in vitro* mammalian cell genotoxicity assays were analysed in both databases. Ames-positive chemicals for which there were data from only a single *in vitro* mammalian cell test were not included in the analyses presented here.

2.1.1. Analysis of rodent carcinogens

Of 318 Ames-positive carcinogens [9], 29 were tested in both mouse lymphoma $Tk^{+/-}$ gene mutation (MLA) and *in vitro* micronucleus (MNvit) tests, and 27 of these gave positive results in at least one of the two mammalian cell tests. The 2 chemicals that did not give any clear positive mammalian cell results were phenacetin and urethane. Phenacetin gave equivocal results in the MLA [11], but this was an old study which did not include many of the requirements of a modern protocol. The MNvit study [12] also only used short treatments. Phenacetin may have given positive results with prolonged treatments in the absence of S9 in either study. However, it is more likely that the metabolic conditions were not optimal. There is evidence that phenacetin may require metabolic activation by hamster liver, and not rat liver [13], in order to give positive results in any of the *in vitro* tests, including the Ames test [14], although there are contradictory data showing it to be non-mutagenic in the presence of rat and hamster liver S9 [15]. Urethane was negative in both the MLA and MNvit studies, but the Ames-positive results have not been confirmed on re-testing in many laboratories, and it is questionable whether urethane is an Ames-positive chemical. Again, the metabolism may be critical. It has been suggested that it may require CYP2E1 for metabolism, but Burke et al. [16] reported urethane as negative with S9 made from rats induced with CYP2E1 inducers. On the other hand, N-nitrosopyrrolidine, a compound known to require CYP2E1, was positive in that study. It is therefore not clear that urethane is a mutagenic carcinogen that is "missed" by the mammalian cell tests.

Of 318 Ames-positive carcinogens, 106 were tested in both MLA and *in vitro* chromosomal aberration (CAvit) tests, and 99 of these gave positive results in at least one of the two *in vitro* mammalian cell tests. The 7 rodent carcinogens that did not give a clear positive result in either MLA or CAvit tests are listed in Table 1. The azo dyes (4 out of 7 compounds) would clearly not be expected to give positive results in mammalian cells unless similar reductive metabolic conditions were applied that gave positive results in the Ames tests. For the other 3 chemicals the positive Ames results appear to be acceptable, but the *in vitro* mammalian cell studies

Table 1
Ames-positive rodent carcinogens and *in vivo* genotoxins not clearly positive in either mammalian cell gene mutation or CAvit tests.

Chemical	CAS No.	Results MLA or *Hprt*/CAvit[a]	Comments
A. Carcinogens			
1-amino-2,4-dibromoanthraquinone	81-49-2	-/-	1-amino-2,4-dibromoanthraquinone gave an acceptable positive for TA1537 and TA98 after pre-incubation without S9. But the MLA was limited by solubility and the CAvit only used 2 hr treatments
C.I. Acid Red 114	6459-94-5	-/-	Azo dye – requires reductive metabolism
C.I. Direct Blue 6	2602-46-2	E/-	Azo dye – requires reductive metabolism
C.I. Solvent Yellow 14	842-07-9	E/-	Azo dye – requires reductive metabolism
D&C Red 9	5160-02-1	-/-	Azo dye – requires reductive metabolism
2,4-diaminophenol 2HCl	137-09-7	-TC/-	2,4-diaminophenol dihydrochloride gave a weak but acceptable positive for TA98 after pre-incubation with S9. But the MLA was an old study (1987) and the CAvit only used short treatments with early sampling times.
Trifluralin	1582-09-8	-/-	Old MLA and CAvit studies. Trifluralin was weakly positive for TA100, but only with hamster liver S9. There are many negative study reports in the published literature using conventional Ames tests. It is also likely that Trifluralin was contaminated with nitrosamine (E. Zeiger, personal communication).
B. *In vivo* genotoxins			
4-acetylaminofluorene	28322-02-3	E/-	Negative for CAvit in normal and genetically engineered V79 cells; see text for further details
Agaritine	2757-90-3	-/-	Very limited data are available. Genotoxic mechanism may involve free-radical damage as TA104 is most sensitive Ames strain. Unable to judge the quality of mammalian cell tests as no details were given in the review paper [15]
Parathion-methyl	298-00-0	U/E	Gave weak but acceptable positive in TA100 and TA1535 after pre-incubation with and without S9. However, there is 1 published positive response in CAvit, and it may be positive in MLA with a modern protocol.
Spy dust	2608-48-2	U/-TC	It was clearly positive in the Ames test with S9. It may be positive in mammalian cells with modern protocols.
Tinidazole	19387-91-8	-/E	Tinadazole is a nitro-imidazole that is clearly positive in TA100. However, 2 out of 3 papers reported positive results in CAvit.

