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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

Table 3
Results from Japanese CSCL and ISHL databases of *in vitro* genotoxicity tests for chemicals tested either for carcinogenicity or *in vivo* genotoxicity (including chemicals overlapping with other databases).

In vitro results for Ames-positive chemicals		No. of chemicals showing *in vitro* profile of results/total			
		Carcinogenicity results		*In vivo* genotoxicity results (not tested for carcinogenicity)	
CAvit or MNvit	Gene mutation (*Tk*$^{+/-}$ or *Hprt*)	Carc	Non-carc	In vivo positive (IVP)	In vivo negative (IVN)
+	+	19/23	3/5	1/1	0/0
+	–	0/23	1/5	0/1	0/0
–	+	3/23	0/5	0/1	0/0
–	–	1/23	1/5	0/1	0/0
+	ND	15/15	5/6	3/4	7/10
–	ND	0/15	1/6	1/4	3/10

+ = positive; - = negative; ND = not determined.

two mammalian cell tests were available for 10 out of the 11 Ames-positive substances: a test for gene mutation and either a test for clastogenicity (CAvit) or the *in vitro* MN test (MNvit). Long-term toxicity studies in rats and mice were available for all substances. Mechanistic data were available for some pesticide active substances in order to demonstrate a non-genotoxic mode of action for carcinogenicity. However, these data were not collected and were not taken into account in the analysis.

Results were categorised as positive, negative or equivocal on a weight of evidence approach since it might be that more than one study was available for some endpoints. Results are detailed in Table 4.

The 7 out of 10 Ames-positive substances that were found positive in at least one mammalian cell test were either rodent carcinogens or genotoxic *in vivo*. By contrast, the 3 Ames-positive substances that were negative in the mammalian cell tests were neither rodent carcinogens nor *in vivo* genotoxic compounds. A further analysis of the Ames test results for these 3 substances is described below:

- Dinocap: the positive results in the Ames test were observed with dinocap technical of low purity. Purified samples of dinocap were not mutagenic in the Ames test. Therefore, positive results in the Ames test might be due to the presence of genotoxic impurities.
- Ethephon: the test substance was only positive in TA 1535 with and without S9.
- Fenitrothion: mutagenicity was observed only in TA 100 and it was enhanced with metabolic activation. Mutagenicity was not observed in a nitroreductase-deficient strain, TA100 NR, and it decreased in a transacetylase-deficient strain, TA100/1,8-DNP6. The results suggest that bacterial nitroreductase activity is necessary for fenitrothion to express mutagenicity.

2.5. Analysis of data from SCCS

V. Rogiers presented data that had been collected at the Vrije Universiteit Brussel (VUB) by herself, Tatyana Y. Doktorova and Gamze Ates, and has been published [49]. For this survey, the VUB's Cosmetics ingredients DataBase (CosDB) was used, which contains toxicological information on cosmetic ingredients that are present in the Annexes of the Cosmetic Directive 76/768/EEC (preservatives, UV-filters, colorants and specially studied substances with a potential risk for human health such as hair dyes). The data was extracted from the opinions generated in the period between 2000 and 2012 by the Scientific Committee on Cosmetic Products and non-food products (SCC(NF)P) and Scientific Committee on Consumer Safety (SCCS) [50]. For the purpose of the workshop, only the genotoxicity test results were evaluated, and in particular the substances showing a positive Ames test were selected.

The survey provided 52 Ames-positive compounds with quality data (evaluated by the experts of the SCC(NF)P and SCCS). Analysis of data from the other *in vitro* genotoxicity assays performed on the Ames-positive compounds showed that the mammalian cell gene mutation test on *Tk*$^{+/-}$ or *Hprt* loci (49/52) and the CAvit (38/52) are the most commonly performed *in vitro* tests. Interestingly, 20 (including equivocal results) out of the 49 mammalian cell gene mutation assays were also positive, whereas for the CAvit, 25 (including equivocal results) out of the 38 compounds also tested positive. A survey of the outcome of the follow-up *in vivo* tests carried out for those compounds showed that a positive Ames test resulted in only 2 compounds that were genotoxic *in vivo* (4% Ames-positive compounds confirmed *in vivo*). The other 50 compounds produced negative results in the follow-up *in vivo* genotoxicity tests (96% "false" positives based on the Ames test). Only 8 out of the 52 scrutinised compounds had carcinogenicity data (2-year rodent carcinogenicity assay).

A more detailed examination of the data showed that 47 out of the 52 Ames-positive compounds were identified as hair dyes (19 semi-permanent hair dyes, 16 oxidative hair dyes and 12 hair dyes that can be used either as a semi-permanent hair dye or as an oxidative hair dye depending on the presence of peroxide). Analysis of the number of "false" positives was performed for each of the 3 separate groups, but the outcome did not differ significantly from the general analysis of the 52 compounds.

