Mutagenesis vol. 25 no. 6 pp. 539–553, 2010
Advance Access Publication 18 August 2010
doi:10.1093/mutage/geq041

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

David Kirkland* and Paul Fowler[1]

Department of Genetic and Molecular Toxicology, Kirkland Consulting, PO Box 79, Tadcaster LS24 0AS, UK and [1]Covance Laboratories Limited, Otley Road, Harrogate HG3 1PY, UK.

*To whom correspondence should be addressed. Department of Genetic and Molecular Toxicology, Kirkland Consulting, PO Box 79, Tadcaster LS24 0AS, UK, Tel: +44 1937 830849; Email: dkirkland@genetoxconsulting.co.uk

Received on May 24, 2010; revised on July 2, 2010; accepted on July 6, 2010

In the analysis by Parry *et al.* [Parry, J. M., Parry, E., Phrakonkham, P. and Corvi, R. (2010) Analysis of published data for top concentration considerations in mammalian cell genotoxicity testing. Mutagenesis, 25, 531–538], 24 rodent carcinogens that were negative in the Ames test were identified that were only positive in mammalian cell tests at concentrations between 1 and 10 mM. These carcinogens can be subdivided into four groups as follows: (1) probable non-genotoxic (non-mutagenic) carcinogens, tumour promoters or negative for genotoxicity *in vivo* (*n* = 10); (2) questionable carcinogens (*n* = 4); (3) carcinogens with a probable genotoxic mode of action (*n* = 5); (4) compounds where carcinogenicity or *in vivo* genotoxicity is unknown or unclear (*n* = 5). It is not expected that *in vitro* mammalian cell tests should give positive results with Group 1 chemicals. Within Groups 2–4, five chemicals were considered a low priority because they could be detected using modified conditions because genotoxicity was associated with precipitate or pH shifts or because non-standard metabolism was required. The remaining nine chemicals were therefore considered most critical in terms of detection of genotoxic activity in mammalian cells. Daminozide was also included because it may have given positive responses between 1 and 10 mM. Many of the reported studies could have given positive results only at >1 mM because 'old' protocols were followed. These 10 chemicals have therefore been retested using modern protocols. Some were negative even up to 10 mM. Others were positive at concentrations <1 mM. Only methylolacrylamide was positive at a concentration >1 mM (2 mM = 202 μg/ml). Low-molecular weight substances may therefore require concentrations >1 mM, but further work is needed. Based on this analysis, it is concluded that the 10 mM upper limit in mammalian cell tests can be lowered without any loss of sensitivity in detecting genotoxic rodent carcinogens. A new limit of 1 mM or 500 μg/ml, whichever is the higher, is proposed.

Introduction

In the accompanying paper by Parry *et al.* (1), a review of the published literature of those rodent carcinogens for which data have been generated in Ames bacterial mutagenicity assays and *in vitro* mammalian cell gene mutation and chromosome damage tests was undertaken. 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 test. Genotoxicity data were available for 553 of these chemicals, which provided the starting point for the analysis of the relationships between the detection of the potential genotoxic activity of rodent carcinogens and the concentration range over which positive responses were detected. The purpose of the review was to investigate whether the current upper limit of testing in mammalian cell tests, namely 10 mM, was still supported or whether there was evidence that the top limit could be lowered, as has recently been proposed as a revision to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) S2 guideline for pharmaceuticals (2). Of the 553 rodent carcinogens, 24 that were negative in the standard Ames test were identified that only gave positive results in mammalian cell tests at concentrations between 1 and 10 mM. These compounds would seem to make the case that the 10 mM upper limit of testing for non-toxic substances is justified. However, it is important to consider these compounds in more detail. For the purposes of setting priorities in terms of this exercise, namely establishing the importance of detecting (or not) positive results in mammalian cell tests with these 24 carcinogens, they have been grouped into four different categories as follows:

1. Probable non-genotoxic (non-mutagenic) carcinogens, tumour promoters or negative for genotoxicity *in vivo*

It is difficult to categorically identify some chemicals as being non-genotoxic carcinogens. Data are often limited or conflicting. Thus, for the following chemicals, based on a weight of evidence approach, it is considered that they are less likely than those in categories two to four to induce tumours via a genotoxic mode of action. Deductive Estimation of Risk from Existing Knowledge (DEREK) has been used as a part of this assessment, although it is recognised that alerts in DEREK are mainly driven by Ames test results, and all of these chemicals were negative in the Ames test. Nonetheless, the following are considered probable non-genotoxic or non-relevant carcinogens or tumour promoters, for the reasons given:

- Chlorendic acid [115-28-6, molecular weight (MW) 389]—there are no DNA adduct or *in vivo* genotoxicity data on chlorendic acid, however there is a positive result for induction of replicative DNA synthesis [RDS, (3)], which may be considered indicative of a non-genotoxic mechanism. In carcinogenicity studies (4), it induced neoplastic nodules of the liver in male and female rats and hepatocellular carcinomas in high-dose female rats. The incidences of acinar cell adenomas of the pancreas, which are rare in F344 rats, were increased in male rats relative to those of controls. In mice, it induced hepatocellular adenomas and carcinomas (with lung

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

© The Author 2010. Published by Oxford University Press on behalf of the UK Environmental Mutagen Society.
All rights reserved. For permissions, please e-mail: journals.permissions@oxfordjournals.org.

metastases) in male mice. Other tumours (alveolar/bronchiolar adenomas of the lung of male rats, alveolar/bronchiolar adenomas or carcinomas in female mice, preputial gland carcinomas in low-dose male rats, sarcomas, fibrosarcomas or neurofibrosarcomas of the salivary gland in male rats) were increased, but were not significantly different from controls, although some of these tumours are uncommon in control animals. According to Environmental Health Criteria (vol. 185, 1996) chlorendic acid has tumour-promoting activity. Chlorendic acid does not have structural alerts for mutagenicity according to DEREK for Windows v11 and is considered a non-mutagenic carcinogen by Tennant (5).

• Clofibrate (637-07-0, MW 243)—clofibrate does not produce DNA adducts (6) and does not induce unscheduled DNA synthesis (UDS) *in vivo* (7). It does not produce structural alerts for mutagenicity according to DEREK for Windows v11. It induces hepatocellular carcinomas in male rats (females not tested) but is not tumourigenic to mice (IARC Monographs, vol. 66). Its tumourigenic activity is considered to be due to peroxisome proliferation, a mechanism that is not expressed in humans (see IARC Technical Publication No. 24, 1994).

• Furfural (98-01-1, MW 96)—there are no adduct data for furfural, it is equivocal for chromosomal aberrations (CAs) *in vivo* [see UK Health and Safety Executive Risk Assessment document, vol. EH72/6, 1997] although it was negative in a National Toxicology Programme (NTP) CA study (4) and is negative for UDS (8). It does induce RDS in mouse livers *in vivo* (9), which is indicative of a non-genotoxic mode of hepatocarcinogenic action. It does give a structural alert for bacterial mutagenicity according to DEREK for Windows v11 but was equivocal in the Ames test according to Zeiger (10) and NTP (4) and is considered a non-mutagenic carcinogen by Tennant (5).

• Isophorone (78-59-1, MW 138)—isophorone did not produce any structural alerts for mutagenicity according to DEREK for Windows v11. It produced tumours of the male rat kidney, which were associated with $\alpha 2\mu$-globulin accumulation. Preputial gland tumours in male rats and clitoral gland adenomas in female rats are also attributed to $\alpha 2\mu$-globulin accumulation. There was equivocal evidence of liver tumour induction in male mice but no tumours in female mice [(11), IARC Monographs vol. 73)]. Importantly, Thier *et al.* (12) found no DNA adducts in liver or kidneys of strains of rats and mice used in the carcinogenicity studies, and, along with Tennant (5), concluded isophorone is a non-genotoxic carcinogen. Also it did not induce micronuclei in rats (13) or CAs in mice *in vivo* after intraperitoneal administration (4).

• Methapyrilene HCl (135-23-9, MW 298)—this compound did not produce DNA adducts in mouse lymphoma cells *in vitro* (14). It does not give structural alerts for mutagenicity according to DEREK for Windows v11. There are no published *in vivo* genotoxicity data. It induces hepatocellular carcinomas and cholangiocarcinomas in male and female rats (15), but this is associated with liver cell proliferation (NTP Toxicity Report, vol. 46, 2000), which seems to be a significant mode of action for tumour induction via a non-genotoxic mechanism.

• Methimazole (60-56-0, MW 114)—there are no DNA adduct data, but methimazole does not produce structural alerts for mutagenicity according to DEREK for Windows v11. In addition, methimazole is negative for both micronuclei and CAs *in vivo* (IARC Monographs, vol. 79). Thyroid follicular cell adenomas were induced in rats and mice (IARC Monographs, vol. 79). In these and other animals, it decreases thyroid peroxidase, which decreases thyroid hormone production and increases cell proliferation by stimulation of thyroid stimulating hormone production (IARC Monographs, vol. 79). This is therefore considered a non-genotoxic mode of carcinogenic action.

• Alpha-methylbenzyl alcohol (98-85-1, MW 123)—this compound produced increased incidences of renal tubular cell adenomas and one adenocarcinoma (in a low-dose animal) in male rats. This was accompanied by dose-related renal toxicity. No tumours were induced in female rats or in mice. There were excessive deaths in the male rat treatment groups and increased deaths in the high-dose female treatment group, which may have decreased sensitivity. However, a World Health Organisation (WHO) committee (16) concluded 'On the basis of the available evidence, the Committee concluded that the higher incidence of benign neoplasms in the kidney of male rats is not relevant to humans'. There are no DNA adduct data, but alpha-methylbenzyl alcohol (AKA 1-phenylethanol) did not induce micronuclei in mice *in vivo* (17). It does not produce structural alerts for mutagenicity according to DEREK for Windows v11 and it was considered a non-mutagenic carcinogen by Tennant (5).

• Methylphenidate hydrochloride (298-59-9, MW 270)—this compound has been widely studied in recent years because of a reported increase in CAs in lymphocytes of children treated with the drug. The positive CA result in CHO cells in the presence of S-9 (4) was not reproduced in human lymphocytes at concentrations up to 10 mM (18) and these same authors found it did not induce micronuclei in the bone marrow or mice. Andreazza *et al.* (19) also found no induction of micronuclei in peripheral blood cells of rats but did find induction of comets in brain cells and lymphocytes. However, this latter finding was considered by Jacobson-Kram *et al.* (20) to probably be due to cytotoxic effects. Methylphenidate did not induce tumours in rats or in transgenic haploinsufficient *p53* mice (4). Liver tumours (mainly hepatocellular adenomas in males and females, but also a few hepatoblastomas, which are quite rare, in females) were induced in normal mice (4), but as commented by Jacobson-Kram *et al.* (20), such mouse liver tumours are common and often associated with non-genotoxic mechanisms. It does not produce structural alerts for mutagenicity according to DEREK for Windows v11. On the basis of the former data and many new results, Jacobson-Kram *et al.* (20) concluded 'The weight-of-evidence from these studies suggests a lack of carcinogenic risk to humans in general, and specifically, a lack of risk based on a genotoxic mechanism. Consistent with this interpretation, the NTP characterised methylphenidate as a nongenotoxic chemical'.