+ = positive; – = negative; E = equivocal; U = uninterpretable according to the reassessment of NTP MLA results by Schisler et al. [27]; TC = technically compromised, *i.e.* test result is questionable due to failure to meet essential standard criteria for an adequate study.

[a] See [9] and [10] for detailed citations.

were old, or deficient in some aspects, and modern, robust studies may give different results.

Thus, it appears that almost all Ames-positive rodent carcinogens (27/29 for MLA + MNvit and 99/106 for MLA + CAvit) are also positive in at least one of the *in vitro* mammalian cell tests. Where the mammalian cell test results are not clearly positive there may be justifiable explanations in terms of metabolic conditions or study design. Thus, there are no clear examples of Ames-positive carcinogens giving negative results in 2 mammalian cell tests covering different endpoints.

2.1.2. Analysis of in vivo genotoxins

Of 202 Ames-positive *in vivo* genotoxins obtained from another published database [10], 36 were tested for both gene mutation (*Tk*$^{+/-}$ or *Hprt*) and MNvit, and 34 were positive in at least one of the two *in vitro* mammalian cell tests. The 2 chemicals that did not give a clear positive result either for gene mutation or micronucleus induction in mammalian cells were 4-acetylaminofluorene and dimethoate. 4-Acetylaminofluorene was reported equivocal in the MLA [17], but unfortunately no details were given, and the original data have not been found. It was reported negative in the MNvit [17–19], but the studies were performed in rat hepatocytes, which is a non-standard method, and negative in the CAvit in normal and genetically engineered V79 cells. It was clearly positive in Ames strain TA98 with S9 in both preincubation and plate test protocols [20]. The *in vivo* genotoxic activity of 4-acetylaminofluorene may be questionable because, although it was reported positive for induction of transgenic mutations in MutaMouse [17], there are negative and equivocal results reported for UDS in liver, CA in bone marrow, and MN in bone marrow and liver (see [10] for citations). Dimethoate was weakly positive in Ames strain TA100 after preincubation in the presence and absence of S9, but it is notable that positive responses (>2-fold) were seen only at very high concentrations (10 and 16 mg/plate). To call it negative in mammalian cells may be harsh because dimethoate was positive in 1 out of 3 MLA tests [21] and gave mixed results in the MNvit [22–24], and some positive results have been reported in the CAvit [25,26].

Of the 202 Ames-positive *in vivo* genotoxins, 51 were tested for both gene mutation (*Tk*$^{+/-}$ or *Hprt*) and CAvit, and 46 were positive in at least one of the two *in vitro* mammalian cell tests. The 5 *in vivo* genotoxins that did not give a clear positive result in either MLA or CAvit tests are listed in Table 1. 4-Acetylaminofluorene has been discussed above. From the comments in Table 1, it can be seen that there are many reasons to suspect that the other 4 chemicals may be positive in mammalian cells if tested according to a current protocol.

Thus, it appears that almost all Ames-positive *in vivo* genotoxins are also positive in at least one mammalian cell test (34/36 for *Tk*$^{+/-}$ or *Hprt* + MNvit and 46/51 for *Tk*$^{+/-}$ or *Hprt* + CAvit), and there are no clear examples of Ames-positive *in vivo* genotoxins giving negative results in 2 mammalian cell tests covering different endpoints. Where the mammalian cell test results are not clearly positive there are justifiable explanations in terms of the reliability of the results or study design, reliability of the positive Ames result, or reliability of the positive *in vivo* result.