Finally, the hypothesis as to whether Ames-positive results could be overruled by 2 negative *in vitro* mammalian cell assays was challenged. It appeared that 9 out of the 52 Ames-positive compounds were associated with 2 negative *in vitro* mammalian cell tests. The concomitant *in vivo* genotoxicity test results were negative as such classifying the Ames test results as being "false" positive. In 7 out of the 9 cases the positive results were obtained in *Salmonella typhimurium* TA98 strain in the presence of S9.

2.6. Review of ECHA database

At the time of the workshop, data on industrial chemicals submitted to the European Chemicals Agency (ECHA) under the REACH legislation were not available. However, lists of chemicals for which carcinogenicity *in vivo*, and *in vitro* genotoxicity data were retrieved from the ECHA's registration database by P. Karamertzanis and F. Le Curieux, and were subsequently analysed by Ph. Vanparys on behalf of EURL ECVAM.

The dossiers considered for the current evaluation were retrieved from ECHA's registration database (http://echa.europa.eu/information-on-chemicals/registered-substances; last accessed 22 July 2014) by applying a set of filters. The first filter that was applied was to select lead and individual dossiers that fulfilled the information requirements of REACH Annex IX or X and were submitted prior to the 2010 registration deadline. This filter produced a set of 1904 dossiers that were subsequently analysed with regard to the genotoxicity, carcinogenicity and classification information they contained. A second filter selected dossiers with a positive

Table 4
Results from Ames-positive pesticides from the European Pesticide Peer Review Database in mammalian cell tests, *in vivo* genotoxicity and carcinogenicity studies.

Pesticide	CAS No.	Gene mutation (*Hprt* or *Tk*$^{+/-}$)	MNvit or CAvit	Some evidence of *in vivo* genotoxicity[a]	Rodent carcinogenicity
Carbendazim	10605-21-7	–	+	+	+
Dichlorvos	62-73-7	+	+	+	E
Dinocap	39300-45-3	–	–	–	–
Ethephon	16672-87-0	–	–	–	–
Fenitrothion	122-14-5	–	–	–	–
Parathion-methyl	298-00-0	+	+	+	–
Phosmet	732-11-6	+	–	–	+
Tri-allate	2303-17-5	–	n.a	–	+
Acetochlor	34256-82-1	+	+	+	+
1,3-dichloropropene	542-75-6	–	+	+	+
2-phenylphenol	90-43-7	+	+	E	+

+ = Positive; – = negative; E = equivocal; n.a. = not available.

[a] For the purposes of this analysis the criterion applied to define an "*in vivo* genotoxic compound" was when there was some indication of genotoxicity *in vivo*.

Ames study. The positive result had to be in an endpoint study record that according to the registrant's assessment was considered reliable (reliability 1 or 2). A reliability score of 1 means that the study is reliable without restriction, while a reliability score 2 is related to studies for which there are some restrictions (*e.g.* no reference to OECD Test Guidelines, deviation from OECD Test Guidelines, significant methodological deficiencies, *etc.*). The algorithm did not attempt to recognise weight of evidence in Ames, *i.e.* one reliable positive result was sufficient to select the dossier, regardless of the outcome of other Ames studies. At this stage, study results based on read-across were also included. This approach led to approximately 400 dossiers with evidence of positive Ames results that were then used to contrast the positive Ames outcome with *in vivo* mutagenicity and carcinogenicity data in the dossier. Because identifying cancer evidence in carcinogenicity studies is not straightforward, the algorithm used the carcinogenicity classification. In some cases a harmonised classification for carcinogenicity was available, (*i.e.* Globally Harmonized System of Classification and Labeling of Chemicals (GHS) categories C1A, C1B or C2), in others the substances had been self-classified by the registrants [51]. The dossiers with evidence of positive Ames results were divided into the following four lists:

- Ames positive chemicals that were negative for carcinogenicity (list 1): at least one reliable carcinogenicity study AND no harmonised and self-classification as C1A, C 1B or C2 [51]. List 1 contained 72 dossiers related to 71 unique substances.
- Ames positive chemicals that were negative for *in vivo* genotoxicity (list 2): at least one reliable *in vivo* genotoxicity study with no positive results, and no harmonised or self-classification as germ cell mutagen M1A, M1B or M2 (as also defined in [51]). List 2 contained 154 dossiers related to 154 unique substances.
- Ames positive chemicals that were positive for carcinogenicity (list 3): the availability of a harmonised or a self-classification as carcinogen C1A, C1B or C2 [51]. List 3 contained 280 dossiers related to 266 unique substances.
- Ames positive chemicals that were positive for *in vivo* genotoxicity (list 4): at least one reliable *in vivo* genotoxicity study, and at least one positive result in a reliable *in vivo* genotoxicity, or the availability of a harmonised or a self-classification as germ cell mutagen M1A, M1B or M2 [51]. List 4 contained 240 dossiers related to 226 unique substances.

