by aromatic nitro compounds and CA-induction by polynitrophenol or precursor. TIMES showed two alerts for CA-induction by both parent chemical and metabolite(s): (1) nitro compounds interact with DNA, and (2) amines, aminophenols, phenyleneamines or hydroxylamines interact with DNA and topoisomerases/proteins. 2,4-Dinitrophenol acts as a metabolic poison by uncoupling oxidative phosphorylation, and this mechanism will have a threshold. It reduced ATP level and induced CAs in CHO and TK cells at cytotoxic concentrations *in vitro* [73]. Dinitrophenol is recognized as a chemical which shows clastogenicity by indirect mechanism, *i.e.*, energy depletion [74]. The weight of evidence suggests the level of concern is negligible.

ID108. 2-Ethylbutyric acid (CAS no. 88-09-5) [MW=116]: 2-Ethylbutyric acid induced CAs after 24-h treatment without S9 mix (5.5%, 5.0%, and 17.0% at 3.4, 6.9, and 10.3 mM (1.2 mg/mL), respectively); relative cell growth, as measured by survival cell count, was 94%, 83% or 62%, respectively [21]. A mouse bone marrow MN test was negative [22]. These data indicate that this chemical is not mutagenic *in vivo* [35]. The level of concern is negligible.

ID109. Ferrous sulfate heptahydrate (CAS no. 7782-63-0) [MW=278]: In the two independent experiments, ferrous sulfate heptahydrate induced CAs after 6-h treatment without S9 mix (19.0% and 39.0% at 5.4 mM (1.5 mg/mL)); relative cell growth, as measured by survival cell count, was 45% and 12%, respectively. Reproducible CA-induction was also observed in the treatments with S9 mix (in the first test, 9.0% and 72.5% at 1.8 (0.5 mg/mL) and 3.6 mM in which relative cell growth was 82% and 45%, respectively; in the second test, 23.0–85.5% at 3.2-5.4 mM in which relative cell growth was 59–19%, respectively) [23]. DEREK did not show any structural alerts. Iron salts are known to induce genotoxicity due to the Fenton reaction and production of oxygen radicals, a mechanism with a threshold [75]. *In vivo*, ferrous sulfate heptahydrate and the other iron salt, ferric chloride hexahydrate (CAS no. 10025-77-1), did not induce micronuclei in the digestive tract including stomach, duodenum and colon after oral administration [47,76]. A mouse bone marrow MN test for ferrous chloride was negative after intraperitoneal injection [47]. No increase in tumor incidence was reported for rats ingesting ferric chloride in drinking water for 2 years [47]. The weight of evidence suggests the level of concern is negligible.

ID110. 2-Hydroxypropanenitrile (CAS no. 78-97-7) [MW=71]: 2-Hydroxypropanenitrile induced CAs weakly (10.0% and 9.5%) after 6-h treatment with and without S9 mix at 10 mM (0.7 mg/mL), respectively [13,50]. Relative cell growth, as measured by monolayer confluence, was about 65% at 10 mM with S9 mix. No structural alerts were shown by DEREK and TIMES. There is no supporting evidence for a reduced level of concern, so some concern still remains.

ID111. 2-Mercaptobenzimidazole (CAS no. 583-39-1) [MW=150]: 2-Mercaptobenzimidazole induced CAs only with S9 mix (11.0% and 11.5% at 5.3 and 10 mM (1.5 mg/mL), respectively) [15]. Relative cell growth, as measured by monolayer confluence, was about 85–95% at 2.5–10 mM. DEREK showed a structural alert for mutagenicity due to a benzimidazole moiety, but that chemical was negative in the Ames test. An alert for CA-induction due to 2-thiobenzimidazole or -benzothiazole was also shown. TIMES showed an alert for CA-induction for both parent chemical and metabolite(s): thiols interact with topoisomerases/proteins. There was no evidence of MN induction in the mouse peripheral blood MN test in a 13-week inhalation study [77]. However, *in vivo* long term MN test by inhalation route will not have resulted in much systemic exposure, compared to an acute MN test by oral or intraperitoneal routes. In addition, the *in vivo* erythrocyte MN test is not definitive as the *in vitro* result was S9-dependent and thus reactive metabolite(s) may not have reached the bone marrow in sufficient concentrations to elicit an effect. The level of concern is minimal.

ID112. N-Methylaniline (CAS no. 100-61-8) [MW=107]: *N*-Methylaniline induced CAs after 24-h treatment without S9 mix (15.0% and 18.2% at 5.5 and 10 mM (1.1 mg/mL), respectively) and after 6-h treatment with S9 mix (12.4% at 10 mM) [15]. Relative cell growth, as measured by monolayer confluence, was about 50% at 10 mM with S9 mix. However, the number of cells analyzed were only 177 or 148 at 10 mM with or without S9 mix, respectively. DEREK did not show any structural alerts, but TIMES showed an alert for CA induction due to possible formation of hydroxyl amine metabolite(s), which can interact with DNA. *N*-Methylaniline yields aniline (CAS no. 62-53-3) in rat and rabbit [78], and aniline induces MN in mice and rats [79]. Aniline is assigned to carcinogen category 2 in the Globally Harmonised System of Classification and Labeling of Chemicals (GHS) classification by the EU regulation [80]. Though *N*-ethylaniline (CAS no. 103-69-5, ID90), a closely related structural analogue, was discussed in a section of the effect of high toxicity (see Section 3.2.1.2.), the definition is not suitable for *N*-methylaniline. Thus, the some level of concern remains. Note that there is a question as to whether aniline is a genotoxic carcinogen, and MN induction may be secondary to methemoglobinemia and regenerative anemia [81].

ID113. p-Nitrophenol sodium salt (CAS no. 824-78-2) [MW=161]: *p*-Nitrophenol sodium salt induced CAs after 6-h treatment without S9 mix (7.5% and 28.0% at 5 and 7.5 mM (1.2 mg/mL), respectively) and with S9 mix (11.5%, 19.0%, 33.5%, and 48.0% at 3.8, 5.0, 6.3, and 7.5 mM, respectively) [21]. Relative cell growth, as measured by monolayer confluence, was 66% or 35% at 5 or 7.5 mM without S9 mix, and 80%, 80%, 61% or 42% at 3.8, 5, 6.3, or 7.5 mM, respectively. TIMES showed three structural alerts for CA-induction for both parent chemical and possible metabolite(s): (1) nitro compounds interact with DNA, (2) amines, aminophenols, or phenyleneamines interact with DNA or topoisomerases/proteins, (3) hydroxylamines interact with DNA. These alerts should be also Ames-positive but *p*-nitrophenol is Ames-negative. DEREK did not show any structural alerts. In addition, *p*-nitrophenol (CAS no. 100-02-7, free base of the chemical) was negative in an *in vivo* mouse bone marrow MN test with intravenous treatment [82]. The weight of evidence suggests the level of concern is negligible.

ID114. Sorbitan monooctadecanoate (CAS no. 1338-41-6) [MW=431]: Sorbitan monooctadecanoate induced CAs with S9 mix (21.0%, 26.0%, and 45.5% at 2.5, 5, and 10 mM (4.3 mg/mL), respectively) in which relative cell growth, as measured by monolayer confluence, was about 85%, 80% or 70%, respectively [16]. No structural alerts were shown by DEREK and TIMES. There was no evidence of carcinogenic potential in rats and mice [83]. The weight of evidence suggests the level of concern is negligible.

ID115. Trimethoxyphosphine (CAS no. 121-45-9) [MW=124]: Trimethoxyphosphine induced CAs at the highest concentration of 10 mM (1.2 mg/mL) with 24-h treatment without S9 mix (4.5%) and with 6-h treatment with S9 mix (7.0%) [19]. Relative cell growth, as measured by survival cell count, was about 85%, 80% or 70%, respectively. No structural alerts were shown by DEREK and TIMES. There is no supporting evidence for a reduced level of concern. Thus, the some level of concern remains.

ID116. Trimethylamine (CAS no. 75-50-3) [MW=59]: Trimethylamine induced CAs after 6-h treatment without S9 mix (9.0%, 22.5%, and 22.5% at 6.4, 8, and 10 mM (0.6 mg/mL), respectively) and with S9 mix (2.0%, 5.5%, and 45.0% at 6.4, 8, and 10 mM, respectively) [20]. Relative cell growth, as measured by monolayer confluence, was 42%, 23% or 6% without S9 mix, or 52%, 42% or 17% with S9 mix, respectively. Extremely toxic doses (less than 25% relative cell growth) increased the frequencies of CAs. A close analogue, dimethylamine (CAS no. 124-40-3), was negative in the standard Ames test, *in vitro* CA test with CHL cells, and *in vivo* rat bone marrow CA test by inhalation for 3 months, examined 15 and 90 days after the end of exposure [84]. However, *in vivo* long term bone

Table 6
Evaluation of level of concern for human health risk assessment on 38 "missed" chemicals.

Possible factors of irrelevant positives	Number of chemicals with different level of concern (Chemical ID)		
	Negligible	Minimal	Some
1. Possible effects of extreme culture conditions (n=15)			
1.1 Low pH (n=7)	6 (IDs 79,80,81,82,83,85)	1 (ID 84)	0
1.2 High toxicity (n=6)	4 (IDs 87,88,89,91)	2 (IDs 86,90)	0
1.3 Precipitation coupled with high toxicity (n=2)	2 (IDs 92,93)	0	0
2. Weak evidence for a positive (n=2)	1 (ID 94)	1 (ID 95)	0
3. Possible other factors (n=21)			
3.1 Induction of polyploidy only (n=1)	1 (ID 96)	0	0
3.2 Selected chemical class with DNA reactivity (n=4)	3 (IDs 97,98,99)	1 (ID 100)	0
3.3 Others (n=16)	8 (IDs 101,103,105, 107,108,109,113,114)	4 (IDs 102,106,111,116)	4 (IDs 104,110,112,115)
Total (n=38)	25	9	4

marrow CA test by inhalation route may not have given much systemic exposure, compare than acute CA test by oral or intraperitoneal route. The level of concern is minimal.

3.3. Level of concern for human health risk assessment on 38 "missed" chemicals

The result of evaluation of the level of concern was summarised in Table 6. Among 38 missed chemicals, four were considered to be of some concern, or nine were considered to be of minimal concern, and remaining 25 were considered to be of negligible concern. Note that the "of some concern" classification is in most cases due to the absence of relevant additional data, and not to available data that suggest a real concern.

3.4. Application of different top concentrations to the "missed" chemicals

The results of application of several top concentration limits to the missed chemicals are shown in Table 7. It would be preferable that the top concentration limit detects the 13 missed chemicals with minimal or some concern and does not detect the 25 missed chemicals with negligible concern. The numbers of chemicals detected at 1 mM or 0.5 mg/mL, whichever is higher, 2 mM or 1 mg/mL, whichever is higher, 4 mM or 2 mg/mL, whichever is lower, and 10 mM or 2 mg/mL, whichever is lower were 2, 8, 3 and 11 for 13 chemicals with some or minimal concern, and 9, 17, 14 and 23 for 25 chemicals with negligible concern, respectively. The top concentration of 2 mM or 1 mg/mL, whichever is higher is the most effective concentration, *i.e.*, relatively higher (8/13) or lower (17/25) detection number among 13 or 25 chemicals, respectively. On the other hand, 1 mM or 0.5 mg/mL, whichever is higher, was not effective (2/13) for detection of 13 chemicals with concern for this data set. The highest concentration of 10 mM or 2 mg/mL, whichever is lower, was good detection (11/13) of 13 chemicals with concern; however, it detected almost all (23/25) of 25 chemicals with negligible concern. Other top concentration employed of 4 mM or 2 mg/mL, whichever is lower, was not effective (3/13) for detection of 13 chemicals with concern.

