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Analysis of published data for top concentration considerations in mammalian cell genotoxicity testing

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The ability of the *in vitro* mammalian cell tests currently used to identify genotoxins has been shown to be limited by a high rate of false-positive results, triggering further unnecessary testing *in vivo*. During an European Centre for the Validation of Alternative Methods workshop on how to improve the specificity of these assays, testing at high concentrations was identified as one possible source of false positives. Thus far, Organisation for Economic Co-operation and Development genotoxicity test guidelines have required testing of chemicals using mammalian cells *in vitro* should be undertaken to concentrations as high as 10 mM (5000 μg/ml). Recently, a draft revision of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use genotoxicity test guidelines has recommended that testing concentrations should be reduced to 1 mM (500 μg/ml). To assess the impact that this lowering would have on the outcome of *in vitro* genotoxicity testing, we established a database of 384 chemicals classified as rodent carcinogens and reported Ames test results and the test concentrations that produced positive results in the mouse lymphoma assay (MLA), *in vitro* chromosome aberration (CA) assay and *in vitro* micronucleus test. Genotoxicity testing results were illustrated for 229 and 338 compounds in the MLA and *in vitro* CA assay, respectively. Of these test compounds, 62.5% produced positive results in the MLA, of which 20.3% required testing between 1 and 10 mM. A total of 58.0% produced positive results in *in vitro* CA assays, of which 25.0% required testing between 1 and 10 mM. If the testing concentration limit for mammalian cell assays was reduced to 1 mM, 24 (6.25%) potential carcinogens would not be detected in any part of the standard *in vitro* genotoxicity test battery (Ames test, MLA and *in vitro* CA assay). Further re-evaluation and/or retest of these compounds by Kirkland and Fowler [Kirkland, D. and Fowler, P. (2010) Further analysis of Ames-negative rodent carcinogens that are only genotoxic in mammalian cells in vitro at concentrations exceeding 1 mM, including retesting of compounds of concern. Mutagenesis 25, 539–553] suggest that the current 10 mM top concentration can be reduced without any loss of sensitivity in detecting rodent carcinogens.

Introduction

The EU legislation REACH (Regulation, Evaluation, Authorisation and restriction of CHemicals) foresees the safety assessment of thousands of chemicals within the next decade (1). Even if animal testing should be undertaken as the last resort, the evaluation of the genotoxic/mutagenic potential appears to be among the end points for which the highest number of *in vivo* tests will be needed (2). This is mainly due to the poor specificity of the standard *in vitro* test battery regarding the discrimination between rodent carcinogens and non-carcinogens (3), thus triggering the follow-up of any positive outcome in the *in vitro* standard test battery with appropriate *in vivo* tests, regardless of the tonnage level of the chemical. Furthermore, the Seventh Amendment to the Cosmetics Directive prohibits any acute *in vivo* genotoxicity tests for cosmetics ingredients since March 2009 (4), thus potentially precluding the development of many new cosmetics ingredients.

Over the past 20 years, there have been considerable efforts to develop *in vitro* methodologies, which can replace experimental animal *in vivo* assays in the identification of potential human mutagens and carcinogens. However, if *in vitro* assays are to effectively replace the use of *in vivo* methods, it is essential that the *in vitro* assays are of high quality and that the data produced are unambiguous in their ability to identify genotoxins and do not generate conclusions which falsely classify some compounds as positive mutagens and potential carcinogens. During an expert workshop organised by the European Centre for the Validation of Alternative Methods (ECVAM) that aimed to identify factors, which may be important in the generation of false-positive results, it was suggested that testing up to high concentrations and to high levels of cytotoxicity, as is currently required in mammalian cell genotoxicity tests, may contribute to the high frequency of false positives (5). In a report of that workshop, the participants recommended that a thorough review of both published and industry-held data was urgently needed to determine whether the current testing limit of 10 mM (or 5000 μg/ml) and the high levels of compound-induced cytotoxicity during testing are necessary for the effective detection of potential *in vivo* genotoxins and DNA-reactive mutagenic carcinogens. Although this type of approach has been questioned by some authors (6), suggestions to lower the current upper limit may be justified in terms of metabolic and cellular processes and relatively low human tissue exposures (5).