There were dossiers that could be found in more than one of these lists. After this automated procedure the lists were manually examined and post-processed. In particular, the following substances were removed: petroleum derivatives (which accounted for the majority of the substances), substances duplicated within the other databases considered for the overall analysis (9 chemicals overlapped with NTP, 11 with the Japanese CSCL Database, 11 with CGX [9], 1 with DG SANCO SSCS and Cosmetics Industry DB), polymers and inorganic compounds with no structure and/or no molecular weight, and mixtures. This resulted in a final list of 73 registered dossiers corresponding to 73 unique substances.

These 73 selected dossiers were downloaded from the ECHA dissemination website and reviewed. At this stage, read-across data were not considered. Tests that were reviewed for the purpose of this analysis were: Ames test, CAvit, MNvit, MLA, *Hprt*, CAviv, MNviv, UDSviv, *in vivo* comet, and transgenic mouse models. For carcinogenicity the study reports were reviewed and the classification (harmonized or self-) was reported when available. A positive, negative, inconclusive or equivocal call was given for each test result. Since for some chemicals more than one study per test was available, a total of 1062 studies were reviewed. Among these studies some were considered not informative for the purpose of this exercise (*e.g.* read-across). Subsequently, for each test method an overall call was given following the same criteria as those used in the construction of a consolidated database (see [52]). The review of the ECHA dossiers relied entirely on the information submitted by the applicant.

In several cases the information reported was not sufficient to allow a firm overall conclusion for a certain test in accordance with the criteria defined. This was the case with negative or unclear results, where no firm conclusion could be made in terms of requirements specified in current OECD Test Guidelines or recommended best practices (*e.g.* studies only without S9 or only with S9, abnormal pH levels or osmolality, not the standard set of bacterial strains, no adequate levels of toxicity, no proof of target cell exposure *in vivo*, *etc.*). Often the submitted studies reported only the conclusions and omitted the original data; in other cases, references were made to reports which were not accessible or where references were not available. Thus, for the above reasons, despite the massive review work, several studies were given an overall "inconclusive" call and were as such not considered informative.

The re-evaluation of the 73 compounds resulted in 29 Ames-positive chemicals, 12 Ames-equivocal, 16 Ames-inconclusive, and 16 Ames-negative. These data were considered insufficient to allow a meaningful analysis of the ECHA results in isolation. However, it was decided to add these data to a new consolidated database [52], but it was necessary to conduct a further search of literature data with the aim of clarifying some of the inconclusive or equivocal calls. ECHA data, which resulted from the subsequent expert and literature review, and added to a consolidated database [52] therefore represent data from 32 chemicals that were submitted to ECHA for the 2010 registration deadline.

2.7. Review of industry data

2.7.1. BASF data on pesticides and industrial chemicals

M. Schulz (BASF, Germany) reported that data on positive Ames studies and *in vivo* follow-ups is rare for pesticides, as a positive Ames test result is in general a cut-off criterion for the development of new chemical entities. Nevertheless, the occurrence of positive findings in bacterial reverse mutation assays with industrial chemicals and pesticides at BASF was extremely low over the last 7 years with only 19 out of 574 compounds (3.3%) giving positive Ames results. It should be noted that from this set of 19 positives, 6 test materials were complex mixtures. Twelve of these 574 substances were tested in the rodent bioassay and failed to induce tumours, whereas 2 of these gave positive results either in the Ames test or in an additional mammalian cell genotoxicity test (*Hprt* mutation or CAvit). Both substances were biocides, so the *in vitro* observations were not that surprising.

2.7.2. Pharmaceutical industry

A. Lynch (GSK, UK) reported that the Ames Test for mutagenicity is a strong predictor of genotoxic carcinogenicity and is a pivotal 'must do' in progressing a drug for non-life threatening indications, where positive results often represent a "no go" decision for further development.

Since 2009, GSK has tested 976 compounds in the Ames test; these have included active pharmaceutical ingredients (APIs) and drug synthesis intermediates, impurities, degradants and, on occasion, human-specific metabolites. Of these, the majority of API's were negative (86.3%). Of the 134 positive API's (13.7%), 37 compounds were positive only in the presence of induced rat liver S9 metabolic activation (27.6% of positives, 3.8% of compounds tested). The development of an Ames-positive API is generally terminated early in the absence of a positive risk/benefit assessment. Such compounds are seldom assessed *in vivo* for genotoxicity or carcinogenicity, and there are only 11 examples of APIs in the GSK database with positive Ames results and additional *in vivo* genotoxicity and/or carcinogenicity data (Table 5). One reason for this is the positive impact of quantitative structural activity relationship (QSAR) assessment on drug design where careful consideration of chemical structure during lead-drug optimisation avoids the synthesis of API with overt mutagenic structures.