• Phenylbutazone (50-33-9, MW 308)—in the carcinogenicity studies on phenylbutazone reported by Kari *et al.* (21), uncommon tubular cell tumours of the kidney were found in 13 exposed rats, 9 in males and 4 in females. These were associated with inflammation, papillary necrosis and mineralisation in both sexes of rats. In female rats, two carcinomas of the pelvis transitional epithelium were found but were associated with dose-related increases in hyperplasia and cysts. Liver tumours were found in male mice but were associated with haemorrhage, centrilobular cytomegaly and karyomegaly, fatty metamorphosis, cellular degeneration and coagulative necrosis. In a separate study in DONRYU rats (22), spontaneous rat tumours were slightly increased but were not statistically significant. There are no DNA adduct data, but it

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

does not produce structural alerts for mutagenicity according to DEREK for Windows v11, and it is negative for micronuclei and CAs *in vivo* (IARC Monographs, Supplement 7), although there is a report of a positive sister chromatid exchange test in bone marrow cells of mice (23). It is considered to be a non-mutagenic carcinogen by Tennant (5). An additional consideration is that this is a human drug, and therefore its testing would be limited according to ICH guidelines (currently suggested as 1 mM).

• 1,1,2-Trichloroethane (79-00-5, MW 133)—this compound produced liver tumours and adrenal pheochromocytomas in male and female B6C3F1 mice and was not tumorigenic in other strains of mice, rats or hamsters. The adequacy of the rat study has been questioned (24), probably because administration was via subcutaneous injection. There is a report of 1,1,2-trichloroethane binding to DNA of liver, kidney lung and stomach of rats *in vivo* (25), but it did not induce pre-neoplastic foci in an initiation/promotion study in rats (26). It also failed to induce tumours in rasH2 transgenic mice in a 26-week study (27). Although genotoxic in mammalian cells *in vitro*, there is strong evidence of an aneugenic mechanism, as predominantly kinetochore-positive micronuclei were induced in MCL-5 and h2E1 cells (28). Although it does give a structural alert (alkylating agent) according to DEREK for Windows v11, it was negative in mouse bone marrow micronucleus tests (29,30) and induced replicative DNA synthesis (9) but not UDS (31) in rodent liver. According to Tennant (5), it is a non-mutagenic carcinogen, and according to a BUA report (32) 'The mostly negative findings in genotoxicity studies as well as in the long-term studies on rats (carcinogenesis study and initiation/promotion test) and the increase of the S-phases in mice liver can be considered as an indication that the formation of tumours in mice does not take place according to a genotoxic mechanism'.

It can be argued that because these compounds induce tumours via a non-genotoxic (non-mutagenic) or non-relevant mechanism or are not genotoxic *in vivo*, we would not expect *in vitro* genotoxicity tests to detect them as positive. Therefore, the fact they may only be detected at concentrations >1 mM is irrelevant.

2. Compounds with questionable carcinogenicity

• Chlorobenzene (108-90-7, MW 113)—chlorobenzene does not have structural alerts for mutagenicity according to DEREK for Windows v11, but it does produce DNA adducts (33,34). There are conflicting reports of induction of MN *in vivo* in bone marrow, it being reported as positive by Mavournin *et al.* (35), but negative by Shelby *et al.* (36), although Shelby and Witt (37) reported it positive for induction of CA in bone marrow. It increased the occurrence of neoplastic nodules of the liver in high-dose (120 mg/kg/day) male F344/N rats, providing some but not clear evidence of carcinogenicity of chlorobenzene in male rats. Carcinogenic effects of chlorobenzene were not observed in female F344/N rats or in male or female B6C3F1 mice (4).

• Ethionamide (536-33-4, MW 166)—there are no DNA adduct data for ethionamide and no *in vivo* genotoxicity data. According to IARC (volume 13), it induced thyroid carcinomas in mice after oral administration; however, there are two negative NTP carcinogenicity studies. In rats, a variety of neoplasms were observed in treated and control groups of each sex, but the lesions were of types commonly found in Fischer 344 rats, and none of the incidences of tumours in dosed animals were statistically significant when compared with controls. In the mice, the incidences of malignant lymphoma were slightly higher in dosed than in control mice but were not significant by any of the statistical tests used. It has no structural alerts for mutagenicity according to DEREK for Windows v11.

• Furosemide (54-31-9, MW 331)—there are no data on DNA adducts with furosemide, but it does not give structural alerts according to DEREK for Windows v11. It is negative for micronuclei *in vivo*, although only males were tested (4). According to Tennant (5), it is considered a non-mutagenic carcinogen. The carcinogenicity of furosemide is complex. The equivocal evidence in male rats for meningiomas might be considered controversial. An article by Sills *et al.* (38) examined the chemicals producing low incidences of brain tumours in F344 rats from NTP studies. For furosemide, there were three tumours in the low-dose animals and none in the high dose. These tumours are extremely rare (only 2 in 2000 control males in the relevant historical controls). These tumours were considered the cause of death, which occurred during weeks 47, 48 and 97. Sills *et al.* (38) say that it was concluded that the unusual tumours may have been related to treatment. The absence of a dose response made it difficult to unequivocally relate them to treatment. Sills *et al.* (38) examined a total of 10 chemicals associated with equivocal evidence (or clear evidence) of carcinogenicity based on brain tumour responses in F344 rats. Most chemicals (7/10) were positive in Ames, so furosemide is an exception. Most chemicals also were associated with tumours at other sites (true for furosemide where there was evidence of mammary carcinomas in female mice). Regarding the kidney neoplasms in male rats, although the kidney is the target organ for pharmacological activity, and one might suspect that kidney tumours might be treatment-related and based on some (non-genotoxic) excessive pharmacologic effects, these tumours were considered to provide only equivocal evidence of carcinogenicity. IARC concluded that furosemide was a Group 3 carcinogen. Of note is the fact that they state that furosemide is metabolised by mouse and human liver microsomes and binds covalently to proteins. This suggests the formation of reactive metabolites.

• Toluene (108-88-3, MW 92)—toluene produces DNA adducts in cultured cells *in vitro* (39), and induces micronuclei *in vivo* (40), but was negative in the Comet assay *in vivo* (41) and does not give structural alerts according to DEREK for Windows v11. Although included as a carcinogen in the Gold database (42), according to IARC, the evidence suggests a lack of carcinogenic activity (IARC Monographs, vol. 71). However, it has been argued that toluene should be reclassified as neither genotoxic nor carcinogenic. Recent authoritative reviews also do not appear to put credence in earlier studies reporting clastogenicity. For example:

From a WHO report from 2004 on toluene in drinking water (WHO/SDE/WSH/03.04/116) 'It has been concluded that toluene has not been demonstrated to be genotoxic'.

From Screening Information Data Set Initial Assessment Profile, 'Toluene is evaluated as not carcinogenic'. This view was confirmed by the European Union Scientific Committee on Cosmetic Products in 2006 'The CSTEE agrees with the assessor that toluene can be considered to be non-genotoxic' and 'The CSTEE agrees with the assessor in that it cannot be concluded that toluene is carcinogenic'.

It could be argued that *in vitro* tests should detect chlorobenzene, ethionamide, furosemide and toluene because they may be carcinogenic, and modes of action are unclear.

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

3. Carcinogens with a probable genotoxic mode of action

As with Group 1, clearly establishing a genotoxic mode of action is not easy, although induction of DNA adducts, where data are available, is considered a significant indicator. Tumours at multiple sites and in more than species are also taken as an indicator. Thus weight of evidence has again been used to identify the following as probably inducing tumours via a genotoxic mode of action. They are therefore considered a higher priority than Group 1 in that it is considered more important that these chemicals would be detected as positive in mammalian cell tests because they are negative in the Ames test.

• Caffeic acid (331-39-5, MW 180)—there are no DNA adduct data for caffeic acid. It is negative for micronuclei *in vivo* (43), but only a single-dose level was used and it is not clear if this was a maximum tolerated dose. Because it is a catechol-containing polyphenol, it probably induces oxidative stress. This may be relevant to its carcinogenic mode of action. After dietary administration, it produced renal cell adenomas in female mice and a high incidence of renal tubular cell hyperplasia in mice of each sex. An increase in the combined incidence of squamous cell papillomas and carcinomas of the forestomach was seen in male mice, and a high incidence of hyperplasia of the forestomach was seen in both males and females. In rats, it produced squamous cell papillomas and carcinomas of the forestomach in animals of each sex and a few renal cell adenomas in males. IARC (volume 56) has classified this compound as Group 2B (possible human carcinogen) and indicated that there are sufficient data in animals. This may be one of those 'two-edged sword' compounds—protective against oxidative stress at low doses and damaging at higher doses. There is one report of *in vitro* oxidative effects of caffeic acid in the presence of copper (44) and this may relate to the pro-oxidant properties of polyphenols, of which caffeic acid is an example.

• 3-(*p*-Chlorophenyl)-1-1-dimethylurea (AKA Monuron; 150-68-5, MW 199)—monuron is clearly carcinogenic in male rats producing tubular cell adenocarcinomas and adenomas of the kidney and neoplastic nodules or carcinomas of the liver (4). There are no data suggesting the tumours are due to a non-genotoxic mechanism (e.g. chronic irritation or cell proliferation). There are no data on DNA adducts, but it induces micronuclei and CAs *in vivo* (36,45). Two papers categorise monuron as a non-genotoxic carcinogen, although it is not clear what was the basis for that conclusion (46,47). Although it is probably a genotoxic carcinogen, it seems there is no consensus in the literature on this.

• Furan (110-00-9, MW 68)—following oral administration, it produced hepatocellular adenomas and carcinomas in mice. In rats, it produced hepatocellular adenomas in animals of each sex and carcinomas in males; a high incidence of cholangiocarcinomas was seen in both males and females. The incidence of mononuclear cell leukaemia was also increased in animals of each sex (IARC Monographs, vol. 63). Furan produces DNA adducts (48,49) and induces CAs *in vivo* after intraperitoneal administration (4), although it is negative for micronuclei (50) and UDS (51) *in vivo*. Induction of cell proliferation may be involved in its carcinogenicity (51), and a recent paper (52) also suggests a non-genotoxic mode of action, but a DNA-reactive component cannot be excluded.

• 2-Mercaptobenzothiazole (149-30-4, MW 167)—some evidence of carcinogenic activity for 2-mercaptobenzothiazole was seen (4) in male F344/N rats (mononuclear cell leukaemia, pancreatic acinar cell adenomas, adrenal gland pheochromocytomas and preputial gland adenomas or carcinomas) and in female F344/N rats (adrenal gland pheochromocytomas and pituitary gland adenomas). No carcinogenic activity was seen (4) in male B6C3F1 mice but equivocal evidence of carcinogenic activity was seen in female B6C3F1 mice (hepatocellular adenomas or carcinomas). It did not induce DNA adducts (53) or micronuclei (54) *in vivo*. The tumours in rats, which are not typical of non-genotoxic carcinogens, suggest it may be a genotoxic carcinogen.

• Styrene (100-42-5, MW 104)—styrene produced some increases in adenomas and carcinomas of the lung in male mice after oral administration, which was suggestive of carcinogenic activity. However, there was no convincing evidence of carcinogenic activity in rats or mice of both sexes (4). On the other hand, styrene-7,8-oxide induces benign and malignant forestomach tumours in rats and mice of both sexes (IARC Monographs, vol. 60). If converted to styrene oxide, styrene produces DNA adducts (55). *In vivo*, the results depend on species and route of administration, presumably governing the likelihood of conversion to the oxide. There were negative results for micronuclei in mice following inhalation exposure (56), although it was positive for micronuclei after intraperitoneal administration (57,58). It also induced comets in mouse lymphocytes, bone marrow, liver and kidney *in vivo* after intraperitoneal exposure (59). However, it was negative for micronuclei in rats after either intraperitoneal (58) or inhalation (60) exposure and negative for micronuclei in hamsters (61) after intraperitoneal injection. Styrene also failed to induce CAs in rats after inhalation exposure (56,62) and intraperitoneal injection (63).