4. Discussion

In this analysis of 249 HPV chemicals tested in the *in vitro* CA test with CHL cells in accordance with Japanese or OECD test guidelines, we singled out 38 chemicals that were positive for CAs at >1 mM but negative at ≤1 mM and negative in the Ames test—chemicals that would be missed in the standard genotoxicity test battery if the highest concentration tested were 1 mM. Based on weight of evidence approach, including evaluations of effects of extreme culture conditions (low pH, high toxicity, or precipitation), *in silico* structural alert analysis, *in vivo* genotoxicity and carcinogenicity test data, mode of action, or information from closely related chemicals, we evaluated the level of concern for human health risk assessment on 38 "missed" chemicals. After an exhaustive review, we identified four chemicals with some concern, nine with minimal concern, and remaining 25 with negligible concern. Several proposals to reduce the top concentration in *in vitro* mammalian cell genotoxicity tests have been made [4,5,12]. Those are as follows: (1) 1 mM or 0.5 mg/mL, whichever is lower, (2) 1 mM or 0.5 mg/mL, whichever is higher, (3) 4 mM or 2 mg/mL, whichever is lower, and (4) 10 mM or 2 mg/mL, whichever is lower. Item (1) is for pharmaceuticals, but the following note is also added; for pharmaceuticals with unusually low molecular weight (*e.g.*, less than 200) higher test concentrations should be considered [12]. The other items are for industrial chemicals. Note that a large percentage of these industrial chemicals had molecular weights of ≤200, with some notable exceptions. On the other hand, such a reduction runs the risk of eliminating genotoxic agents in the hazard identification stage [2]. Thus, several top concentration limits including 2 mM or 1 mg/mL, whichever is higher, were applied to 38 missed chemicals. It will be preferable that the top test concentration allows the detection of 13 chemicals with minimal or some concern, but cannot detect 25 chemicals with negligible concern. The top concentration of 2 mM or 1 mg/mL, whichever is higher, is most effective, *i.e.*, relatively higher (8/13) or lower (17/25) detection among 13 or 25 chemicals, respectively. Other top concentration, 1 mM or 0.5 mg/mL, whichever is higher [4], was not effective (2/13) for detecting chemicals with concern, but good (*i.e.*, low, 9/25) for chemicals with negligible concern. The other two top concentrations (4 mM or 2 mg/mL, whichever is lower, and 10 mM or 2 mg/mL, whichever is lower) did not show enough response to one of both groups of chemicals; 10 mM or 2 mg/mL, whichever is lower, detected almost all (23/25) chemicals with negligible concern, and 4 mM or 2 mg/mL, whichever is lower, was not effective (3/13) for 13 chemicals with concern. Therefore, we propose 2 mM or 1 mg/mL, whichever is higher, as the top concentration limit for industrial chemicals. If the top concentration were reduced to 2 mM or 1 mg/mL, whichever is higher, the percent of positives would be reduced to 37.8% (94/249) in the dataset of 249 HPV chemicals; current percent of positives was 46.6% (116/249) including 6 chemicals positive at >10 mM. Approximately 80% (204/249) of the analyzed chemicals had molecular weight <300; this means that more than 3.3 mM will be selected as top concentration of 1 mg/mL for majority of chemicals in the dataset (Table 8). In case of chemicals with molecular weight of >1000, top concentration of more than 2 mg/mL will be selected.

Conclusion from our analysis is not based on the carcinogenicity data, unlike in the case of analysis by Parry or Kirkland [3,4]; unfortunately, our dataset did not contain sufficient

Table 7
Application of different top concentrations to 38 missed chemicals (13 with minimal or some concern and 25 with negligible concern).

ID no.	Chemical name	CAS	MW	LEC (mM)	LEC (mg/mL)	Detection at different top concentration limit			
						1 mM or 0.5 mg/mL, whichever is higher	2 mM or 1 mg/mL, whichever is higher	4 mM or 2 mg/mL, whichever is lower	10 mM or 2 mg/mL, whichever is lower
13 missed chemicals with minimal or some concern									
84	Methyl acetoacetate	105-45-3	116.1	10.0	1.2	No	No	No	Yes
86	1,3-Bis(2-methylphenyl)guanidine	97-39-2	239.3	2.5	0.6	No	Yes	Yes	Yes
90	*N*-Ethylaniline	103-69-5	121.2	9.1	1.1	No	No	No	Yes
95	1,3,5-Tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)isocyanuric acid	27676-62-6	784.1	3.2	2.5	No	No	No	No
100	Ethenyltrimethoxysilane	2768-02-7	148.2	5.0	0.8	No	Yes	No	Yes
102	C.I. Fluorescent brightner 271	41267-43-0	1347.1	3.7	5.0	No	No	No	No
104	Dibutyl adipate	105-99-7	258.4	2.5	0.7	No	Yes	Yes	Yes
106	*N,N*-Dimethylbenzylamine	103-83-3	135.2	3.8	0.4	Yes	Yes	Yes	Yes
110	2-Hydroxypropanenitrile	78-97-7	71.1	10.0	0.7	No	Yes	No	Yes
111	2-Mercaptobenzimidazole	583-39-1	150.2	5.3	0.8	No	Yes	No	Yes
112	*N*-Methylaniline	100-61-8	107.2	5.5	0.6	No	Yes	No	Yes
115	Trimethoxyphosphine	121-45-9	124.1	10.0	1.2	No	No	No	Yes
116	Trimethylamine	75-50-3	59.1	6.4	0.4	Yes	Yes	No	Yes
	Number of chemicals detected among the 13 chemicals					2	8	3	11
25 missed chemicals with negligible concern									
79	3-Aminobenzenesulfonic acid	121-47-1	173.2	2.4	0.4	Yes	Yes	Yes	Yes
80	2-Amino-5-chloro-4-methylbenzenesulfonic acid	88-53-9	221.5	9.0	2.0	No	No	No	Yes
81	2-Amino-5-methylbenzenesulfonic acid	88-44-8	187.2	5.1	1.0	No	Yes	No	Yes
82	Glycerol triacetate	102-76-1	218.2	10.0	2.2	No	No	No	No
83	4-Hydroxybenzoic acid	99-96-7	138.1	5.1	0.7	No	Yes	No	Yes
85	1-Naphthylacetic acid	86-87-3	186.2	9.1	1.7	No	No	No	Yes
87	*tert*-Butyl-methacrylate	585-07-9	142.2	2.8	0.4	Yes	Yes	Yes	Yes
88	*o*-Dichlorobenzene	95-50-1	147.0	1.6	0.2	Yes	Yes	Yes	Yes
89	Dicyclohexylamine	101-83-7	181.3	3.3	0.6	No	Yes	Yes	Yes
91	2-Hydroxyethyl methacrylate	868-77-9	130.2	5.0	0.7	No	Yes	No	Yes
92	4-Methylbenzoic acid	99-94-5	136.2	8.8	1.2	No	No	No	Yes
93	Triphosphoric acid aluminium salt	13939-25-8	317.9	6.3	2.0	No	No	No	Yes
94	4,4′-Sulfonyldiphenol	80-09-1	250.3	1.6	0.4	Yes	Yes	Yes	Yes
96	1,2-Dicyanobenzene	91-15-6	128.1	2.5	0.3	Yes	Yes	Yes	Yes
97	2-(Diethylamino)ethyl methacrylate	105-16-8	185.3	3.2	0.6	No	Yes	Yes	Yes
98	Methacrylic acid, monoester with propane-1,2-diol	27813-02-1	144.2	5.0	0.7	No	Yes	No	Yes
99	(Methacryloyloxyethyl) trimethylammonium chloride	5039-78-1	207.7	10.0	2.1	No	No	No	No
101	2-Chlorophenol	95-57-8	128.6	2.0	0.3	Yes	Yes	Yes	Yes
103	1,4-Dibromobenzene	106-37-6	235.9	2.3	0.6	No	Yes	Yes	Yes
105	2-(Di-*n*-butylamino)ethanol	102-81-8	173.3	1.9	0.3	Yes	Yes	Yes	Yes
107	2,4-Dinitrophenol	51-28-5	184.1	6.5	1.2	No	No	No	Yes
108	2-Ethylbutyric acid	88-09-5	116.2	3.4	0.4	Yes	Yes	Yes	Yes
109	Ferrous sulfate heptahydrate	7782-63-0	278.0	1.8	0.5	Yes	Yes	Yes	Yes
113	*p*-Nitrophenol sodium salt	824-78-2	161.1	3.8	0.6	No	Yes	Yes	Yes
114	Sorbitan monooctadecanoate	1338-41-6	430.6	2.5	1.1	No	No	Yes	Yes
	Number of chemicals detected among the 25 chemicals					9	17	14	23

Table 8
Comparison of selection of top test concentration for chemicals with different molecular weight in 2 mM or 1 mg/mL, whichever is higher.

Molecular weight	Selection of 2 mM or 1 mg/mL (whichever is higher)		
100	2 mM (0.2 mg/mL)	<	1 mg/mL (10 mM)
300	2 mM (0.6 mg/mL)	<	1 mg/mL (3.3 mM)
500	2 mM (1 mg/mL)	=	1 mg/mL (2 mM)
800	2 mM (1.6 mg/mL)	>	1 mg/mL (1.3 mM)
1000	2 mM (2 mg/mL)	>	1 mg/mL (1 mM)

Underlines show concentration to be selected.

carcinogenicity information, so we determined the biologic relevancy of *in vitro* CA induction based on the weight of evidence approach. Results from *in vitro* CA test with CHL cells only might lead to biased conclusions. However, the strength of our study is the high reliability of the test results due to the fact that all data were generated according to national or international test guideline under GLP conditions. Therefore, our analysis would be helpful to discuss on top concentration issues. In this analysis, many "irrelevant" positives by extreme culture conditions (low pH, high toxicity, and precipitation) were also identified. Note that CHL cells are often described as among the most sensitive cells, *i.e.*, effects observed at lower concentrations as compared to the other cell lines. The recently suggested improvements in testing are important to reduce irrelevant positives, in addition to defining the top concentration. Data from *in vitro* mammalian genotoxicity tests, using the criteria defined by this paper, should be helpful in genotoxic hazard identification.

Conflict of interest

There are no conflicts of interest.

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Investigating the Relationship between in Vitro–in Vivo Genotoxicity: Derivation of Mechanistic QSAR Models for in Vivo Liver Genotoxicity and in Vivo Bone Marrow Micronucleus Formation Which Encompass Metabolism

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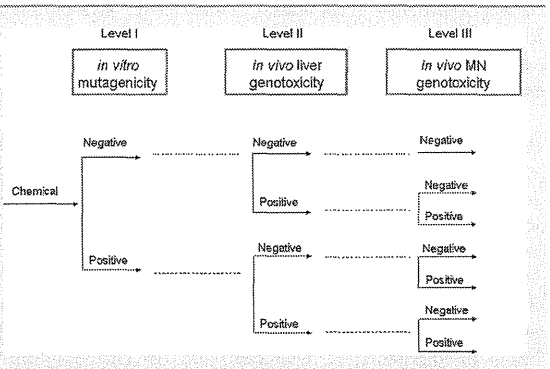
S *Supporting Information*

ABSTRACT: Strategic testing as part of an integrated testing strategy (ITS) to maximize information and avoid the use of animals where possible is fast becoming the norm with the advent of new legislation such as REACH. Genotoxicity is an area where regulatory testing is clearly defined as part of ITS schemes. Under REACH, the specific information requirements depend on the tonnage manufactured or imported. Two types of test systems exist to meet these information requirements, in vivo genotoxicity assays, which take into account the whole animal, and in vitro assays, which are conducted outside the living mammalian organism using microbial or mammalian cells under appropriate culturing conditions. Clearly, with these different broad experimental categories, results for a given chemical can often differ, which present challenges in the interpretation as well as in attempting to model the results in silico. This study attempted to compare the differences between in vitro and in vivo genotoxicity results, to rationalize these differences with plausible hypothesis in concert with available data. Two proof of concept (Q)SAR models were developed, one for in vivo genotoxicity effects in liver and a second for in vivo micronucleus formation in bone marrow. These "mechanistic models" will be of practical value in testing strategies, and both have been implemented into the TIMES software platform (http://oasis-lmc.org) to help predict the genotoxicity outcome of newly untested chemicals.



INTRODUCTION

Terms of Reference: Genotoxicity versus Mutagenicity. Carcinogenicity and mutagenicity are among the toxicological end points that pose the highest concern for human health and are subject to regulatory testing for hazard and risk assessment. Much of the data that are currently available in the public domain have thus been derived from tests conducted to investigate potentially harmful effects on genetic material, that is, genotoxicity or mutagenicity. Since both terms, mutagenicity and genotoxicity, will be referenced in this paper, working definitions are given. According to academic definitions, genetic alterations that are fixed and can be inherited are termed mutations. These include different types of events such as base substitutions and deletions, structural chromosomal aberrations (CAs) (break and rearrangements), and numerical CAs (loss or gain of chromosomes, i.e., aneuploidy). The assays established to evaluate these events are described in brief. Genotoxicity is considered as a broader term—aside from mutations, it also encompasses other alterations of genetic material that are not fixed and are not inherited, such as DNA damage. Genotoxicity may or may not be transformed into mutations by the cell's machinery during cell replication, and it may be an indication of potential carcinogenesis associated with the exposure to a chemical agent. Appropriate in vivo experimental test systems used to evaluate genotoxicity include the bone marrow in vivo micronucleus test (MNT) assay, the unscheduled DNA synthesis (UDS) assay, and the alkaline single-cell gel electrophoresis assay (Comet assay). These tests are relevant to assess DNA-damaging and DNA-repair processes in specific organs of investigation in the whole animal such as liver. Therefore, the term *liver genotoxicity* was regarded as appropriate for the purposes of this study, although, overall, a wide array of other

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events aside from mutations are encompassed in these test systems.