Reactive chemistry is still the mainstay of small molecule drug synthesis and the generation of the final API can often require several intermediate steps. The control of drug synthesis intermediates and impurities in the final API is therefore an important consideration for worker safety and for patients, respectively. Where possible, manufacturing procedures are put in place to eliminate and/or control levels of contamination. Ames-positive drug synthesis intermediates and impurities are generally controlled to the threshold of toxicological concern (TTC) and these compounds are also rarely assessed for *in vivo* genotoxicity or carcinogenicity. The methods on which the TTC value is based are generally considered to be extremely conservative as they involve a simple linear extrapolation from the dose giving a 50% tumour incidence (TD_{50}) to a 1 in 10^{-6} incidence using TD_{50} data for the most sensitive species and most sensitive site *i.e.* several worst case assumptions [53]. As indicated in the European Medicines Agency (EMA) guideline on limits of genotoxic impurities in pharmaceutical products [54], a TTC value of 1.5 μg/day is expected to result in no more than a 10^{-5} lifetime cancer risk. Compounds which cannot be controlled to the TTC either require extensive follow-up testing with additional *in vivo* (geno)toxicology studies to determine their potential carcinogenic risk, or alternative synthetic routes can be considered which eliminates the compound of concern. There are 19 examples of intermediates/impurities in the GSK database with Ames-positive results and additional *in vivo* genotoxicity and/or carcinogenicity data (Table 5).

Ames-positive degradation products and/or human specific metabolites represent another category of compounds which present a significant challenge. These compounds are often only identified later on during drug development. One such degradant is contained in the GSK database of Ames-positive compounds (Table 5).

2.7.3. Cosmetics industry

Genotoxicity data that were generated in conjunction with the submission of safety dossiers for hair dye ingredients as mandated by the European Commission (currently SCCS) were presented by S. Pfuhler (The Procter & Gamble Company, Cincinnati, OH). The majority of the data that went into the industry dossier submissions was generated between 2003 and 2009, and those studies were performed in compliance with OECD Test Guidelines and GLP. The dossiers were reviewed by the SSCS and the outcomes (the so-called opinions) are published on the respective website of the European Commission [50]. Thus, most of the data here presented are related to substances also reviewed in Section 2.5.

An overview of all genotoxicity data for those dyes that have shown unfavourable (*i.e.* positive) Ames results ($n = 27$) was presented to the workshop. For all dyes, data from one to three mammalian cell-based *in vitro* studies was available, as well as data from at least one *in vivo* follow-up study (mostly two). All *in vivo* follow-up studies were negative with one exception that will be discussed later, thereby supporting the assumption that the positive results observed in the Ames test may not be relevant for the safety assessment of these compounds. The profile of the *in vitro* mammalian cell results, on the other hand, was very inconsistent. The mammalian cell data do not seem to give a clear picture in terms of their value in predicting the *in vivo* genotoxicity potential of these dyes, however there were several dyes that have a signature that seems worth mentioning. Five out of 27 Ames-positive hair dyes exhibited a negative profile in the mammalian cell-based studies performed. In these cases the *in vitro* mammalian cell data agreed with the negative *in vivo* data, which supports the value of such a data constellation for predicting the absence of a genotoxic potential *in vivo*.

Table 5
Summary of Ames-positive compounds from the GSK database with *in vivo* genotoxicity and/or carcinogenicity data.

Tot No.	DEREK alerts	Positive BlueScreen-GreenScreen (total)	Positive Ames (total)		*In vitro* mammalian positive (total)			Positive *in vivo*			Carc (Total)	Comments
			+S9	-S9	MLA ($Tk^{+/-}$)	CA	Other	MN	Comet	UDS		
38	23	12 (13)	28	29	14 (22)	6 (8)	2 (4)	0 (38)	0 (5)	1 (6)	3 (6)	11 API 19 interm. 1 degradant

Interm. = intermediate; API = active pharmaceutical ingredient.

Table 6
Factors affecting chemical accessibility, metabolism and toxicity in bacteria, mammalian cells and intact mammals.