It could be argued that these Ames-negative chemicals should be detected in *in vitro* tests and therefore the need to test >1 mM in mammalian cell tests needs to be investigated further.

4. Compounds where the mode of carcinogenic action is unknown or *in vivo* genotoxicity is unknown or unclear

• Allyl isovalerate (2835-39-4, MW 142)—allyl isovalerate induced squamous cell papillomas of the forestomach in male mice and increased the incidence of lymphomas in female mice. In rats of both sexes, increases in the incidence of mononuclear cell leukaemia were observed (IARC Monographs, vol. 71). There are no DNA adduct data for allyl isovalerate, but it was considered a non-mutagenic carcinogen by Tennant (5). It did not induce CA in mice *in vivo* after intraperitoneal injection (4) but there are no other *in vivo* data. Its metabolism to glycidol and glycidaldehyde would suggest it may be DNA reactive.

• Benzofuran (271-89-6, MW 118)—there are no DNA adduct data on benzofuran and no *in vivo* genotoxicity data. Although Tennant (5) concluded it was a non-mutagenic carcinogen, renal tumours were induced in female rats (which are rarely found spontaneously) and liver, lung and forestomach tumours were induced in mice (4) that could not be attributed to a non-genotoxic mechanism. The tumour profile is suggestive of some kind of DNA reactivity, although it could also reflect species-specific metabolism that renders mice and rats differentially sensitive in a manner similar to coumarin. Data are not available to draw a conclusion to support this or another non-genotoxic mode of action.

• CI Direct Blue 15 (2429-74-5, MW 993)—it produced benign and malignant tumours of the skin, Zymbal gland, liver, small intestine and oral cavity as well as leukaemia in male and

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

female rats and tumours of the large intestine and preputial gland in males and of the uterus and clitoral gland in females (IARC Monographs, vol. 57). There are no DNA adduct data and no *in vivo* genotoxicity data on CI Direct Blue 15.

• FD&C Red 1 (3564-09-8, MW 494)—it is carcinogenic in rats following oral administration, producing liver cell tumours. It also produced bladder tumours in mice following its implantation in the urinary bladder (IARC Monographs, vol. 8). There are no DNA adduct data and no *in vivo* genotoxicity data on FD&C Red 1.

• Methylolacrylamide (924-42-5, MW 101)—following oral dosing, it increased the incidences of Harderian gland adenomas, hepatocellular adenomas and carcinomas and alveolar–bronchiolar lung adenomas and carcinomas in male and female mice and increased the incidence of benign granulosa cell tumours of the ovary in female mice. In rats, no increase in tumour incidence was observed (IARC Monographs, vol. 60). There are no DNA adduct data on methylolacrylamide. It was negative for micronuclei in male mouse bone marrow (4) and rat bone marrow (64) *in vivo* after intraperitoneal injection. However, it produced a weak micronucleus response in mouse peripheral blood after intraperitoneal injection (64). The difference in response of rats and mice in the study of Paulsson *et al.* (64) may reflect different exposures to the epoxy metabolite (higher in mice) or may reflect the statistical sensitivity of the analysis of micronuclei since many more cells were scored from the mouse peripheral blood samples than from the rat bone marrow samples. Its similarity to acrylamide would probably suggest a DNA reactive mode of action.

It could be argued that in the absence of clear *in vivo* genotoxic action and unknown mode of carcinogenic action, it is not known whether we should expect *in vitro* mammalian cell tests to detect these rodent carcinogens that are negative in standard Ames tests. However, a cautious approach would argue that the above chemicals should probably be detected by *in vitro* genotoxicity tests.

Priorities for testing

The chemicals in Group 1 should not be considered as justifying test concentrations up to 10 mM, i.e. we should not expect that our *in vitro* tests detect non-genotoxic carcinogens, tumour promoters or compounds clearly negative for genotoxicity *in vivo*. Thus, the chemicals in Groups 2–4 are the main priority for determining whether concentrations >1 mM are needed.

The *in vitro* data, including CA and mouse lymphoma assay (MLA) results, for the above 14 compounds in Groups 2–4 are further reviewed in detail. The following comments can be made:

• Allyl isovalerate (2835-39-4)—the MLA was only conducted in the absence of S-9 (4). The only positive response in the MLA, i.e. where mutant frequency (MF) exceeded the global evaluation factor (GEF) (65) occurred in one of two cultures treated at 400 μg/ml (2.8 mM) where relative total growth (RTG) was reduced to <10%. In the NTP protocol, only short treatments were used, and allyl isovalerate may have given mutagenic responses at lower concentrations or lower levels of toxicity following a 24-h continuous treatment.

In the CA study in CHO cells (4), allyl isovalerate was only positive in the presence of S-9, and the lowest positive concentration was 300 μg/ml (2.1 mM). As the NTP protocol consisted only of short treatments and early sampling (on this occasion at 12.5 h), it is conceivable that allyl isovalerate would induce CA at lower concentrations following continuous treatments and/or later sampling. Thus, allyl isovalerate may be clastogenic at <1 mM if tested in a modern protocol.

• Benzofuran (271-89-6)—benzofuran was negative for CA (4) but induced significant (i.e. exceeded the GEF) *tk* mutations in the MLA (4) at 150 and 175 μg/ml (1.27 and 1.48 mM). These concentrations produced toxicity that was on the borderline of acceptability (i.e. 10% RTG in one replicate but <10% RTG in the other). The NTP study did not include a continuous treatment, which would now be included if borderline results were obtained. Thus, benzofuran may be mutagenic at concentrations <1 mM if tested in a modern protocol.

• Caffeic acid (331-39-5)—in the CA study of Whitehead *et al.* (66), CHO cells were treated for 3 h in the absence and presence of S-9 (and also in the presence of mouse intestinal cells) and harvested 20 h later. Thus, the protocol is similar to a current Organisation for Economic Co-operation and Development (OECD) protocol, although no continuous treatment in the absence of S-9 was included. For caffeic acid, the lowest positive concentration was 260 μg/ml (1.44 mM). The next lowest concentration tested was 240 μg/ml (1.33 mM), which gave 6% cells with aberrations. As there was no solvent (negative) control in these studies, it is not known if this response was positive or not. For CHO cells, it would probably be equivocal. However, only 100 cells per dose were scored, which is less than currently recommended. The result is therefore questionable.

In the CA study of Hanham *et al.* (67), CHO cells were again treated for 3 h in the absence of S-9 only and harvested 20 h later. The lowest positive concentration was 2.77 mM. After allowing for auto-oxidation of caffeic acid to occur before treatment, the lowest positive concentration was reduced to 0.69 mM. Again only 100 cells per dose were scored, which is less than currently recommended, and no continuous treatment in the absence of S-9 was included.

In the MLA study of Fung *et al.* (68), the lowest positive response (i.e. exceeding the GEF) was in the absence of S-9 and probably at 371 μg/ml (2.06 mM). As the study was not optimised for detection of small colony mutants, and the mechanism of caffeic acid genotoxicity probably involves hydrogen peroxide production, some clastogenic activity may have been missed. Only short treatments were used in this study, and a current protocol would also include a 24-h treatment.

In the MLA study of McGregor *et al.* (69), the lowest positive concentration was 300 μg/ml (1.66 mM), but the mutant response was quite strong, and the next lowest concentration was 100 μg/ml (0.56 mM). Thus, caffeic acid may well have given a positive response at a concentration of ~200 μg/ml, which is <1 mM. As above, only short (4 h) treatments were used, and positive responses may well have been seen at lower concentrations if longer treatment times had been used.

In all of these studies, the positive responses with caffeic acid are so close to 1 mM, it may be positive at <1 mM if tested in modern protocols, where both CA and MLA studies would include a 24-h treatment in the absence of S-9 and an MLA study would be optimised for detection of small colony mutants.

• Chlorobenzene (108-90-7)—in the NTP study (4), chlorobenzene did not induce CA at concentrations up to

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

500 μg/ml (4.44 mM), which was highly toxic. However, in the MLA (4) in the absence of S-9, the lowest positive concentration (i.e. induced MF exceeds the GEF) for chlorobenzene was 125 μg/ml (1.11 mM). At the next lowest concentration (100 μg/ml, 0.89 mM), there was an increase in MF, but it did not reach the GEF. Given that the lowest significant positive response with a 3-h treatment was so close to 1 mM, it is highly likely that a positive response would be seen at <1 mM with a 24-h treatment. Chlorobenzene may therefore be positive in the MLA if a 24-h treatment in the absence of S-9 is included.

- 3-(*p*-Chlorophenyl)-1-1-dimethylurea (AKA monuron; 150-68-5)—although negative in the MLA (4), monuron induced CA in CHO cells in the presence of S-9 (4). The lowest concentration tested (1300 μg/ml; 6.6 mM) gave the highest frequency of CA. Therefore, the lowest effective concentration was not determined and may have been <1 mM. Also, as the NTP protocol only employed short treatments, in this case followed by sampling at ~20 h, it is not known whether continuous treatments in the absence of S-9 would have produced positive responses at lower concentrations. Thus, monuron may be clastogenic if tested in a modern protocol, including a continuous treatment in the absence of S-9.
- CI Direct Blue 15 (2429-74-5)—the MLA study with CI Direct Blue 15 was conducted under a US Environmental Protection Agency contract, and the data are not available for review. However, CI Direct Blue 15 is an azo-dye and is positive in an Ames test with reductive or anaerobic incubation (10). It can be argued, therefore, that this carcinogen should be detected in an Ames test suitably modified to take account of the structural alerts. Therefore, the conditions under which it is detected in a mammalian cell test would not be relevant.
- Ethionamide (536-33-4)—whether ethionamide is genotoxic is debatable. In a CA study in CHL cells (according to a Japanese protocol), the lowest positive concentration for ethionamide was 400 μg/ml (2.4 mM) following a continuous 48-h treatment (70). The detailed data are not available for review and therefore further comment on this result is not possible. In the NTP CA test (4), ethionamide was negative at concentrations up to 1600 μg/ml (9.6 mM), but the early sampling times in the NTP protocol would probably have been too early to detect clastogenic effects with this chemical. Hilliard *et al.* (71) repeated the study with later sampling times and found positive responses ~6 to 8 mM, but precipitation was seen at these concentrations. They concluded that ethionamide did not induce CA at soluble concentrations and that both CA and toxicity occur only when precipitate is present. Ethionamide was considered an example of chemicals that induce misleading positive results in *in vitro* mammalian cell tests by Kirkland *et al.* (72). It can therefore be argued that ethionamide, as a non-carcinogen, should not be expected to produce genotoxic responses in mammalian cells *in vitro* and should not be used to justify testing at concentrations up to10 mM.
- FD&C Red 1 (3564-09-8)—this is an azo dye, and the Ames test was positive when the Prival modification (FMN + hamster S-9) was used (73). Therefore, as with CI Direct Blue 15, knowledge of the chemical structure would lead to positive results in a modified Ames test, and the fact that the MLA was only positive at 3.44 mM (73) is irrelevant.
- Furan (110-00-9)—in the CA study (4), a positive response was obtained in the absence of S-9 at 160 μg/ml (=2.35 mM). A marginal positive response was seen at 100 μg/ml (1.47 mM). Higher concentrations were needed to induce positive responses in the presence of S-9. Because the NTP protocol was designed to sample cells at the first mitosis, cultures were sampled at 12–13 h after the start of treatment. This is much earlier than the 1.5 cell cycles recommended in current guidelines, which would result in sampling at ~20 h after the start of treatment. Thus, furan may well be positive at <1mM if tested in a current CA protocol.