Current Quantitative Structure–Activity Relationship (QSAR) Approaches. The importance of assessing genotoxicity coupled with the availability of experimental data has prompted many in silico studies. James and Elisabeth Millers's "electrophilic theory" introduced a chemical concept to help rationalize the mode of action of genotoxic carcinogens.[1] This prompted many evaluations to derive so-called structural alerts (SA), simple yet effective means of encoding qualitative mechanistic understanding for predicting potential mutagenicity/carcinogenicity. Seminal efforts include SA for carcinogenicity by John Ashby,[2] who subsequently extended his list with additional SA.[3] Bailey et al. compiled a set of 33 SAs for regulatory use within the U.S. Food and Drug Administration (FDA), which was predominantly based on the Ashby alerts.[4] Kazius et al. evaluated a mutagenicity database comprising 4337 mutagens and nonmutagens taken from the Toxnet database (http:/toxnet.nlm.nih.gov/) and derived 29 SAs for mutagenicity with associated detoxification fragments.[5] Some of these alerts exist in software platforms to enable routine use; for example, 17 SAs for mutagenicity are implemented into the OASIS tissue metabolism simulator (TIMES) software.[6] Benigni et al. combined the published information from Ashby, Bailey et al., and Kazius et al. with additional information from the OncoLogic (U.S. EPA) software (http://www.epa.gov/oppt/sf/pubs/oncologic.htm)[7] to arrive at a list of 33 SA for carcinogens and mutagens.[8]

Current quantitative strategies include (Q)SARs and expert systems. Two types of (Q)SAR models, local and global, exist to estimate the mutagenic potential of chemicals. Local (Q)SARs provide estimated results for closely related (congeneric) chemical structures. Such models are most predictive, but only if the essential features of the model domains are clearly represented. Models based on physicochemical descriptors with clear mechanistic meaning are particularly helpful in rationalizing genotoxic outcome as exemplified by Chung et al.[9] Other local models are based on mathematical representations of chemical structure, for example, topological indices, and thus are more difficult to interpret.[10]

Global (Q)SARs aim to provide mutagenicity estimations for a diverse (noncongeneric) set of chemicals. Such (Q)SARs may be additionally encoded into expert systems. For example, TOPKAT empirically makes predictions for a range of different end points including Ames mutagenicity and rodent carcinogenicity.[11] Other expert systems such as TIMES attempt to provide clear mechanistic meaning through the use of SAs, which address the reactivity toward DNA and/or proteins.[12,13] TIMES also includes 3D QSARs to underpin some of the available SAs. All of the aforementioned (Q)SARs have typically been derived on Ames (*Salmonella* mutagenicity data). TIMES includes a platform for in vitro CA data in addition to that for Ames.[13] There is a paucity of models for in vivo genotoxicity, but as highlighted in the survey by Benigni et al., there is only one publically available model for in vivo micronucleus.[14] The scarcity of such models may be due in part to experimental data being less readily available but also due to the complexicity of how to rationalize and interpret the outputs from the different test systems.

Our own investigation aims to fill in the above in vitro–in vivo genotoxicity gap by considering both the available test systems and how they are currently applied to formulate an approach for modeling in vivo genotoxicity. For convenience, we considered the REACH ITS[15] for mutagenicity since this described the typical assays used and how their outcomes should be interpreted for subsequent decision making. The actual experimental test systems are assumed to be reasonably familiar and are only briefly described in the next section.

Experimental Assays and Data for Rodent Mutagenicity and Genotoxicity. Integrated testing strategies, notably those described in the REACH Technical guidance,[15] outline the in vitro and in vivo systems that are most frequently used to evaluate the mutagenic potential of chemical substances. The in vitro systems include the bacterial reverse mutation test (Ames), an in vitro mammalian cell gene mutation test [such as the mouse lymphoma or hypoxanthine–guanine phosphoribosyltransferase (*hprt*) assay], the in vitro mammalian chromosome aberration (CA) test, and the in vitro MNT.[15] The Ames test uses amino acid-requiring strains of bacteria to detect (reverse) gene mutations (point and frameshift mutations). The in vitro mouse lymphoma assay (MLA), when correctly performed, detects structural chromosome aberrations, aneuploidy, and recombination events (e.g., such as gene conversion) that result in loss of heterozygosity. The *hprt* test identifies chemicals that induce gene mutations in the *hprt* gene of established cell lines. The in vitro mammalian CA test detects structural chromosome aberrations and increases in polyploidy. The in vitro MNT has the potential to detect both clastogenic (chromosome aberrations) and aneugenic (chromosome lagging due to dysfunction of mitotic apparatus) chemicals.

The scheme under REACH can be summarized as follows. As a first tier, three in vitro tests are recommended, which includes an Ames test, a mouse micronucleus/CA, and a mouse lymphoma/HRPT assay. If the results from all three tests are negative, then no more testing is merited, and a conclusion of nongenotoxicity can be made for the substance under study. If one or more tests are positive, then in vivo testing may be instigated. Obviously metabolism, pharmacokinetics, and toxicokinetics factors [absorption, distribution, metabolism, excretion (ADME)] are all inherent features in the in vivo genotoxicity tests, although the genetic end points for the tests address different genetic mechanisms. The UDS in vivo assay is used to evaluate the role of DNA repair. The in vivo Comet assay is a sensitive technique for the detection of DNA strand breaks; thus, it can be used for measuring DNA strand breaks in any tissue of an animal. Site-specific effects at contact tissues or the target tissue where the test compound accumulates or induces toxicity can be readily assessed. The specificity of the contact tissue under investigation is also feasible for the transgenic rodent gene mutation test (TGR), which measures gene mutations in vivo. However, the in vivo MNT is probably the most widely used test.[16] When performed appropriately, it detects both clastogenicity and aneugenicity.[17] The frequency of micronucleated polychromatic erythrocytes is traditionally determined from bone marrow samples, but with the emerging automated scoring methods, the emphasis is moving to assessing the induction of micronuclei in immature erythrocytes in peripheral blood samples.[18]

Most of the established in vitro mutagenicity tests, which are used for regulatory purposes, exhibit relatively high sensitivity for detection of genotoxic carcinogens.[19] However, particularly those based on cultured mammalian cells are thought to produce a remarkably high occurrence of irrelevant positive results (i.e., exhibit low specificity), when compared with rodent carcinogenicity.[19,20] To increase the specificity of predictions, regulators tend to interpret in vitro positive results in an in vivo perspective, that is, in vivo confirmation of in vitro mutagens.

In addition, in vivo tests can also be utilized to identify chemicals producing in vivo only positive results (i.e., chemicals for which mutagenicity is not or poorly detected in vitro). Only a very limited number of chemicals have been found to be genotoxic in vivo and not in the standard in vitro tests. Most of these are pharmaceuticals such as atovaquone (95233-18-4), which is designed to affect pathways of cellular regulation, including cell cycle regulation. One of the most preferred in vivo assays, complementing genotoxicity test batteries, is the in vivo bone marrow MNT. The preference of this assay is attributed to both its wide mutagenicity range assessment (clastogenicity and aneugenicity) and its remarkably high specificity in concordance with the genotoxic carcinogenicity model, although it shows low sensitivity.[14,21] Therefore, it may be appropriate to include a second in vivo test if a positive in vitro result has not been adequately confirmed by the in vivo bone marrow MNT test. The UDS test is one complement to the bone marrow MNT since it is a surrogate in vivo gene mutation assay[21] measuring DNA excision repair of induced DNA damage. The utility of the Comet and the TGR assays to detect genotoxic damage in specific tissues, specifically DNA strand breaks and gene mutations has also been recognized.[15] Thus, an evaluation of in vivo genotoxicity potential could involve integrating outcomes from MNT and either UDS, Comet, and TGR tests depending on the outcomes that have been observed in vitro. UDS, Comet, and TGR can also be undertaken to address in vivo liver genotoxicity. Such tissue-specific assays are useful in in vivo follow-up tests especially since the liver is an organ of high metabolic capacity and therefore is frequently subjected to significant toxic overload.

Aims of the Study. Bearing in mind the way in which these different assays are integrated together, our goal was to investigate the in vitro and in vivo relationship, the so-termed in vitro–in vivo "gap" to inform the development of mechanistic (Q)SAR model(s). A large body of data covering in vitro mutagenicity, in vivo (liver) genotoxicity, and in vivo bone marrow MNT test results was collected for the same set of substances. The scope of the investigation can be summarized in the following three questions: (a) To what extent are in vitro mutagenic chemicals in vivo (liver) genotoxic, that is, what in vivo detoxification pathways exist? (b) To what extent are in vivo (liver) genotoxic chemicals in vivo bone marrow MNT positive? (c) Are there in vitro nonmutagenic chemicals that are in vivo liver or bone marrow genotoxic; that is, what in vivo bioactivation pathways exist? These questions were structured into a workflow (Figure 1) and enabled a stepwise evaluation of the in vitro–in vivo gap.

MATERIALS AND METHODS

Compilation of Data Set. Our training set comprised 557 chemicals ("557 list") with in vivo MNT data (Appendix I of the Supporting Information lists the substances and their overall calls). In vitro mutagenicity and in vivo (liver) data were collected for the same set of substances to the extent possible. This helped maximize the overlap between chemicals with various genotoxicity effects and the in vivo MNT data set. Documented in vitro mutagenicity data from multiple literature sources were identified for 397 noncongeneric chemicals within the training set (Appendix II of the Supporting Information). Positive calls were categorized by the digit 1, negative calls by 0, and N/A signified "no data available", based on the literature searches that were performed. Our in vitro data comprised that from the Ames assay, the CA assay, and the MLA, since these are the typical assays considered under REACH. Out of necessity and as typically the case for modeling efforts, reported study results were accepted as

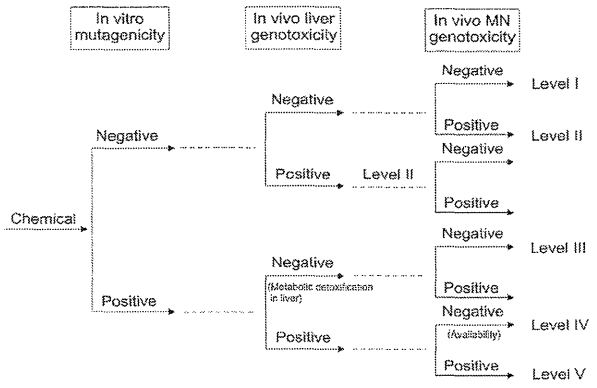


Figure 1. Workflow outlining the in vivo–in vitro gap.

reported, although an extensive effort was made in expert judgment and evaluation of the data quality and correctness of the calls.

Ames results with the rat liver S9 metabolic activation system were available for 283 noncongeneric chemicals. Of these chemicals, 109 (38%) were associated with positive calls and 174 (62%) with negative calls. Documented in vitro CA test data were identified for 296 chemicals, of which 186 (63%) were positive and 110 (37%) were considered negative. Data from 194 chemicals had been assessed in the in vitro MLA. The majority of the chemicals tested positive (148 chemicals, i.e., 76%) and 46 chemicals (24%) tested negative. For the 397 in vitro mutagenicity data, these comprised 267 positive calls (68%) and 124 negative calls (32%), and six calls were inconclusive. These substances were ethylene dichloride (107-06-2), sulfan blue (129-17-9), thiabendazole (148-79-8), methyl parathion (298-00-0), dibutylnitrosamine (924-16-3), C.I. direct black 38 (1937-37-7). In these six cases, only Ames and in vitro CA test outcomes were available with positive calls in Ames and negative calls in in vitro CA tests.

Results from in vivo Comet, UDS, and TGR assays were also collected to help evaluate in vivo liver genotoxic potential. Data were available for 185 diverse chemicals, which are listed in Appendix III of the Supporting Information. The Comet assay provided liver genotoxicity assignments for 127 (69%) of the 185 chemicals. Of the 127 chemicals, 78 (61%) were positive, and 49 (39%) were negative. The TGR comprised rodent liver genotoxicity data for 34 (18%) of the 185 chemicals; 27 (80%) of these were reported as positive, and 7 (20%) were negative. The in vivo UDS assay was associated with the least amount of liver genotoxicity data, only 24 (13%) of the 185 chemicals had overall calls, and five of them were observed to be positive in this assay (21%), and 19 were (79%) negative in this assay. Overall, of the 185 substances with liver assignments, 109 were associated with positive calls (59%) and 76 with negative calls (41%). The "557 list" included almost equal numbers of positive (267 chemicals, i.e., 48%) and negative (290 chemicals, i.e., 52%) MNT assignations performed in either bone marrow or peripheral blood. Figure 2 summarizes the distribution of assignments in each of the test systems.

The evaluation of this investigation was often hampered by conflicting in vivo MNT data available in the public domain. The compromised quality of these MNT data was attributed to the fact that many chemicals had been evaluated in the early 1980s; when species (rat vs mouse) and gender (male vs female) differences may not always have been considered, etc. To date, the validity of the in vivo MNT data has only been verified for chemicals where the in vitro mutagenicity outcome appeared to be negative, relative to the in vivo case (in either liver or bone marrow), where the genotoxicity result was positive. Expert judgment was relied upon to consider whether there were factors resulting in inconsistent in vitro results as compared with the in vivo situation, for example, rodent species differences, nonphysiological culture conditions, etc.