Bacteria	Mammalian cells	Mammals
Circular DNA (no nucleus)	Nuclear chromosomes with associated proteins and histones	Nuclear chromosomes with associated proteins and histones
Cell wall	Plasma membrane	Plasma membrane
Single cell, exposure *via* immediate environment	Single cell or monolayers, exposure *via* solution	Tissue and organs, exposure *via* ADME (bloodstream)
Limited xenobiotic metabolism	Limited xenobiotic metabolism	Extensive xenobiotic metabolism
Limited antioxidant activity	Some antioxidant activity	Full antioxidant activity
Response to stress/toxicity is mutation	Response to stress/toxicity is cell death	Response to stress/toxicity is cell death
Default to a toxic environment is survival through mutation	Default to a toxic environment is cell death	Default to a toxic exposure is cell death
Response to toxicity is dose dependent	Response to toxicity is dose-dependent with a low threshold	Response to toxicity is dose-dependent with highest possible threshold
DNA damage repair is geared to survival/mutation	DNA damage repair is geared to fidelity	DNA damage repair is geared to fidelity

By contrast, the only dye that exhibited positive results *in vivo* (1-hydroxy-2-amino-5-metyhylbenzene; mouse micronucleus assay) has a very different *in vitro* profile, and gave positive effects in all mammalian cell assays that were performed. While the *in vivo* positive result, observed in a study using oral gavage, is pointing towards a genotoxic hazard it is important to mention that this dye will be activated by phase I hydroxylation but detoxified by N-acetylation, a phase II reaction prevalent in the skin [55–57]. The fact that this dye produced an increase in the frequency of bone marrow micronuclei after oral exposure and was positive in the mammalian cell assays, however, is supportive of the use of an *in vitro* only follow-up strategy for Ames-positive compounds, in that two mammalian cell studies with favourable outcomes can be taken as an indication of absence of *in vivo* genotoxic potential.

2.8. Metabolic considerations

N. Gooderham presented his views on "The role of metabolism in genotoxicity – the dynamic dimension". The requirement for metabolism in mammals has almost certainly evolved through competition between plants and animals for survival. As part of a defence mechanism to dissuade mobile competitors (insects and higher order species) from consuming them, plants synthesise chemicals that are distasteful or harmful to higher order organisms. In many cases these chemicals have no nutritional value and can be inherently toxic. Thus during evolution, mammals developed strategies to eliminate or reduce their exposure to these foreign chemicals (xenobiotics) when consumed as part of their diet. Such compounds are generally lipophilic and once they have access to the body, they would accumulate in fatty tissue. Mammalian metabolism has evolved to convert these lipophilic materials to polar derivatives to facilitate their excretion and elimination from the organism. The chemical diversity of these xenobiotics is enormous, thus the enzyme systems that have evolved to deal with this array of structures have broad substrate specificity at the expense of the high catalytic efficiency normally associated with biochemistry. In the higher order species such as mammals, the diversity of structure that metabolism can process is truly remarkable. These metabolic systems transform xenobiotics, dependent upon the chemical's physiochemical characteristics (*e.g.* lipophilicity, pKa), using and manipulating functional groups with the objective of bestowing polarity on the molecule [58]. In understanding the mammal's ability to achieve this objective, we can define discrete processes involved as the chemical journeys through the body, specifically absorption, distribution, metabolism and elimination (ADME).

Each of these steps involves interaction of the xenobiotic with biological biomolecules and the entire process is highly dynamic. There are competing pathways and thus multiple metabolic products (metabolites), kinetic considerations, functionalisation and conjugation reactions, competing chemistry, and enzyme inhibition/induction effects [59]. All of these elements can alter the metabolic fate, and thus the toxicity, of xenobiotics. Other considerations include qualitative issues such as pathway deficiency (*e.g.* sulphation in the pig), enzyme polymorphism, precursor deficiency (*e.g.* sulphate levels), and tissue and species specificity [60].

Metabolism is usually geared to detoxication as the compound is rendered polar and thus excretable, but sometimes metabolism results in a more damaging product being generated, leading to toxicity. Detoxication and activation processes occur within the same temporal frame and at any specific time the balance between activation and deactivation determines toxicity and hence a toxicity threshold.

Since mammalian metabolism capacity is the product of evolution, there are significantly different responses to xenobiotics in bacteria compared to mammals. Specifically, there can be species specific metabolism, differing detoxication thresholds, differing oxidative damage responses, chemical reactivity of the xenobiotic with a species-specific biomolecule resulting in the formation of a reactive product, and species-specific responses to toxicity (see Table 6).

Thus, bacteria and mammals have very different metabolism capability and this ability to activate or deactivate xenobiotics has led to the evolution of different outcome strategies to address toxicity (Table 6). The bacterial response to toxicity is mutation to adapt and survive, whereas the mammalian cell default to toxicity is cell death (apoptosis/necrosis). Examples of metabolic differences between bacteria and mammalian cells include bacterial alkyl hydroperoxide reductase, used as a defence against DNA oxidative damage [61]; high levels of reductive enzymes, compared to mammalian cells, permitting bacteria to efficiently activate nitro and azo compounds to electrophilic metabolites [62]; limited phase 2 conjugation capacity, although bacteria are usually proficient in glutathione S-transferase activity [63]. In current *S. typhimurium* bacterial mutagenicity assays, to circumvent this limited metabolism capacity, the incubation of bacteria with xenobiotics is supplemented by adding mammalian hepatic S9, a partially purified tissue preparation rich in enzymes involved in xenobiotic metabolism. Traditionally this S9 is prepared from the livers of rats treated with mixtures of chemicals known to induce hepatic drug metabolism. Typically the inducer will be Aroclor 1254 (a mixture of polychlorinated biphenyls) or a phenobarbital/β-naphthoflavone mixture. Both treatments preferentially induce oxidative liver enzymes, particularly cytochromes P450. Inclusion of induced rat liver S9 in bacterial mutagenicity assays generates a highly oxidising catalytic environment that favours oxidative

activation and bears little resemblance to human metabolic capacity. The induced S9 preparation has limited phase 2 (conjugation metabolism) activity due to deficient concentrations of obligatory co-factors. It should also be noted that the metabolising potential of S9 is species specific.

Intact mammalian cells have an active phase 1 and phase 2 metabolism capacity. Hepatocytes would express the best complement of enzymes, but are technically difficult to isolate and use. Furthermore, since primary hepatocytes are not in a proliferative phase, there is a small window of opportunity (isolation to 4–6 h) after which the metabolic capacity is compromised. Using established cell lines is a potential alternative, but these invariably are enzyme deficient, especially in regards to the range of cytochromes P450, or if engineered, express an unbalanced enzyme profile.

These fundamental metabolism differences between bacteria, mammalian cell lines and intact animals are illustrated by responses to the food-derived heterocyclic amine 2-amino-3,8-dimethylimidazo[4,5-b]quinoxaline (MeIQx). MeIQx is carcinogenic in lifetime bioassays at high doses (400–600 ppm in the diet) in both rats and mice [64,65]. In *S. typhimurium* bacterial mutation assays, MeIQx is negative in the absence of S9, but is a remarkably potent mutagen in the presence of Aroclor-induced rat liver S9 and human liver S9 [66]. In mammalian cell genotoxicity assays, MeIQx generally gives a weak response (UDS in rat and hamster hepatocytes; *Hprt* locus in CHO cells; *Hprt* locus in V79 cells; SCE in CHO cells; micronuclei in HepG2 cells) [67,68]. In *in vivo* genotoxicity assays, the response is again generally weak requiring high doses (micronuclei and SCE in C57Bl/6 mice) or negative [69]. In other *in vivo* genetic damage assays, such as formation of GST-P positive foci in rats, the trend is repeated with positive foci only being scored at high doses [70]. In these experiments, there was evidence of a dose threshold effect for GST-P foci, yet in the same animals ^{32}P-postlabelling analysis suggested clear dose-dependency of adduct formation even at very low dose exposures [71]. This has led Wei et al. [72] to propose that a practical threshold dose exists for this genotoxic carcinogen. These dose-dependent effects can be rationalised on the basis of switching the MeIQx metabolic profile. Under the metabolic conditions used in the Ames test and in high dose *in vivo* studies, oxidative metabolism of MeIQx to its genotoxic N-hydroxy metabolite is favoured. Analysis of the kinetics of MeIQx metabolism in the rat indicates that at steady state the catalytic efficiency (K_{cat}/K_m) for the primary oxidative pathways is C5-OH > N-OH > C8-OH. At low doses of MeIQx the substrate is limiting and C5-oxidation predominates. Formation of the N-hydroxy metabolite (genotoxic) is limited by substrate availability and removal by C8-oxidation and glucuronidation. At high doses, the oxidative pathways are saturated and the N-hydroxy metabolite accumulates due to substrate availability and inefficient C8-oxidation and limiting glucuronidation.

These considerations indicate that it is possible to have an Ames positive result under conditions that favour high levels of oxidative metabolism, yet performing similar experiments in mammalian cells results in less genotoxic potential or perhaps negative results. This paradigm is continued *in vivo* unless the doses of chemical used exceed the capacity of key detoxication pathways, thereby leading to metabolic switching to toxication pathways and resulting genotoxicity. Under these circumstances genotoxicity shows a pragmatic threshold that is governed by the kinetic considerations of competing metabolic pathways.