In the MLA study of McGregor *et al.* (74), the lowest concentration that would be considered positive under current criteria was 2116 μg/ml (31 mM) and such a high level would not be tested under current guidelines. Again only short (4 h) treatments were used in the absence of S-9; the study was not optimised for detection of small colony mutants. Furan may well be mutagenic at lower concentrations following 24-h treatment. Thus, furan may be mutagenic at <1 mM in the MLA if tested with longer treatment times in the absence of S-9, in a study optimised for detection of small colony mutants.

- Furosemide (54-31-9)—in the MLA (4), the lowest concentration giving a positive response (i.e. exceeds GEF) was 1500 μg/ml (4.5 mM), However, when these data were reviewed by Mitchell *et al.* (75), there was an implication that the positive response may have been due to pH shift. With modern protocols, shifts in pH that might give rise to artefactual-positive responses would be avoided. In the CA study (4), the lowest positive concentration was 2513 μg/ml (7.6 mM). However, this was associated with precipitate and no concurrent cytotoxicity measures were included. Modern protocols would avoid testing of precipitates. Ishidate (76) also reported it positive for CA at 2000 μg/ml, but no details were given so it is not known whether precipitate or pH shifts were involved. It can therefore be argued that furosemide should not be expected to produce genotoxic responses in mammalian cells *in vitro* when using a modern protocol with adequate controls for pH shift and precipitation.
- 2-Mercaptobenzothiazole (149-30-4)—in the MLA, a weak positive response was reported (induced MF less than GEF) at concentrations up to 20 μg/ml (<1 mM), where there was 70–80% reduction in RTG (4). However, it induced CA in the presence of S-9 at 351 μg/ml (2.1 mM), which was the lowest concentration scored (4). It may well be clastogenic at lower concentrations.
- Methylolacrylamide (924-42-5)—in the CA study (4), a positive response (16% cells with aberrations) was obtained at the lowest concentration tested, 250 μg/ml (~2.5 mM) in the absence of S-9. As the NTP protocol, only employed short treatments, in this case followed by sampling at ~20 h, it is not known whether continuous treatments in the absence of S-9 would have produced positive responses at lower concentrations. Thus, methylolacrylamide may be clastogenic at concentrations <1 mM if tested in a modern protocol, including a continuous treatment in the absence of S-9.
- Styrene (100-42-5)—according to Matsuoka *et al.* (77), styrene was only tested in CHL cells at one concentration (2.4 mM) and gave 19% cells with aberrations after a 3-h treatment in the presence of S-9. It is not stated whether other concentrations were tested but not reported. There are other reports of styrene only being detected as positive at concentrations >1 mM (77,78), but in these tests, no metabolic activation was included. When activated either by red blood cells or Clophen-induced S-9 (78–80), styrene was positive in

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

the range 0.1–1 mM. The metabolic activation conditions are therefore critical to its conversion to styrene oxide and its detection as a clastogen. As discussed by Scott and Preston (81), the activation by P450-dependent monooxygenases needs to exceed deactivation by epoxide hydrolase. It is possible that the usual induced S-9 preparations contain too much epoxide hydrolase and are not optimal. It can be argued that simple *in vitro* tests cannot be expected to detect every type of chemical and that styrene has such specific metabolic requirements (i.e. inhibition of epoxide hydrolase) that it would be inappropriate to expect standard genotoxicity tests to detect styrene as genotoxic at concentrations <1 mM when the activation conditions are not optimal.

• Toluene (108-88-3)—in the MLA (74), the lowest concentration giving a positive result by current criteria was 250 μg/ml (2.7 mM). This was in the absence of S-9, but only a 4-h treatment was used. It is possible that toluene would be mutagenic at lower concentrations following a continuous treatment in the absence of S-9, as required by current recommendations.

Thus, more than one-third of the Ames-negative rodent carcinogens that are only detected as positive in mammalian cells at concentrations in the range 1–10 mM are non-genotoxic carcinogens. There are therefore only 14 chemicals where it can be argued that one should expect the *in vitro* tests to detect a positive response. As discussed in detail above, five of these are considered of low priority in the current context for the following reasons:

CI Direct Blue 15 and FD&C Red 1 are azo dyes that are readily detected in a modified Ames test.

Furosemide and ethionamide are only clastogenic at precipitating concentrations, and mutagenic responses in the MLA with furosemide were associated with pH shifts. They should therefore be considered non-genotoxic *in vitro* rather than being seen as examples that justify testing to 10 mM.

Styrene can be detected at concentrations <1 mM if activation by monooxygenases exceeds deactivation by epoxide hydrolase. Activation conditions therefore need to be optimised for its conversion to styrene oxide and its detection as a clastogen.

The reported mammalian cell tests on the nine remaining carcinogens—allyl isovalerate, benzofuran, caffeic acid, chlorobenzene, 3-(*p*-chlorophenyl)-1-1-dimethylurea, furan, 2-mercaptobenzothiazole, methylolacrylamide and toluene—were all ‘old’ and either used too early sampling times, only used short treatments or were not optimised in other ways. There is a high chance that these chemicals would be positive at lower concentrations (possibly <1 mM) if retested according to current recommendations. In addition, in the paper by Parry *et al*. (1), it can be seen that daminozide was only clearly positive at concentrations just >10 mM, and therefore, it may be worth retesting in case with prolonged treatments; it becomes positive <10 mM.

These 10 chemicals have therefore been tested in MLA and/or CA tests using modern protocols, and these tests are described below.

Materials and methods

All test chemicals and reagents were purchased from Sigma–Aldrich (Poole, Dorset, UK) unless otherwise stated.

Chemicals were dissolved in dimethyl sulphoxide with the exception of daminozide, which was dissolved in sterile ultrapure water (Baxter, Newbury, Berkshire, UK).

Aroclor-1254-induced rat liver S-9 was obtained from Molecular Toxicology Incorporated, Boone, NC, USA and was prepared as follows—glucose-6-phosphate (180 mg/ml), NADP (25 mg/ml), potassium chloride (KCl) (150 mM) and rat liver S-9 were mixed in the ratio 1:1:1:2. An aliquot of this mix was added to each cell culture to achieve a final concentration of 2% (v/v).

Mouse lymphoma assay

The MLA was carried out as previously described by Clements (82). L5178Y/$tk^{+/-}$−3.7.2C mouse lymphoma cells were a gift from M. Fellows (AstraZeneca, Alderley Park, UK) who originally obtained them from Dr J. Cole (MRC Cell Mutation Unit, University of Sussex, UK). They were confirmed as free of mycoplasma by polymerase chain reaction (PCR).

Cells were cultured in suspension in RPMI 1640 (Gibco, Paisley, UK) medium supplemented with 10% heat-inactivated horse serum (Gibco), 2 mM/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM/l sodium pyruvate and 2.5 μg/ml amphotericin B (Invitrogen, Paisley, UK). Pluronic acid was added at a concentration of 0.5 mg/ml to prevent clumping of cells. For the 3-h treatment, serum concentration was lowered to 5%, but then raised to 20% when cells were transferred into 96-well plates for the mutation assay. Cultures were routinely diluted to a concentration of 2×10^5 cells/ml to prevent overgrowth.

At least 1×10^7 cells (3-h treatment in the absence and presence of Aroclor-1254-induced rat liver S-9) or 4×10^6 cells (24-h treatment in the absence of S-9) were treated with a range of concentrations of test chemical in a final culture volume of 20 ml. After incubation at 37 ± 1°C with 5% (v/v) CO_2, cultures were centrifuged at 200 g for 5 min, washed and resuspended in fresh supplemented RPMI 1640 medium.

Cultures were maintained for 2 days after the end of treatment to allow for $tk^{-/-}$ mutation expression. During the expression period, subculturing was performed as required to keep cell density at approximately 1×10^6 cells per ml (1×10^7 cells per flask). From observations on recovery and growth of the cultures during the expression period, a minimum of four test concentrations, plus negative and positive controls, were selected to be plated for viability and trifluorothymidine (TFT) resistance. The maximum concentration was selected based on a reduction in RTG of ∼80%, or was 10 mM, or was the highest non-precipitating concentration, whichever was the lower.

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

Table I. CA data from allyl isovalerate treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h +S-9		20 h −S-9	
		% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps
0	0	0	1	0	2
59.06	0.41	0	2		
78.75	0.56	0	15***		
105	0.75	3	40***		
187	1.3			27	2
249	1.8			28	0
332	2.3			58	3

***$P < 0.001$.

Table II. MLA data from benzofuran treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		24 h −S-9	
		RTG (%)	MF[a]	RTG (%)	MF[a]	RTG (%)	MF[a]
0	0	100	79.01	100	72.64	1.00	104.24
11.81	0.1	105	63.43			124	68.16
23.63	0.2			55	71.70		
35.43	0.3	82	58.21			93	78.90
47.25	0.4			41	91.91		
59.05	0.5	50	76.34			53	77.59
70.88	0.6			30	111.7		
82.67	0.7	36	85.50			26	106.71
94.5	0.8			22	132.1		
106.3	0.9	24	129.16			11	143.81
118.1	1	36	93.34	12	180.6	11	124.61
236.3	2	0	274.53[b]			0	636.54[b]
472.5	4	Toxic		Toxic		Toxic	
1181	10	Toxic		Toxic		Toxic	

[a]5-TFT resistant mutants per 10^6 viable cells.
[b]Increases in mutation frequency above the GEF.

Table III. MLA data from Caffeic acid treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		24 h −S-9	
		RTG (%)	MF[a]	RTG (%)	MF[a]	RTG (%)	MF[a]
0	0	100	77.59	100	78.36	100	95.21
18.02	0.1					102	103.34
36.04	0.2					71	154.75
54.06	0.3					47	217.56
72.08	0.4					28	285.30[b]
90.1	0.5					23	327.00[b]
108.1	0.6					14	352.77[b]
126.1	0.7					8	379.20[b]
144.1	0.8	23	156.98	56	98.91	4	486.25[b]
162.2	0.9					2	434.95[b]
180.2	1	18	152.45	64	95.89	2	549.90[b]
360.3	2	11	135.52	27	117.13	Toxic	
540.5	3	9	171.29	13	127.27	Toxic	
720.6	4	7	205.60[b]	10	148.65	Toxic	
900.8	5	5	186.78	6	143.33	Toxic	
1081	6	Toxic		6	144.25	Toxic	
1261	7	Toxic		Toxic		Toxic	

[a]5-TFT resistant mutants per 10^6 viable cells.
[b]Increases in mutation frequency above the GEF.

To assess viability, at the end of the expression period cells in the selected cultures were diluted to 8 cells/ml. Adjusted cell culture (0.2 ml) was placed into each well of 2 × 96-well plates (averaging 1.6 cells per well). Plates were incubated at 37 ± 1°C with 5% (v/v) CO_2 for 1–2 weeks. Wells containing viable clones were identified by eye using background illumination and counted.

For the mutation assay, cell concentrations were adjusted at the end of the expression period to 1 × 10^4/ml, and the selective agent TFT was added to a final concentration of 3 μg/ml. Adjusted cell culture (0.2 ml) was transferred into each well of 4 × 96-well plates (2 × 10^3 cells per well). Plates were incubated at 37 ± 1°C with 5% (v/v) CO_2 for 1–2 weeks. Wells containing mutant clones were identified by eye using background illumination and counted, and MF was calculated as follows:

$$\mathrm{MF} = \frac{-\ln P(0) \text{ for mutant plates}}{\text{Number of cells per well}^* \times (\text{viability}/100)}.$$

*Number of cells per well is 2000 cells per well on average on all mutant plates.

