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S-Conjugate-dependent Toxicity: Alternatives to Animal Studies

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Abstract

Studies on the mechanisms of glutathione *S*-conjugate-dependent toxicity are a major focus of the research in our laboratory. These studies aim to elucidate the mechanism of the selective nephrotoxicity of a range of halogenated olefins or haloalkenes that undergo conversion to toxic metabolites by the cysteine conjugate β-lyase pathway. The β-lyase pathway is a multiorgan, multistep pathway that involves glutathione transferase-catalyzed glutathione *S*-conjugate formation in the liver, enzyme-catalyzed hydrolysis of the glutathione *S*-conjugates to the corresponding cysteine *S*-conjugates, active uptake of the *S*-conjugates by the kidney, and bioactivation by renal cysteine conjugate β-lyase. Although animal experiments are conducted to determine the nephrotoxicity of haloalkenes, a range of alternatives to the use of intact animals has also been employed in these studies. These alternatives include studies with purified and expressed enzymes, isolated rat and human hepatocytes, isolated rat renal proximal tubular cells, tissue culture, chemical models, Fourier-transform ion cyclotron resonance mass spectrometry, and computational chemistry. The use of the alternatives has allowed a reduction in the number of animals used and, in some cases, replacement of animals with *in vitro* models.

Introduction

Our laboratory investigates the mechanisms of glutathione *S*-conjugate-dependent toxicity. One specific aim of these studies is to elucidate the mechanism of the selective kidney toxicity or nephrotoxicity of a range of haloalkenes (Figure 1). Haloalkenes are widely used in industry, and some are formed as degradation products of anesthetic agents. Research on the mechanism of haloalkene-induced nephrotoxicity has demonstrated that haloalkenes undergo bioactivation by the cysteine conjugate β-lyase pathway, which is a

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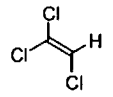

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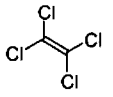

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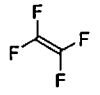
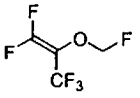


Tetrafluoroethylene

Chlorotrifluoroethylene



2-(Fluoromethoxy)-1,1,3,3,3-pentafluoro-1-propene (Compound A)

Fig. 1.
Examples of haloalkenes that undergo bioactivation by the cysteine conjugate β-lyase pathway.

multiorgan, multistep pathway; reviews about the β-lyase pathway have been published (*1,2*). Bioactivation is the process whereby nontoxic chemicals are enzymatically converted to toxic metabolites (*3*). The β-lyase pathway is shown in Figure 2. The initial step in the β-lyase-dependent bioactivation of haloalkenes involves hepatic glutathione transferase-catalyzed glutathione *S*-conjugate formation. For most haloalkenes, the microsomal glutathione transferase is the most important catalyst of glutathione *S*-conjugate formation. The glutathione *S*-conjugates thus formed are excreted in the bile and undergo sequential γ-glutamyltransferase- and dipeptidase-catalyzed hydrolysis to the corresponding cysteine *S*-conjugates. The cysteine *S*-conjugates may undergo β-lyase-catalyzed bioactivation or may be metabolized to mercapturates (*S*-substituted *N*-acetyl-L-cysteines), which may undergo acylase-catalyzed hydrolysis to regenerate the cysteine *S*-conjugates.

The glutathione and cysteine *S*-conjugates are actively taken up by amino acid transporters in the kidney, where they undergo bioactivation by renal cytosolic and mitochondrial cysteine conjugate β-lyases. Transport of cysteine *S*-conjugates into the kidney and renal β-lyase-catalyzed bioactivation are the target-organ determining steps in the nephrotoxicity of haloalkenes. Chloroalkene- and bromine-containing, fluoroalkene-derived cysteine *S*-conjugates are mutagenic, cytotoxic, and nephrotoxic, whereas bromine-lacking, fluoroalkene-derived cysteine *S*-conjugates are cytotoxic and nephrotoxic, but are not mutagenic.