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines on genotoxicity testing (7) has recommended that pharmaceuticals should be assessed for their potential mammalian cell genotoxicity *in vitro* by testing up to concentrations of 10 mM or 5000 μg/ml (whichever is lower). This advice has recently been changed in the current draft guideline revision to a recommendation for a maximum

test concentration of 1 mM or 500 μg/ml (whichever is lower). The rationale for this change is based on the well-documented concentrations of pharmaceuticals that are normally achieved in human blood following dosing at therapeutic levels and is an attempt to limit the number of false positives in *in vitro* mammalian cell tests (8). While the ICH guideline revision for pharmaceuticals still has to be definitively adopted, the debate on top testing concentration in *in vitro* genotoxicity assays has been broaden to all chemicals.

As a follow-up work sponsored by ECVAM, the present review summarises the results of the analysis of existing data on *in vitro* mammalian cell tests. The objectives were as follows:

- to address the appropriateness of the 10 mM (or 5000 μg/ml) top concentration currently recommended for genotoxicity testing of chemicals,
- to identify the chemicals that are positive in mammalian cell tests at concentrations >1 mM and
- to review Ames test data to see if chemicals for which high concentrations are needed to produce positive results in mammalian cells would be detected in other parts of the standard battery like the bacterial test.

In this study, a review of the published literature of those rodent carcinogens for which there have been data generated in Ames bacterial mutagenicity assays and *in vitro* mammalian cell gene and chromosome mutation tests was undertaken. The Carcinogenicity and Genotoxicity eXperience (CGX) database generated by Kirkland *et al*. (3) on rodent carcinogens was used as the initial basis for this analysis.

Publications describing studies which aimed to detect genetic damage in chromosome aberration (CA) tests and gene mutations in the mouse lymphoma tests were re-evaluated whenever available. The standard CA test yielded the greatest amount of information on clastogenic potential, but some data concerning chromosome mutation were also available from the *in vitro* micronucleus (MN) assays for those chemicals classified as rodent carcinogens in the CGX database (9). Genotoxicity data were available for 553 of these chemicals, which provided the starting point for our analysis of the relationships between the detection of the potential genotoxic activity of rodent carcinogens and the concentration range over which studies are performed.

Materials and methods

Generation of the database

The CGX database (3), which includes the results of *in vitro* mammalian cell genotoxicity tests for rodent carcinogens, provided the basis for the production of our database. In particular, we used Appendix A v2, which corresponds to the updated version of the database (9). Of the 553 compounds for which genotoxicity data were available, 384 were identified for which data on the different mammalian genotoxicity tests were reported. Ames test data were available for the majority of them. The corresponding publications were evaluated, which contain *in vitro* genotoxicity test data using (i) the mouse lymphoma assay (MLA), (ii) the *in vitro* CA assay, (iii) the *in vitro* MN test and (iv) sister chromatid exchange (SCE) analysis. However, as SCE is not recommended in current guidelines, we have not included data from this assay in our main analysis.

For most compounds, raw data from the original publications or sources (e.g. online databases) were re-evaluated according to current guidelines. When original data were not available, more recent papers were searched for in the literature and included in the analysis. Alternatively, conclusions from expert reviews such as Mitchell *et al*. (10) and Ishidate *et al*. (11) were accepted.

A new database was generated including the name and the CAS number of each compound, genotoxicity test results, together with testing concentrations and experimental conditions when available (e.g. with or without metabolic activation, cell line used), and the respective reference(s). The general criterion used for the inclusion of data in this database was that they should have been generated with protocols that followed or complied with the Organisation for Economic Co-operation and Development test guidelines and current recommendations (12–18). In cases where several publications were available, sometimes with both positive and negative datasets for a compound, the most reliable ones were taken into consideration and the study(ies) that fulfilled current guideline criteria overruled other studies for giving an overall call for that compound. When no conclusion could be drawn, the final call was kept as inconclusive.

Reporting of results

Three main categories of responses were used to give an overall call for each test and each compound:

+: clearly positive response in all or in the majority of the studies or datasets reported,

−: clearly negative response in all the studies or datasets reported or in the majority of them when inconclusive/equivocal results were also mentioned and

I or E: the inconclusive/equivocal call was used when results were not consistent between datasets from different studies (inconclusive) or within one study (equivocal). The equivocal call was also used for weak-positive responses without statistically significant effects or when not all the criteria for a positive response were met [e.g. a dose-related increase in mutant frequency in MLA results but below the Global Evaluation Factor (GEF)]. Finally, the equivocal call was used when negative results were not adequately obtained (e.g. chemicals only tested without metabolic activation).