To illustrate the structural diversity of the training set, the 557 list was profiled against the set of DNA and protein binding alerts available within the OECD Toolbox v2.1. The distribution chart is

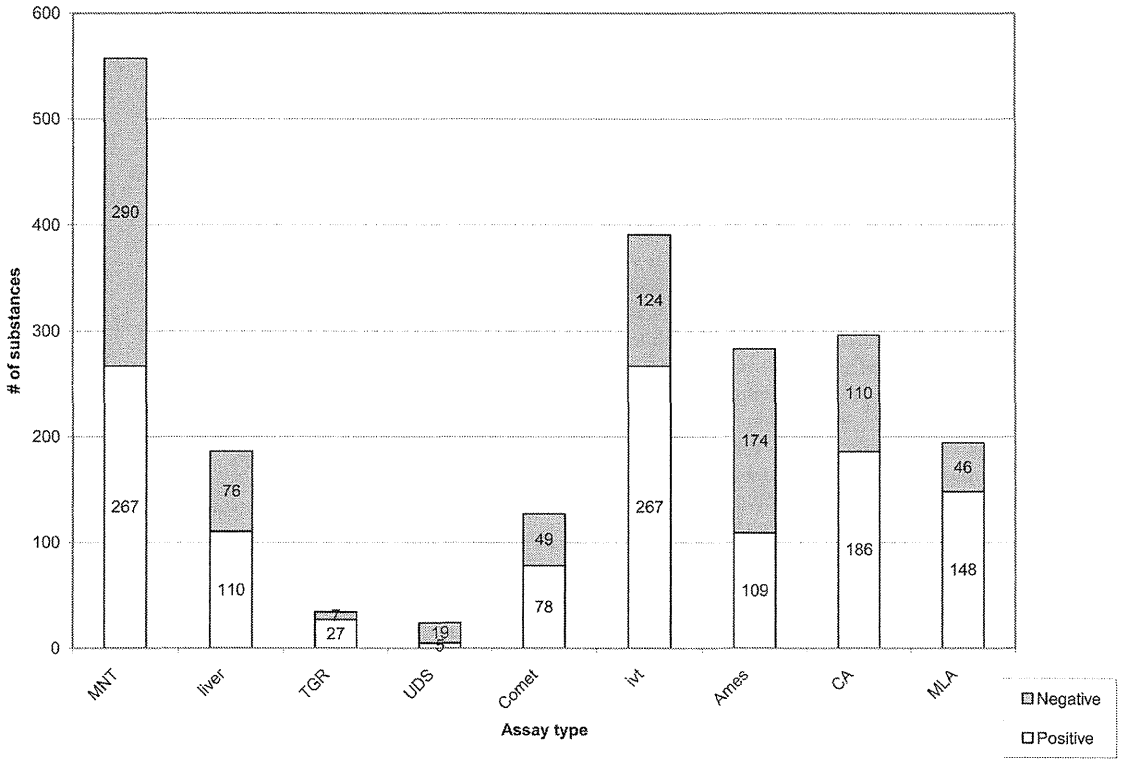


Figure 2. Distribution of the overall calls for each of the test assays under study.

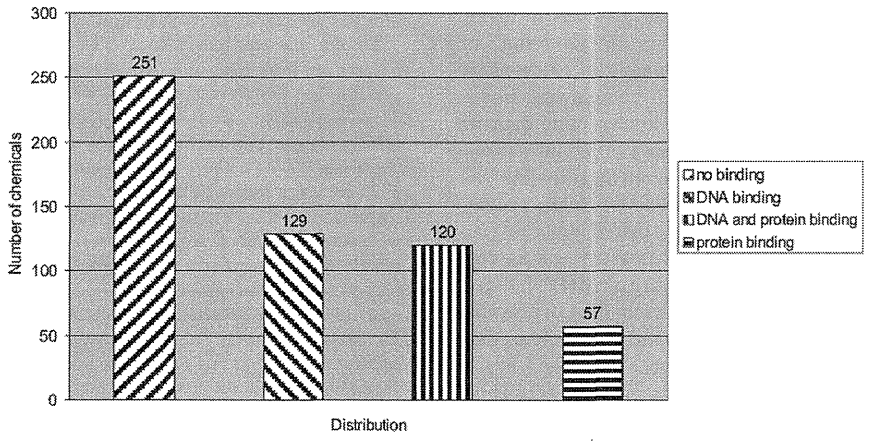


Figure 3. Distribution of training set chemicals across DNA and protein binding alerts.

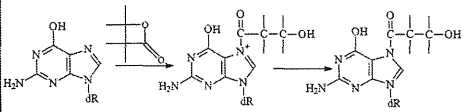
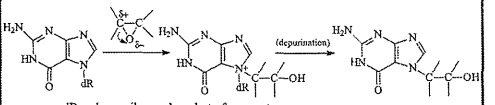
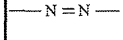
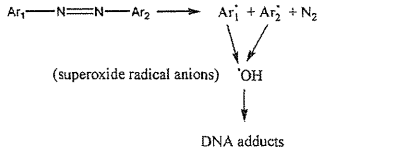
shown in Figure 3. The results reveal that 251 (45%) of the 557 chemicals possess no DNA and/or protein binding alerts. One hundred twenty-nine of the remaining 306 (55%) chemicals have one or more DNA binding alerts, 57 chemicals have a protein binding alert, and 120 chemicals have both DNA and protein binding alerts. This distribution shows a broad spread of chemical mechanisms as depicted by the SAs triggered.

Our modeling approach sought to use the existing TIMES formalism and refine the components that had been originally developed to estimate Ames and in vitro CA. Here, we provide a brief overview of these components.

Modeling Reactivity to DNA and Proteins. According to the working hypothesis, interaction of chemicals with DNA and/or with specific proteins (such as histone, topoisomerase, spindle protein tubulus, and DNA repair enzymes) encompasses a diversity of genotoxic events, which can damage mammalian cells. For example, the formation of micronuclei arises as a result of the covalent interaction between chemicals with DNA and/or specific proteins. Accordingly, a reactivity component for an in vivo model, which predicts genotoxic effects such as formation of micronuclei or liver damages, should be based on the assessment of the potential of that chemical to interact with DNA and/or proteins.

TIMES models predicting the outcomes in Ames and the CA test have previously been published.[12,13] It has been established that the Ames test primarily accounts for the direct interaction of chemicals with DNA, whereas the in vitro CA test assesses both DNA and protein (e.g., histone, topoisomerase, spindle protein tubulus, and DNA repair enzymes) binding. This implies that Ames mutagenic chemicals should be CA positive, but the converse is not necessarily true. A recent comparative analysis of in vitro mutagenic data for a large

Table 1. Alerting Groups and Descriptors Used in COREPA Models for Estimating Their Reactivity Associated with Supporting Mechanistic Information[a]

#	Alerting group	Chemical class	Descriptors in the COREPA model*	Interaction mechanism	Reference
1		Lactones	-	 dR – deoxyribose phosphate fragment Ring opening S_N2 reaction	(77)
2		Epoxides	*MW* E_{HOMO}	 dR – deoxyribose phosphate fragment Ring opening S_N2 reaction	(78)
3		Azo compounds	log K_{OW} Van der Waals surface	 (superoxide radical anions) $^{\cdot}OH$ DNA adducts Radical mechanism by reactive oxygen species (ROS) formation	(78)

[a]*E_{HOMO}, the energy of the highest occupied molecular orbital (eV); *MW*, molecular weight (Da); log K_{OW}, octanol–water partitioning coefficient (mol L_0^{-1} $mol^{-1}L_w$); and van der Waals surface area (Å^2).

number of chemicals confirmed this assumption. Eighty percent of chemicals that elicited bacterial mutagenicity (based on Ames test results) also induced CA, whereas only 60% of chemicals that induced CA were found to be active in the Ames test.[22,23] To distinguish these two mechanisms, the reactivity component of the newly derived models for MNT and liver genotoxicity was structured into two parts. The first part accounted for the interaction of chemicals with DNA. More than 60 alerting groups (being considered as a part of a future publication) were used to simulate covalent interaction with DNA. The use of each alert had been justified by the mechanistic interpretation of that interaction. Some alerts were additionally underpinned by mechanistically based COmmon REactivity PAttern (COREPA) 3D QSAR models.[24,25] Examples of these DNA binding alerts are presented in Table 1. The SAs are described together with physicochemical property/molecular parameter exclusion/inclusion rules. Supporting reaction mechanism information is also provided.

As seen from Table 1, the SAs can be categorized into two types: (1) those eliciting mutagenicity without the need for modulating factors (#1 in Table 1) and (2) those for which specific molecular parameter(s) define the degree of activation (#2 and #3 in Table 1).

The second part of the reactivity component accounts for the interaction of chemicals with specific proteins. More than 50 SAs were proposed that were associated with protein interaction (http://www.oasis-lmc.org/). Examples of protein binding alerts associated with parameters for reactivity and their supporting reaction mechanism information are presented in Table 2. These are characterized similarly—either requiring modulating factors (#1, #2, and #3 in Table 2) or not (#4 in Table 2).

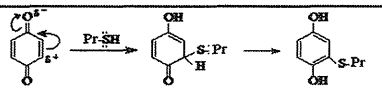
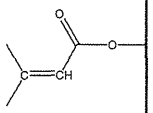
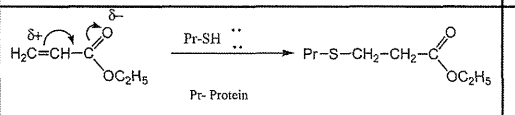
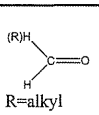
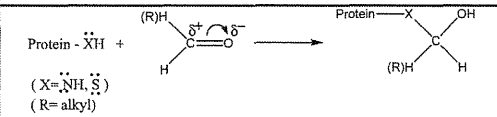
Most of the DNA binding alerts are also able to bind proteins. An example to demonstrate the mechanism by which a DNA binding alert interacts with proteins is presented for quinones in Figure 4.

Quinones are well-known mutagens, and they are included in the list of DNA-causing alerts. Topoisomerases are enzymes that participate in all stages of replication, functional activity, and structural maintenance of DNA. The inhibition of these enzymes by quinones is considered to elicit CA26. This is an example of how the same alert can elicit different outcomes depending on the interaction target. The structure of the reactivity component used in the in vivo genotoxicity models is provided in Figure 5.

A new chemical is first submitted to the reactivity component that encompasses the alerts associated with DNA interactions. A positive prediction for mutagenicity is assigned if the requirements for interaction with DNA are met, indicating that the ultimate mutagenic effect is due to this interaction mechanism. Regardless of whether the chemical meets the requirements for direct interaction with DNA, it is then forwarded to the second part of the reactivity component, which investigates the ability of the chemical to interact with proteins. This is to flag those cases where mutagenicity may arise by both mechanisms (direct interaction with DNA and interaction with protein) simultaneously. If the chemical passes through both parts of the reactivity component without being flagged for activity, a prediction of "unable to produce mutagenicity" is noted.

Conformational Analysis by Genetic Algorithm. To derive 3D QSARs, the flexibility of chemicals needs to be taken into account since this will give rise to the formation of many different conformers, and their reactivity profiles would accordingly differ. Common practice is to calculate molecular parameters for the lowest energy conformation, even though this necessarily may not be the form that drives the response and therefore not the most relevant one to study.[27] Given a systematic conformational analysis search would be computationally intensive (since the number of conformers would increase exponentially with the number of degrees of freedom), LMC derived a procedure to address the issue of conformation space using a genetic algorithm, which minimizes 3D similarity among generated conformers.[28] This made addressing the conformation space practical, even for large and very flexible chemicals. A procedure was also developed to saturate the conformation space, that is, to ensure consistency in the reproducibility of generated conformers and their distribution in the structural space.[28] This allowed the conformational space of chemicals to be populated with an optimal number of conformers.

Table 2. Alerting Groups for Protein Binding, Parameters for Reactivity, and Supporting Interaction Mechanisms[a]

#	Alerting group	Chemical class	Descriptors* in the model.	Interaction mechanism	Reference
1		Quinones	*MW*		(79)
3		Acrylates	log K_{OW}	 Pr- Protein Michael addition	(80)
4	 R=alkyl	Aldehydes	-	 (X=NH, S) (R= alkyl) Schiff base formation	(81)

[a] *MW*, molecular weight (Da); logK_{OW}, octanol–water partitioning coefficient (mol L_0^{-1} mol$^{-1}L_w$).

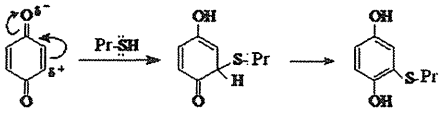


Figure 4. Interaction mechanism of quinones with proteins (Pr).

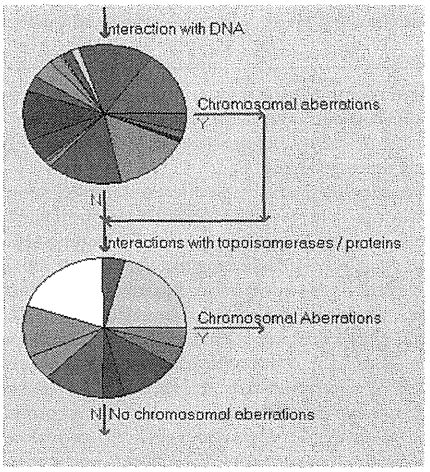


Figure 5. Structure of the reactivity component of the in vivo genotoxicity models.

TIMES. The TIMES platform comprises SA, 3D QSARs, and a metabolism simulator. This simulator comprises a list of hierarchically ordered transformations and a substructure matching engine for their implementation. The modeling is based on a probabilistic approach[29] whereby a hierarchy of transformations is defined by the probabilities of transformations determined in such a way as to reproduce a database of documented metabolic transformations or data for their rate of disappearance. The transformation probabilities are related to the feasibility of occurrence of various metabolic reactions. It is assumed that the transformations are independent and performed sequentially. Each molecular transformation consists of parent submolecular fragments, transformation products, and inhibiting masks. The latter play the role of reaction inhibitors. If a functional group assigned as a mask is attached to the target fragment, the execution of the transformation on the parent chemical is prevented. The presence of groups that can promote or inhibit metabolic reactions significantly increases the number of principal transformations. Currently, 343 principal transformations are used to model rat liver metabolism in vitro. The simulator starts by matching the parent molecule with the reaction fragment associated with the transformation having highest probability of occurrence. When a match is identified, the molecule is metabolized, and transformation products are treated as parent molecules for the next degradation step. The procedure is repeated for the newly formed chemicals until the product of probabilities of consecutively performed transformations reaches a user-defined threshold. The mathematical formalism defining the amount of metabolite, formation, and metabolism probabilities is described elsewhere.[6,29–31] The intent with this study was to refine the existing structure–activity and structure–metabolism rules within TIMES to account for the differences observed between the in vitro and the in vivo results. Where a realistic and feasible hypothesis could be generated and substantiated with data, these would inform the refinement of existing rules or introduction of new transformation rules.