2.9. Supplementary tests

The possible role of additional *in vitro* approaches as follow-up tests was presented by Ph. Vanparys. A core *in vitro* genotoxicity battery comprising the Ames test plus the MNvit is sufficient to detect rodent carcinogens [10]. The expectation is that in the future for chemical testing, more results will be generated with the combination of the Ames test and MNvit. For most industrial sectors, development of compounds positive in the Ames test and MNvit will be stopped before going into *in vivo* tests. But compounds positive in the Ames test and negative in the MNvit will trigger additional testing. In his opinion, compounds positive in the Ames test only in the absence of an exogenous metabolic activation system (S9 mix) should be further tested in the MLA or *Hprt* test. When found negative in the *in vitro* mammalian gene mutation assays, it is reasonable to assume that the compound has no mutagenic potential in mammalian cells. Compounds found positive in the Ames test only in the presence of S9-mix should be further tested in the Ames test with a battery of different S9-mix systems to explore species differences in the metabolising pattern. If the positive findings are related to a metabolising pattern not relevant for humans, the next step is to assess the compound in an *in vitro* mammalian gene mutation test. When negative in this assay, it is also reasonable to assume that the compound has no mutagenic potential in mammalian cells. In order to define whether mutagenic potential can lead to transformations, a cell transformation assay (CTA) preferentially on Syrian hamster embryo cells (SHE) cells can be performed.

Genotoxicity may result from a DNA-reactive or non DNA-reactive mechanism of action. Transcriptomics are very useful to define the mechanism of action and to define whether the particular mechanism can lead to genotoxicity *in vivo* and to carcinogenicity [73,74]. Transcriptomics can also be very helpful for the determination of the relevance of positive findings in the *in vitro* genotoxicity tests. It would be useful if it was possible to provide clear guidance on follow-up testing, but each data set would need to be considered on a case-by-case basis.

3. Discussion

Following the individual presentations the participants formed 2 breakout groups. The groups were asked to address the following questions, respectively:

1. Are there any general trends that can be concluded from the data regarding the “risk” predicted by different patterns of *in vitro* results (whether mammalian cell tests are positive or negative in the face of Ames-positive results)? Do different patterns of *in vitro* results signify different follow-up approaches (particularly if and when *in vivo* tests are needed)?
2. What *in vitro* tests, if any, can be eliminated without diminishing the predictivity for *in vivo* genotoxicity or cancer of the standard battery? What *in vitro* tests can be added to help with the decision-making process? In what circumstances would you select additional *in vitro* studies, and which ones?

Breakout group 1 looked at the data provided in the individual databases and made some initial analyses. They noted the following:

- The incidence of carcinogens among the Ames-positive results is ~70%, but this includes genotoxic and non-genotoxic carcinogens
- The incidence of Ames-positive non-carcinogens is ~24%
- The incidence of Ames-positive compounds with negative results in 2 mammalian cell assays is:
  - 1.2% (1/86) for *in vivo* genotoxic compounds
  - 3.3% (9/211) for carcinogens
  - 21.4% (15/70) for *in vivo* non-genotoxic compounds
  - 22.6% (12/53) for non-carcinogens.

Thus the group concluded that, on the basis of this limited analysis, if an Ames-positive compound shows negative results in

well-performed *in vitro* mammalian cell tests for both chromosomal damage and gene mutations, the compound tested is unlikely to be an *in vivo* genotoxin or a carcinogen. If supporting evidence from mechanistic studies is available then further (*in vivo*) testing should not be necessary. Thus, if a 2 test battery of Ames and MNvit is used, and gives positive results in the Ames test but negative results in the MNvit, then a second mammalian cell assay (with gene mutation endpoints) should be performed.

However, the group noted that bias could result from the small sample sizes of Ames-positive chemicals giving negative results in 2 mammalian cell tests. There could also be sample bias associated with the number of tests of a particular assay type. It was noted that there was over-representation of carcinogens and of certain chemical classes in some of the databases, and that these databases consisted of a large number of old mammalian cell studies in which the criteria for positive or negative outcomes would most likely have been based on criteria no longer used.

Breakout group 2 noted that for an Ames-positive result it is important to investigate the response pattern, *e.g.*, are the results positive in the absence or presence of S9, in which strains are the responses seen, and at which magnitude. Based on these results is it possible to generate a hypothesis why the Ames test is positive? Addressing such a question should include analysis of the chemical structure (are there structural alerts?), and try to understand if there are metabolic pathways that are unlikely to be relevant for humans (*e.g.*, nitroreduction in bacteria). Another possible explanation is that many of the azo dyes are not very pure, and the positive Ames test result for these chemicals may be produced by low levels of mutagenic impurities that do not need azo reduction for their mutagenicity. Because of the lower sensitivity of many of the mammalian cell assays, these impurities may not be present at levels sufficient for the mammalian cell response.

This group also discussed the situation where a chemical is positive in the Ames test but negative in the MNvit. In such a case, it is important to check the purity of the test material, because a trace contaminant may have a serious impact on the results.

Such results also need to be considered in light of differences in metabolism between the Ames and mammalian cell tests, for example:

- Different S9 concentrations are used, most usually 10–30% S9 mix in the Ames test, but 2–4% S9 mix in the *in vitro* mammalian cell tests.
- The duration of exposure is longer in the Ames test as compared to the mammalian cell tests.
- The intrinsic metabolism of the bacterial and mammalian cells may produce different metabolic profiles with a test chemical.
- The S9 mix used favours oxidative metabolism. Bacteria may be less able to defend against such oxidative metabolites whereas mammalian cells may have sufficient phase II metabolism to promote detoxification.