In addition, the proportions of small and large colony mutants were determined for the negative and positive controls and for concentrations of test chemical showing significant increases in MF over the negative control. The significance of increases in MF was determined by comparison with concurrent controls, and whether the GEF (126 mutants per 10^6 viable cells) recommended by Moore *et al.* (65) was exceeded. A linear trend test was also performed. A test chemical was clearly mutagenic if the MF at any test concentration exceeded the sum of the mean control MF plus GEF and there was a significant linear trend test.

In all experiments, the mean MFs in the negative (vehicle) controls were within the normal range (50–170 mutants per 10^6 viable cells) and positive controls showed increases in MF of at least 300 mutants per 10^6 viable cells)

CA assay

CHO cells were a gift from Dr S. Galloway (Merck Research Laboratories, West Point, PA, USA) and were confirmed free of mycoplasma by PCR. Cells were maintained in McCoy's 5A medium (Gibco) supplemented with 10% (v/v) heat-inactivated foetal calf serum (Invitrogen) and 100 μg/ml gentamycin. Cells were subcultured regularly at low density (∼3 to 5 × 10^5 cells per flask) to prevent overgrowth and maintain low aberration frequencies. Cell monolayers were removed from stock cultures using trypsin/EDTA solution (Invitrogen).

Prior to the start of treatment, baseline cell counts were performed to be used for the estimation of toxicity [expressed as relative population doubling (RPD) relative to controls] at the time of cell harvest.

Populations of CHO cells were treated with test chemicals for 3 h in the presence and absence of S-9 followed by 17 h recovery (3 + 17 h ± S-9) or for 20 h in the absence of S-9 (20 + 0 – S-9). After incubation at 37 ± 1°C with 5% (v/v) CO_2, cultures were washed with 0.85% saline solution (Baxter) and freshly supplemented McCoy's 5A medium was added. All cultures were incubated at 37°C with 5% CO_2. Approximately 1.5 h prior to harvest, colchicine was at a final concentration of 1 μg/ml.

At harvest 200 μl of cell suspension from all cultures was taken for determination of cell number using a Coulter Counter (Beckman, High Wycombe, Buckinghamshire, UK). The remaining cells were centrifuged at 200 g for 5 min and resuspended in 4 ml 0.075 M KCl and incubated at 37°C ± 1°C for 5 min. Cells were then fixed in cold methanol:glacial acetic acid (3:1, v/v). Several drops of fixed cell suspension were transferred on to

Table IV. CA data from Caffeic acid treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		20 h −S-9	
		% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps
0	0	0	2	0	2	0	6
180.2	1	37	8*			46	29***
360.3	2	51	19***	5	2	73	27***
540.5	3	61	10*	18	6	79	
720.6	4	66		55	31***	80	
1802	10	80		73	16***		

*$P < 0.05$.
***$P < 0.001$.

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

microscope slides and allowed to air dry before staining for 5 min in filtered 4% (v/v) Giemsa in pH 6.8 buffer. They were then rinsed, dried and mounted with coverslips.

RPD was determined as follows:

$$\frac{\text{Number of population doublings in treated cultures}}{\text{Number of population doublings in control cultures}} \times 100,$$

where population doubling = [log (post-treatment cell number/initial cell number)]/log 2.

An extensive range of concentrations was tested but concentrations for CA analysis were chosen on the basis of toxicity (expressed as RPD). The highest concentration for chromosome analysis was one at which RPD was reduced to ~50% of control levels or 10 mM or was the lowest concentration that produced precipitation, whichever was the lower. In the tables that follow, data are only presented for the concentrations that were scored. At each dose level, 200 metaphases were analysed for CAs, unless damage was severe in which case scoring ceased once 10 cells with structural aberrations (excluding gaps) had been found. Only cells with 19–23 (modal number ± 2) chromosomes were considered acceptable for analysis. Cells with >23 chromosomes (polyploid, hyperdiploid or endoreduplicated cells) were noted and recorded separately. Structural aberrations were classified according to the International System for Human Cytogenetic Nomenclature scheme whereby a gap is defined as a discontinuity less than the width of the chromatid with no evidence of displacement of the fragment and a deletion is defined as a discontinuity greater than the width of the chromatid and/or evidence of displacement of the fragment.

Positive responses were considered to be those in which the frequency of structural aberrations excluding gaps gave statistically significant increases, using Fishers exact test, and exceeded the laboratory's historical control range.

In each experiment, acceptable levels of heterogeneity were observed, the proportion of cells with structural aberrations in negative controls fell within the normal range, and positive controls gave statistically significant increases in the proportion of cells with structural aberrations excluding gaps.

Table V. MLA data from Chlorobenzene treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		24 h −S-9	
		RTG (%)	MF[a]	RTG (%)	MF[a]	RTG (%)	MF[a]
0	0	100	75.39	100	58.86	100	99.36
67.56	0.6	79	73.10	22	299.25[b]	94	75.59
78.82	0.7	75	84.60	25	314.70[b]	94	61.34
90.08	0.8	85	67.69	32	295.18[b]	71	86.35
112.6	1	80	59.34	27	338.70[b]	72	68.52
225.2	2	20	93.59	Toxic		24	74.59
337.8	3	Toxic		Toxic		Toxic	
450.4	4	Toxic		Toxic		Toxic	

[a]5-TFT resistant mutants per 10^6 viable cells.
[b]Increases in mutation frequency above the GEF.

Results

Allyl isovalerate

Allyl isovalerate was re-evaluated for induction of CA in CHO cells; these data are presented in Table I. Following 3-h treatments in the absence of S-9 toxicity was seen at and below 1 mM, however, the toxicity response curve was very steep meaning that there were no suitable concentrations for CA analysis. Because of the positive response seen in the presence of S-9, this experiment was not repeated. Following 3-h treatments in the presence of S-9, there were clear increases in CA at 78.75 and 105.0 μg/ml (0.55 and 0.74 mM). Following 20-h treatments in the absence of S-9, there were no increases in structural CA at concentrations up to 332.2 μg/ml (2.34 mM), which induced 58% reduction in RPD. Thus, allyl isovalerate does induce CA at concentrations <1 mM (<100 μg/ml) when tested in the presence of S-9 in a modern protocol.

Benzofuran

Benzofuran was re-evaluated for induction of *tk* mutations in the MLA. The data are presented in Table II. Following 3-h treatments in the absence and presence of S-9 and 24-h treatment in the absence of S-9, there was significant cytotoxicity at 1 mM and 100% cytotoxicity at 2 mM. Only at 2 mM (236.3 μg/ml) were there increases in MF that exceeded the GEF, but this concentration would be excluded from evaluation because of the excessive (>90%) toxicity. Therefore, there were no increases in *tk* MF that exceeded the GEF at any concentration that produced an acceptable level of toxicity. Benzofuran is therefore not confirmed as a mammalian cell genotoxin in a modern protocol satisfying current recommendations for testing.

Caffeic acid

Caffeic acid was re-evaluated for induction of CA in CHO cells and for induction of *tk* mutations in the MLA.

The results from the MLA are shown in Table III. Significant cytotoxicity was seen at concentrations of ≥3 mM following 3-h treatment in the presence of S-9, but there was no increase in MF that exceeded the GEF. A significant increase in MF (exceeding the GEF) was seen at 4 mM following 3-h treatment in the absence of S-9, but cytotoxicity (reduction in RTG) was >90%, so this data would be excluded from evaluation. However, increases in MF were seen over a wider range of concentrations following 24-h treatment in the absence of S-9,

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

Table VI. CA data from monuron treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		20 h −S-9	
		% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps
0	0	0	0	0	0	0	0
84.47	0.4	7	0			13	3
150.2	0.8			21	0	26	2
200.2	1	9	0				
267	1.3			27	2	40	3
356	1.8					67	1
475	2.4	49	2				
844	4.3	100		47	4		
1125	5.7			100			

with the lowest concentration giving an increase that exceeded the GEF being 0.4 mM (72.08 μg/ml).

The results from the CA test are shown in Table IV. It can be seen that caffeic acid induced biologically significant levels of CA in all parts of the study, the lowest clastogenic concentrations being 4 mM following 3-h treatments, but being 1 mM (180.2 μg/ml) following 20-h treatment in the absence of S-9.These responses were associated with relatively high cytotoxicity (~50% reduction in RPD).

Thus, caffeic acid is genotoxic in mammalian cells (MLA and CA) at concentrations <1 mM (i.e. <200 μg/ml in the CA test and <100 μg/ml in the MLA) when prolonged treatments in the absence of S-9, as required in a modern protocol, are included.

Chlorobenzene

Chlorobenzene was re-evaluated for induction of *tk* mutations in the MLA. The results are shown in Table V. Toxicity limited the highest tested dose in the absence of S-9 (3- and 24-h treatment) to 2 mM; however, there were no increases in MF greater than the GEF with these treatments. In the presence of S-9, there were significant increases in MF that exceeded the GEF from concentrations of 0.6–1 mM (67.56–112.6 μg/ml) confirming that chlorobenzene is genotoxic at concentrations <1 mM (and <100 μg/ml) when tested with metabolic activation.

*3-(p-*Chlorophenyl)-1-1-dimethylurea (AKA monuron)

Monuron was re-evaluated for induction of CA in CHO cells. The results are shown in Table VI. Following 3-h treatment in the absence and presence of S-9 and 20-h treatment in the absence of S-9, no increases in CA were seen at concentrations up to 843.8 μg/ml (4.25 mM), which induced ~50% reduction in RPD.

Daminozide

Daminozide was re-evaluated for induction of CA in CHO cells and for induction of *tk* mutations in the MLA. The MLA results are shown in Table VII. Following 3-h treatment in the absence and presence of S-9 and following 24-h treatment in the absence of S-9, there was little or no toxicity induced (maximum 38% reduction in RTG). There were no increases in MF up to 10 mM. The CA data are shown in Table VIII. Again, there was little or no cytotoxicity up to 10 mM in any of the treatments (maximum 25% reduction in RPD following 20-h treatment in the absence of S-9). There were no increases in CA. Thus, the genotoxicity of daminozide in mammalian cells has not been confirmed at concentrations up to 10 mM.

Table VII. MLA data from daminozide treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		24 h −S-9	
		RTG (%)	MF[a]	RTG (%)	MF[a]	RTG (%)	MF[a]
0	0	100	66.99	100	78.81	100	71.95
128.1	0.8	80	66.08	95	105.87	100	54.32
160.2	1	86	65.61	131	64.58	98	57.40
320.3	2	96	61.54	127	68.44	105	59.77
480.5	3	104	77.57	125	67.50	103	65.85
640.7	4	82	90.82	123	79.30	101	55.06
800.9	5	91	72.81	117	75.43	89	33.33
961	6	78	80.28	118	78.50	86	52.60
1121	7	84	82.99	107	84.21	93	50.49
1281	8	101	73.79	123	88.37	80	60.84
1442	9	80	88.46	124	78.18	83	70.36
1602	10	62	85.27	117	90.11	68	59.43

[a]5-TFT resistant mutants per 10^6 viable cells.

Furan

Furan was re-evaluated for induction of CA in CHO cells and for induction of *tk* mutations in the MLA. The MLA data are shown in Table IX. There was little toxicity induced following 3- or 24-h treatment in the absence of S-9, and there was no increase in MF up to 10 mM. Treatments for 3 h in the presence of S-9 did induce cytotoxicity and significant increases in MF. The lowest concentration inducing an increase in MF that exceeded the GEF was 0.8 mM (54.46 μg/ml) where there was also significant but acceptable (according to current guidelines) toxicity, RTG being reduced to 16% of control.