The test concentration ranges for which data were available were evaluated. Many of the datasets only provided classifications such as positive, negative and inconclusive/equivocal and in these cases, the original publications were consulted whenever possible and decisions were made as to the lowest effective concentration reported in the case of positive results and the highest tested concentration reported in the case of negative and inconclusive/equivocal results.

Further information regarding the test conditions, including the cell line tested and whether metabolic activation was used or not, were mentioned whenever available.

MLA data

Sources. The MLA data reviewed come from Mitchell *et al*. (10), the National Toxicology Program (NTP) database (19) and additional publications. Whenever possible, and for most of the papers cited in Mitchell *et al*. (10), original data were checked.

Interpretation of the results. For the evaluation of the data, the quality criteria developed by the US EPA (Environmental Protection Agency) review panel were applied (10). For a positive MLA response, these criteria can be summarised as: positive responses with concentration-related increase in mutant frequency with relative total growth (RTG) of at least 10%. In addition, whenever possible, the original data were checked for compliance to IWGT (International Workshop on Genotoxicity Testing) recommendations on the acceptance criteria for MLA results (14–18): more specifically, for a positive call, data with an RTG <10% were excluded since generally not considered relevant, the induced mutant frequency had to meet or exceed the GEF (= 90×10^{-6} or 126×10^{-6} cells for the agar and the microwell versions of the assay, respectively) and a positive dose-related increase had to be observed. The trend test was taken into consideration whenever available, otherwise only the statistical significance of individual concentrations was considered. For the purpose of this study, datasets with mutant frequencies for negative controls lower than those recommended (i.e. $<35 \times 10^{-6}$ cells for the agar method or $<50 \times 10^{-6}$ cells for the microwell method) were included in the analysis and considered positive (instead of inconclusive/equivocal) when the other criteria for positive calls were fulfilled.

EPA also recommends testing compounds up to 10 mM (or 5000 μg/ml). Only a limited number of the chemicals reported as negative or inconclusive/equivocal achieved this criterion, and those not tested up to this concentration were still included in the database. However, information on whether they were tested up to their limit of solubility was not always available.

In vitro CA data

Sources. The *in vitro* CA data reviewed were from Ishidate *et al*. (11), the NTP database (19) and additional publications.

Interpretation of the results. The acceptability criteria used for a positive *in vitro* CA result were the induction of >5% of cells with CAs as well as a statistically significant dose–response trend test, in accordance with those used in the NTP database (19). For some compounds, original data were not available but results were reviewed by Ishidate *et al*. (11); in those cases, criteria used by the authors were judged as being quite conservative and thus suitable for the present analysis of positive testing concentrations.

For compounds reported as *in vitro* CA negative in the original or review publications, the decision was taken to give them an *in vitro* CA inconclusive/equivocal call when they had been tested only in the 'without metabolic activation' condition (i.e. for calciferol, haematoxylin, hexachlorobenzene, phenyl glycidyl ether, phorbol, propylthiouracil, senkirkine, thioacetamide and vinylidene chloride).

In vitro MN test data

This is a relatively more recent assay than the MLA and *in vitro* CA assay, and thus has a smaller number of published results. Review papers such as Matsushima *et al*. (20) and Miller *et al*. (21) and additional publications were used to collect the *in vitro* MN data.

Ames test data

As part of the standard test battery to detect mutagenic compounds, Ames test data were also reviewed. For the present study, only the Ames test call is reported. Those data were taken mainly from the CGX database (9) and the NTP database (19).

Analysis of the data

Data gathered on the MLA, *in vitro* CA assay, *in vitro* MN test and Ames test are provided in the supplementary Table, available at *Mutagenesis* Online. We have analysed this new database in a variety of ways with the aim of providing an information source that can be used to set maximum testing concentrations for various chemicals.