RESULTS AND DISCUSSION

Workflow for Genotoxicity at Different Levels of Biological Organization. While the full set of data comprised 557 chemicals, a set of data where results from all assays were available were required to develop the mechanistic (Q)SAR models. Overall, calls for in vitro, liver genotoxicity, and in vivo MNT were available for 162 chemicals. Table 3 shows the list of 162 chemicals. A hierarchical workflow (Figure 6) outlines the results.

The first tier of in vitro tests comprises 162 chemicals that were either positive or negative in Ames, CA, and MLA. Four chemicals were assigned as inconclusive since Ames and CA data were found to be conflicting. All four were Ames positive but CA negative. The four chemicals were ethylene dichloride (107-06-2), thiabendazole (148-79-8), dibutylnitrosamine (924-16-3), and C.I. direct black 38 (1937-37-7). These were excluded from further study. Thirty-two (20%) of the 158 chemicals remaining were found to be in vitro negative, and 126 (80%) were found to elicit in vitro positive responses. Substances were categorized as negative if two or more results were negative and positive if they were positive in at least one of the three tests.

The 32 (20%) nonmutagenic chemicals in vitro were investigated in both liver and MNT in vivo tests. Thirty of the 32 in vitro nonmutagenic chemicals were confirmed negative in vivo

Table 3. List of the 162 Chemicals and Their Summary Calls Both in Vitro and in Vivo Test Systems

CAS	name	ivt	liver	MNT
50-06-6	phenobarbital	1	1	1
50-32-8	benzo(*a*)pyrene	1	1	1
50-55-5	reserpine	0	0	0
51-03-6	piperonyl butoxide	1	0	0
51-79-6	urethane	1	1	1
52-24-4	thio-TEPA	1	1	1
56-04-2	methylthiouracil	0	0	0
56-23-5	carbon tetrachloride	0	0	0
56-57-5	4-nitroquinoline 1-oxide	1	1	1
56-75-7	chloramphenicol	0	0	0
57-14-7	dimazine	1	1	1
57-22-7	vincristine	1	0	1
57-30-7	phenobarbital, sodium	0	0	0
57-50-1	sucrose	0	0	0
57-57-8	propiolactone	1	1	0
57-97-6	7,12-dimethylbenz(A)anthracene	1	1	1
58-08-2	caffeine	1	0	0
58-89-9	lindane	0	0	0
59-05-2	methotrexate	1	1	1
59-89	*N*-nitrosomorpholine	1	1	1
60-09-2-3	*p*-aminoazobenzene	1	1	1
60-11-7	4-dimethylaminoazobenzene	1	1	1
60-35-5	acetamide	0	0	0
60-57-1	dieldrin	1	1	1
62-44-2	acetophenetidin	1	0	1
62-53-3	aniline	1	1	1
62-55-5	thioacetamide	1	0	1
64-86-8	colchicine	1	0	1
66-27-3	methyl methanesulfonate	1	1	1
67-20-9	nitrofurantion	1	1	0
67-66-3	chloroform	1	0	0
67-68-5	dimethyl sulfoxide	0	0	0
68-12-2	dimethylformamide	0	0	0
70-25-7	*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine	1	1	1
71-43-2	benzene	1	1	1
75-07-0	acetaldehyde	1	1	1
75-09-2	methylene chloride	1	1	0
75-25-2	bromoform	1	0	0
75-56-9	propylene oxide	1	0	1
79-06-1	acrylamide	1	1	1
79-34-5	1,1,2,2-tetrachloroethane	1	1	1
81-07-2	saccharin	0	0	0
84-16-2	hexestrol	1	0	0
89-65-6	erythorbic acid	0	0	0
90-43-7	2-phenylphenol	1	1	0
91-20-3	naphthalene	1	0	0
91-59-8	2-naphthalenamine	1	1	1
91-64-5	coumarin	1	0	0
91-94-1	3,3′-dichlorobenzidine	1	1	1
92-52-4	biphenyl	1	1	0
92-67-1	4-biphenylamine	1	1	1
92-87-5	benzidine	1	1	1
95-50-1	1,2-dichlorobenzene	1	0	0
95-53-4	*o*-toluidine	1	1	0
95-80-7	2,4-diaminotoluene	1	1	0
95-83-0	4-chloro-1,2-diaminobenzene	1	1	1
96-09-3	styrene oxide	1	1	0
96-12-8	1,2-dibromo-3-chloropropane	1	1	1
96-45-7	ethylenethiourea	1	1	0
97-53-0	eugenol	1	0	0
97-56-3	*o*-aminoazotoluene	1	1	0
99-56-9	1,2-diamino-4-nitrobenzene	1	0	0
100-41-4	ethylbenzene	1	0	0
100-42-5	styrene	1	1	0
100-51-6	benzyl alcohol	1	0	0
100-75-4	1-nitrosopiperidine	1	1	0
101-14-4	4,4′-methylenebis(2-chlorobenzenamine)	1	1	1
101-77-9	4,4′-methylenebis(aniline)	1	1	1
103-33-3	aminoazobenzene	1	1	1
103-90-2	acetaminophen	1	1	1
104-55-2	cinnamaldehyde	1	0	0
105-11-3	*p*-quinone dioxime	1	0	0
105-60-2	hexahydro-2 h-azepin-2-one	0	0	0
106-46-7	1,4-dichlorobenzene	0	1	1
106-93-4	ethylene dibromide	1	1	0
106-99-0	butadiene	1	0	0
107-06-2	ethylene dichloride	no conclusion	1	0
107-13-1	acrylonitrile	1	0	0
108-88-3	toluene	0	0	0
108-95-2	phenol	1	1	0
110-00-9	furan	1	1	0
110-44-1	sorbic acid	0	0	0
110-86-1	pyridine	0	0	0
117-39-5	quercetin	1	0	0
117-81-7	bis(2-ethylhexyl)phthalate	0	0	0
118-96-7	2,4,6-trinitrotoluene	1	0	0
119-53-9	benzoin	1	0	0
119-93-7	tolidine	1	1	1
120-47-8	ethylparaben	1	0	0
120-71-8	*p*-cresidine	1	0	0
121-79-9	propyl gallate	1	0	0
123-91-1	1,4-dioxane	0	0	0
124-48-1	chlorodibromomethane	1	1	0
126-72-7	tris(2,3-dibromopropyl) Phosphate	1	1	1
128-37-0	butylated hydroxytoluene	1	0	0
128-44-9	saccharin, sodium	0	0	0
134-32-7	1-naphthylamine	1	1	1
136-40-3	phenazopyridine hydrochloride [USAN]	1	1	1
139-13-9	triglycollamic acid	1	1	0
140-11-4	benzyl acetate	0	0	0
140-88-5	ethyl acrylate	1	1	0
142-04-1	aniline HCl	1	1	1
147-94-4	cytosine arabinoside	1	0	1
148-79-8	thiabendazole	no conclusion	1	1
148-82-3	melphalan	1	1	1
301-04-2	lead acetate	1	0	0
305-03-3	chlorambucil	1	1	1
309-00-2	aldrin	1	0	0
366-70-1	procarbazine hydrochloride	1	1	1
427-51-0	cyproterone acetate	0	1	0
446-86-6	azathioprine	1	1	1
492-80-8	auramine	1	1	0
501-30-4	kojic acid	1	0	0
532-32-1	sodium benzoate	1	0	0
542-75-6	1,3-dichloropropene [BSI:ISO]	1	1	0
602-87-9	5-nitroacenaphthene	1	1	1
604-75-1	oxazepam	1	1	0
609-20-1	2,6-dichloro-*para*-phenylenediamine	1	1	1

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Table 3. continued

CAS	name	ivt	liver	MNT
621-64-7	*N*-nitroso(di-*n*-propyl)amine	1	1	0
624-18-0	*p*-phenylenediamine·2HCl	1	0	0
637-07-0	clofibrate	1	0	0
684-93-5	methylnitrosourea	1	1	1
759-73-9	*N*-ethyl-*N*-nitrosourea	1	1	1
816-57-9	propylnitrosourea	1	1	1
842-07-9	1-phenylazo-2-naphthol	1	1	1
924-16-3	dibutylnitrosamine	no conclusion	1	0
930-55-2	1-nitrosopyrrolidine	1	1	0
1116-54-7	2,2′-(nitrosoimino)bisethanol	1	1	0
1120-71-4	1,3-propane sultone	1	1	1
1162-65-8	aflatoxin B1	1	1	1
1634-04-4	methyl *tert*-butyl ether	1	0	0
1746-01-6	tetrachlorodibenzodioxin	0	0	0
1937-37-7	C.I. direct black 38	no conclusion	1	1
2353-45-9	fast green FCF	0	0	0
2611-82-7	new coccine	0	0	0
2650-18-2	C.I. acid blue 9	1	1	0
2783-94-0	FD&C yellow	1	0	0
2784-94-3	HC blue no. 1	1	0	0
2835-95-2	5-amino-*o*-cresol	1	1	0
2921-88-2	chlorpyrifos	1	1	0
3564-09-8	Ponceau 3R	1	1	0
3688-53-7	furylfuramide	1	1	1
4418-26-2	sodium dehydroacetate	1	0	1
5064-31-3	nitrilotriacetic acid, trisodium salt	0	0	0
5307-14-2	2-nitro-4-phenylenediamine	1	1	0
6369-59-1	2,5-diaminotoluene sulfate	1	0	0
6441-77-6	phloxine	0	0	0
6923-22-4	monocrotophos	1	1	1
10595-95-6	*N*-nitrosomethylethylamine	1	1	0
11121-48-5	rose bengal	0	0	0
13552-44-8	4,4′-methylenedianiline 2HCl	1	1	1
15972-60-8	alachlor	1	1	1
16423-68-0	C.I. acid red 51	1	1	0
18883-66-4	streptozotocin	1	1	1
20830-81-3	daunamycin	1	1	1
33229-34-4	HC blue no. 2 [AKA ethanol, 2,2′((4-(2-hydroxyethylamino)-3-nitrophenyl)imino)di-]	0	0	0
33419-42-0	etoposide	1	1	1
62450-07-1	1-methyl-5*H*-pyrido[4,3-*b*]indol-3-amine	1	1	1
67774-32-7	polybrominated biphenyl mixture	0	0	0
77439-76-0	3-chloro-4-dichloromethyl-5-hydroxy-2-furanone	1	1	0
93957-54-1	fluvastatin	0	0	0
93957-55-2	fluvastatin sodium	0	0	0

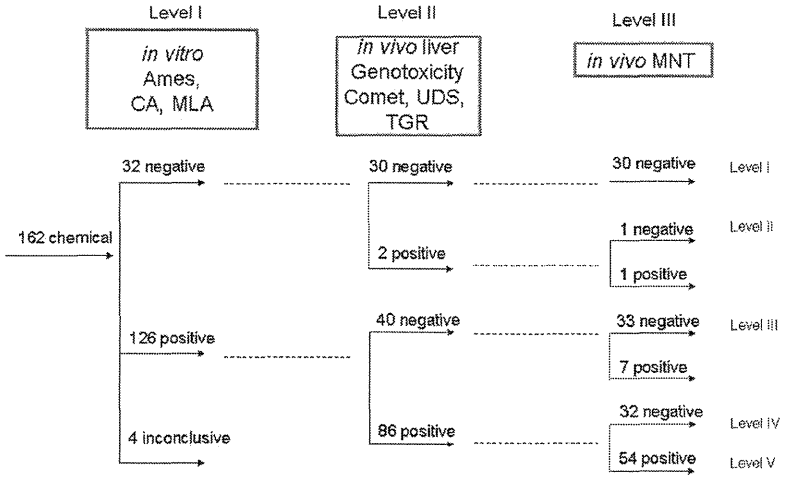


Figure 6. Workflow for the 162 chemicals with results in all test systems.

in liver and in the MNT. The two in vitro nonmutagens, 1,4-dichlorobenzene (104-46-7) and cyprotenone acetate (427-51-0), were found to be in vivo liver positive. Only 1,4-dichlorobenzene was found to be positive in the MNT.

A similar comparison was made for the 126 in vitro mutagens. Of these, 40 (32%) in vitro mutagenic chemicals were observed to be in vivo liver nongenotoxic. This suggested that in vitro mutagenicity was not necessarily a predictor of positive in vivo liver effect. The remaining 86 (68%) of the 126 in vitro mutagenic chemicals produced in vivo liver positive effects. Fifty-four (63%) of these 86 chemicals appeared to confirm this response by a positive genotoxic outcome in bone marrow. In contrast, the other 32 of these 86 chemicals (37%) were negative in bone marrow. These chemicals might conceivably have been "exhausted" en route from the liver to bone marrow. Forty liver nongenotoxic chemicals were also investigated. Thirty-three (83%) of these 40 chemicals confirmed the negative response observed in liver with a negative outcome in the MNT. The other seven chemicals (17%) were positive in the MNT. These data were reviewed in more detail to put forward plausible hypothesis to rationalize the inconsistent results.

In Vitro Nonmutagenic, In Vivo Genotoxic Cases. The in vitro nonmutagenic but in vivo genotoxic chemicals were critically evaluated. Several factors that could result in irrelevant in vitro–in vivo assignments were considered. For instance, an in vitro negative response could be due to shortcomings in the way that the experiments were performed, for example, limited solubility of the chemicals, elevated (or low) incubation temperatures, etc. Similarly, an in vivo positive response could be due to in vivo-specific experimental factors such as higher

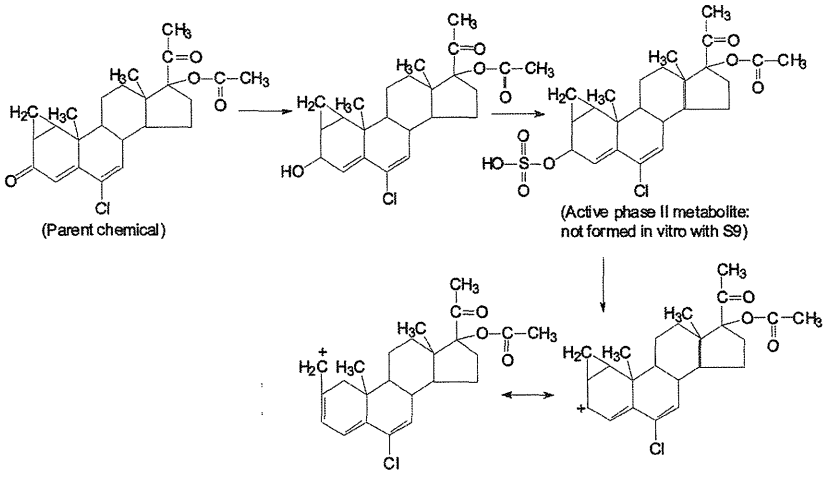


Figure 7. Mechanism of cyproterone acetate bioactivation in the liver.

exposure concentrations in vivo than in vitro, route of exposure, extrahepatic activation (e.g., in kidney, gallbladder), etc. In addition to factors driven by experimental design and/or conduct, rodent species differences when comparing data from in vitro and in vivo systems could also be a consideration.

Tweats et al.[32] have investigated the impact of differences between in vitro and in vivo metabolic activation and enzyme expression for urethane. Enzyme differences between both systems have also been found to be responsible for the in vivo bioactivation of procarbazine,[33] hydroquinone, and benzene.[34] The in vitro assignation of these and other small hydrophobic compounds strongly depend on the type of P450 isoenzymes expressed. Ghanayem et al.[35] showed that P450 2E1 (CYP 2E1) is involved in the in vitro oxidative activation of acrylamide, urethane, benzene, acrylonitrile, vinyl chloride, styrene, 1-bromopropane, trichloroethylene, dichloroethylene, acetaminophen, and butadiene. In the presence of other P450s, some of these chemicals would be negative for mutagenicity. Therefore, aside from the incubation conditions, the general artificiality of the in vitro systems should also be considered when comparing in vitro and in vivo studies.

As noted already and reflected in Figure 6, only 1,4-dichlorobenzene (104-46-7) and cyproterone acetate (427-51-0) belonged to the category of chemicals that were in vitro negative but in vivo liver positive. 1,4-Dichlorobenzene was additionally found to be positive in the MNT. This MNT result was that from Mohtashamipur et al.[36] Subsequent searching in the literature identified two other studies that by Morita et al.[37] and one reported by the NTP.[38] Neither demonstrated any micronuclei formation in mouse bone marrow. Moreover, Tegethoff[39] who attempted to recreate the conditions of Mohtashamipur et al.[36] failed to reproduce the study. The potential of 1,4-dichlorobenzene to elicit in vivo liver damage was also investigated. A positive result in the Comet assay was reported in mice, whereas a negative result was reported in mice in the UDS test.[40] Thus, on a weight of evidence basis, it is more likely that 1,4-dichlorobenzene is not genotoxic in liver and bone marrow and hence presumably not bioactivated.

Cyproterone acetate (427-51-0) has been found to be negative in vitro but does cause genotoxicity in liver in vivo. Aside from metabolic detoxification, phase II metabolic sulfation catalyzed by sulfotransferase enzymes play a significant role in rat in vivo metabolic bioactivation pathway of cyproterone acetate.[41] The authors suggested that the reactive species formed from cyproterone acetate are short-lived and genotoxic when formed within the target cells only. However, the external metabolic activation in vitro did not include phase II sulfation, due to the lack of detoxification cofactors in artificial S9 systems. Even if reactive sulfoconjugates were to be formed externally, mutations may not necessarily be induced in the indicator cells, since sulfoconjugates could be short-lived and rather hydrophilic; that is, they would not be able to cross the membrane of these target cells. Thus, the nonmutagenicity of cyproterone acetate in even the most relevant in vitro test systems in the presence of S9[42] can be attributed to artificiality of the latter. The bioactivation of cyproterone acetate in the liver is outlined in the scheme in Figure 7.

On the basis of our data set, there was only a single example of an in vitro negative chemical that was an in vivo genotoxin and that was a pharmaceutical. Therefore, it seems fair to conclude that if an untested chemical provides no indication for mutagenicity (i.e., does not contain SAs associated with DNA and/or protein interaction), it could also be assigned as "preliminary in vivo non-genotoxic".

In Vitro Mutagenic, In Vivo Liver Nongenotoxic MNT Positive Cases. Direct in vivo bone marrow metabolic activation (i.e., when bone marrow genotoxic metabolites were not observed in other tissues) has been relatively poorly investigated as compared with liver bioactivation. Within our data set, seven substances had negative in vivo liver genotoxicity outcomes yet in vivo MNT positive outcomes. All seven substances were positive in vitro. The seven substances were vincristine (57-22-7), acetophenetidin (62-44-2), thioacetamide (62-55-5), colchicine (64-86-8), propylene oxide (75-56-9), cytosine arabinoside (147-94-4), and sodium dehydroacetate (4418-26-2).

Vincristine (57-22-7) is a spindle fiber disrupting agent that induces aberrant mitoses, resulting in chromosome loss (aneuploidy) and production of MN.[43] The lack of detectable DNA damage in the Comet assay in either mice or rats is consistent with the fact that the vincristine interacts with microtubulin protein, rather than DNA, as a primary cellular target. Thus, the difference in the capacity of the Comet and MNT to detect genotoxicity could explain the in vivo data discrepancy. A closer inspection of the available mutagenicity data for acetophenetidin (62-44-2) showed that it was negative in Ames with mouse or rat S9 liver homogenate fractions but elicited a positive result when hamster S9 was used. The relative high

activity of N→O acetyltransferase in hamster S9[44,45] as compared with that in mouse or rat could explain the conflicting Ames results, since DNA adduct formation could be realized.[46] Acetophenetidin (62-44-2) was positive in an in vitro CA experiment, suggesting that it could act through a protein interaction.[47] However, DNA adduct formation is also facilitated, and this was experimentally shown to be the case based on the available in vivo Comet assay results, which showed no effects in liver but positive effects in the kidney.[48] In vivo, species differences were also observed in the bone marrow, with positive results in mice but negative findings in rats.[49−51]

It has been shown that thioacetamide (62-55-5) requires metabolic activation by CYP2E1. Thioacetamide *S*-oxide and thioacetamide *S,S*-dioxide are the reactive metabolites, which covalently bind to the macromolecules (DNA, RNA, and proteins). The differences in the activity of metabolizing enzymes in rats and mice could account for the discrepancies in the in vitro and in vivo systems.

Colchicine (64-86-8) was positive in the in vitro CA yet negative in Ames, suggesting that its preferential mode of action is via a protein interaction. This might explain the differences between the positive MNT and the negative Comet assay. Propylene oxide (75-56-9) and sodium dehydroacetate (4418-26-2) showed in vitro−in vivo data discrepancy because of the difference in route of administration of pathway of oral (Comet) vs intraperitoneal (MNT). Cytosine arabinoside (147-94-4) showed a difference in test capacity with a positive assignment in tests detecting protein interaction, such as the in vitro CA. Overall, in vivo bioactivation directly in bone marrow was not considered to be relevant for the seven chemicals identified since other more plausible justifications could be made to account for their positive MNT results.

In Vitro Mutagenic, In Vivo Liver Genotoxic MNT Negative Cases. Thirty-two substances were found to be mutagenic in vitro and in vivo liver genotoxic yet negative in the bone marrow MNT. Table 4 lists the substances together with their respective calls.

Conceivably, this pathway in the workflow represents a "bioexhaustive" detoxification route where either reactive metabolites of liver genotoxic chemicals are "bioexhausted" en route to the bone marrow due to off target reactions or are simple short-lived intermediates that are formed in the liver. One example is that of styrene. Styrene itself is nonelectrophilic but is metabolized to styene-7,8-oxide, which binds covalently to DNA and does show activity in various in vitro and in vivo assays for genetic effects. An evaluation of the remaining substances with respect to their MNT data is ongoing as part of our continuing efforts.

Deriving a (Q)SAR Model for in Vivo MNT. The in vivo MNT model was developed by combining the existing TIMES reactivity module (as already described earlier) with a new in vivo metabolism simulator. The working hypothesis assumed that the availability of parent chemicals or their metabolites in the target tissue were not rate limiting; hence, no differences would be expected between the in vitro and in vivo call; that is, the toxicodynamic model for in vitro should also be valid in vivo. Thus, the reactivity module developed for modeling in vitro CA mutagenicity should be suitable as part of the newly derived in vivo model for MNT.

A new in vivo metabolic simulator (i.e., transformation table) was developed comprising a set of structurally generalized molecular transformations (source and product fragments). A database of 220 in vivo metabolic pathways of chemicals was compiled and formed the training set used to derive the rat in vivo metabolic simulator. Experimentally observed in vivo metabolic pathways of diverse chemicals were extracted from the primary literature from journals including *Drug Metabolism and Disposition, Xenobiotica, Toxicological Sciences, Journal of Biological Chemistry, Biochemical Pharmacology,* etc. The following criteria were applied for studies to be incorporated into the final database:

- Metabolism studies conducted in vivo only,
- Rodent species: rats only,
- Experimental system: the whole organism,
- No enzyme inducers or inhibitors should be administered to the experimental animals.

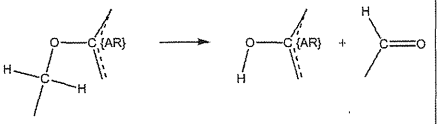
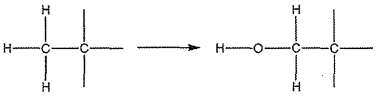
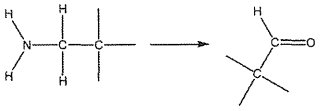
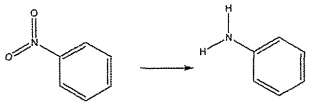
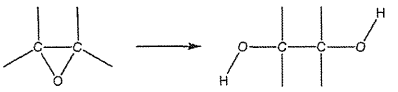
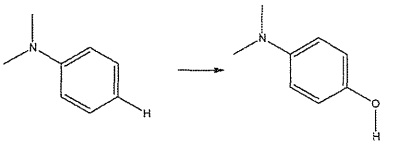
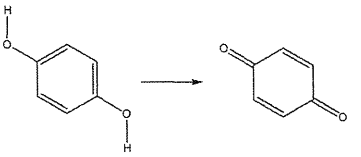
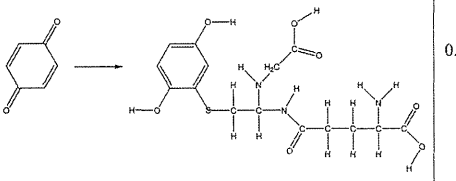
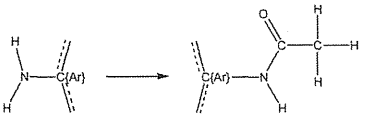
The current version of the metabolism simulator contains 506 structurally generalized molecular transformations, which were subdivided into the following types:

- 26 abiotic (nonenzymatic) transformations (e.g., tautomerization, acyl halide hydrolysis, geminal diol dehydration, etc.), which occur for the most part spontaneously.
- 415 phase I enzymatic transformations (e.g., aliphatic C-oxidation, epoxidation, aromatic C-hydroxylation, ester hydrolysis, amide hydrolysis, dehalogenation, etc.)
- 65 phase II enzymatic transformations (e.g., O-glucuronidation, glutathione conjugation, sulfation, acetylation, etc.)

Table 4. List of the 32 Chemicals That Are in Vitro Positive and Positive in Vivo in Liver but Negative in the MNT

CAS	name	ivt	liver	MNT
57-57-8	propiolactone	1	1	0
67-20-9	nitrofurantion	1	1	0
75-09-2	methylene chloride	1	1	0
90-43-7	2-phenylphenol	1	1	0
92-52-4	biphenyl	1	1	0
95-53-4	*o*-toluidine	1	1	0
95-80-7	2,4-diaminotoluene	1	1	0
96-09-3	styrene oxide	1	1	0
96-45-7	ethylenethiourea	1	1	0
97-56-3	*o*-aminoazotoluene	1	1	0
100-42-5	styrene	1	1	0
100-75-4	1-nitrosopiperidine	1	1	0
106-93-4	ethylene dibromide	1	1	0
108-95-2	phenol	1	1	0
110-00-9	furan	1	1	0
124-48-1	chlorodibromomethane	1	1	0
139-13-9	triglycollamic acid	1	1	0
140-88-5	ethyl acrylate	1	1	0
492-80-8	auramine	1	1	0
542-75-6	1,3-dichloropropene [BSI:ISO]	1	1	0
604-75-1	oxazepam	1	1	0
621-64-7	*N*-nitroso(di-*n*-propyl)amine	1	1	0
930-55-2	1-nitrosopyrrolidine	1	1	0
1116-54-7	2,2′-(nitrosoimino)bisethanol	1	1	0
2650-18-2	C.I. acid blue 9	1	1	0
2835-95-2	5-amino-*o*-cresol	1	1	0
2921-88-2	chlorpyrifos	1	1	0
3564-09-8	Ponceau 3R	1	1	0
5307-14-2	2-nitro-4-phenylenediamine	1	1	0
10595-95-6	*N*-nitrosomethylethylamine	1	1	0
16423-68-0	C.I. acid red 51	1	1	0
77439-76-0	3-chloro-4-dichloromethyl-5-hydroxy-2-furanone	1	1	0

Table 5. List of Selected Principal Transformations[a]

#	Principal transformations	P*
1	Epoxidation (*Phase I*) 	0.95
2	Oxidative O-Dealkylation (*Phase I*) 	0.95
3	Aliphatic C-Oxidation (*Phase I*) 	0.95
4	Oxidative Deamination (*Phase I*) 	0.95
5	Nitro Group Reduction (*Phase I*) 	0.95
6	Epoxide Hydration (*Phase I*) 	0.95
7	Aromatic C-Hydroxylation (*Phase I*) 	0.95
8	Quinone Formation (*Phase I*) 	0.90
9	Glutathione Conjugation (*Phase II*) 	0.85
10	Acetylation (*Phase II*) 	0.95

[a]*P, probability of transformation. In general, it defines the priority of application of these transformations.

A list of some of the principal transformation reactions included in the current version of the simulator is presented in Table 5. As seen from the table, transformations are characterized by their probabilistic assessment. The probability values depend on the commonality of a given metabolic transformation in the training metabolism data set. Nonenzymatic (abiotic, spontaneous) transformations had the highest probability value of 1.00. Values less than 1.00 were assigned to enzymatic transformations with lower priority in their application.

The database compiled was subsequently implemented into MetaPath (LMC), a software tool partially supported by U.S. EPA (Athens, United States) under grant CR-83199501-0. The collected database of metabolic pathways and expert knowledge were then used to determine the principal transformations and train the system to simulate in vivo metabolism of training chemicals.

The first attempt to model in vivo bone marrow MN formation of the training set chemicals in the "557 list" (note at this stage this was prior to any critical data analysis) involved combining the MNT reactivity module with the newly developed in vivo rat liver metabolism simulator (in the early prototype version of the model, the in vivo logic had not yet been considered). The performance of this model was poor—a sensitivity of 76% and specificity of 37%, possibly due to inadequate simulation of the presence of parent chemicals or their liver metabolites in the remotely located bone marrow. The in vivo simulator was then adjusted to reproduce more phase II conjugation reactions at certain "branches" of the metabolic generation "tree". In vitro, all generated metabolites are theoretically available to interact (almost stochastically) with macromolecules present in the incubation medium and thus have the potential to elicit a mutagenicity effect.[22] In vivo, enzymes are aggregated in multienzyme complexes, and the cells could be protected from reactive metabolites via shuttling intermediates between consecutive enzymes. Thus, the product of one enzymatic reaction may become a substrate of the subsequent enzymatic reaction. In this study, no attempts were made to investigate the metabolic hierarchy in detail; instead, we have tried to identify those metabolic pathways (occurring mainly in liver) where metabolites could be "trapped" and thus unavailable to react with macromolecules. The identification of these metabolic detoxification pathways was thought to help explain if only in part the poor availability of chemicals in the target organ and thus define the contribution of metabolism factors to the final outcome. An example illustrating the difference between in vitro and in vivo (liver) availability of epichlorohydrin is presented in Figure 8. In vitro studies show that epichlorohydrin is predominantly hydrolyzed into 3-chloro-1,2-propanediol

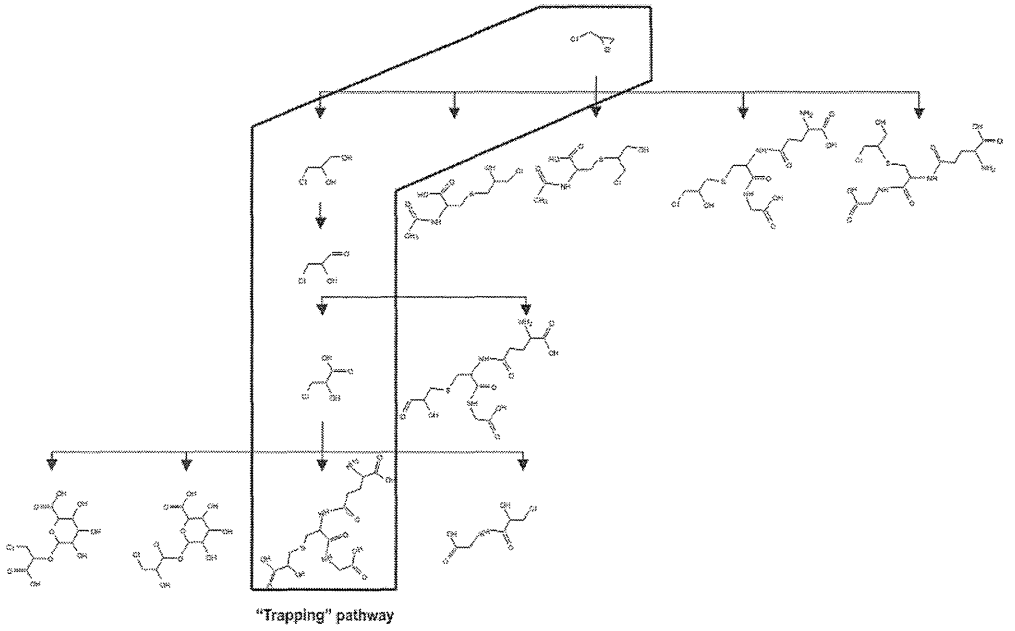


Figure 8. Metabolic tree of the epichlorohydrin (106-89-8). In vitro mutagenic parent and metabolite (3-chloro-1,2-propanediol) are considered as "trapped" in in vivo detoxification pathways.

712 by the microsomal epoxide hydrolase(s) of mouse liver. The
713 authors considered the role of glutathione conjugation in the in
714 vitro metabolic reactions as not being significant.[52] Therefore,
715 it may be assumed that the availability of epichlorohydrin, as a
716 direct-acting mutagen, and its metabolite 3-chloro-1,2-propane-
717 diol is high enough in the in vitro environment to induce muta-
718 genicity by interaction with DNA. In the in vivo environment,
719 within 20 min of oral or intraperitoneal administration of epi-
720 chlorohydrin in mice, the parent compound is no longer detec-
721 table in the blood, while the level of 3-chloro-1,2-propanediol
722 reaches a peak. The latter was measurable up to 5 h following
723 exposure; thus, the biotransformation of epichlorohydrin was
724 partly associated with both the enzymatic and the nonenzymatic
725 hydrolysis. Phase II conjugation with glutathione takes place via
726 mediation of phase II glutathione transferases; a direct conju-
727 gation of epichlorohydrin with glutathione in vivo has also been
728 observed.[52] Therefore, both the parent compound and the in
729 vitro mutagenic metabolite 3-chloro-1,2-propanediol can be con-
730 sidered as "trapped" in in vivo metabolic phase II detoxification
731 pathways, reducing their availability in liver, where no liver
732 genotoxicity in vivo is observed (Figure 8).
733 With liver as the target organ in our modeling exercise, we
734 assumed that the effect of metabolic detoxification was an im-
735 portant prerequisite to assess the availability of chemicals in the
736 liver and, hence, the appearance of ultimate genotoxicity effect.
737 However, modeling of genotoxic effects at a remote tissue such
738 as the bone marrow requires more ADME factors to be taken
739 into account. For instance, highly reactive parent chemicals
740 and/or metabolites can be involved in off-target protein reac-
741 tions along their path from liver to the bone marrow.[53] An
742 example illustrating "bioexhausting" detoxification of chemicals
743 unavailable in the remote bone marrow to elicit genotoxicity is
744 provided for the 5-amino-*o*-cresol in Figure 9.
745 This industrial chemical was found to induce in vivo liver
746 genotoxicity,[54] but evidence exists to suggest that the remote bone
747 marrow remains undamaged by this chemical.[55] The metabolism

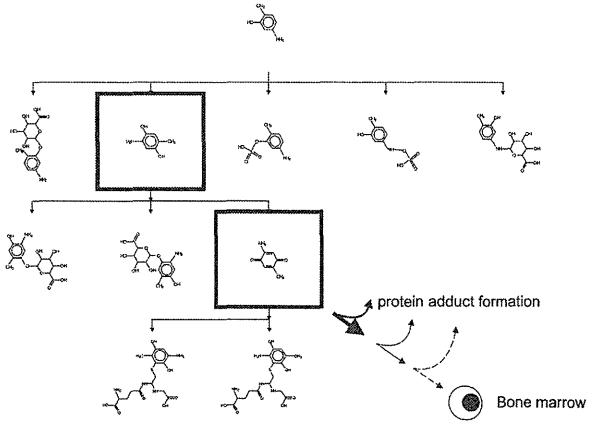


Figure 9. Simulated metabolic tree of 5-amino-*o*-cresol (2835-95-2). The in vivo liver reactive metabolites (2-amino-5-methyl-1,4-benzenediol and 2-amino-5-methyl-1,4-benzoquinone) were considered as "bioexhausted" approaching the bone marrow.

and disposition study of the 5-amino-*o*-cresol indicated that 748
the presence of 1,4-dihydroxy-substituted metabolite lead to 749
possible formation of another reactive intermediate, that is, a 750
quinone.[56] The parent chemical and its metabolites are then 751
partially detoxified in liver and might exert some in vivo geno- 752
toxicity therein. The liver reactive entities were presumably 753
involved in off-target protein reactions approaching to the bone 754
marrow and thus were deficient in the remote tissue to exert 755
genotoxicity. Along with the overall genotoxicity predictions 756
of the 5-amino-*o*-cresol, Supporting Information about the 757
applicability domain is also provided in the standard MNT 758
report presented in Table 6.[57] 759

As with any model, characterizing its scope by way of an 760
applicability domain is critical to ensure appropriate subsequent use. 761

Table 6. Reported in Vitro and in Vivo Genotoxicity Outcome of the Parent 5-Amino-*o*-cresol and Its Metabolites (2-Amino-5-methyl-1,4-benzoquinone) Provided in the MNT Model

		in vivo MNT		in vitro			subdomains						
							structural domain				mechanistic domain		
	CAS NAME SMILES	obsd effect	pred. effect	pred. effect	active fragment	type of in vivo detoxification	general requirements	correct fragment	incorrect fragment	unknown fragment	alert performance	interpolation space	total domain
	2835-95-2												
parent	5-amino-*o*-cresol	nongenotoxic	nongenotoxic	mutagenic to bacteria (Ames test)	amines	bio exhausting	in domain	in domain (100%)	in domain (0%)	in domain (0%)	in domain	in domain	in domain
	c1(C)c(O)cc(N)cc1												
	2-amino-5-methyl-1,4-benzenediol		nongenotoxic	mutagenic to bacteria and proteins	amines, aminophenols, and phenyleneamines	bio exhausting					in domain	in domain	in domain
	c1(O)c(N)cc(O)c(C)c1												
metabolites	2-amino-5-methyl-1,4-benzoquinone			mutagenic to proteins	quinones	bio exhausting					in domain	in domain	
	C1(N)C(=O)C=C(C)C(=O)C=1		nongenotoxic										

The applicability domain includes three different levels: general parametric requirements, structural domain, and mechanistic domain. The first two domain levels have been provided for parent chemicals only, whereas the mechanistic domain is provided for parents and metabolites. The general parametric requirements encompass ranges of two molecular parameters:

- Molecular weight *MW* (in Da) (18, 1255),
- log K_{OW} (mol L_O^{-1} mol^{-1} L_W) (−20, 15).

The structural domain was based on atom-centered fragments extracted from correctly and incorrectly predicted training set chemicals. This domain level account for the atom type, hybridization, and attached H-atoms. To determine a fragment, first neighbors were selected. However, if the neighbor is a heteroatom, then the diameter of the fragment is increased to three consecutive heteroatoms or to the first sp^3 carbon atoms. The mechanistic domain included both performance of an alerting group, which is hypothesized to produce reactivity and the domain of explanatory variables determining the parametric requirements for the functional groups to elicit their reactivity.[57] The performance of an alerting group is considered to be reasonable if it exceeds the model-defined threshold of 60%.