Scientists in the U.S. must comply with federal regulations and policies that govern the use of vertebrate animals. The guiding principles about the use of vertebrate animals include: a reduction in the number of animals used, a refinement of techniques to limit animal use, and a replacement of animals with models that do not involve the use of animals. These are the "three Rs" of animal use. The regulations require that all investigations that involve vertebrate animals must be approved by an Institutional Animal Care and Use Committee (IACUC), which must contain both scientists and members of the public. Significantly, the IACUC can withhold approval of a protocol, but cannot dissapprove a protocol. Hence, the investigator and the IACUC must negotiate until a satisfactory protocol is developed. In developing a protocol for IACUC review, the investigator must provide assurance that a sufficient number of animals is used to answer the scientific question being asked, but that an excessive number of animals is not used, that procedures are used that limit pain and discomfort, that alternative models or techniques have been considered, and that acceptable methods of euthanasia are employed.

The use of vertebrate animals in biomedical research is extensive and will continue to be extensive for the foreseeable future. Indeed, the development of gene manipulation strategies that allow the development of transgenic and knock-out animals has increased the use of mice. Moreover, research aimed at developing

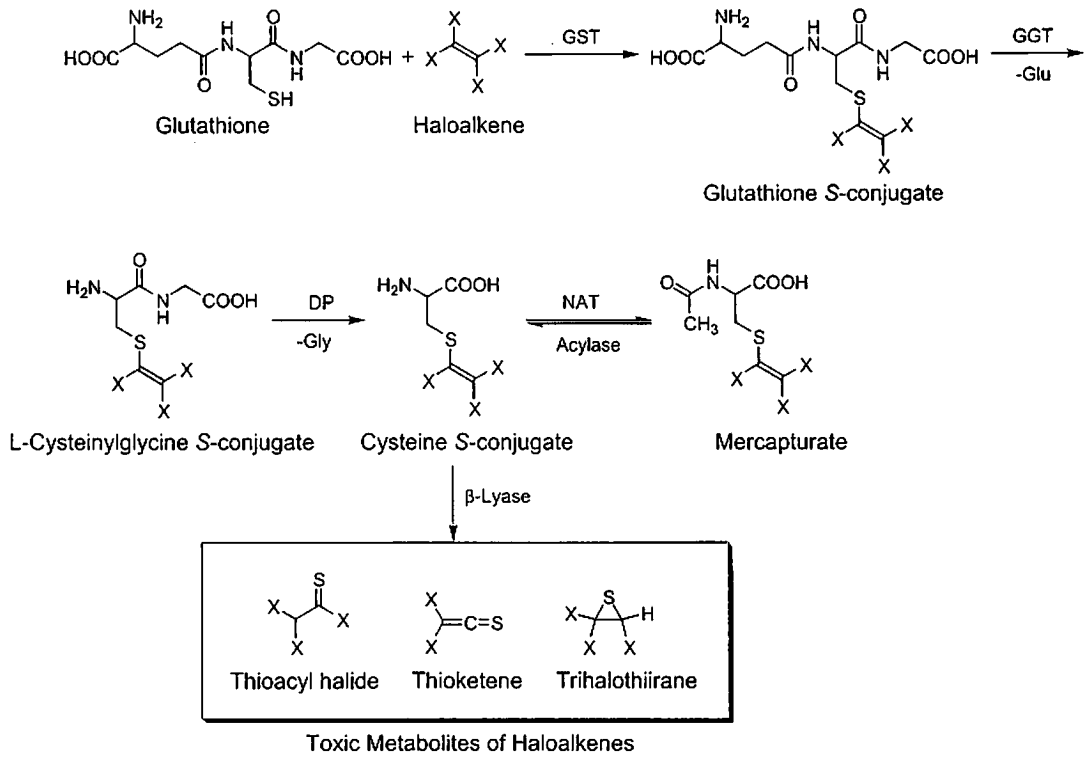


Figure 2.

Cysteine conjugate β-lyase pathway for the bioactivation of haloalkenes. GST, glutathione transferase; GGT, γ-glutamyltransferase; DP, dipeptidases; NAT, *N*-acetyltransferase. (See Figure 1 for structures of nephrotoxic haloalkenes that undergo cysteine conjugate β-lyase-dependent bioactivation.)

methods for gene therapy requires the use of vertebrate animals and will continue to be an active area of investigation for many years to come.

Vertebrate animals are used in our research on *S*-conjugate-induced toxicity, but not in large numbers. The β-lyase pathway involves several critical steps that occur in multiple organs. *In vitro* systems that do not involve the use of animals are not presently available that allow modeling of this multistep, multiorgan pathway. Although important advances have been made in the development of *in vitro* techniques, *in vivo* studies are necessary to ensure that the results of *in vitro* studies reflect events that occur in intact animals.

A range of alternatives to animals is used in our laboratory to study *S*-conjugate-induced toxicity. These alternatives include studies with purified and expressed enzymes, isolated rat and human hepatocytes, isolated rat renal proximal tubular cells, tissue culture, chemical models, Fourier-transform ion cyclotron resonance mass spectrometry, and computational chemistry. Examples of each of these alternatives will be given below along with an indication of how they respond to the "three Rs" of animal use.

Use of purified and expressed enzymes

Purified and expressed enzymes are used to study the individual steps in the β-lyase pathway. Enzymes involved include the microsomal glutathione transferase, γ-glutamyltransferase, aminopeptidase M, cysteinylglycine dipeptidase, and renal mitochondrial and cytosolic β-lyases. Although most purified enzymes are obtained from vertebrate animals, the high specific activity of purified enzymes

Table 1. Biosynthesis of S-(2-chloro-1,1,2-trifluoroethyl)glutathione in rat and human hepatocytes and in HepG2 cells (data are from Tanaka and Anders (15)).

Cell Source	*S*-(2-Chloro-1,1,2-trifluoroethyl)glutathione formed (nmol/mg protein/min)
Rat	26.1 ± 0.9
Donor 1	24.4
Donor 2	19.8
Donor 3	15
Donor 4	2.6
HepG2	5.3 ± 0.2

allows the conduct of a large number of experiments with a limited amount of purified protein. An expression system for the microsomal glutathione transferase has been described (*4*). In our laboratory, we have purified the human cytosolic β-lyase and have also conducted studies with human surgical waste (*5*). These studies demonstrated that the activity of human renal β-lyase is much lower than in the rat. Investigations into the enzymology of renal β-lyases show the presence of at least four renal β-lyases: low molecular-weight cytosolic and mitochondrial enzymes and high molecular-weight cytosolic and mitochondrial enzymes (*6,7*). Cytotoxic cysteine *S*-conjugates induce mitochondrial dysfunction (*8*), and the enzymology studies mentioned above demonstrated that a high molecular-weight mitochondrial β-lyase is likely responsible for the cytotoxicity of cysteine S-conjugates (*7*).

The sequences of the cDNAs encoding the low molecular-weight cytosolic β-lyase, which is identical with glutamine transaminase K, are available (*9-12*). The cDNA encoding the low molecular-weight cytosolic β-lyase has been expressed in COS-1 cells (*9*). The availability of cDNAs encoding all renal β-lyases will allow development of expression systems and detailed studies of the enzymology of the bioactivation of cysteine *S*-conjugates.

The use of purified and expressed enzymes allows both a reduction and replacement of animals in studying *S*-conjugate-induced toxicity.

Use of isolated rat and human hepatocytes

The initial step in the β-lyase pathway is the biosynthesis of haloalkene-derived glutathione *S*-conjugates. This reaction is preferentially catalyzed by the microsomal glutathione transferase (*13*), which is most abundantly expressed in the liver (*14*). Isolated rat hepatocytes with high viability can be obtained in high yield by the collagenase perfusion method. We have used isolated rat hepatocytes to study the stereochemistry of the reaction of chlorotrifluoroethylene (*13*); these studies showed the microsomal glutathione transferase catalyses the stereoselective addition of glutathione to chlorotrifluoroethylene and that the microsomal glutathione trans-

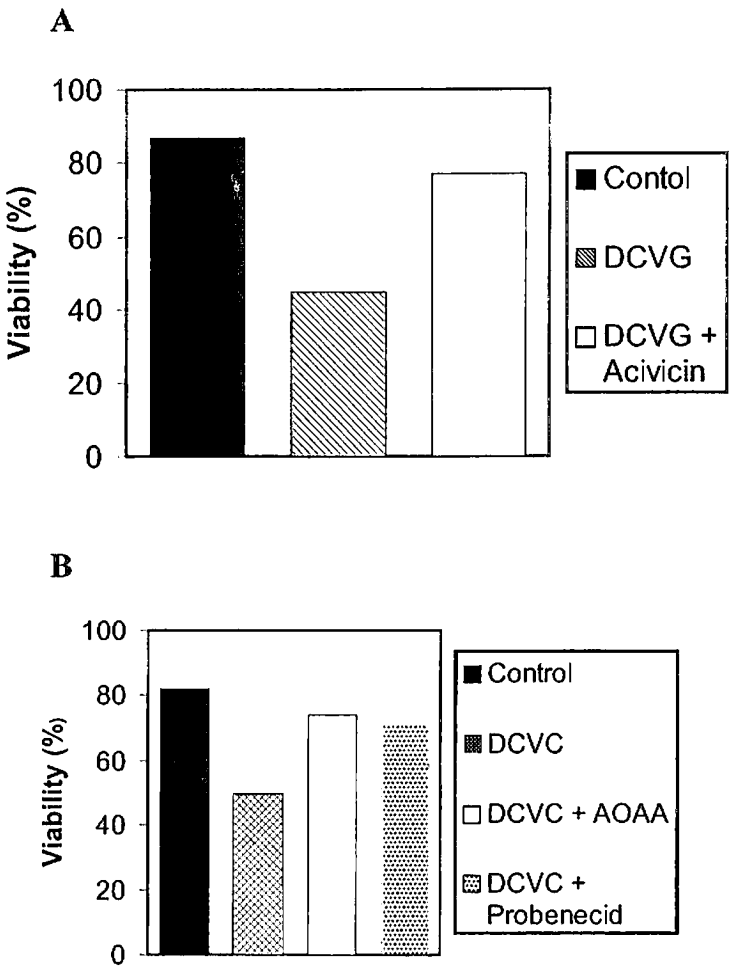


Figure 3. Effect of (aminooxy)acetic acid (AOAA) and probenecid on the cytotoxicity of S-(1,2-dichlorovinyl)glutathione (DCVG, panel A) and S-(1,2-dichlorovinyl)-L-cysteine (DCVC, panel B) in isolated rat renal proximal tubular cells. Data are from Lash and Anders (16).

ferase is responsible for about 85% of glutathione *S*-conjugate formation in rat hepatocytes.

Human hepatocytes are a highly relevant model system, and cells can be prepared from livers that are unsuitable for transplantation. In our experience, the yield and viability of human hepatocytes is usually lower that obtained with rat liver. We have used isolated human hepatocytes to study the reaction of glutathione with chlorotrifluoroethylene to give *S*-(2-chloro-1,1,2-trifluoroethyl) glutathione (*15*). These studies showed that the rate of glutathione *S*-conjugate formation is similar in rat and human hepatocytes and is higher in hepatocytes than in the liver-derived HepG2 cell line (Table 1).

The use of rat hepatocytes allows a reduction in the number of animals used, and the use of human hepatocytes allows replacement of experimental animals in studying *S*-conjugate biosynthesis.

Use of isolated rat renal proximal tubular cells

Cysteine *S*-conjugates are cytotoxic in isolated rat renal proximal tubular (IRPT) cells. Hence, IRPT cells are a useful model to study

S-conjugate-induced cytotoxicity. IRPT cells can be obtained in good yield (about 20 million per rat) and high viability by a collagenase perfusion method. We have used IRPT cells to explore the mechanisms of glutathione and cysteine S-conjugate-induced cytotoxicity (*16*) (Figure 3). One strategy used in defining the mechanism of S-conjugate-induced cytotoxicity was the use of selective inhibitors of key steps in the β-lyase pathway. For example, the γ-glutamyltransferase inhibitor acivicin blocks the conversion of glutathione S-conjugates to cysteine S-conjugates and is therefore expected to block cysteine S-conjugate-induced cytotoxicity. As shown in Figure 3A, acivicin blocks the toxicity of the glutathione S-conjugate of trichloroethylene, S-(1,2-dichlorovinyl)glutathione. (Aminooxy)acetic acid inhibits the pyridoxal phosphate-dependent β-lyase, which catalyzes the final step in the bioactivation of cysteine S-conjugates; accordingly, (aminooxy)acetic acid may be expected to block the cytotoxicity of cysteine S-conjugates. The data in Figure 3B show that (aminooxy)acetic acid blocks the cytotoxicity of S-(1,2-dichlorovinyl)-L-cysteine. Also, the uptake of cysteine S-conjugates by IRPT cells requires active transport, and probenecid blocks the cytotoxicty of S-(1,2-dichlorovinyl)-L-cysteine (Figure 3B). Both acivicin and (aminooxy)acetic acid are highly toxic and difficult to use effectively in intact animals. Hence, a particular advantage of IRPT cells is that highly toxic inhibitors can be used to dissect individual steps in the bioactivation of cysteine S-conjugates. The use of IRPT cells allows a reduction in the number of animals used to study S-conjugate-induced toxicity.