2.1.3. Analysis of non-carcinogens

In the CGX database [9], 170 of 183 non-carcinogens had Ames test results, and 130 of these were negative in the Ames test. Of the 40 non-carcinogens that were positive in the Ames test, 22 had been tested in mammalian cell tests covering 2 different endpoints, and 18 had given unexpected positive results in at least one of the two mammalian cell tests; 4 were negative for both endpoints. The 4 non-carcinogens found positive in the Ames test and negative in the *in vitro* mammalian cell assays were:

- C.I. Food Red 3 (C.I. Acid Red 14)
  - uninterpretable in MLA according to the re-evaluation by Schisler et al. [27], and negative in CAvit but only short treatment times were used [25].
- Calcium cyanamide
  - originally negative in MLA [25], but positive according to the re-evaluation by Schisler et al. [27], and negative in CAvit but only short treatments and early sampling times were used [25].
- Dioxathion
  - uninterpretable in MLA according to the re-evaluation by Schisler et al. [27], and negative in CAvit but only short treatment times were used [25].
- Parathion-methyl
  - negative in MLA but testing was limited by acidic pH shift [11], and negative in CAvit but only short treatment times were used [25].

C.I. Food Red 3 is an azo dye, and requires reductive metabolism to exert its genotoxic effects. For the other 3 substances, the mammalian cell studies do not meet current requirements. There are therefore as many uncertainties regarding the reliability of the negative mammalian cell results with the non-carcinogens as there are with the carcinogens and *in vivo* genotoxins. A similar analysis of chemicals that are negative for *in vivo* genotoxicity was not performed at this time.

2.1.4. Summary of the above analyses

More than 93% of Ames-positive rodent carcinogens and *in vivo* genotoxins are also positive in at least 1 mammalian cell test when MLA and CAvit, or MLA and MNvit are considered. Most of those not clearly positive in either of 2 mammalian cell tests covering different endpoints were:

- Either tested in old or non-standard protocols and may be positive in a current protocol,
- or have given some published positive mammalian cell results,
- or there are insufficient details to determine robustness of negative results,
- or need some specialised metabolism (*e.g.* azo dyes),
- or the reported Ames positive results are not convincing.

2.1.5. When negative results are obtained in two in vitro mammalian cell assays

There were no convincing examples of an Ames-positive carcinogen or an *in vivo* genotoxin giving negative results in 2 robust *in vitro* mammalian cell tests covering different endpoints (gene mutation and either micronuclei or chromosomal aberrations). The numbers of Ames-positive non-carcinogens that had mammalian cell data from 2 different endpoints were much lower. There were also negative results in mammalian cells that may be considered unreliable.

Considering the data at face value (*i.e.* ignoring the uncertainties with some of the published data discussed above), and accepting the results as published, the frequencies with which 2 negative mammalian cell test results have been found were different for the 3 subsets of chemicals analysed. These frequencies were:

- 9 out of 135 (7%) Ames-positive rodent carcinogens
- 6 out of 87 (7%) Ames-positive *in vivo* genotoxins
- 4 out of 22 (18%) Ames-positive non-carcinogens.

Based on the above analysis it was concluded that if a compound is positive in the Ames test, but gives negative results for 2 mammalian cell tests covering different endpoints, it is more likely to be a non-carcinogen than either a rodent carcinogen or

an *in vivo* genotoxin (18% *vs* 7%), and that this is worth further investigation with a broader database of chemicals.

2.2. Review of NTP database

The NTP database was assembled by E. Zeiger from NTP-contracted test results between 1979 and 2013. The advantage of this database is that the *in vitro* mammalian cell assays were performed by standard protocols in only 5 laboratories, with little variation over the years (with the exception of S9 concentrations), and the data were evaluated by standardised criteria. Also, all of the test data are publicly available through the web at http://tools.niehs.nih.gov/ntp_tox/index.cfm?fuseaction=ntpsearch.searchhome&showMessage=true.

The following analyses were performed considering only the clear positive and negative results. Chemicals that had equivocal results in the genetic toxicity assays or in the rodent cancer assay were not included in the analyses, although their presence in the database is noted. There were no test results available from the MNvit test.

2.2.1. Analysis of carcinogenicity data

There were 237 Ames test positive chemicals tested for rodent carcinogenicity; 183 (77%) were carcinogenic, 39 (16%) were non-carcinogenic, and 15 (6%) were equivocal. *In vitro* mammalian cell test data (CAvit, MLA) were available for 204 of the 237 chemicals. MNviv data were available for 95 Ames-positive chemicals tested for carcinogenicity. Although the presence of non-carcinogenic Ames-positive chemicals have been identified and discussed [6], an analysis of results obtained in the *in vitro* mammalian cell for such "misleading" Ames-positives indicators of carcinogenic or *in vivo* mutagenic potential, has not been previously performed.