It should also be noted that the bone marrow hematopoietic cells possess low biotransformation capacity; therefore, reactive species with short half-lives may be unable to reach them. Among the different chemical classes, aromatic amines, *N*-nitroso compounds, nitroimidazoles, and haloalkanes are known to be difficult for the detection of possible genotoxic effects in the bone marrow.[58] The absence of some parent chemicals and/or metabolites in the bone marrow could also be associated with some specific physicochemical properties such as high hydrophilicity, volatility, etc., hampering their transport to this tissue.[59]

The performance of the prototype MNT model and the correlation between in vitro and in vivo genotoxicity outcomes were assessed by a number of "false positive" and "false negative" chemicals when the model was applied to the training set chemicals on the "557 list". Initially, the in vivo MNT model illustrated very low specificity and had not taken into account in vivo detoxification. This was confirmed by the analysis of the "false positives" of the model for which in vitro mutagenicity data were also available (Figure 10); 90% of the in vivo "false positives" have been documented to be mutagenic in vitro. It was assumed that the in vitro active chemicals and/or their active metabolites characteristic for the "static" in vitro incubation conditions are not freely available in vivo to cause damage. The majority of these metabolites are considered to be "trapped" across in vivo detoxification pathways. Note that the implementation of the "trapping" metabolic detoxification pathways in the in vivo model was introduced to predict genotoxicity in liver only as the principal organ for xenobiotic metabolism. However, modeling in vivo liver genotoxicity is not always a good predictive tool for the bone marrow MNT, since, as mentioned above, the presence of chemicals in a remote organ such as the bone marrow depends on other ADME factors. Thus, a second type of in vivo detoxification pathways, accounting for the deficiency of the chemicals to be active in the bone marrow, was added to the MNT model. These detoxification pathways have been used to explain negative in vivo MNT of chemicals, which are known to cause in vivo liver genotoxicity. To date, 76 "trapping" and 52 metabolic detoxification pathways,

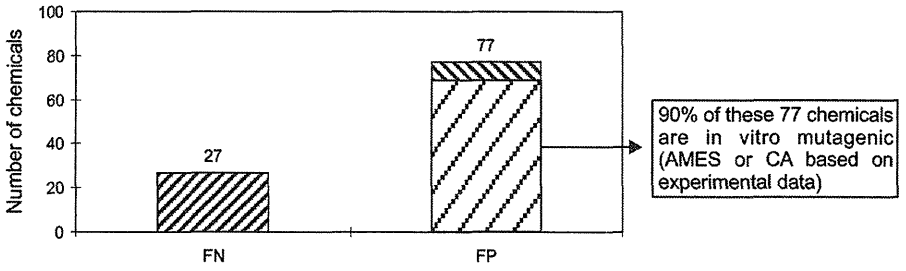


Figure 10. In vivo MNT model estimations: false negatives (FN) and false positives (FP). An analysis based on chemicals with available overlapping in vitro–in vivo experimental data.

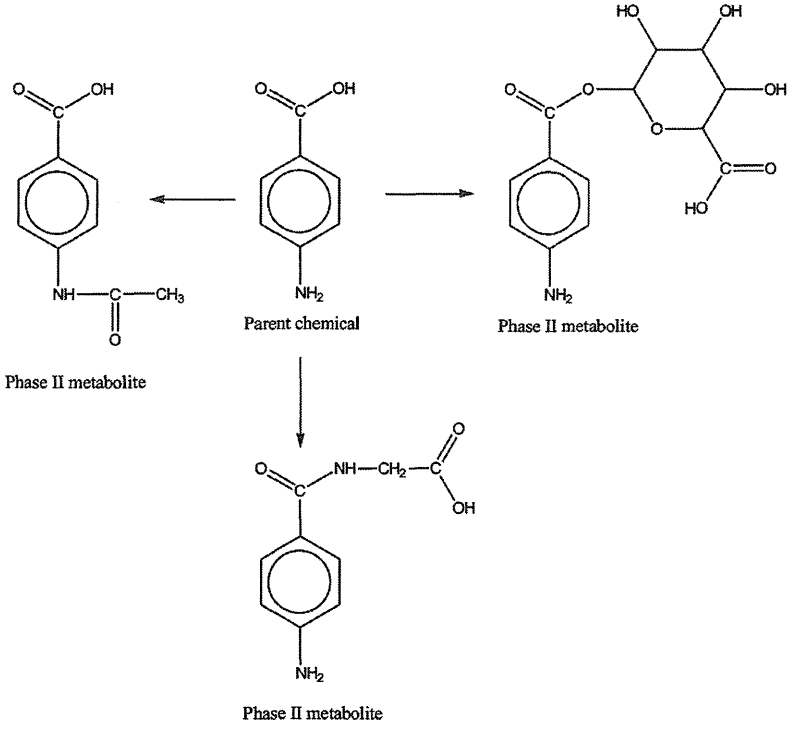


Figure 11. Highly polar substituents (e.g., COOH, SO_3H, COOR, phosphate, thiophosphate, etc.) on the aromatic amine trigger in vivo phase II detoxification and excretion directly.

accounting for the chemicals with negative in vivo genotoxicity as determined by the bone marrow MNT, have been implemented into the model to provide some insight on both the liver and the bone marrow detoxification mechanisms. The following chemical classes were studied to elucidate the contribution of in vivo metabolic transformations to negative bone marrow MNT test results: aromatic amines, organic halides, nitro compounds, epoxides, ureides, isocyanates, hydroxylamines, pyranones, quinoneimines, and thiols. An example, demonstrating the effect of in vivo metabolism on the potential genotoxicity of polar aromatic amines in the bone marrow, is presented in Figure 11. It is shown that the lack of demonstrated in vivo genotoxicity is a consequence of the presence of polar functional groups in aromatic amines that hamper the occurrence of the CYP-mediated in vivo phase I N-hydroxylation as bioactivation reaction. For aromatic amines with highly polar substituents in their molecules, the in vivo enzymatic activities favor the phase II metabolic detoxification reactions leading to excretion, and the specific pharmacokinetics factors clearly contribute to this outcome. As a result, phase I bioactivation reactions of N-hydroxylation, otherwise occurring in vitro environment, is assumed to be "suppressed" in in vivo systems.

Correlation between in vitro and in vivo genotoxicity results was also assessed within the subset of 27 "false negatives" for which documented mutagenicity data were available. Table 7 lists these substances.

In the performed critical data analysis, 24 of these 27 chemicals were assigned to be nonmutagenic according to Ames and in vitro CA tests. The Ames result for indomethacin (53-86-1) was inconclusive. The only positive CA was for diethylstilbestrol (56-53-1). No CA result was available for procarbazine hydrochloride (366-70-1). The results indicate that the in vivo toxicodynamic model (which is assumed to be same in vitro) "logically" evaluates these chemicals to be nongenotoxic, since no SAs associated with DNA and/or protein interactions exist in their molecular structures. Such an observation in turn prompted a reanalysis of the in vivo bioactivation capacity of these 27 chemicals. A search for additional mutagenicity data

Table 7. List of the 27 Chemicals That Were False Negatives in the MNT Model

CAS	name	Ames	CA	MLA	QA-ed ivt	in vivo liver	QA-ed in vivo MNT
87-29-6	cinnamyl anthranilate	0	0	inconclusive	inconclusive	N/A	inconclusive
108-88-3	toluene	0	0	0	0	0	0
115-96-8	tris(2-chloroethyl) phosphate	0	0	N/A	0	N/A	inconclusive
116-06-3	aldicarb	0	0	1	1	N/A	1
117-81-7	bis(2-ethylhexyl)phthalate	0	0	0	0	0	0
1163-19-5	decabromobiphenyl ether	0	0	0	0	N/A	inconclusive
127-47-9	retinol acetate	0	0	N/A	0	N/A	0
366-70-1	procarbazine hydrochloride	0	N/A	1	1	N/A	1
103-84-4	acetanilide	0	0	N/A	0	N/A	0
53-86-1	indomethacin	inconclusive	0	N/A	inconclusive	N/A	1
56-53-1	diethylstilbestrol	0	1	1	1	N/A	1
64-77-7	tolbutamide	0	0	0	0	N/A	1
62-55-5	thioacetamide	0	0	1	1	0	1
58-89-9	lindane	0	0	N/A	0	N/A	0
94-75-7	2,4-dichloro-phenoxyacetic acid	0	0	N/A	0	N/A	0
78-79-5	isoprene	0	0	N/A	0	N/A	1
56-72-4	coumaphos	0	0	0	0	N/A	0
79-11-8	chloroacetic acid	0	0	1	1	N/A	1
123-91-1	1,4-dioxane	0	0	0	0	0	0
79-01-6	trichloroethylene	0	0	1	1	N/A	1
108-90-7	chlorobenzene	0	0	1	1	N/A	1
95-50-1	1,2-dichlorobenzene	0	0	1	1	N/A	0
106-46-7	1,4-dichlorobenzene	0	0	N/A	0	1	1
87-61-6	1,2,3-trichlorobenzene	0	0	N/A	0	N/A	1
120-82-1	1,2,4-trichlorobenzene	0	0	N/A	0	N/A	1
108-70-3	1,3,5-trichlorobenzene	0	0	N/A	0	N/A	1
2058-46-0	oxytetracycline·HCl	0	0	1	1	N/A	1

was undertaken using in vitro data for the MLA to supplement the Ames and the CA data. The following seven substances were associated with positive MLA data: aldicarb (116-06-3), thioacetamide (62-55-5), chloroacetic acid (79-11-8), trichloroethylene (79-01-6), chlorobenzene (108-90-7), 1,2-dichlorobenzene (95-50-1), and oxytetracycline·HCl (2058-46-0). Cinnamyl anthranilate (87-29-6) had an inconclusive MLA result. This left 16 substances that were in vitro negative. In contrast to the analysis based on available documented data across the three levels, this investigation was hampered by lack of in vivo liver genotoxicity data assessed by Comet, UDS, or the TGR tests. Data to evaluate in vivo liver genotoxicity was only found for four substances: negative outcomes for toluene (108-88-3), bis(2-ethylhexyl)phthalate (117-81-7), 1,4-dioxane (123-91-1), and a positive outcome for 1,4-dichlorobenzene (106-46-7). This left 12 substances for which a critical analysis was undertaken of the available in vivo bone marrow MNT data. Further review of MNT data for tris(2-chloroethyl) phosphate (115-96-8)[60] and decabromobiphenyl ether (1163-19-5)[61] revealed them to have inconclusive findings. Retinol acetate (127-47-9), acetanilide (103-84-4), lindane (58-89-9), 2,4-dichloro-phenoxyacetic acid (94-75-7), and coumaphos (56-72-4) were now found to be associated with negative MNT data.[62−66] This left five chemicals with positive MNT results, which were presumably in vivo bioactivated. These chemicals are listed as follows: tolbutamide (64-77-7), isoprene (78-79-5), 1,2,3-trichlorobenzene (87-61-6), 1,2,4-trichlorobenzene (120-82-1), and 1,3,5-trichlorobenzene (108-70-3) and are discussed in turn. The toxic metabolite of tolbutamide *n*-butyl isocyanate appears to be efficiently detoxified in vivo as glutathione conjugate S-(*n*-butylcarbamoyl)glutathione in rats.[67] The positive result in MNT was only found in mouse strain C57BL/6J. The discrepancies between the in vivo and the in vitro results could be related to the possibility of the formation the toxic metabolite *n*-butyl isocyanate, which depends on the activity of the corresponding enzymes in different species (rat, mouse, and hamster).

Isoprene (IP) was metabolized to IP-1,2-oxide (2-ethenyl-2-methyloxirane) and IP-3,4-oxide (propen-2-yloxirane) by CYP450 enzyme system, with CYP2E1 having the highest activity in the formation of isoprene monoepoxides and the corresponding diepoxide. Isoprene monoepoxides were found to be nonmutagenic, while isoprene diepoxide was mutagenic and genotoxic. Among the two monoepoxides, IP-1,2-oxide is the main metabolite (90−95% of the dose used) but is less stable (half-life at 37 °C, 85 min), because of its high reactivity toward hydrolysis. Buckley et al.[68] showed that the stable metabolite IP-3,4-oxide (half-life at 37 °C, 73 h) could be further oxidized to the mutagenic diepoxide. Irrespective of the fact that the ratio between IP-1,2-oxide and IP-3,4-oxide was found to be similar in all rodent species,[69] the positive genotoxic results were obtained only in mouse bone marrow cells, which is in agreement with higher activity of CYP2E1 in mice than in rats.

A number of considerations can be made to account for the discrepancies observed in the in vitro and in vivo genotoxicity of trichlorobenzenes. Two key reasons are provided here:

(1) Bacterial tester strains usually employed in the Ames test are not sufficiently sensitive to detect chlorinated benzenes and/or their metabolites. According to Claxton et al.,[70] the *Salmonella* assay is not very responsive to mutagens within halogenated cyclic and aromatic compounds. Because the most reactive metabolites of trichlorobenzenes are their benzoquinone derivatives, the choice of suitable *Salmonella typhimurium* tester strains is very important. Hakura et al.[71] established that the mutagenicity