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Can *in vitro* mammalian cell genotoxicity test results be used to complement positive results in the Ames test and help predict carcinogenic or *in vivo* genotoxic activity? II. Construction and analysis of a consolidated database☆

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ABSTRACT

A Workshop sponsored by EURL ECVAM was held in Ispra, Italy in 2013 to consider whether the *in vitro* mammalian cell genotoxicity test results could complement and mitigate the implications of a positive Ames test response for the prediction of *in vivo* genotoxicity and carcinogenicity, and if patterns of results could be identified. Databases of Ames-positive chemicals that were tested for *in vivo* genotoxicity and/or carcinogenicity were collected from different sources and analysed individually (Kirkland et al., in this issue). Because there were overlaps and inconsistent test results among chemicals in the different databases, a combined database which eliminated the overlaps and evaluated the inconsistencies was considered preferable for addressing the above question. A database of >700 Ames-positive chemicals also tested *in vivo* was compiled, and the results in *in vitro* mammalian cell tests were analysed. Because the database was limited to Ames-positive chemicals, the majority (>85%) of carcinogens (103/119) and *in vivo* genotoxins (83/88) were positive when tested in both *in vitro* gene mutation and aneugenicity/clastogenicity tests. However, about half (>45%) of chemicals that were not carcinogenic (19/28) or genotoxic *in vivo* (33/73) also gave the same patterns of positive mammalian cell results. Although the different frequencies were statistically significant, positive results in 2 *in vitro* mammalian cell tests did not, *per se*, add to the predictivity of the positive Ames test. By contrast, negative results for both *in vitro* mammalian cell endpoints were rare for Ames-positive carcinogens (3/119) and *in vivo* genotoxins (2/88) but, were significantly more frequent for Ames-positive chemicals that are not carcinogenic (4/28) or genotoxic *in vivo* (14/73). Thus, in the case of an Ames-positive chemical, negative results in 2 *in vitro* mammalian cell tests covering both mutation and clastogenicity/aneugenicity endpoints should be considered as indicative of absence of *in vivo* genotoxic or carcinogenic potential.

Abbreviations: EURL ECVAM, EU Reference Laboratory for Alternatives to Animal Testing; GLP, Good Laboratory Practices; IARC, International Agency for Research on Cancer; MLA/tk, mouse lymphoma $Tk^{+/-}$ gene mutation assay; *Hprt*, hypoxanthine-guanine phosphoribosyl transferase locus; MNvit, *in vitro* micronucleus test; CAvit, *in vitro* chromosomal aberration test; MNviv, *in vivo* micronucleus test; NTP, National Toxicology Program; CAviv, *in vivo* chromosomal aberration test; Carc, carcinogenicity; TGR, *in vivo* transgenic rodent mutation assay; UDSviv, *in vivo* unscheduled DNA synthesis test; DNAviv, *in vivo* DNA strand breakage (*e.g.*, comet or alkaline elution) assay; vitChrom, combined MNvit and CAvit data; vivChrom, combined MNviv and CAviv data.

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1. Introduction

In all regulatory genetic toxicity testing schemes, the first test performed is the bacterial reverse mutation assay generally conducted in *Salmonella typhimurium* strains (the Ames test). A positive response in the test leads to the presumption that the chemical will be shown to be a carcinogen or *in vivo* genotoxin. However, it has been demonstrated previously that approximately 20–30% of chemicals positive in the Ames test are non-carcinogens [1] and a higher percentage are not genotoxic *in vivo* [1]. A number of reasons can be postulated, or scientific rationale demonstrated, for this lack of concordance. On a mechanistic basis, mutagenicity is not carcinogenicity, and the induction of a mutation is only one of a number of obligate steps in the progression of a normal cell and tissue to malignancy. Other aspects that have to be considered are that *in vivo* tests, which require absorption, distribution and metabolism of the test substance tend to be less sensitive than *in vitro* tests where the test substance is applied directly to the target cells. There are a number of other reasons why chemicals mutagenic in the Ames test may not be active *in vivo*, including differences in metabolic profile, and these have been described in the companion publication [2].

In addition to the Ames test, chemicals are also tested in *in vitro* mammalian cells for mutation (usually the mouse lymphoma $Tk^{+/-}$ or mammalian cell *Hprt* system, hereafter called MLA) and/or chromosome aberrations (CAvit) or micronuclei (MNvit) in hamster, rodent, or human cells in culture. Chemicals that are mutagenic in the Ames test (*i.e.*, DNA-reactive) also tend to be positive in the *in vitro* mammalian cell assays [1,3]. One question that has persisted for many years is whether the *in vitro* mammalian cell genotoxicity test results could complement or mitigate a positive Ames test response for the prediction of *in vivo* genotoxicity and carcinogenicity, and if such patterns of results can be identified.