The CA data are shown in Table X. It can be seen that, again, there was little toxicity at 10 mM in the 3- and 20-h treatment in the absence of S-9. However, furan did induce toxicity following 3-h treatment in the presence of S-9, and increases in CA were seen. The lowest concentration giving a biologically significant increase in CA was 4 mM (272.3 μg/ml).

Thus, furan is detected as genotoxic in mammalian cells (notably in the MLA) at concentrations <1 mM (and <100 μg/ml), although this is only found in the presence of S-9 and accompanied by >80% toxicity.

2-Mercaptobenzothiazole

2-Mercaptobenzothiazole was re-evaluated for induction of CA in CHO cells, but only using a 3-h treatment in the presence of S-9 as this was the most relevant on the basis of the published data as discussed above. The results are shown in Table XI. Although significant cytotoxicity was induced at 1 and 2 mM, there were no increases in CA.

2-Mercaptobenzothiazole is therefore not confirmed as a mammalian cell clastogen.

Table VIII. CA data from daminozide treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		20 h −S-9	
		% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps
0	0	0	2	0	1	0	0
160.2	1	0	1	0	4	0	2
800.9	5	0	2	0	1	4	1
1602	10	0	3	0	2	16	1

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

Methylolacrylamide

Methylolacrylamide was re-evaluated for induction of CA in CHO cells and for induction of *tk* mutations in the MLA. The MLA data are shown in Table XII. Significant increases in MF were seen in all parts of the study, often at moderate levels of toxicity. The lowest concentration inducing an increase in MF that exceeded the GEF was 2 mM following 24-h treatment in the absence of S-9. However, given the large increase in MF between 1 and 2 mM (202.2 μg/ml), it is highly likely that methylolacrylamide produces significant increases in MF at concentrations closer to 1 mM.

The CA data are shown in Table XIII. Significant increases in CA were seen in all parts of the study, often at moderate levels of toxicity. The lowest concentration inducing a significant increase in CA was 2 mM (202.2 μg/ml) following 21-h treatment in the absence of S-9. Again, the magnitude of the increase in CA between 1 and 2 mM suggests that methylolacrylamide is likely to induce significant CA at concentrations closer to 1 mM.

Thus, methylolacrylamide is genotoxic at lower concentrations (possibly <200 μg/ml) in a modern protocol where prolonged treatments in the absence of S-9 are included.

Toluene

Toluene was re-evaluated for induction of *tk* mutations in the MLA. The results are given in Table XIV. Toluene was toxic to the mouse lymphoma cells, RTG being reduced to 10, 7 and 16% at 2.8, 2.8 and 3.2 mM following 3 h in the absence of S-9, 3 h in the presence of S-9 and 24 h in the absence of S-9, respectively. However, even at these high levels of cytotoxicity, there were no increases in MF that exceeded the GEF. Therefore, toluene is not confirmed as a mammalian cell genotoxin.

Discussion

In this study, 10 chemicals were selected as priorities for retesting. These chemicals were previously tested in a MLA or a CA assay in CHO cells (many as part of the NTP programme). Of the 10 chemicals tested, five failed to induce either *tk* mutations or CA despite testing up to levels inducing significant toxicity (50% reduction in RPD or 10–20% RTG) or a 10 mM maximum. Daminozide was found to be non-toxic up to the maximum tested concentration of 10 mM, monuron, 2-mercaptobenzothiazole and toluene all induced significant levels of toxicity which limited the highest analysable concentration to <5 mM for all chemicals. For these four chemicals, the previously published genotoxic outcome has not been reproduced. Benzofuran gave positive results in a mouse lymphoma test, however, as the increases in mutation frequency were observed at very high levels of toxicity (0% RTG) which would not normally be considered suitable for scoring, these data were discounted and it was concluded that the previously published genotoxic outcome for benzofuran was also not reproduced.

The majority of the data used to draw conclusions of genotoxic potential (1) is from the NTP database. Many of the original tests followed old protocols that would not comply with today's recommendations, In the NTP CA studies, the concentrations scored were much higher than that could be achieved in the current tests. Since there was no concurrent measure of cytotoxicity, the NTP study may have used concentrations that induced >50% cytotoxicity which could explain why when retesting with modern protocols and including concurrent measures of cytotoxicity such as RPD (83), the positive responses have not been reproduced. Recent focus on the importance of accurate toxicity estimation and

Table IX. MLA data from furan treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		24 h −S-9	
		RTG (%)	MF[a]	RTG (%)	MF[a]	RTG (%)	MF[a]
0	0	100	81.27	100	69.55	100	83.90
6.807	0.1			113	51.51		
13.61	0.2			93	75.84		
20.42	0.3			78	74.54		
27.23	0.4			65	90.44		
34.04	0.5			51	110.09		
40.84	0.6			26	160.48		
47.65	0.7			25	187.38		
54.46	0.8			16	300.33[b]		
61.26	0.9			13	384.39[b]		
68.07	1	94	79.37	8	372.28[b]	118	53.32
136.1	2	104	80.15	Toxic		90	72.37
204.2	3	111	60.67	Toxic		94	69.38
272.3	4	110	68.25	Toxic		92	59.96
340.4	5	87	88.46	Toxic		82	80.28
408.4	6	65	88.63	Toxic		56	71.08
476.5	7	73	77.35	Toxic		52	97.84
544.6	8	71	73.44	Toxic		35	139.67
612.6	9	36[c]	135.79[c]	Toxic		7	195.97
680.7	10	72	86.65	Toxic		45	93.37

[a]5-TFT resistant mutants per 10^6 viable cells.
[b]Increases in mutation frequency above the GEF.
[c]Treatment excluded from analysis due to excessive heterogeneity.

Table X. CA data from furan treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		20 h −S-9	
		% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps
0	0	0	2	0	2	0	0
68.07	1	0	0	23	0	0	0
272.3	4			45	12***		
340.4	5	3	0	64	14***	0	1
680.7	10	13	1			7	0

***$P < 0.001$.

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

choice of toxicity measure (84,85) highlights the observation that certain measures of toxicity can lead to selection of artificially high concentrations in *in vitro* tests. The NTP data were based on highest analysable concentration rather than a pragmatic limit of toxicity, which almost certainly led to higher concentrations being analysed. In addition, the NTP CA protocols sampled cells in their first mitosis after treatment, often as early as 12 h after the start of treatment; modern protocols routinely use a later sampling time of between 1.5 and 2 cell cycles (OECD Guideline 473). Modern MLA protocols use the GEF when determining mutation induction, whereas the NTP mouse lymphoma studies compared average mutation frequencies between solvents and treated cultures. Thus, in the case of five of the chemicals tested (benzofuran, monuron, daminozide, 2-mercaptobenzothiazole and toluene), a genotoxic response was not seen when testing to current limits in a modern protocol. Thus, for these five chemicals, whatever the mechanism of carcinogenicity, they are not genotoxic in bacteria or mammalian cells *in vitro*.

The remaining five chemicals were all confirmed as genotoxic in either CA or MLAs using modern protocols. Of these, allyl isovalerate, caffeic acid, chlorobenzene and furan were all positive in either a MLA or CA assay at concentrations <1 mM (also <200 μg/ml). The only chemical that gave positive responses >1 mM was methylolacrylamide, which was positive in both the mouse lymphoma and CA tests at concentrations of ≥2 mM (202.2 μg/ml). Although this is the only example from the current set that would require testing >1 mM, there may be other examples, and further testing of relevant carcinogens and *in vivo* genotoxins is needed. Methylolacrylamide has a molecular weight of only 101. Thus, although 1 mM may be an acceptable upper limit for high-molecular weight substances, for low-molecular weight substances 1 mM may be too low. We therefore propose an approach, which would accommodate these potential differences. A limit of 1 mM or 500 μg/ml, whichever is the higher, would have detected all of the carcinogens that were re-evaluated in this study and allows higher concentrations to be tested with low-molecular weight substances.

A paper by Muller and Sofuni (86) proposed several solutions to reduce top concentrations including, reducing the upper limit of toxicity to a degree whereby 25% fewer positive results would be seen, many of which were not correlated with positive *in vivo* data and could be considered false positives and lowering the upper concentration limits irrespective of cytotoxicity. At the time of publication, there were limited data supporting these solutions. A recent ECVAM workshop (87) also argued that reducing the top concentration for testing would reduce 'misleading' or 'false' positive results *in vitro*, and consequently reduce the numbers of animals needed in follow-up *in vivo* testing.

A recent paper by Elespuru *et al.* (88) argued that 'modification of test parameters to reduce the number of positive results without regard to risk is inappropriate'. However, these authors also 'donot believe agents negative in rodent (cancer) bioassays and positive in *in vitro* tests are necessarily false positives. While there are insufficient data to deny this categorically, there are clearly many examples of chemicals that produce positive results *in vitro* that are considered by experts not to be predictive of genotoxic potential in rodents or humans *in vivo* (72). Each such misleading positive result leads to additional testing, often requiring unnecessary use of animals. It is therefore incumbent on the scientific community to try to improve the specificity of the

Table XI. CA data from 2-mercaptobenzothiazole treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9	
		% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps
0	0	0	1
134	0.8	40	2
167	1	37	3
335	2	89	1

Table XII. MLA data from methylolacryamide treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		24 h −S-9	
		RTG (%)	MF[a]	RTG (%)	MF[a]	RTG (%)	MF[a]
0	0	100	97.5	100	68.1	100	78.6
101.1	1	114	121.3	97	88.4	66	152.6
202.2	2	107	172.2	87	103.9	5	662.8[b]
303.3	3	74	261.6[b]	64	175.5	0	2631.23[b]
404.4	4	50	460.3[b]	59	208.9[b]	Toxic	
505.5	5	21	714.3[b]	38	340.6[b]	Toxic	
606.6	6	13	847.3[b]	29	449.1[b]	Toxic	
707.7	7	3	1382.9[b]	20	521.1[b]	Toxic	
808.8	8	Toxic		11	711.8[b]	Toxic	
909.9	9	Toxic		6	817.2[b]	Toxic	

[a]5-TFT resistant mutants per 10^6 viable cells.
[b]Increases in mutation frequency above the GEF.

Table XIII. CA data from methylolacryamide treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		20 h −S-9	
		% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps	% cytotoxicity (reduction in RPD)	% cells with aberrations excluding gaps
0	0	0	0	0	2	0	1
101.1	1	2	1	24	1	7	3
202.2	2	24	5*	39	2	33	32***
303.3	3	28	27***	51	7	78	40***
404.4	4	83	40***	76		93	

***$P<0.001$.

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

Table XIV. MLA data from toluene treatments in the presence and absence of metabolic activation

Treatment (μg/ml)	Concentration mM	3 h −S-9		3 h +S-9		24 h −S-9	
		RTG (%)	MF[a]	RTG (%)	MF[a]	RTG (%)	MF[a]
0	0	100	69.01	100	79.37	100	63.38
36.85	0.4	89	60.52	118	45.76	95	40.04
73.7	0.8	81	60.31	82	57.4	95	38.53
110.6	1.2	68	62.56	70	69.01	85	36.33
147.4	1.6	56	53.75	48	75.61	75	38.71
184.3	2	47	48.80	38	77.86	64	38.36
221.1	2.4	36	70.23	23	100.28	44	37.55
258	2.8	10	78.51	7	90.57	29	46.18
294.8	3.2	Toxic		Toxic		16	50.33
331.7	3.6	Toxic		Toxic		5	92.31
368.5	4	Toxic		Toxic		Toxic	

[a]5-TFT resistant mutants per 10^6 viable cells.

commonly used genotoxicity tests without compromising sensitivity. One approach is to challenge the need for testing to such high concentrations as 10 mM, as in the accompanying paper of Parry *et al*. (1) and this paper.