1. In the first instance, we focused the analysis on the MLA and *in vitro* CA assay results by classifying the compounds into positive, negative and inconclusive/equivocal and then ranked the compounds on the basis of testing concentrations, for the respective test methods.
2. Then, we considered only the compounds reported positive in each of the two mammalian cell tests and categorised them following the testing concentration that induced the positive response (<1 mM, 1–10 mM and >10 mM).
3. In order to determine if the rodent carcinogenic compounds could be identified in other parts of the standard *in vitro* test battery, we reviewed the Ames test data.
4. For each mammalian cell test, we identified, among the compounds positive at concentrations between 1 and 10 mM, those that were Ames test-negative or equivocal. The compounds inducing a positive response at a concentration >10 mM were not considered in the further analysis since they would not be detected according to current recommendations for top testing concentration to 10 mM and would thus be considered as negative.
5. Finally, taking into consideration both the MLA and *in vitro* CA assay data, we identified the compounds negative or equivocal in the Ames test, which required testing at concentrations between 1 and 10 mM to be detected by at least one of the mammalian cell tests.

Results

The present analysis mainly focuses on the MLA and the *in vitro* CA assay for which most data were available. For the majority of the compounds, it was possible to compile results for both the MLA and the *in vitro* CA assay, in addition to Ames test data. For the remaining chemicals, data were available for only one of the mammalian cell tests. Only a limited number of data were available for the analysis in *in vitro* MN test.

Distribution of the different responses in mammalian cell genotoxicity tests

Adequate MLA and *in vitro* CA assay data were identified for 229 and 338 chemicals, respectively. The number of compounds inducing positive, negative or inconclusive/equivocal responses in those assays are shown in Table I. Similar proportions of positive calls were found for both tests (62.5 and 58.0% for MLA and *in vitro* CA assay, respectively). It can be observed that very few compounds that gave a negative call in either test were tested up to 10 mM (supplementary Table is available at *Mutagenesis* Online). For a high percentage of the negative chemicals, the highest concentration tested was <1 mM (for 42.9 and 39.1% of MLA- and CA-negative compounds, respectively).

Distribution of concentrations producing positive responses in mammalian cell genotoxicity tests

For both mammalian cell assays, there was a wide range of treatment concentrations, which produced positive responses. We have illustrated the positive response data for the studies undertaken with the MLA and *in vitro* CA assay in graphical formats in Figure 1 and Figure 2, respectively. In these figures, the lowest concentrations, which produced positive responses in each assay are displayed over treatment concentrations of 0.000001–100 mM. Taking a closer look at the distribution of these data (Table II), it can be seen that similar proportions of positive results were produced in the assays over the different concentration ranges, the largest percentages being represented by responses already positive at concentrations <1 mM (75.5 and 70.4% for MLA and *in vitro* CA assay, respectively). Whereas ~4.5% of the chemicals needed testing at concentrations >10 mM in either method to be detected, 20.3 and 25.0% of the MLA and *in vitro* CA-positive compounds, respectively, were positive at concentrations between 1 and 10 mM.

Analysis of mouse lymphoma data versus Ames test data

Table III indicates the distribution of the 143 MLA-positive chemicals according to the testing concentration and to the

Table I. Distribution of the chemicals according to the overall call in the MLA and *in vitro* CA assays

Overall call	MLA (%)	*In vitro* CA assay (%)
Positive	143 (62.5)	196 (58.0)
Negative	42 (18.3)	105 (31.1)
Inconclusive/equivocal	44 (19.2)	37 (10.9)
Total	229 (100)	338 (100)

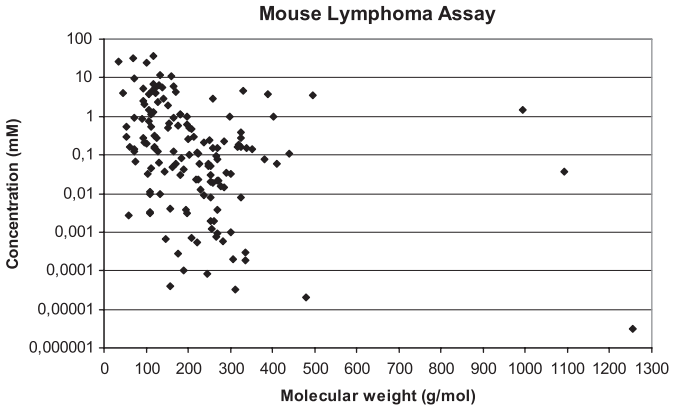

Fig. 1. Distribution of testing concentrations, which produced positive results in MLA in relation to the molecular weight of the chemicals tested.