Among the 39 non-carcinogens, 17 chemicals were tested in both the *in vitro* cytogenetics (CAvit) and mouse lymphoma (MLA) assays; 11 were positive in both, 3 (fenaminosulf; HC Blue 2; 4-nitro-*o*-phenylenediamine) were negative in CAvit but positive in MLA, and 1 (*N*-(1-naphthyl)ethylenediamine 2HCl) was positive in CAvit but negative in MLA. Only 2 chemicals (calcium cyanamide and dioxathion), were negative in both mammalian cell tests. Moreover, there were MNviv test data for 8 of these non-carcinogens; 5 (2-chloroethanol, 2-chloromethylpyridine HCl, 2,4-dimethoxyaniline HCl, glutaraldehyde and 8-hydroxyquinoline) were negative in MNviv and 2 (methyl methacrylate and 4-nitro-*o*-pheylenediamine) were judged equivocal. Therefore 88% (15/17) of the Ames-positive non-carcinogens found in the NTP database were also positive in at least one *in vitro* mammalian cell assay, and no non-carcinogens were negative in both *in vitro* assays.

The NTP database contains 9 phenylenediamines and N-substituted phenylenediamines that were tested for rodent carcinogenicity and in the CAvit and/or MLA tests. Four (2-chloro-*p*-phenylenediamine sulfate, 4-nitro-*o*-phenylenediamine, HC Blue 2 and *p*-phenylenediamine HCl) were non-carcinogens and two of the three were positive in both CAvit and MLA. The 3 carcinogens (2,6-dichloro-*p*-phenylenediamine, HC Blue 1 and 2-nitro-*p*-phenylenediamine) that were tested in CAvit and MLA were positive in both tests. The contrasting results for the carcinogen HC Blue 1, which was positive in both CAvit and MLA, and the structurally analogous non-carcinogen HC Blue 2, which was negative in both CAvit and MLA, are notable and interesting.

As was noted previously [6], a large proportion of the Ames-positive non-carcinogens in the NTP database are benzenamines or *N*-substituted benzenamines. Of the 17 non-carcinogens tested in both *in vitro* mammalian cell tests, 9 (41%) are in this structural class. With the exception of 4-nitro-*o*-phenylenediamine and HC Blue 2, the chemicals in this class of non-carcinogens were positive in all *in vitro* mammalian cell tests.

Table 2
The predictivity of *in vitro* mammalian cell test results for *in vivo* MN results for Ames-positive chemicals in the NTP database.

Test combination	MNviv +ve	(%)	MNviv -ve	(%)
Single test				
CAvit+	28/69	(41)	3/14	(21)
CAvit-	3/14	(21)	11/14	(79)
MLA+	14/41	(34)	1/1	
MLA-	1/1		0	
Two tests				
CAvit+ MLA+	12/36	(33)	0	

+ = positive; – = negative.

The discrepancy between the *in vitro* data and the *in vivo* cancer responses of 2,4- and 2,6-toluenediamine does not appear to be related to differences between their *in vitro* and *in vivo* metabolism, based on comparisons between the mutagenic non-carcinogen, 2,6-toluenediamine and the mutagenic hepatocarcinogen, 2,4-toluenediamine. Moreover, both induced micronuclei in mouse bone marrow cells *in vivo* showing that the 2,6-isomer was non-carcinogenic despite its being genetically active *in vivo*.

Subsequent studies by Cunningham et al. [28] confirmed this conclusion by showing that 2,6-toluenediamine is metabolised by the rat to proximate mutagens. In apparent contrast to this finding, hepatocellular proliferation was induced in the rat by the 2,4-isomer but not by 2,6-toluenediamine after 8 days oral dosing [29]. Also, both isomers produced equivalent mutagenic responses in the livers of Big Blue mice following 30 days in the diet, but the response of the carcinogenic 2,4-isomer was significantly higher after 90 days administration [30]. This difference in the Big Blue responses may not have been noted if the 28-day OECD Test Guideline [31] had been used.

There were 9 non-carcinogens that were tested in at least one *in vitro* mammalian cell test and in MNviv. They were all positive in at least one *in vitro* test and, with one exception (2,6-toluenediamine), were all negative or equivocal in MNviv. In comparison, there were 36 Ames-positive carcinogens that were tested in both *in vitro* mammalian cell tests and in MNviv. All were positive in at least one mammalian cell test with one exception, CI Basic Red 9 HCl, which was negative in CAvit and equivocal in MLA. Of these 36 chemicals, 25 (including CI Basic Red 9 HCl) were negative or equivocal in MNviv.

When the MNviv results were examined to determine if they could distinguish the Ames-positive carcinogens from the non-carcinogens, 31/33 (94%) carcinogens were positive; a negative response in MNviv was not predictive for carcinogenicity, *i.e.*, 16% (8/51) were non-carcinogenic.

These results show that the responses in the *in vitro* mammalian cell tests, or the combination of *in vitro* and MNviv, cannot be used to distinguish Ames-positive carcinogens from Ames-positive non-carcinogens. It is also clear from this database that the use of MNviv provides no added value to the predictivity for cancer of the *in vitro* mammalian cell results.

2.2.2. Analysis of in vivo genotoxicity data

There were 139 Ames-positive chemicals with *in vivo* MN test results; 45 of these were also positive in MNviv, and 83 were negative. Among the 139 Ames-positive chemicals, 96 were tested in at least one *in vitro* mammalian cell test, and 99 had rodent cancer test results.

A positive CAvit result predicted a positive MNviv result for 28/69 (41%) chemicals (Table 2), whereas 11/14 (79%) of the chemicals negative in CAvit were negative in MNviv. There were 3 chemicals negative in CAvit that were positive in MNviv (3′-azido-3deoxythymidine, dimethylvinyl chloride, and propylene

glycol mono-t-butyl ether); dimethylvinyl chloride was positive in MLA. Similar to CAvit, 14/41 (34%) of the chemicals positive in MLA were positive in MNviv; there was only one chemical (butadiene) that was negative in MLA that was tested in MNviv, and the MNviv result was positive. The negative result in the MLA test may be the result of butadiene's volatility.

There were 46 Ames-positive chemicals tested in both *in vitro* mammalian cell tests (CAvit and MLA) that were also tested in MNviv, 36 of which were positive in both *in vitro* tests; 33% (12/36) were also positive *in vivo*. There were no chemicals that were negative in both *in vitro* tests that were tested in MNviv. Among the chemicals tested in MNviv, there were no chemicals positive in CAvit that were negative in MLA. However, four chemicals (CI Disperse Yellow 3, dimethylvinyl chloride, tribromomethane, and chlorodibromomethane) were negative in CAvit and positive in MLA; only dimethylvinyl chloride was positive in MNviv.

Based on these data, a positive response in CAvit or MLA, or in both tests, is not predictive of the *in vivo* MN response. In contrast, negative responses in these *in vitro* tests virtually assure that the *in vivo* MN response will be negative.

There can be a number of reasons for the high proportion of negative results in the *in vivo* tests among the chemicals that are positive in the *in vitro* assays and for carcinogenicity. The positive responses *in vitro*, and in the chronic rodent cancer assay, are evidence that the substances are genotoxic. The negative responses in the MNviv assay strongly suggest that the test chemical, or its mutagenic metabolite, is not reaching the bone marrow target cells at sufficient concentration to produce the genetic damage, *i.e.*, not achieving concentrations equivalent to those that produced positive responses *in vitro*. Another possibility is that the lowered sensitivity of the MNviv test is the result of cells with damaged chromosomes being required to complete a full round of replication, which is not possible for cells with severely damaged chromosomes, before the micronuclei can be visualised.