Although only 10 chemicals have been retested so far, based on the analysis of Parry *et al*. (1) and the further evaluations presented here, the top concentration for testing in mammalian cells can be reduced from the current 10 mM without any loss of sensitivity in detecting rodent carcinogens. This is in line with the proposed revisions to the ICH S2 guideline for human pharmaceuticals. We therefore propose a new top limit of 1 mM or 500 μg/ml, whichever is the higher, since this would have detected all of the chemicals retested in this study.

Funding

Covance laboratories with additional contribution from COLIPA, the European Cosmetic and Toiletry Association.

Acknowledgements

Our thanks go to Marilyn Aardema and her colleagues at P&G for valuable interpretation of the carcinogenicity data and to Mike O'Donovan and his colleagues at AstraZeneca for performing the DEREK for Windows analyses.

Conflict of interest statement: None declared.

References

1. Parry, J. M., Parry, E., Phrakonkham, P. and Corvi, R. (2010) Analysis of published data for top concentration considerations in mammalian cell genotoxicity testing. *Mutagenesis*, **25**, 531–538.
2. ICH S2(R1). Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use. Consultation Step 3. EMEA/CHMP/ICH/126642/2008, London, UK.
3. Uno, Y., Takasawa, H., Miyagawa, M., Inuoe, Y., Murata, T. and Yoshikawa, K. (1994) An *in vivo-in vitro* replicative DNA synthesis (RDS) test using rat hepatocytes as an early prediction assay for nongenotoxic hepatocarcinogens: screening of 22 known positives and 25 noncarcinogens. *Mutat. Res.*, **320**, 189–205.
4. NTP http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm (accessed 1 July, 2010).
5. Tennant, R. W. (1993) A perspective on nonmutagenic mechanisms in carcinogenesis. *Environ. Health Perspect.*, **101** (Suppl. 3), 231–236.
6. Gupta, R. C., Goel, S. K., Earley, K., Singh, B. and Reddy, J. K. (1985) ^{32}P-postlabeling analysis of peroxisome proliferator-DNA adduct formation in rat liver *in vivo* and hepatocytes *in vitro*. *Carcinogenesis*, **6**, 933–936.
7. Nilsson, R., Beije, B., Preat, V., Erixon, K. and Ramel, C. (1991) On the mechanism of the hepatocarcinogenicity of peroxisome proliferators. *Chem. Biol. Interact.*, **78**, 235–250.
8. Lake, B. G., Edwards, A. J., Price, R. J., Phillips, B. J., Renwick, A. B., Beamand, J. A. and Adams, T. B. (2001) Lack of effect of furfural on unscheduled DNA synthesis in the *in vivo* rat and mouse hepatocyte DNA repair assays and in precision-cut human liver slices. *Food Chem. Toxicol.*, **39**, 999–1011.
9. Miyagawa, M., Takasawa, H., Sugiyama, A., Inoue, Y., Murata, T., Uno, Y. and Yoshikawa, K. (1995) The *in vivo-in vitro* replicative DNA synthesis (RDS) test with hepatocytes prepared from male B6C3F1 mice as an early prediction assay for putative nongenotoxic (Ames-negative) mouse hepatocarcinogens. *Mutat. Res.*, **343**, 157–183.
10. Zeiger, E. (1997) Genotoxicity database. In Gold, L. S. and Zeiger, E. (eds), *Handbook of Carcinogenic Potency and Genotoxicity Databases,* CRC Press Inc., Boca Raton, FL, USA, pp. 687–729.
11. WHO (1995) Isophorone. WHO working Group. *Environ. Health Criteria*, **174**, 84.
12. Thier, R., Peter, H., Wiegand, H. J. and Bolt, H. M. (1990) DNA binding study of isophorone in rats and mice. *Arch. Toxicol.*, **64**, 684–685.
13. O'Donoghue, J. L., Haworth, S. R., Curren, R. D. *et al.* (1988) Mutagenicity studies on ketone solvents: methyl ethyl ketone, methyl isobutyl ketone, and isophorone. *Mutat. Res.*, **206**, 149–161.
14. Casciano, D. A., Talaska, G. and Clive, D. (1991) The potent hepatocarcinogen methapyrilene induces mutations in L5178Y mouse lymphoma cells in the apparent absence of DNA adduct formation. *Mutat. Res.*, **263**, 127–132.
15. Lijinsky, W., Reuber, M. D. and Blackwell, B. N. (1980) Liver tumors induced in rats by oral administration of the antihistaminic methapyrilene hydrochloride. *Science*, **15**, 817–819.
16. WHO (1993) *Alpha-methylbenzyl Alcohol*. WHO, Geneva, Switzerland. pp. 57–64. WHO Food Additives Series 32.
17. Engelhardt, G. (2006) *In vivo* micronucleus test in mice with 1-phenylethanol. *Arch. Toxicol.*, **80**, 868–872.
18. Suter, W., Martus, H. J. and Elhajouji, A. (2006) Methylphenidate is not clastogenic in cultured human lymphocytes and in the mouse bone marrow micronucleus test. *Mutat. Res.*, **607**, 153–159.
19. Andreazza, A. C., Frey, B. N., Valvassori, S. S. *et al.* (2007) DNA damage in rats after treatment with methylphenidate. *Prog. Neuropsychopharmacol. Biol. Psychiatry*, **31**, 1282–1288.
20. Jacobson-Kram, D., Mattison, D., Shelby, M., Slikker, W., Tice, R. and Witt, K. (2008) Methylphenidate and chromosome damage. *Cancer Lett.*, **260**, 216–218.
21. Kari, F., Bucher, J., Haseman, J., Eustis, S. and Huff, J. (1995) Long-term exposure to the anti-inflammatory agent phenylbutazone induces kidney tumors in rats and liver tumors in mice. *Jpn. J. Cancer Res.*, **86**, 252–263.
22. Meakawa, A., Onodera, H., Tanigawa, H., Furuta, K., Kanno, J., Matsuoka, C., Ogiu, T. and Hayashi, Y. (1987) Long-term studies on carcinogenicity and promoting effect of phenyl butazone in DONRYU rats. *J. Natl Cancer Inst.*, **79**, 577–584.
23. Giri, A. K. and Mukhopadyay, A. (1998) Mutagenicity assay in Salmonella and in vivo SCE in bone marrow cells of mice for 4 pyrazolone derivatives. *Mutat. Res.*, **420**, 15–25.
24. Battershill, J. M. and Fielder, R. J. (1998) Mouse-specific carcinogens: an assessment of hazard and significance for validation of short-term carcinogenicity bioassays in transgenic mice. *Hum. Exp. Toxicol.*, **17**, 193–205.
25. Mazzullo, M., Colacci, A., Grilli, S., Prodi, G. and Arfellini, G. (1986) 1,1,2-Trichloroethane: evidence of genotoxicity from short-term tests. *Jpn. J. Cancer Res.*, **77**, 532–539.
26. Story, D. L., Meierhenry, E. F., Tyson, C. A. and Milman, H. A. (1986) Differences in rat liver enzyme-altered foci produced by chlorinated aliphatics and phenobarbital. *Toxicol. Ind. Health*, **2**, 351–362.
27. Yamamoto, S., Urano, K. and Nomura, T. (1998) Validation of transgenic mice harboring the human prototype c-Ha-ras gene as a bioassay model for rapid carcinogenicity testing. *Toxicol. Lett.*, **102–103**, 473–478.
28. Doherty, A. T., Ellard, S., Parry, E. M. and Parry, J. M. (1996) An investigation into the activation and deactivation of chlorinated hydrocarbons to genotoxins in metabolically competent human cells. *Mutagenesis*, **11**, 247–274.
29. SIDS (2004) *1,1′,2-Trichloroethane (Post SIDS assessment Report). Screening Information Data Set for High Production Volume Chemicals.* 10. http://www.chem.unep.ch/irptc/sids/oecdsids/TRICHLOROET.pdf (accessed 26 July, 2010).
30. Crebelli, R., Carere, A., Leopardi, P., Conti, L., Fassio, F., Raiteri, F., Barone, D., Cinelli, S. and Vericat, J. A. (1999) Evaluation of 10 aliphatic