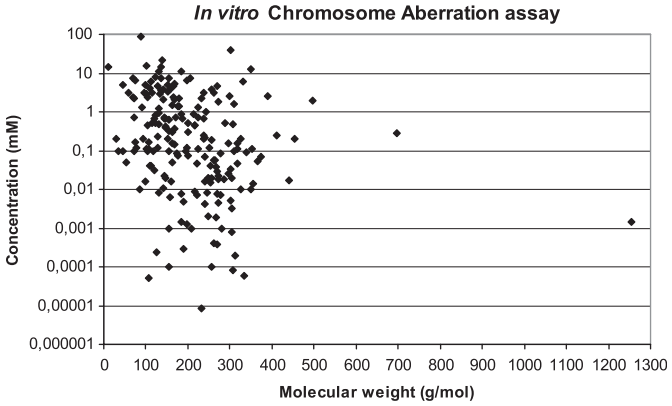

Fig. 2. Distribution of testing concentrations, which produced positive results in CA assays in relation to the molecular weight of the chemicals tested.

Table II. Distribution of the chemicals according to the lowest concentrations tested that produced positive results in the MLA and *in vitro* CA assays

Lowest concentrations giving positive results	MLA (%)	*In vitro* CA assay (%)
≤1 mM	108 (75.5)	138 (70.4)
1–10 mM	29 (20.3)	49 (25.0)
>10 mM	6 (4.2)	9 (4.6)
Total	143 (100)	196 (100)

Table III. Distribution of the chemicals according to the call for Ames test and to the lowest concentration tested that produced positive results in the MLA

Ames test call	Lowest concentration giving positive results in the MLA			
	≤1 mM	1–10 mM	>10 mM	Total
Positive	73 (83.0/67.6)	13 (14.7/44.8)	2 (2.3/33.3)	88 (100/61.5)
Negative	33 (67.3/30.6)	12 (24.5/41.4)	4 (8.2/66.7)	49 (100/34.3)
Equivocal	1 (20.0/0.9)	4 (80.0/13.8)	0	5 (100/3.5)
No data	1 (100/0.9)	0	0	1 (100/0.7)
Total	108 (75.5/100)	29 (20.3/100)	6 (4.2/100)	143 (100/100)

(% of the row/% of the column).

Ames test call. The largest group with 73 compounds was represented by the Ames test-positive compounds that were consistently detected in the MLA at concentrations <1 mM. This testing concentration range also allowed the identification of 33 genotoxic compounds that induced a negative response in the Ames test. Among the 29 chemicals, which produced positive results in the MLA at concentrations from 1 to 10 mM, 16 (55.2%) were negative or equivocal in the Ames test. These compounds are listed in Table IV, in order of testing concentrations. Moreover, six chemicals were MLA-positive only when tested >10 mM (Table III): two of them were Ames test-positive, whereas the remaining four were not detected in the bacterial test.

Analysis of in vitro CA data versus Ames test data

Table V indicates the distribution of the 196 *in vitro* CA-positive chemicals according to the minimum testing concentration which produced positive results and to the Ames test call. As was also shown in the MLA, the largest group was represented by the 102 Ames test-positive compounds that were consistently detected in the *in vitro* CA assay at concentrations <1 mM. Thirty-six compounds, which were Ames test negative, equivocal or with no data were also detected positive in this range of concentrations. Forty-nine chemicals needed testing concentrations between 1 and 10 mM to be detected, of which 20 (40.8%) compounds could not be detected by the Ames test (Table VI). Nine chemicals were reported *in vitro* CA positive at concentrations >10 mM, among which four were negative in the Ames test (Table V).

Analysis of mammalian cell test data versus Ames test data

When combining the MLA and the *in vitro* CA data (Table IV and Table VI, respectively), 29 compounds were identified which were negative in the Ames test and positive in either mammalian cell assay over a concentration range between 1 and 10 mM. Among those, five compounds (i.e. benzaldehyde, acetaldehyde, acrylamide, 2-chloro-2-methylpropene and hydroquinone) were positive at concentrations <1 mM in one of the two tests. Consequently, a final list was established, including 24 compounds that needed testing at concentrations between 1 and 10 mM to be detected in at least one of the two mammalian cell tests, either because (i) both assays gave positive results only at concentrations >1 mM (seven chemicals) or because (ii) only one of the two tests was positive (6 chemicals MLA-positive only/11 chemicals *in vitro* CA positive only) (Table VII). This subgroup of compounds, which would not have been detected by the standard test battery if the top dose would have been reduced to 1 mM, represents 6.25% of the total number of chemicals included in the database and 22% of the Ames test-negative/equivocal compounds.

Analysis of in vitro MN data

In vitro MN data were available for 52 chemicals (supplementary Table is available at *Mutagenesis* Online), of which 5 were *in vitro* MN negative and 47 positive. All *in vitro* MN-positive compounds were found positive at concentrations <1 mM (Figure 3). Among the 21 compounds which were negative in the Ames test, 2 were also negative in the *in vitro* MN test, i.e. nafenopin tested to 0.1 mM and tetrachloroethylene tested to 1.51 mM, while 19 compounds were positive in the *in vitro* MN test at concentrations <1 mM.

Discussion

The detection and regulation of potential human carcinogens is currently based upon the application of test batteries of bacterial and mammalian cell culture assays to detect genotoxic activity, supplemented with *in vivo* assays involving the use of rodents. The aim of these test batteries is to provide a comprehensive assessment of genotoxic potential. However, recent data (3) suggest that the current testing strategy suffers from a poor specificity that can lead to unnecessary follow-up *in vivo* studies. As part of ECVAM efforts to reduce, refine and replace animal use in toxicity assessment, the present study was undertaken to determine whether reducing the top dose concentration currently used in *in vitro* mammalian cell genotoxicity assays could be a constructive way of improving the specificity of the whole test battery.

While compiling and re-evaluating existing data for each test, care was taken to include the most relevant studies and

Table IV. List of chemicals positive in the MLA within the concentration range of 1–10 mM and equivocal or negative in the Ames test

Chemical	CAS no.	Lowest concentration giving positive results in the MLA (mM)	Ames call
Methapyrilene hydrochloride	135-23-9	1.01	E
Chlorobenzene	108-90-7	1.11	–
Caffeic acid	331-39-5	1.11	–
Benzofuran	271-89-6	1.27	–
C.I. Direct blue 15	2429-74-5	1.51	E
Furfural	98-01-1	2.10	E
Toluene	108-88-3	2.44	–
Allyl isovalerate	2835-39-4	2.81	–
FD & C red 1 (Ponceau 3R)	3564-09-8	3.44	–
Benzaldehyde	100-52-7	3.77	–
Chlorendic acid	115-28-6	3.86	–
Acetaldehyde	75-07-0	3.98	–
α-Methylbenzyl alcohol	98-85-1	4.10	–
Furosemide	54-31-9	4.55	–
Isophorone	78-59-1	5.79	–
Acrylamide	79-06-1	9.80	E

E, equivocal; –, negative.

Table V. Distribution of the chemicals according to the call for Ames test and to the lowest concentration tested that produced positive results in the *in vitro* CA assay

Ames test call	Lowest concentration giving positive results in the *in vitro* CA assay			
	≤1 mM	1–10 mM	>10 mM	Total
Positive	102 (75.0/73.9)	29 (21.3/59.2)	5 (3.7/55.6)	136 (100/69.4)
Negative	31 (58.5/22.5)	18 (34.0/36.7)	4 (7.5/44.4)	53 (100/27.0)
Equivocal	3 (60.0/2.2)	2 (40.0/4.1)	0	5 (100/2.6)
No data	2 (100/1.4)	0	0	2 (100/1.0)
Total	138 (70.4/100)	49 (25.0/100)	9 (4.6/100)	196 (100/100)

(% of the row/% of the column).