2.3. Review of Japanese CSCL and ISHL databases

T. Morita reported on data from the Ames test and CAvit obtained from the Japanese Chemical Substances Control Law (CSCL) and the Industrial Safety and Health Law (ISHL) databases. The CSCL database (its other name is Japan Existing Chemical Data Base) [32] includes 277 chemicals with results from the Ames and *in vitro* CA test with CHL cells (as of January 2012). The ISHL database consists of 5 data books, "Mutagenicity Test Data of Existing Chemical Substances", *i.e.*, Data book, 1996 [33]; Suppl. 1997 [34]; Suppl. 2, 2000 [35]; Suppl. 3, 2005 [36]; and Suppl. 4, 2008 [37]. The ISHL database includes 412 chemicals with results from Ames test and CAvit with CHL cells. Both tests in the two databases were conducted in accordance with Good Laboratory Practices (GLP) and Japanese and/or OECD Test Guidelines. For further genotoxicity data searches, the following documents or databases were used: OECD Screening information data set (OECD SIDS) [38], EU risk assessment reports (EU RAR) [39], monographs from IARC [40], NTP database [25], and a publication from Morita *et al.* (1997) [41]. For carcinogenicity data searches, the following databases were used; IARC [42], Carcinogenicity potency database (CPDB) [43], EU Classification, Labelling and Packaging (EU CLP) list [44], "Maximale Arbeitsplatz-Konzentration" (MAK) list [45], NTP database [25], and Japanese Ministry of Health and Labour and Welfare (MHLW) database [46].

The results of the analyses were as follows.

2.3.1. Total database including overlapping chemicals

Among 689 chemicals from the CSCL and ISHL databases, a total of 64 chemicals were found which were Ames-positive and had results from CAvit. About half (29) of them also had data from another mammalian cell test(s) (MLA or *Hprt* mutation). Of these 64 chemicals, 49 had been tested for carcinogenicity, and 15 had been tested for *in vivo* genotoxicity but not tested for carcinogenicity.

Of the 49 Ames-positive chemicals tested for carcinogenicity there were:

- 23 carcinogens that had data in 2 mammalian cell tests (CAvit or MNvit plus $Tk^{+/-}$ or *Hprt* gene mutation);
- 15 carcinogens that had data in only 1 mammalian cell test (CAvit);
- 5 non-carcinogens that had data in 2 mammalian cell tests (CAvit or MNvit plus $Tk^{+/-}$ or *Hprt* gene mutation);
- 6 non-carcinogens that had data in only 1 mammalian cell test (CAvit).

Of the 15 Ames-positive chemicals with *in vivo* genotoxicity data, but not tested for carcinogenicity, there were:

- 1 chemical positive *in vivo* that had data in 2 mammalian cell tests (CAvit or MNvit plus $Tk^{+/-}$ or *Hprt* gene mutation);
- 4 chemicals positive *in vivo* that had data in only 1 mammalian cell test (CAvit);
- 0 chemicals negative *in vivo* that had data in 2 mammalian cell tests (CAvit or MNvit plus $Tk^{+/-}$ or *Hprt* gene mutation);
- 10 chemicals negative *in vivo* that had data in only 1 mammalian cell test (CAvit).

The results for these 64 chemicals are summarised in Table 3. It can be seen that almost all (97%, 37/38) Ames-positive carcinogens were positive in at least 1 mammalian cell test. The database of chemicals tested for *in vivo* genotoxicity was much smaller, but nonetheless 4/5 (80%) Ames-positive *in vivo* genotoxic chemicals were also positive in at least 1 mammalian cell test.

2.3.2. Database with some overlapping chemicals removed

T. Morita then excluded the 15 chemicals that overlapped with the CGX and *in vivo* genotoxin databases discussed above (see Section 2.1). This left 49 chemicals which were Ames-positive and had data from *in vitro* mammalian cell tests. The 15 chemicals that overlapped had all been tested for carcinogenicity, and so this left 34 that had been tested for carcinogenicity, together with the 15 (discussed above) that had been tested for *in vivo* genotoxicity but not for carcinogenicity.

Of the remaining 34 Ames-positive chemicals tested for carcinogenicity there were:

- 8 carcinogens that had data in 2 mammalian cell tests (CAvit or MNvit plus $Tk^{+/-}$ or *Hprt* gene mutation);
- 15 carcinogens that had data in only 1 mammalian cell test (CAvit);
- 5 non-carcinogens that had data in 2 mammalian cell tests (CAvit or MNvit plus $Tk^{+/-}$ or *Hprt* gene mutation);
- 6 non-carcinogens that had data in only 1 mammalian cell test (CAvit).

The analysis of this slightly smaller database gave similar results to that containing overlapping chemicals in that almost all (96%, 22/23) Ames-positive carcinogens were positive in at least 1 mammalian cell test.

2.4. Review of European Pesticide Peer Review database

J.M. Parra Morte presented an analysis of data collected from 186 pesticide peer-reviewed active substances at the European Union level [47,48]. Only 11 were positive in the Ames test. Data from