Modifications to the standard assays were also considered as being useful. Standard S9 is supplemented with co-factors for phase I activation enzymes. It may be useful to include an additional arm to a standard study that includes co-factors for phase II metabolism. To further understand whether the results using rat liver S9 are relevant, metabolic profiling could be performed in induced rat hepatocytes and compared with human hepatocytes.

With Ames-positive mammalian-cell negative chemicals it is also worth considering whether the test compound induces oxidative stress. Bacteria and mammalian cells have different capabilities to cope with specific oxidative stress. Some Ames strains are more susceptible to oxidative stress (*e.g.* TA97, TA100, TA102), and positive responses in these strains can give a hint that oxidative stress may be responsible for the positive findings. An option would therefore be to modify the Ames test design by addition of anti-oxidants, although care should be taken that this does not affect metabolic activation by S9. Another option would be to perform specific biochemical assays addressing oxidative stress.

The use of the 3D-skin models may be useful to follow-up Ames-positive results with dermally exposed chemicals. A negative comet assay in a 3D-skin model would tend to confirm the negative mammalian cell result showing that there is no primary DNA-damage. Alternatively co-culture systems (using 3D-skin and mammalian cells) can be used for this purpose as well.

CTAs can provide some additional information for weight of evidence assessments to help in decision making especially when no *in vivo* testing is allowed. Test systems, where the target is a stress gene (*e.g.*, Greenscreen/Bluescreen with GADD45) may also be useful. However, these test systems have not yet been evaluated in the context of Ames test positive responses/mammalian test negatives, as addressed in this workshop. Toxicogenomic approaches may also provide some additional information on mode of action.

The strengths and weaknesses of these different follow-up approaches are summarised in Table 7. In general, these assays can be sub-divided into assays that give mechanistic information and assays that might be necessary to confirm a conclusion or negative mammalian cell results.

Table 7
Strengths and weaknesses of different follow-up approaches to Ames-positive MNvit-negative chemicals.

Assay type	Strengths	Weaknesses
3-D skin comet	*In vivo*-like	Not validated yet.
Metabolism studies	Help explain the positive Ames result and its relevance.	Cost, time, specialised laboratories.
Toxicogenomics	Mechanistic information	Cost, time, specialised laboratories. Not validated yet.
Repair assays	Mechanistic information	Not validated yet.
Cell transformation assay	Correlates well with rat carcinogens.	The mechanisms underlying the transformations are not known.
Greenscreen/Bluescreen	Useful in screening. Detects a wide variety of DNA-damage.	Respond to non-specific DNA-damage.
In silico studies for metabolism (*e.g.* Meteor)	Alerts for activation pathways. Meteor can give all the possible metabolites. May help to define what to do next.	Meteor does not distinguish between the metabolites (specific for bacteria and specific for mammalian cells).
In silico studies for structural alerts	Specific structural alerts. May help define which tests to select.	Applicability domain limited (new structures).

4. Conclusions and recommendations

In the final plenary discussion the workshop participants agreed that, based on the individual presentations and the initial analysis of results across the different databases, there appear to be patterns of results in mammalian cell tests that are consistent across databases and indicate whether an Ames-positive chemical is likely or not likely to be carcinogenic or genotoxic *in vivo*. In particular it appears that negative results in mammalian cell tests covering clastogenic/aneugenic and gene mutation endpoints could indicate that an Ames-positive chemical is not likely to be carcinogenic or genotoxic *in vivo*. However, there were relatively few Ames-positive chemicals in the individual databases that were negative in mammalian cell tests *in vitro* and for carcinogenicity. There was consensus that because of their regulatory implications, these preliminary results were worth following up. Given that the individual databases contained overlapping chemicals with occasionally contradictory results, it was recommended that a consolidated database be constructed that would eliminate duplication, and that this be analysed. The construction and analysis of the consolidated database is reported separately [52].

It was also acknowledged that, even if the analysis of the consolidated database shows that negative mammalian cell test results could be indicative that an Ames-positive chemical is not likely to be carcinogenic or genotoxic *in vivo*, it is unlikely that a firm conclusion could be reached, or *in vivo* testing be avoided, reduced or delayed, without further data. The suggestions discussed above for follow-up studies to investigate metabolism, oxidative stress, and effects in other *in vitro* test systems should be considered.

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Conflict of interest

The authors have declared that there are no conflicts of interest.

Disclaimer

This document represents the consensus of the participants' views expressed as individual scientists and does not necessarily represent the policies and procedures of their respective institutions.

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