To address this question, a consolidated database was constructed from individual databases containing data from the scientific literature, regulatory agencies, and other government and industry databases, that were presented at a workshop hosted and sponsored by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), Ispra, Italy from 23 to 25 January 2013, described and discussed in the companion paper [2]. This consolidated database contained 726 chemicals that were positive or equivocal in the Ames test that were also tested in at least one *in vivo* genetic toxicity test or for rodent carcinogenicity.

The consolidated database was analysed to identify the patterns of responses in the *in vitro* mammalian cell tests that might distinguish an Ames-positive carcinogen or *in vivo* genotoxin from an Ames-positive non-carcinogen or non-genotoxin. Where possible, reasons for negative results in mammalian cells, and for the discordance between the *in vitro* and *in vivo* test results, are discussed.

2. Construction of the database

The evaluation of the individual databases presented at the workshop [2] showed that many chemicals appeared in more than one database, and therefore the possibility that significant bias in the workshop's overall conclusions could have accrued from a small number of chemicals replicated in different databases, many of which came from the same original source, had to be considered. We therefore consolidated the data from the different sources into a master database. In compiling this database we took the following actions:

- Excluded substances such as complex hydrocarbons, gasoline fractions, paraffins, *etc.*, where the structure was not known and there was no CAS No. We also excluded asbestos fibres, mosquito coil smoke and motorcycle exhaust particles since the chemical nature and purity of the test substances could not be defined. However, some complex mixtures (*e.g.*, diesel exhaust, tobacco protein) were included because the datasets were comprehensive and (as far as we could establish) equivalent material was used in each test.
- Since the objective of the exercise was to identify whether results in *in vitro* mammalian cell assays (MLA, CAvit, MNvit) with Ames-positive chemicals could help predict *in vivo* genotoxic or carcinogenic activity, or lack thereof, chemicals for which there were no valid *in vivo* results for carcinogenicity or the genotoxicity endpoints – chromosomal aberrations (CAviv), micronuclei (MNviv), UDS (UDSviv), transgenic mutations in rodents (TGR), or DNA strand breakage (DNAviv) – were excluded.
- We combined free bases and simple acid salts of 75 chemical substances into 36 single entries since it is expected that free bases and their hydrochloride or sulphate salts, for example, would behave as identical chemical substances in the aqueous environments present in the *in vitro* and *in vivo* studies under consideration.
- We combined R- and S-isomers of 8 chemicals into 4 single entries where similar results had been obtained on testing the isomers separately.
- In addition to combining the results from the individual databases, information from the peer-reviewed literature or expert publications (*e.g.*, review articles, IARC monographs) was used to fill in missing test data or resolve apparent differences in results among the different databases reporting on the same chemicals.

For each substance it was necessary to confirm the overall activity in the Ames test. Substances that were clearly negative or inconclusive in the Ames test were excluded from the database. It should be noted that some individual Ames tests with negative results could be considered "inconclusive" (see later for further explanation), for example, if the full complement of strains had not been used. However, if all of the required strains had been tested and had given negative results across several different studies, an overall negative call was given, and such chemicals were excluded from the database. Chemicals were included regardless of whether a positive finding was noted only in the absence or only in the presence of S9 and regardless of the number of specific bacterial tester strains (including only in *S. typhimurium* strains TA102 or TA104, or *Escherichia coli*) that showed a response. A positive call was made whether rat, hamster or mouse S9 was used, and regardless of the S9 concentration. This provided a database of 726 substances, representing 747 unique CAS numbers when the salts and isomers (see above) are considered, with clear positive or equivocal Ames test results, and this database has been submitted as a supplementary electronic file.

2.1. Criteria for "overall calls" within the database

Prior to analysis of the database, it was necessary to define an "overall call" for each endpoint (chromosomal aberrations, mammalian cell gene mutation, micronuclei, carcinogenicity, *etc.*) for each chemical *in vitro* and *in vivo* because, in some cases, the same chemical was listed with a different call in different individual databases. We decided to limit the overall calls to only 4 categories, namely positive (+ or weak+), negative (−), equivocal (E) or inconclusive (I). Certain rules had to be applied in arriving at these overall calls, and these are described below. Weak+ responses were evaluated as + in the analyses below.

Overall calls were made for carcinogenicity as follows: