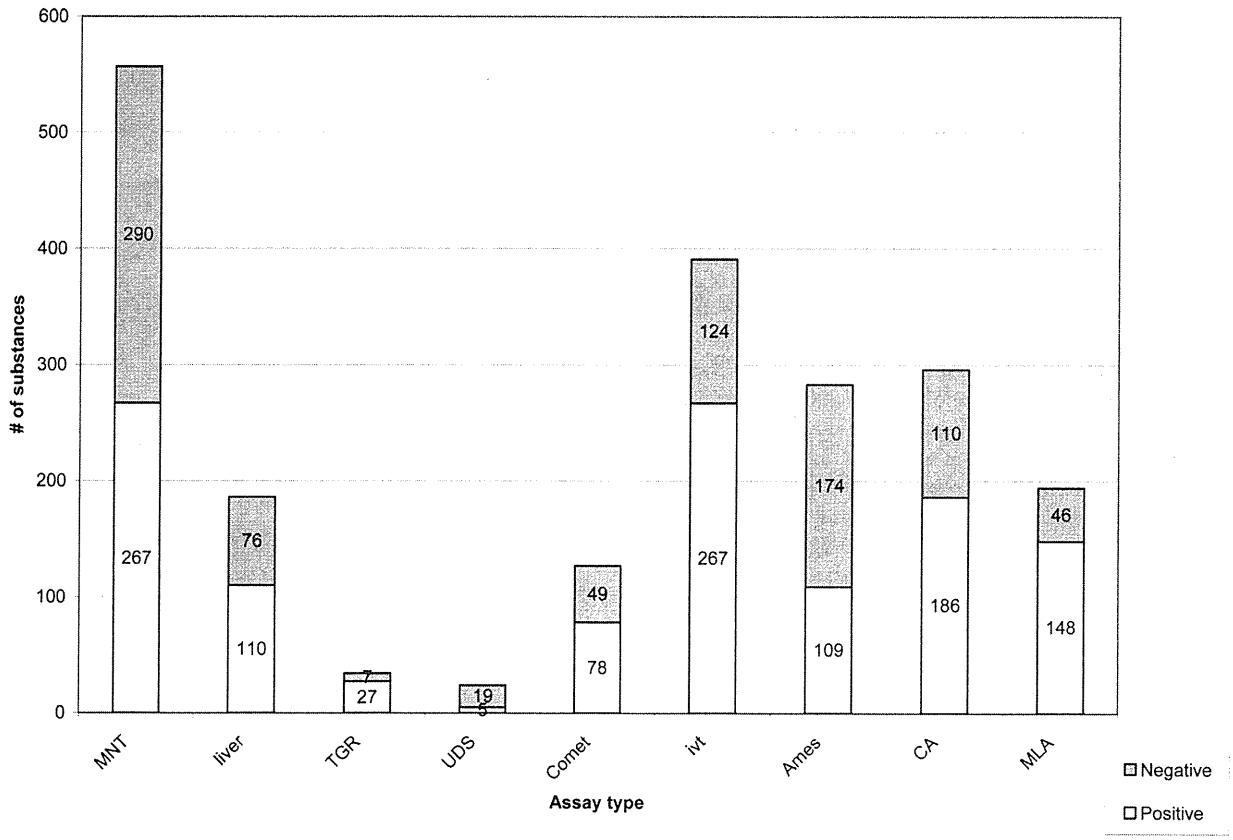


**Figure 2.** Distribution of the overall calls for each of the test assays under study.

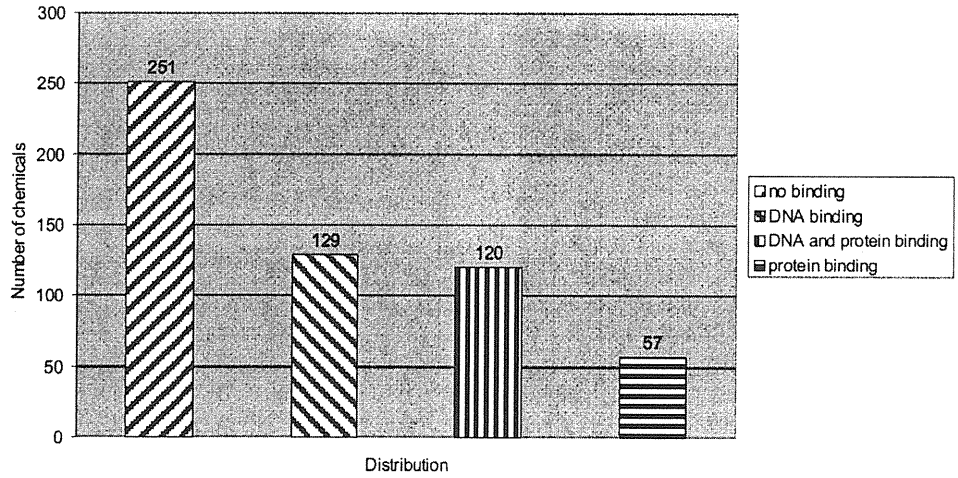


**Figure 3.** Distribution of training set chemicals across DNA and protein binding alerts.

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


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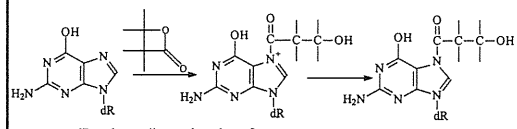
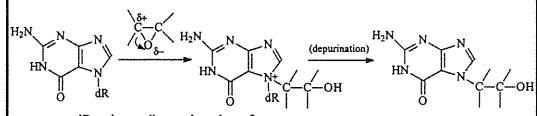
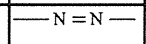
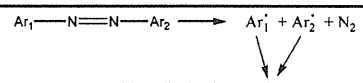
**Table 1. Alerting Groups and Descriptors Used in COREPA Models for Estimating Their Reactivity Associated with Supporting Mechanistic Information**[a]

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


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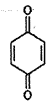
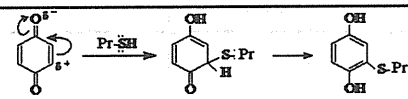
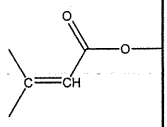
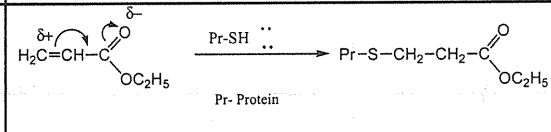
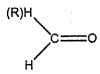
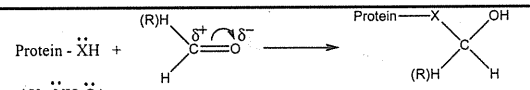
Table 2. Alerting Groups for Protein Binding, Parameters for Reactivity, and Supporting Interaction Mechanisms[a]

| # | 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); log$K_{OW}$, octanol–water partitioning coefficient (mol $L_0^{-1}$ $mol^{-1}L_w$).

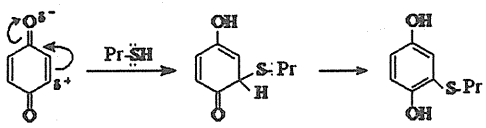


Figure 4. Interaction mechanism of quinones with proteins (Pr).

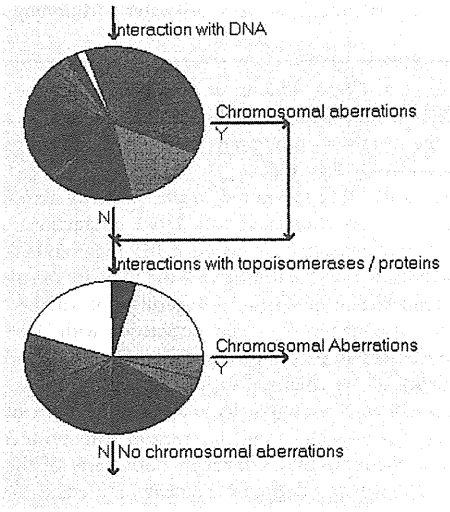



Figure 5. Structure of the reactivity component of the in vivo genotoxicity models.

**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 |

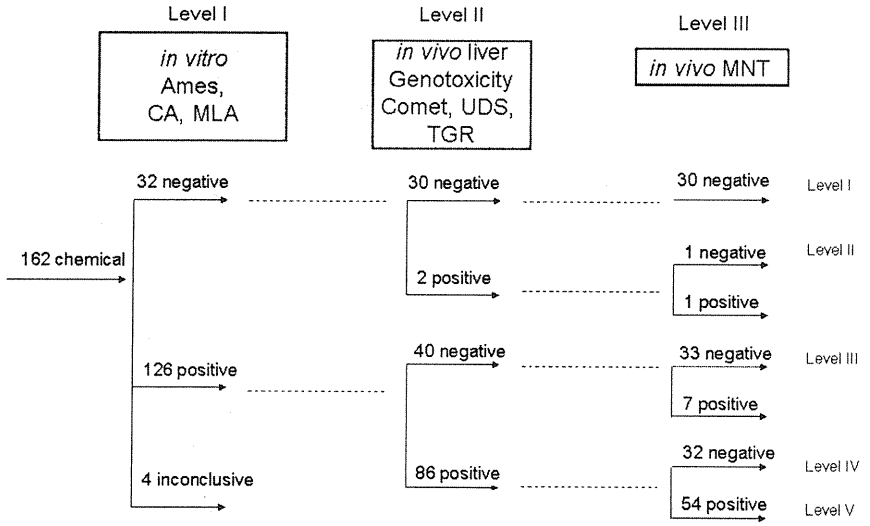


**Figure 6.** Workflow for the 162 chemicals with results in all test systems.

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


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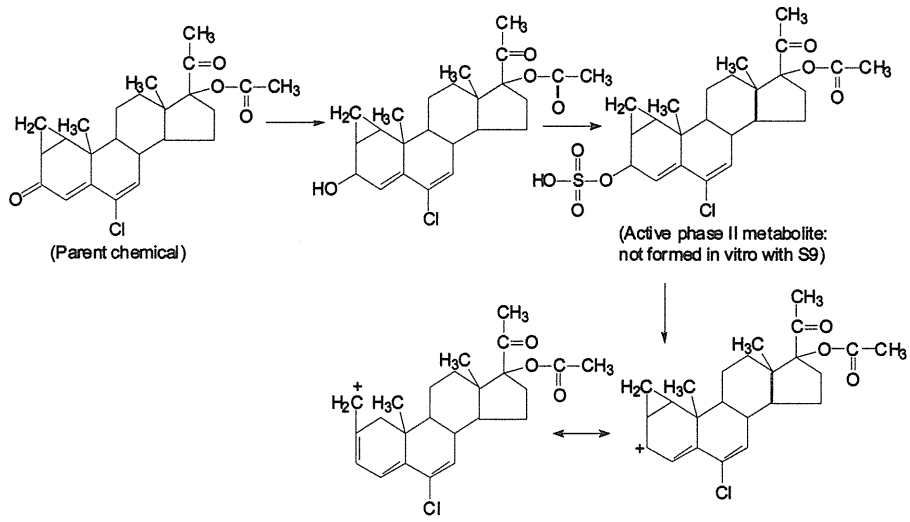



**Figure 7.** Mechanism of cyproterone acetate bioactivation in the liver.

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 (aneuplody) 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.

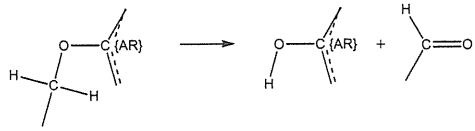
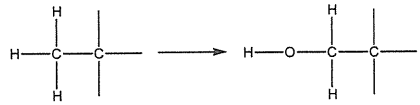
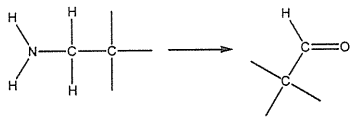
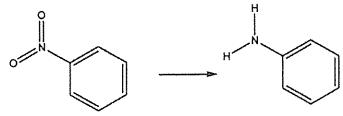
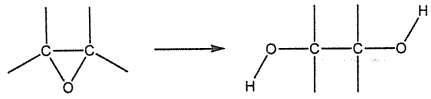
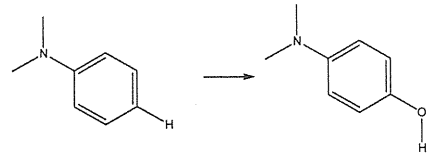
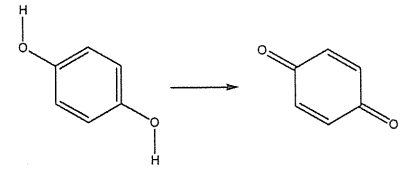
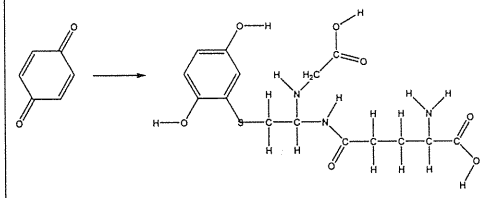
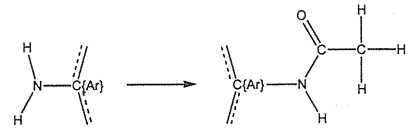
The current version of the metabolism simulator contains 506 structurally generalized molecular transformations, which were subdivided into the following types:

- 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 | iv | 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

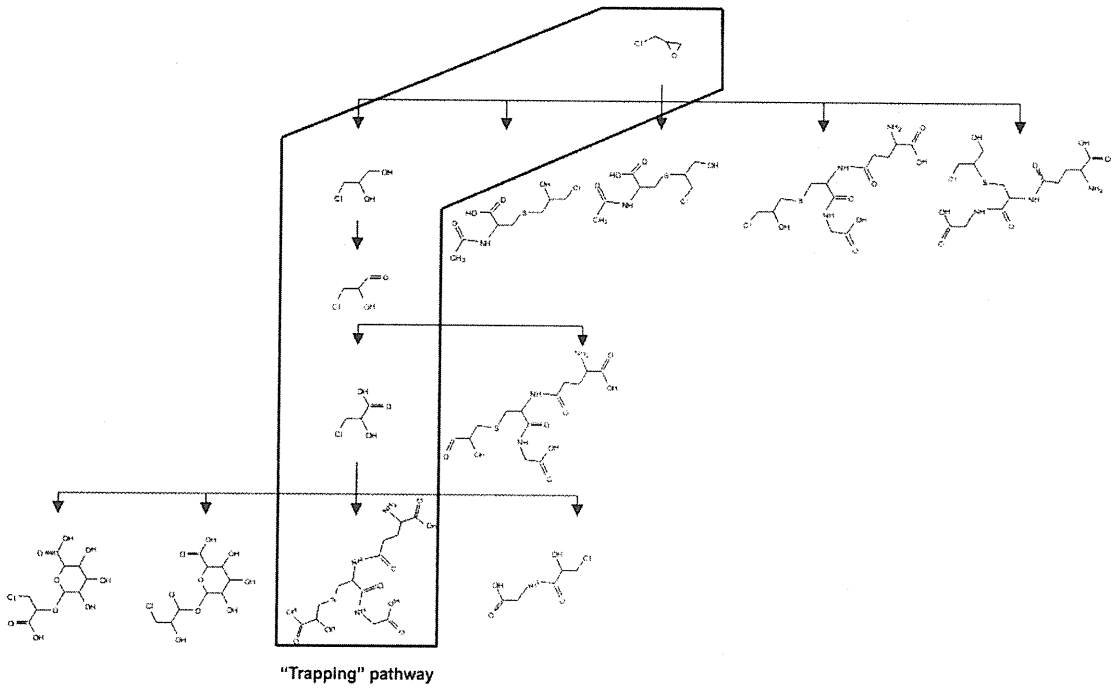



**Figure 8.** Metabolic tree of the epichlorohydrin (106-89-8). In vitro mutagenic parent and metabolite (3-chloro-1,2-propanediol) are considered as "trapped" in in vivo detoxification pathways.