Use of tissue culture

Cultured pig kidney-derived LLC-PK1 cells have been used extensively to study S-conjugate-induced toxicity. Such cells mimic most, but not all, of the processes seen in IRPT cells. For example, the transport of cysteine S-conjugates appears to be different in LLC-PK1 cells compared with IRPT cells (*17,18*). Similar to results obtained with IRPT cells, the cytotoxicity of cysteine S-conjugates is blocked by inhibitors of the β-lyase pathway that can be used only with difficulty in intact animals (19,20). An important advantage of cultured cells is the ability to use them as a platform to construct stably transfected cell lines. As mentioned above, activity of the microsomal glutathione transferase is low in kidney cells, which possess all other steps in the β-lyase pathway. Accordingly, we constructed a stably transfected LLC-PK1 cell line that expresses elevated microsomal glutathione transferase activity (21). This cell line shows increased glutathione S-conjugate formation compared with wild-type cells or with cells transfected with a plasmid that lacked the cDNA for the microsomal glutathione transferase (Figure 4). Similarly, hexachlorobutadiene, which undergoes β-lyase-dependent bioactivation (22), is more cytotoxic in LLC-PK1 cells that express high microsomal glutathione transferase activity than in wild-type cells.

The use of LLC-PK1 cells allows replacement of animals in studying S-conjugate-induced toxicity.

Use of chemical models

The β-lyases are pyridoxal phosphate-dependent enzymes that catalyze both transamination and β-elimination reactions of cysteine S-conjugates (for a review of the enzymology of β-lyases, see (*23,24*)). Two chemical models that mimic the activity of β-lyases have been used in our studies. A Cu^{2+}/pyridoxal model is available that simulates β-lyase-catalyzed reactions (*25*). A model that uses N-dodecylpyridoxal in cetyltrimethylammonium micelles catalyzes both β-elimination and transamination reactions of cysteine S-conjugates, depending on the reaction conditions (*26*). We have used the N-dodecylpyridoxal model to degrade cysteine S-conjugates in