Table VI. List of chemicals positive in the *in vitro* CA assay within the concentration range of 1–10 mM and equivocal or negative in the Ames test

Chemical	CAS no.	Lowest concentration giving positive results in the *in vitro* CA assay (mM)	Ames call
Clofibrate	637-07-0	1.03	–
3-Chloro-2-methylpropene (technical grade)	563-47-3	1.32	–
Caffeic acid	331-39-5	1.40	–
Phenylbutazone	50-33-9	1.63	–
2-Mercaptobenzothiazole	149-30-4	2.10	–
Allyl isovalerate	2835-39-4	2.11	–
Furan	110-00-9	2.35	–
Ethionamide	536-33-4	2.40	–
Styrene	100-42-5	2.40	–
Chlorendic acid	115-28-6	2.50	–
Methapyrilene hydrochloride	135-23-9	2.51	E
1,1,2-Trichloroethane	79-00-5	2.83	–
N-Methylolacrylamide	924-42-5	2.94	–
Furfural	98-01-1	3.12	E
Methimazole	60-56-0	3.20	–
Hydroquinone	123-31-9	4.10	–
Methylphenidate HCl	298-59-9	4.63	–
Furosemide	54-31-9	6.00	–
3-(*p*-Chlorophenyl)-1-1-dimethylurea (AKA monuron)	150-68-5	6.54	–
α-Methylbenzyl alcohol	98-85-1	8.15	–

E, equivocal; –, negative.

from our review of the literature, the following comments can be made:

- Whenever possible, we applied the current criteria to the original data and made our decision for the overall call for each compound. But due to the retrospective design of this study, one limitation was access to these data while some of them were already reported and interpreted by other authors.
- Data on the purity of the chemicals evaluated was not always available and the possible presence of impurities could have

Table VII. List of chemicals equivocal or negative in the Ames test and requiring a testing concentration within the range of 1–10 mM to be positive in the MLA and/or the *in vitro* CA assay

Chemical	CAS no.	MLA call	MLA testing concentration[a] (mM)	CA call	*In vitro* CA testing concentration[a] (mM)	Ames call
Allyl isovalerate	2835-39-4	+	2.81	+	2.11	−
Caffeic acid	331-39-5	+	1.11	+	1.40	−
Chlorendic acid	115-28-6	+	3.86	+	2.50	−
Furfural	98-01-1	+	2.10	+	3.12	E
Furosemide	54-31-9	+	4.55	+	6.00	−
Methapyrilene hydrochloride	135-23-9	+	1.01	+	2.51	E
α-Methylbenzyl alcohol	98-85-1	+	4.10	+	8.15	−
Benzofuran	271-89-6	+	1.27	—	2.34	−
C.I. Direct blue 15	2429-74-5	+	1.51	—	2.52	E
Toluene	108-88-3	+	2.44	—	17.36	−
Chlorobenzene	108-90-7	+	1.11	E	4.50	−
Isophorone	78-59-1	+	5.79	I	9.04	−
FD & C red 1 (Ponceau 3R)	3564-09-8	+	3.44	nd	nd	−
3-(*p*-Chlorophenyl)-1-1-dimethylurea (AKA monuron)	150-68-5	I	5.54	+	6.54	−
Ethionamide	536-33-4	I	4.81	+	2.40	−
2-Mercaptobenzothiazole	149-30-4	E	0.12	+	2.10	−
Furan	110-00-9	+	31.12	+	2.35	−
Clofibrate	637-07-0	nd	nd	+	1.03	−
Methimazole	60-56-0	nd	nd	+	3.20	−
N-Methylolacrylamide	924-42-5	nd	nd	+	2.94	−
Methylphenidate HCl	298-59-9	nd	nd	+	4.63	−
Phenylbutazone	50-33-9	nd	nd	+	1.63	−
Styrene	100-42-5	nd	nd	+	2.40	−
1,1,2-Trichloroethane	79-00-5	nd	nd	+	2.83	−

E, equivocal; I, inconclusive; +, positive; −, negative; nd, no data.
[a]Lowest concentration tested in the case of positive results/highest concentration tested in the case of negative or equivocal/inconclusive results.

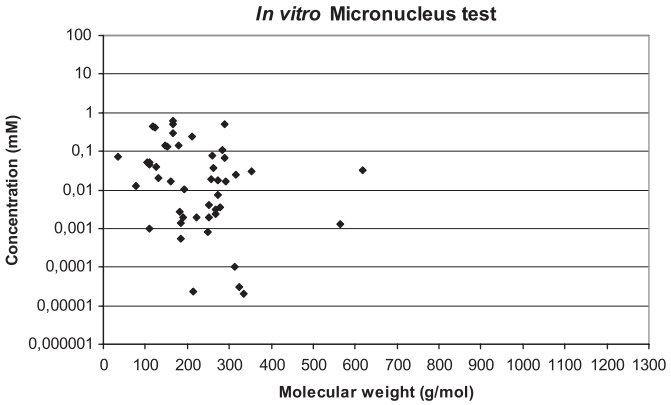

Fig. 3. Distribution of testing concentrations, which produced positive results in *in vitro* MN tests in relation to the molecular weight of the chemicals tested.

influenced the molarity consideration quite substantially. Although such information is of interest, assuming non-genotoxicity of these impurities, one can expect the corrected chemical concentrations that resulted positive in the different mammalian cell tests to be even lower than those reported in the present study.