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

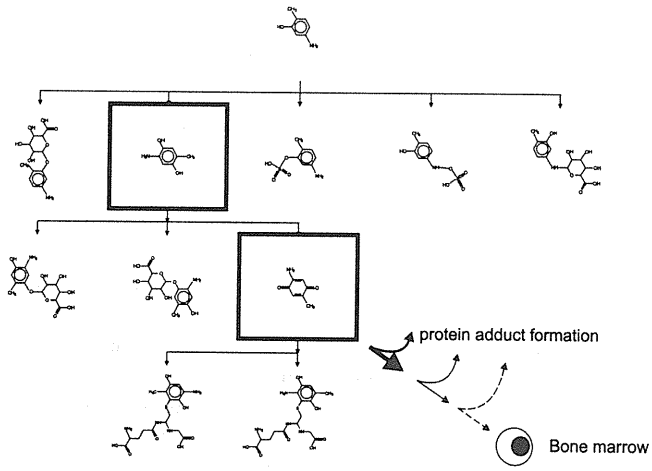



**Figure 9.** Simulated metabolic tree of 5-amino-*o*-cresol (2835-95-2). The in vivo liver reactive metabolites (2-amino-5-methyl-1,4-benzenediol and 2-amino-5-methyl-1,4-benzoquinone) were considered as "bioexhausted" approaching the bone marrow.

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


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**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 | amines | bio exhausting | in domain | in domain (100%) | in domain (0%) | in domain (0%) | general requirements | correct fragment | incorrect fragment | unknown fragment | in domain | in domain | 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 | correct fragment | incorrect fragment | unknown fragment | in domain | in domain | 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 | | | | | | | | | | | | | | | | | | | |
| | C1(N)C(=O)C=C(C)C(=O)C=1 | | nongenotoxic | mutagenic to proteins | | quinones | bio exhausting | | | | | | | | | in domain | in domain | | | |

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,

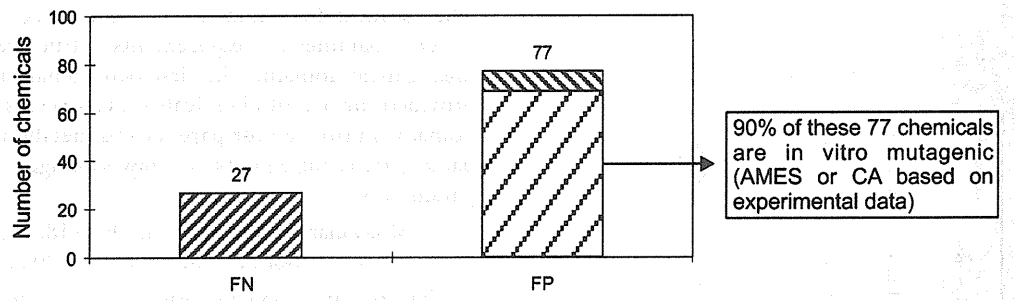


**Figure 10.** In vivo MNT model estimations: false negatives (FN) and false positives (FP). An analysis based on chemicals with available overlapping in vitro–in vivo experimental data.

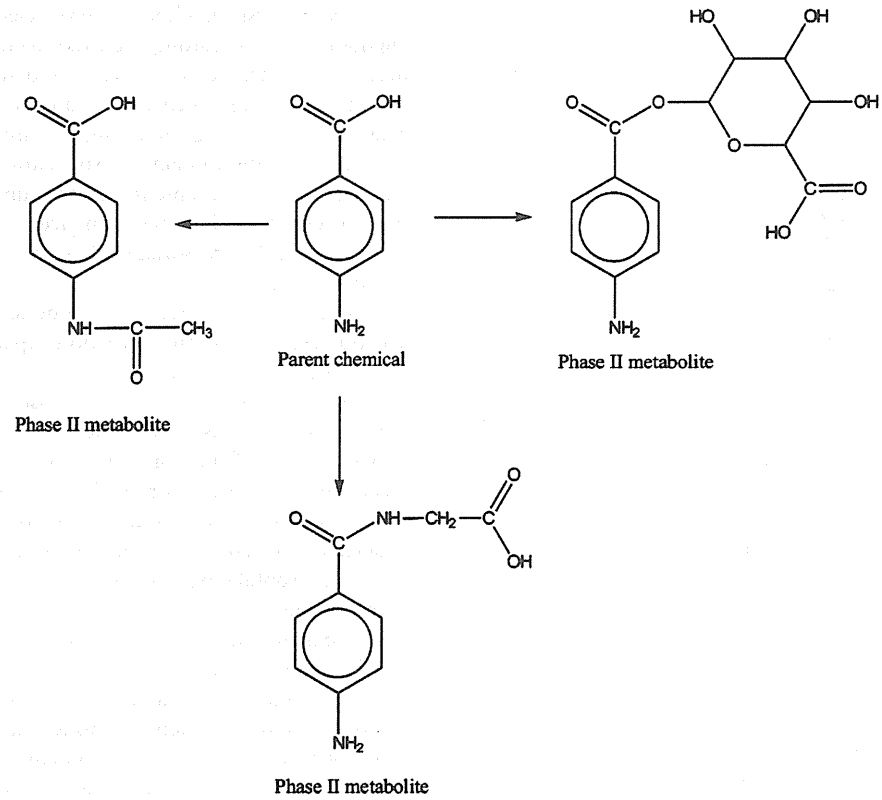


**Figure 11.** Highly polar substituents (e.g., COOH, $SO_3H$, COOR, phosphate, thiophosphate, etc.) on the aromatic amine trigger in vivo phase II detoxification and excretion directly.

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

of benzoquinones (the main reactive metabolites of trichlorobenzenes) was effectively detected with the *S. typhimurium* strains TA104 and TA2637. TA104 was most sensitive to oxidative mutagens, while TA2637 was effective in detecting bulky DNA adducts.

(2) Different activity levels of the specific enzymes in rats and mice responsible for the metabolic activation of chlorinated benzenes. According to the investigation of Hissink et al., the rank order for total in vitro conversion of chlorobenzenes to oxidized metabolites and covalently bound metabolites was mouse > rat ≫ human.[72] Moreover, conversion-dependent covalent binding to proteins was observed for all chlorinated benzenes, in which benzoquinones amounted to about 10−30% of the total metabolites formed.[73] Den Besten et al.[74] were found that cytochrome P4503A1 showed the highest activity toward trichlorobenzenes both with regard to the formation of corresponding chlorophenols and protein-bound metabolites. Thus, the activity of CYP3A1 strain to produce reactive benzoquinone metabolites from trichlorobenzenes seems to be higher in mice than in rats.

The critical review of the observed genotoxicity within the list of 162 workflow chemicals has changed the MNT predicted outcome of some of the "original" 557 training set chemicals. Thus, after including the in vivo metabolic detoxification "logic", the newly developed MNT model exhibited an improved performance: sensitivity of 82% (i.e., 217 correctly predicted genotoxic chemicals out of the total number of 266 documented genotoxins), specificity of 61% (i.e., 170 correctly predicted nongenotoxic chemicals out of total number of 281 observed nongenotoxins), and concordance of 71%. To calculate the model concordance, the chemicals for which explicit model prediction could not be provided (there were 10 chemicals that failed to achieve the user defined threshold of 70%) were excluded from the "557 list". Thus, the model concordance of 71% is based on relation between the total number of correct predictions (genotoxic and nongenotoxic, i.e., 387) out of 547 chemicals.

**Derivation of the Model for in Vivo Liver Genotoxicity.** The modeling of in vivo liver genotoxicity is based on documented data effects for 185 diverse chemicals assessed by the UDS, Comet, and TGR assays (Appendix III in the Supporting Information). The model shared the reactivity and, to a certain extent, the metabolism components of the in vivo MNT model. On the basis of the selection of liver as the target organ of this investigation, the "bioexhausting" component of the detoxification stage usually associated with targets (such as bone marrow) remote from the liver is not considered herein. The liver model was derived directly following the logic of the workflow presented in Figure 1. According to this logic, two possible genotoxicity outcomes are feasible for the in vitro nonmutagenic chemicals. Most of these in vitro negative chemicals are not expected to elicit in vivo liver genotoxicity, whereas bioactivation reactions producing liver damaging metabolites can occur for a limited set of nonmutagens. The fate of the in vitro mutagenic chemicals was also implemented in this logic. Thus, for some in vitro mutagens (e.g., aromatic amines possessing polar functional groups), the parent chemicals or their metabolites or both could be "trapped" in liver detoxification pathways; as a result, they will not elicit genotoxic effect in the target organ. For example, *p*-aminobenzoic acid is found to be liver nongenotoxic, being readily absorbed by the gastrointestinal tract.[75] The liver is the principle site of glycine phase II conjugation; thus, this chemical was not subjected to N-hydroxylation phase I bioactivation reactions such as aromatic amine N-hydroxylation. Bearing in mind metabolic consideration mainly, if in vitro mutagenic chemicals were not involved in liver "trapping" detoxification, they would be considered to be in vivo liver genotoxins. At the present time, 76 "trapping" detoxification pathways have been implemented into the liver genotoxicity model and contribute to its sensitivity of 85% (i.e., 90 correctly predicted genotoxic chemicals of 106 observed liver genotoxins) and specificity of 49% (i.e., 35 correctly predicted nongenotoxic chemicals of 72 observed nongenotoxins). Seven chemicals for which the model cannot provide explicit predictions were excluded from the model statistics. The poor specificity is attributed to the fact that the model was derived in the progression of our in vitro−in vivo investigation, and thus, identification of new "trapping" detoxification pathways according to the "185" list of chemicals is needed before this model is really acceptable for use. This search is ongoing. Some of the most commonly applied "trapping" pathways in detoxification on training set chemicals are as follows:

- Nitroarene reduction → N-acetylation pathway,
- Oxidative O-dealkylation → glucuronidation pathway,
- Oxidative O-dealkylation → sulfation pathway,
- Epoxide hydration → glutathione conjugation pathway, etc.

The nitroarene reduction → N-acetylation pathway is involved in the liver "trapping" detoxification of 4-nitrobenzoic acid as illustrated in Figure 12.

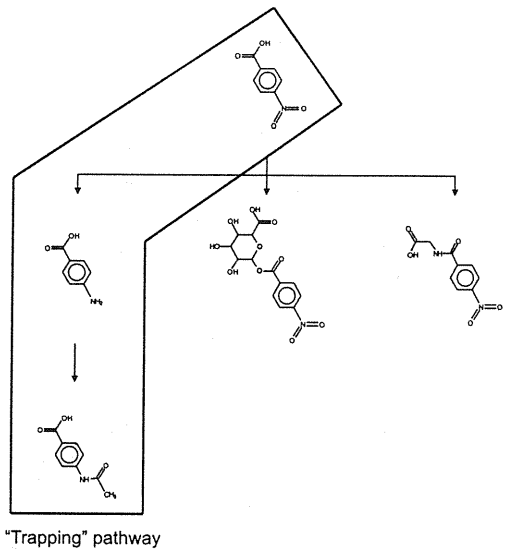


**Figure 12.** Simulated metabolic tree of 4-nitrobenzoic acid (62-23-7). The parent chemical and its metabolite 4-aminobenzoic acid are considered to be "trapped" in a liver detoxification pathway.

4-Nitrobenzoic acid was found to be excreted in rat urine as 4-aminobenzoic acid and its conjugates after oral and intraperitoneal administration.[76] Currently, the "false positive" chemicals are subjected to an expert analysis of their genotoxic potential; eventually, this will result in an expanded list of "trapping" detoxification pathways in liver.

## SUMMARY AND CONCLUSIONS

A workflow relating genotoxicity effects at three different levels of biological organization has been constructed to facilitate


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the systematic evaluation of empirical data. This required the collection of a large amount of data for in vitro mutagenicity (Ames, CA, and MLA); in vivo liver genotoxicity (UDS, Comet, and TGR); and in vivo bone marrow genotoxicity (MNT) of diverse chemicals. The database has been subjected to a critical analysis to minimize as many inconsistencies as possible between the different sources.

A number of levels of the in vitro–in vivo relationship can be derived in the workflow (as depicted in Figure 1). A first level begins with the in vitro negative (nonmutagenic) chemicals, for which two possible in vivo genotoxicity outcomes appear to be feasible. The majority of these chemicals are not expected to produce in vivo genotoxic damage neither in liver nor in the remote bone marrow (level I). However, for a small minority of the nonmutagenic chemicals, in vivo bioactivation reactions can take place to produce reactive metabolites capable to induce in vivo genotoxicity (level II). The principle organ for in vivo metabolic activation is assumed to be liver; no examples for direct bone marrow activation were identified. According to the adopted in vitro–in vivo relationship developed in this work, in vitro negative results can usually be used as sufficient evidence for a lack of in vivo genotoxicity.

The fate of in vitro positive chemicals in vivo is also described. First, because of in vivo detoxification "logic", in vitro positive chemicals could be deactivated in liver; subsequently, no in vivo MNT effect is expected in the bone marrow for these chemicals (level III). The in vivo detoxification "logic" is simulated by introducing so-called "trapping" metabolic pathways. In contrast with the in vitro-generated metabolites, which are freely available to interact with macromolecules, the metabolites in vivo are "trapped" by being engaged in enzyme complexation (channeling effects) and subsequently are unable to interact with DNA and proteins. In vitro positives would also be in vivo liver positive if parent compounds and/or metabolites are not engaged in detoxification pathways. Considering this, there are two options: in vivo liver positives could be "bioexhausted" (e.g., extremely reactive chemicals involved in off-target protein reactions approaching to the bone marrow) and thus lack in vivo MNT effects (level IV), or alternatively, the in vivo liver genotoxic chemicals are in vivo MNT positive if available at the remote target (level V).

The development of the genotoxicity workflow is based on the main assumption that any differences in vitro and in vivo for the same chemicals can be attributed to differences in their bioavailability in the organs of investigation rather than their reactivity. In other words, parent compounds and/or metabolites, which are reactive toward DNA and proteins, could have different in vitro/in vivo effects due to differences in their availability in target organs.

On the basis of the scheme, two models for in vivo genotoxicity have been developed. The models have been combined on the same platform: a new in vivo metabolism simulator explicitly describing the in vivo detoxification effects and a reactivity module based on the electrophilicity of chemicals toward DNA and proteins. Given the accuracy of experimental data (approximately 75–80%), the in vivo MNT model exhibited a reasonable performance: sensitivity of 82% and specificity of 61%.

On the other hand, the in vivo liver genotoxicity model was developed as an outcome of the relationships established in the scheme. According to these relationships, in vitro mutagenic chemicals that are not involved in "trapping" detoxification pathways are considered capable of causing DNA and/protein damage and hence in vivo liver genotoxic effects. Thus, the overall performance of the current model appears to be relatively low (sensitivity of 85% and specificity of 49%). This insufficiency is attributed to the fact that the model is indirectly derived as a result of the in vitro–in vivo gap investigation, rather than from a training set of chemicals. Hence, the current model does not claim to be complete and will require further work (which is ongoing) before it is acceptable for use. By deriving it, we rather aimed to demonstrate the feasibility of the workflow for modeling complex genotoxicity end points. Further work will be focused on collecting more experimental data and performing further in-depth analysis on the training set chemicals to rationalize their detoxification pathways. While the workflow has been derived using genotoxicity information, the approach could be potentially generalized to examine in vitro–in vivo relationships for other complex end points.

## ■ ASSOCIATED CONTENT

### Supporting Information

Appendices I–III containing the set of chemicals and their corresponding calls in the various in vitro and in vivo liver genotoxicity and MNT tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ABBREVIATIONS

MNT, micronucleus test; CA, chromosomal aberration; MLA, mouse lymphoma assay; UDS, unscheduled DNA synthesis; TGR, transgenic rodent gene mutation assay; TIMES, tissue metabolism simulator; QSAR, quantitative structure–activity relationship; SA, structural alerts; ADME, absorption, distribution, metabolism, excretion; *hprt*, hypoxanthine–guanine phosphoribosyltransferase

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# A chemical category approach of genotoxicity studies for branched alkylphenols

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A chemical category is a group of chemicals whose toxicological properties are expected to be similar or follow a regular pattern as a result of structural similarity. The category approach is beneficial for decreasing in the resource of risk assessment for huge amount of unevaluated existing chemicals, and also in the use of all kinds of animal tests including even *in vivo* genotoxicity tests from a point of view of the animal welfare. The present paper reports the results of *in vivo* micronucleus tests of *o-sec*-butylphenol (CAS : 89-72-5) and 2-isopropyl-5-methylphenol (CAS : 89-83-8) and discusses genotoxic potential of seven alkylphenols, *o-sec*-butylphenol, 2-isopropyl-5-methylphenol, *p-sec*-butylphenol (CAS : 99-71-8), 2-*tert*-butylphenol (CAS : 88-18-6), 2, 4-di-*tert*-butylphenol (CAS : 96-76-4), 4-*tert*-butylphenol (CAS : 98-54-4) and 6-*tert*-butyl-*m*-cresole (CAS : 88-60-8) by the category approach. Based on the negative results of *in vivo* micronucleus tests, it can be concluded that these category chemicals are not likely clastogenic *in vivo*. Further *in vivo* micronucleus assays on untested substances may not be required by using the category approach, but further supporting information such as physicochemical profiles and (Q) SAR predictions may be necessary to strengthen the rationale for the category approach.

Keywords : category approach, alkylphenol, genotoxicity

## Introduction

A chemical category is a group of chemicals whose toxicological properties are expected to be similar or follow a regular pattern as a result of structural similarity. A category approach is used in many chemical programmes such as the OECD High Production Volume (HPV) programme[1], the US HPV Challenge programme[2] and the EU Existing Substances programme[3]. The overall data set can allow the estimation of the hazard for the untested endpoints. Data gap filing can be done from one or more tested chemicals to an untested chemical. The category approach is effective for hazard identification and hazard estimation, and it is beneficial for decreasing in the resource of risk assessment for huge amount of unevaluated existing chemicals, and also in the use of all kinds of animal tests including even *in vivo* genotoxicity tests from a point of view of the animal welfare.

Structurally similar alkylphenols shown in Table1 are listed in the most recent OECD HPV List of chemicals to be investigated for environment and human health effects[4] and were selected as target substances for the Safety Examination of Existing Chemicals in Japan in order to obtain reliable information in compliance with the OECD Test Guidelines and in accordance with the principles for GLP[5]. Of these chemicals, 4-*tert*-butylphenol (CAS : 98-54-4) and 6-*tert*-butyl-*m*-cresole (CAS : 88-60-8) were already assessed under the OECD HPV programme[6]. In the OECD HPV programme, screening information data sets (SIDS) for at least two different genotoxic endpoints have been required for the initial assessment[7], and the Ames assays and *in vitro* chromosome aberration assays for these chemicals were performed.

Table 1 shows summary results of genotoxicity studies of the branched alkylphenols. All the chemicals showed negative results in the Ames assays with and

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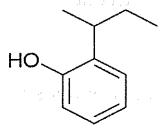
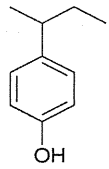
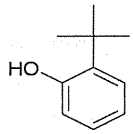
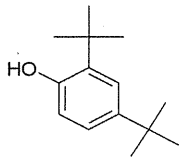
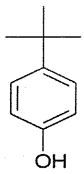
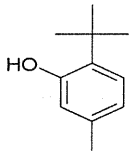
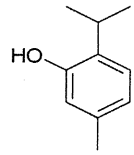
**Table 1** Summary results of genotoxicity studies of branched alkylphenols conducted under the Safety Examination of Existing Chemicals in Japan

| Substance name (CAS No.) | Structure | S9 | Ames | In vitro Chromosome Aberration | | In vivo micronucleus |
|---|---|---|---|---|---|---|
| | | | | [C][a] | [P][b] | |
| *o-sec*-Butylphenol (89-72-5) |  | w/o | − | + | − | [current study] |
| | | with | − | + | − | |
| *p-sec*-Butylphenol (99-71-8) |  | w/o | − | ± | − | |
| | | with | − | ± | − | |
| 2-*tert*-Butylphenol (88-18-6) |  | w/o | − | − | − | −[c] |
| | | with | − | + | + | |
| 2,4-di-*tert*-Butylphenol (96-76-4) |  | w/o | − | − | − | |
| | | with | − | + | − | |
| 4-*tert*-Butylphenol [c] (98-54-4) |  | w/o | − | − | + | − |
| | | with | − | + | + | |
| 6-*tert*-Butyl-*m*-cresole [c] (88-60-8) |  | w/o | − | − | − | −[d] |
| | | with | − | + | − | |
| 2-Isopropyl-5-methylphenol (89-83-8) |  | w/o | − | − | − | [current study] |
| | | with | − | + | − | |

[a] [C] : Clastogenicity ; [b] [P] : Polyploidy ; [c] The initial assessment of the chemical was already assessed under the OECD HPV programme ; [d] The in vivo micronucleus test was carried out by the Chemicals Evaluation and Research Institute, Japan. ; [e] The result form the German Chemical Society-Advisory Committee on Existing Chemicals of Environmental Relevance (Beratergremium fur Umweltrevante Alstoffe : BUA)[21]

without metabolic activation. On the other hand, an equivocal result on *p-sec*-butylphenol (CAS : 99-71-8) and positive results on the other six chemicals were observed for clastogenicity in the *in vitro* chromosome aberration assays with and/or without metabolic activation. Polyploidy was also observed for 2-*tert*-butylphenol (CAS : 88-18-6) and 4-*tert*-butylphenol with and/or without metabolic activation. However, *in vivo* micronucleus tests on 4-*tert*-butylphenol (not publically available) and 6-*tert*-butyl-*m*-cresole[6] showed negative results, and these findings seem to suggest that these branched alkylphenols can be non-genotoxic *in vivo* although *in*

*vitro* genotoxicity is equivocal.

These branched alkylphenols are widely used as antioxidants in rubbers, plastics, foods and oils to inhibit or slow oxidative process, and they are also used as intermediates for synthesis of resins, plasticizers, surface-active agents, perfumes and other products[8]. Consumer exposure to these branched alkylphenols can occur through the use of products containing these chemicals. The similarities in structure, use and *in vitro* genotoxicity seem to support grouping these chemicals into one category. To ascertain robustness of the chemical category of these branched alkylphenols on genotoxicity, additional *in vivo* micronucleus tests were assessed on 2-isopropyl-5-methylphenol (CAS : 89-83-8) as a target of isopropyl substitutions and *o-sec*-butylphenol (CAS : 89-72-5) as a target of *sec*-butyl substitutions. The present paper reports the results of *in vivo* micronucleus tests of 2-isopropyl-5-methylphenol and *o-sec*-butylphenol and discusses genotoxic potential of these chemicals by the category approach.

## Materials and Methods

*In vivo micronucleus test in mouse bone marrow*

The test was performed according to the Guideline for Screening Mutagenicity Testing of Chemicals, Japan and OECD TG 474[9] and in compliance with GLP requirements[5]. 2-Isopropyl-5-methylphenol (Purity> 98% ; Lot No.CAN1119) was obtained from Wako Pure Chemical Industries, Ltd., Japan and cyclophosphamide (CAS No.50-18-0 ; Lot No.73H0846) obtained from Sigma Chemical Co. was used as a positive control. Crj : BDF1 mice, 8-weeks of age, from Charles River Laboratories, Japan were used after more than 1 week acclimatization. Mice were housed in a temperature-and humidity-controlled room (23±1℃ ; 55±5%) with a light-dark (12 h-12 h) cycle. In a dose finding study, 5 male and 5 female mice were singly given 2-isopropyl-5-methylphenol by gavage at 500, 750, 1000, 1250, 1750 or 2000 mg/kg bw, in which deaths were observed at 1500 and 1750 mg/kg bw in females while no death was observed in males for four days (data not shown). Subsequently, a single dose of 2-isopropyl-5-methylphenol at 1500-2000 mg/kg bw caused deaths in males in a preliminary study (data not shown) ; therefore, 1250 mg/kg bw was set as the highest dose in the main test. Sampling time was set at 24 h after administration according to the preliminary study, in which no differences were observed in a sampling time of 24, 48, or 72 h.

Mice (5/sex/dose) were received single oral gavage administration of 2-isopropyl-5-methylphenol at 0 (control : olive oil), 156.3, 312.5, 625, or 1250 mg/kg bw. Positive control mice (5/sex) received single oral doses of cyclophosphamide at 50 mg/kg bw. All groups of mice were killed 24 h after treatment. Bone marrow samples were prepared according to the method of Schmid[10,11] for the control, 312.5, 625, or 1250 mg/kg bw groups. Samples were stained with 0.04 mg/mL acridine orange. According to the method of Hayashi et al.[12], the incidence of micronuclei was determined. Two thousand polychromatic erythrocytes (PCE) for each animal were observed for the incidence of micronucleated erythrocytes, and the proportion of PCE among the total erythrocyte population was also determined from a sample of 500 total erythrocytes for each animal.

Data were analyzed using the Fisher's exact test with Bonferroni correction for group mean comparisons[13]. Dose-dependent increases of the number of micronucleated polychromatic erythrocyte per total number of PCE (MNPCE) was detected using the Cochran-Armitage test[14]. Proportion of PCE among the total erythrocyte population was analyzed by the t-test with Bonferroni correction.

*In vivo micronucleus test in rat bone marrow*

The test was performed according to OECD TG 474[9] and Guideline for Genotoxicity Tests on Drugs[15], and in compliance with GLP requirements[5]. *o-sec*-Butylphenol (Purity 99.15%) was obtained from Honshu Chemical Industry, Japan and cyclophosphamide monohydrate (CAS No.6055-199-2 ; Lot No.036K1225) obtained from Sigma-Aldrich Co. was used as a positive control. Crl : CD (SD) rats, 7-weeks of age, from Charles River Laboratories, Japan were used after one week of acclimatization. Rats were housed in a temperature-and humidity-controlled room (21.8-22.9℃ ; 46.6-62.2%) with a light-dark (12 h-12 h) cycle. The animals were given commercial food and water *ad libitum*. In a dose finding study, 3 male and 3 female rats were given *o-sec*-butylphenol by gavage at 150, 300, 600, 1200 mg/kg bw once a day for two days (24 h interval), in which deaths were observed at 1200 mg/kg bw/day and clinical changes were observed at 600 mg/kg bw/day in both sex (data not shown). Therefore, 600 mg/kg bw was set as the highest dose.