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

halogenated hydrocarbons in the mouse bone marrow micronucleus test. *Mutagenesis*, **14**, 207–215.
31. Madle, S., Dean, S. W., Andrae, U. *et al.* (1994) Recommendations for the performance of UDS tests *in vitro* and *in vivo*. *Mutat. Res.*, **312**, 263–285.
32. BUA (1995) 1,1,2-Trichlorethane (April 1994). In *Beratergremium fuer umweltrelevante Altstoffe (BUA)*, Hirzel Verlag, Stuttgart, Germany, Vol. 152, pp. 176.
33. Krewet, E., Müller, G. and Norpoth, K. (1989) The excretion of chlorophenylmercapturic acid chlorophenols and a guanine adduct in the urine of chlorobenzene-treated rats after Phenobarbital pretreatment. *Toxicology*, **59**, 67–79.
34. Gut, I., Nedelcheva, V., Soucek, P., Stopka, P., Vodicka, P., Gelboin, H. V. and Ingelman-Sundberg, M. (1996) The role of CYP2E1 and 2B1 in metabolic activation of benzene derivatives. *Arch. Toxicol.*, **71**, 45–56.
35. Mavournin, K. H., Blakey, D. H., Cimino, M. C., Salamone, M. F. and Heddle, J. A. (1990) The *in vivo* micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat. Res.*, **239**, 29–80.
36. Shelby, M. D., Erexson, G. L., Hook, G. J. and Tice, R. R. (1993) Evaluation of a three-exposure mouse bone marrow micronucleus protocol: results with 49 chemicals. *Environ. Mol. Mutagen.*, **21**, 160–179.
37. Shelby, M. D. and Witt, K. L. (1995) Comparison of results from mouse bone marrow chromosome aberration and micronucleus tests. *Environ. Mol. Mutagen.*, **25**, 302–313.
38. Sills, R. C., Hailey, J. R., Neal, J., Boorman, G. A., Haseman, J. K. and Melnick, R. L. (1999) Examination of low-incidence brain tumor responses in F344 rats following chemical exposures in National Toxicology Program carcinogenicity studies. *Toxicol. Pathol.*, **27**, 589–599.
39. Gaikwad, N. W. and Bodell, W. J. (2003) Formation of DNA adducts in HL-60 cells treated with the toluene metabolite p-cresol: a potential biomarker for toluene exposure. *Chem. Biol. Interact.*, **145**, 149–158.
40. Mohtashamipur, E., Norpoth, K., Woelke, U. and Huber, P. (1985) Effects of ethylbenzene, toluene, and xylene on the induction of micronuclei in bone marrow polychromatic erythrocytes of mice. *Arch. Toxicol.*, **58**, 106–109.
41. Plappert, U., Barthel, E. and Seidel, H. J. (1994) Reduction of benzene toxicity by toluene. *Environ. Mol. Mutagen.*, **24**, 283–292.
42. Gold, L. S. (2004) *The Carcinogenic Potency Project*. http://potency.berkeley.edu/cpdb.html (accessed 26 July, 2010).
43. Raj, A. S., Heddle, J. A., Newmark, H. L. and Katz, M. (1983) Caffeic acid as an inhibitor of DMBA-induced chromosomal breakage in mice assessed by bone-marrow micronucleus test. *Mutat. Res.*, **124**, 247–253.
44. Yamanaka, N., Oda, O. and Nagao, S. (1997) Prooxidant activity of caffeic acid, dietary non-flavonoid phenolic acid, on Cu2+-induced low density lipoprotein oxidation. *FEBS Lett.*, **45**, 186–190.
45. Sharma, G. P., Sobti, R. C., Chaudhry, A., Gill, R. K. and Ahluwalia, K. K. (1987) Mutagenic potential of a substituted urea herbicide, Monuron. *Cytologia*, **52**, 841–846.
46. Elcombe, C. R., Odum, J., Foster, J. R., Stone, S., Hasmall, S., Soames, A. R., Kimber, I. and Ashby, J. (2002) Prediction of rodent nongenotoxic carcinogenesis: evaluation of biochemical and tissue changes in rodents following exposure to nine nongenotoxic NTP carcinogens. *Environ. Health Perspect.*, **110**, 363–375.
47. Vinken, M., Doktorova, T., Ellinger-Ziegelbauer, H. *et al.* (2008) The carcinoGENOMICS project: critical selection of model compounds for the development of omics-based *in vitro* carcinogenicity screening assays. *Mutat. Res.*, **659**, 202–210.
48. Byrns, M. C., Vu, C. C. and Peterson, L. A. (2004) The formation of substituted 1, N6-etheno-2′-deoxyadenosine and 1, N2-etheno-2′-deoxyguanosine adducts by cis-2-butene-1,4-dial, a reactive metabolite of furan. *Chem. Res. Toxicol.*, **17**, 1607–1613.
49. Byrns, M. C., Vu, C. C., Neidigh, J. W., Abad, J. L., Jones, R. A. and Peterson, L. A. (2006) Detection of DNA adducts derived from the reactive metabolite of furan, cis-2-butene-1,4-dial. *Chem. Res. Toxicol.*, **19**, 414–420.
50. Durling, L. J., Svensson, K. and Abramsson-Zetterberg, L. (2007) Furan is not genotoxic in the micronucleus assay *in vivo* or *in vitro*. *Toxicol. Lett.*, **169**, 43–50.
51. Wilson, D. M., Goldsworthy, T. L., Popp, J. A. and Butterworth, B. E. (1992) Evaluation of genotoxicity, pathological lesions, and cell proliferation in livers of rats and mice treated with furan. *Environ. Mol. Mutagen.*, **19**, 209–222.
52. Kellert, M., Brink, A., Richter, I., Schlatter, J. and Lutz, W. K. (2008) Tests for genotoxicity and mutagenicity of furan and its metabolite *cis*-2-butene-1,4-dial in L5178Y $tk^{+/-}$ mouse lymphoma cells. *Mutat. Res.*, **657**, 127–132.
53. Brewster, D. W., Mirly, K. J., Wilson, A. G. E. and Barnett, J. W., Jr. (1989) Lack of in vivo DNA binding of mercaptobenzothiazole to selected tissues of the rat. *Biochem. Biophys. Res. Commun.*, **165**, 342–348.
54. Toxikologische Bewertung (2000) 2-Mercarptobenzothiazol. Berufsgenossenschaft der chemischen Industrie, Heidelberg, Germany Vol.70, p. 143.
55. Uziel, M., Munro, N. B., Katz, D. S., Vo-Dinh, T., Zeighami, E. A., Waters, M. D. and Griffith, J. D. (1992) DNA adduct formation by 12 chemicals with populations potentially suitable for molecular epidemiological studies. *Mutat. Res.*, **277**, 35–90.
56. Kligerman, A. D., Allen, J. W., Bryant, M. F., Campbell, J. A., Collins, B. W., Doerr, C. L., Erexson, G. L., Kwanyuen, P. and Morgan, D. L. (1992) Cytogenetic studies of mice exposed to styrene by inhalation. *Mutat. Res.*, **280**, 35–43.
57. Norppa, H. (1981) Styrene and vinyltoluene induce micronuclei in mouse bone marrow. *Toxicol. Lett.*, **8**, 247–252.
58. Simula, A. P. and Priestly, B. G. (1992) Species differences in the genotoxicity of cyclophosphamide and styrene in three *in vivo* assays. *Mutat. Res.*, **271**, 49–58.
59. Vaghef, H. and Hellman, B. (1998) Detection of styrene and styrene oxide-induced DNA damage in various organs of mice using the comet assay. *Pharmacol. Toxicol.*, **83**, 69–74.
60. Kligerman, A. D., Allen, J. W., Erexson, G. L. and Morgan, D. L. (1993) Cytogenetic studies of rodents exposed to styrene by inhalation. *IARC Sci. Publ.*, **127**, 217–224.
61. Penttilae, M., Sorsa, M. and Vainio, H. (1980) Inability of styrene to induce nondisjunction in *Drosophila* or a positive micronucleus test in the Chinese hamster. *Toxicol. Lett.*, **6**, 119–123.
62. Preston, R. J. and Abernethy, D. J. (1993) Studies of the induction of chromosomal aberration and sister chromatid exchange in rats exposed to styrene by inhalation. *IARC Sci. Publ.*, **127**, 225–233.
63. Sharief, Y., Brown, A. M., Backer, L. C., Campbell, J. A., Westbrook-Collins, B., Stead, A. G. and Allen, J. W. (1986) Sister chromatid exchange and chromosome aberration analyses in mice after *in vivo* exposure to acrylonitrile, styrene, or butadiene monoxide. *Environ. Mutagen.*, **8**, 439–448.
64. Paulsson, B., Grawè, B. and Tőrnquist, M. (2002) Hemoglobin adducts and micronucleus frequencies in mouse and rat after acrylamide or N-methyloacrylamide treatment. *Mutat. Res.*, **516**, 101–111.
65. Moore, M. M., Honma, M., Clements, J. *et al.* (2006) Mouse lymphoma thymidine kinase gene mutation assay: follow-up meeting of the International Workshop on Genotoxicity Testing—Aberdeen, Scotland, 2003—Assay acceptance criteria, positive controls, and data evaluation. *Environ. Mol. Mutagen.*, **47**, 1–5.
66. Whitehead, F. W., San, R. H. C. and Stich, H. F. (1983) An intestinal cell-mediated chromosome aberration test for the detection of genotoxic agents. *Mutat. Res.*, **111**, 209–217.
67. Hanham, A. F., Dunn, B. P. and Stich, H. F. (1983) Clastogenic activity of caffeic acid and its relationship to hydrogen peroxide generated during autooxidation. *Mutat. Res.*, **116**, 333–339.
68. Fung, V. A., Cameron, T. P., Hughes, T. J., Kirby, P. E. and Dunkel, V. C. (1988) Mutagenic activity of some coffee flavor ingredients. *Mutat. Res.*, **204**, 219–228.
69. McGregor, D. B., Riach, C. G., Brown, A., Edwards, I., Reynolds, D., West, K. and Willington, S. (1988) Reactivity of catecholamines and related substances in the mouse lymphoma L5178Y assay for mutagens. *Environ. Mol. Mutagen.*, **11**, 523–544.
70. Ishidate, M., Jr., Harnois, M. C. and Sofuni, T. (1988) A comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell cultures. *Mutat. Res.*, **195**, 151–213.
71. Hilliard, C. A., Armstrong, M. J., Bradt, C. I., Hill, R. B., Greenwood, S. K. and Galloway, S. M. (1998) Chromosome aberrations *in vitro* related to cytotoxicity of nonmutagenic chemicals and metabolic poisons. *Environ. Mol. Mutagen.*, **31**, 316–326.
72. Kirkland, D., Kasper, P., Müller, L., Corvi, R. and Speit, G. (2008) Recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests: a follow-up to an ECVAM workshop. *Mutat. Res.*, **653**, 99–108.
73. Cameron, T. P., Hughes, T. J., Kirby, P. E., Fung, V. A. and Dunkel, V. C. (1987) Mutagenic activity of 27 dyes and related chemicals in the *Salmonella* microsome and mouse lymphoma $TK^{+/-}$ assays. *Mutat. Res.*, **189**, 223–261.
74. McGregor, D. B., Brown, A., Cattanach, P., Edwards, I., McBride, D., Riach, C. and Caspary, W. J. (1988) Responses of the L5178Y tk^{+}/tk^{-} mouse lymphoma cell forward mutation assay: III. 72 coded compounds. *Environ. Mol. Mutagen.*, **12**, 85–154.

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022

75. Mitchell, A. D., Auletta, A. E., Clive, D., Kirby, P. E., Moore, M. M. and Myhr, B. C. (1997) The L5187/tk$^{+/-}$ mouse lymphoma specific gene and chromosomal mutation assay. A phase III report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat. Res.*, **394**, 177–303.
76. Ishidate, M., Jr. (1987) *Chromosomal Aberration Test In Vitro*. L.I.C, Inc., Tokyo, Japan.
77. Matsuoka, A., Hayashi, M. and Ishidate, M., Jr. (1979) Chromosomal aberration tests on 29 chemicals combined with S-9 mix *in vitro*. *Mutat. Res.*, **66**, 277–290.
78. Norppa, H., Tursi, F. and Einisto, P. (1985) Erythrocytes as a metabolic activation system in mutagenicity tests. In Janiaud, P., Averbeck, D. and Moustacchi, E. (eds), *Mutagenèse et toxicology genetique*. Editions INSERM, Paris, France, pp. 35–48.
79. Jantunen, K., Maki-Paakkanen, J. and Norppa, H. (1986) Induction of chromosome aberrations by styrene and vinylacetate in cultured human lymphocytes: dependence on erythrocytes. *Mutat. Res.*, **159**, 109–116.
80. Pohlova, H., Rossner, P. and Sram, R. J. (1985) Cytogenetic analysis of human peripheral blood lymphocytes in culture exposed *in vitro* to styrene and styrene oxide. *J. Hyg. Epidemiol. Microbiol. Immunol.*, **28**, 269–274.
81. Scott, D. and Preston, R. J. (1994) A re-evaluation of the cytogenetic effects of styrene. *Mutat. Res.*, **318**, 175–203.
82. Clements, J. (2000) The mouse lymphoma assay. *Mutat. Res.*, **455**, 97–110.
83. Greenwood, S. K., Hill, R. B., Sun, J. T., Armstrong, M. J., Johnson, T. E., Gara, J. P. and Galloway, S. M. (2004) Population doubling: a simple and more accurate estimation of cell growth suppression in the in vitro assay for chromosomal aberrations that reduces irrelevant positive results. *Environ. Mol. Mutagen.*, **43**, 36–44.
84. Lorge, E., Hayashi, M., Albertini, S. and Kirkland, D. (2008) Comparison of different methods for an accurate assessment of cytotoxicity in the in vitro micronucleus test: I. Theoretical aspects. *Mutat. Res.*, **655**, 1–3.
85. Fellows, M. D., O'Donovan, M. R., Lorge, E. and Kirkland, D. (2008) Comparison of different methods for an accurate assessment of cytotoxicity in the in vitro micronucleus test: II: practical aspects with toxic agents. *Mutat. Res.*, **655**, 4–21.
86. Muller, L. and Sofuni, T. (2000) Appropriate levels of cytotoxicity for genotoxicity tests using mammalian cells in vitro. *Environ. Mol. Mutagen.*, **35**, 202–205.
87. Kirkland, D., Pfuhler, S., Tweats, D. *et al.* (2007) How to reduce false positive results when undertaking *in vitro* genotoxicity testing and thus avoid unnecessary follow-up animal tests: report of an ECVAM workshop. *Mutat. Res.*, **628**, 31–55.
88. Elespuru, R. K., Agarwal, R., Atrakchi, A. H., Bigger, C. A. H. *et al.* (2009) Current and future application of genetic toxicology assays: the role and value of in vitro mammalian assays. *Toxicol. Sci.*, **109**, 172–179.

Downloaded from https://academic.oup.com/mutage/article/25/6/539/1335467 by guest on 16 August 2022