- For the purpose of this work, as many positive data as possible were evaluated to define the lowest effective concentration for each test and each chemical. This is the reason why, for example, we included in our analysis MLA datasets whose negative control values were too low and should have been rejected according to current recommendations. Applying strictly this rule would have resulted in more inconclusive/equivocal compounds and would have limited the impact of the present study. It is also interesting to note that most of the MLA experiments reported in the NTP database do not fulfil the new criteria developed by the IWGT (14–18).
- For many compounds, a high variability was also observed between the results from different studies using the same testing conditions. When considering those different studies, chemicals could have fallen into different concentration categories, especially <1 mM or from 1 to 10 mM. In this respect, we tried to be as exhaustive as possible and to take into consideration the most reliable data.

This is the first attempt to determine, based on data from the literature, whether the current top dose recommended for *in vitro* genotoxicity testing is justified or not. In the long term, the correctness of our compound calls, particularly for the negative and inconclusive calls, should be confirmed whenever further data are obtained, using improved protocols.

The critical question that arises from this analysis relates to the maximum test concentrations, which ensure detection of genotoxic compounds, without inducing too many 'false'-positive results, but also without negatively impacting on the overall ability of the standard testing battery to detect rodent genotoxins and potential human carcinogens. We focused the analysis on the MLA, *in vitro* CA assay and Ames test for which the highest amount of data was available. Table VII lists the 24 compounds with known carcinogenic potential that could not be detected by the commonly used test battery if the maximum testing concentration was lowered from 10 to 1 mM. This subset of compounds represents 6.25% of the total number of chemicals evaluated.

It would be interesting to understand how critical is the risk of missing such a percentage of potentially genotoxic compounds. To this end, it is important first to assess the

biological significance of these results before recommending or not a change in current guidelines for the top testing concentration. The next step of this analysis is thus to determine whether these 24 compounds are relevant positives, i.e. if, by their mode of action, they should have been detected as genotoxic carcinogens. Moreover, since some of the data reviewed were obtained following old protocols, it can also be questioned whether the testing of these chemicals would give positive result at the same concentrations using current recommendations. These issues have been addressed by Kirkland and Fowler in the accompanying paper (22), who further re-evaluated and/or retested these compounds and concluded that the 10 mM upper limit for non-toxic chemicals in mammalian cell tests is not justified and can be lowered without any loss of sensitivity in detecting genotoxic rodent carcinogens.

In vitro MN data were also reported in the present study even if judged too limited to be taken into consideration in the final analysis. Interestingly, although the *in vitro* MN dataset is far smaller than those for MLA and *in vitro* CA assay, *in vitro* MN test appears to have a better performance in terms of testing concentrations, as all positive responses were obtained at concentrations <1 mM. Such results may be due to the fact that many of these studies are more recent and are more consistent with current testing requirements, therefore a clear positive or negative call is easier to make. They could also be related to the possible higher sensitivity of the *in vitro* MN assay due to the scoring of more cells compared with the *in vitro* CA assay and/or the fact that it detects both structural and numerical chromosome changes. However, our database does not report all the *in vitro* MN data that are available and the publications considered may also represent a selected group of positive chemicals. A more comprehensive evaluation of *in vitro* MN results is thus needed before this trend could be confirmed. If this would be the case, it would further support a revision of the currently used top concentration in mammalian cell genotoxicity testing.

Supplementary data

The supplementary Table is available at *Mutagenesis* online.

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Dedication

Dedicated to the memory of Jim who died on June 15, 2010. He will be missed personally and professionally by many for his enthusiastic contributions as a scientist, innovative thinker and as a driving force for progress in the field of genetic toxicology.

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