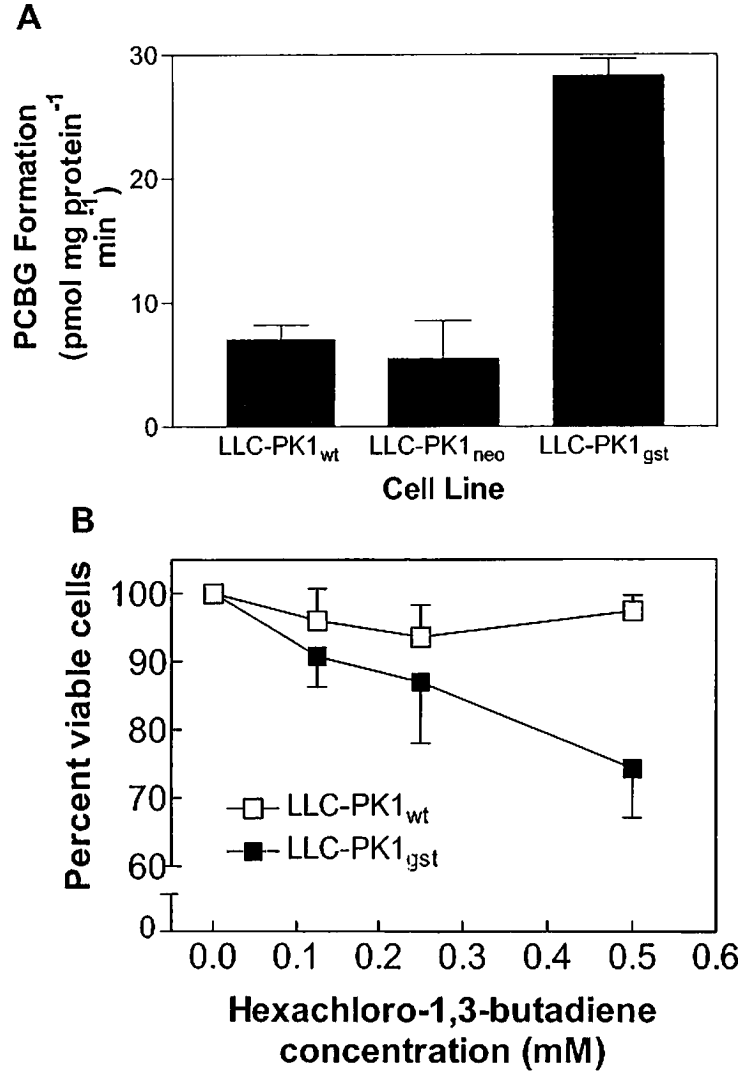


Figure 4. Biotransformation of hexachorobutadiene to *S*-(pentachlorobutadienyl)glutathione in wild-type LLC-PK1 cells (LLC-PK1$_{wt}$), in LLC-PK1 cells transfected with plasmid only (LLC-PK1$_{neo}$), or in cells stably transfected with the cDNA for human microsomal glutathione transferase (LLC-PK1$_{gst}$) (panel A) and cytotoxicity of hexachlorobutadiene in LLC-PK1$_{wt}$ (□) or in LLC-PK1$_{gst}$ (■) cells (panel B). Modified from Otieno and Anders (*21*).

studying the stereochemistry of glutathione *S*-conjugate formation (*13*). Both model systems have been used to study the bioactivation of cysteine *S*-conjugates of 2-(fluoromethoxy)-1,1,3,3,3-pentafluoro-1-propene (Compound A) (Z. Tong and M. W. Anders, unpublished observations).

In other studies, we developed stable, synthetically accessible precursors of reactive metabolites formed during the β-lyase-catalyzed bioactivation of cysteine *S*-conjugates (*27*); these studies allowed demonstration of the formation of thioketenes as *S*-conjugate-derived reactive intermediates.

The use of these chemical models allows replacement of animals in studying *S*-conjugate-induced toxicity.

Use of Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS)

The cytotoxicity of cysteine *S*-conjugates is associated with the formation of reactive intermediates that covalently modify cellular macromolecules. As mentioned above, chemical models and precursors of reactive intermediates have been used to study *S*-conjugate bioactivation. These strategies suffer from the disadvantage that the evidence for reactive-intermediate formation is indirect in that it depends on trapping reactions or on rationalizing reaction mechanisms from stable product formation. FT-ICR MS is a powerful tool for studying chemical reactions in the gas phase. In a dual-cell FT-ICR mass spectrometer, a reagent can be generated in one cell and allowed to react with a neutral molecule, *e.g.*, *N*-acetyl cysteine *S*-conjugate methyl ester, in the analyzer cell. The products formed in such ion-molecule reactions can be identified by their mass spectra, and collision-induced reactions can be used to study the formation of additional products. We have used FT-ICR MS studies to demonstrate thioketene formation from cysteine *S*-conjugates (*28*). Similarly, FT-ICR MS was used to study the fate of Compound A-derived cysteine *S*-conjugates (*29*). Although it may be argued that gas-phase reactions do not mimic condensed-phase reactions, the objective of mechanistic bioactivation studies is to gain insight into the chemical processes involved. Thus, FT-ICR MS experiments have proven valuable in studying *S*-conjugate bioactivation reactions.

The use of FT-ICR MS in studying *S*-conjugate-induced toxicity allows replacement of animal studies.

Use of computational chemistry

The formation and bioactivation of *S*-conjugates depend on enzyme-catalyzed chemical reactions that are amenable to simulation by computational chemistry. Both semi-empirical and *ab initio* calculations are used and are made possible by the availability of inexpensive, high-speed workstations. Although not concerned with *S*-conjugate-induced toxicity, we have used computational chemical methods to model the cytochrome P450-dependent biotransformation of hydrochlorofluorocarbons (*30*) and to develop strategies for the design of safer chemicals (*31*). Theoretical predictions about the reaction of thiolates with haloalkenes and about the formation of cysteine *S*-conjugate-derived reactive intermediates have been reported (*32,33*). We are presently developing a predictive model for the reaction of glutathione with a range of haloalkenes; preliminary data indicate that the calculated potential energy profiles for the reaction of ethanethiolate (as a surrogate for glutathione) with trichloroethylene reflect the experimentally observed glutathione transferase-catalyzed product formation (L. J. Jolivette and M. W. Anders, unpublished data).

Computational chemistry studies allow replacement of animals in studying *S*-conjugate-induced toxicity.

Conclusions

A range of alternatives to the use of animals has been exploited to study *S*-conjugate metabolism and toxicity. These strategies have been most successful in reducing the number of animals used or in the replacement of animals with expressed enzymes, cell lines, chemical models, FT-ICR MS, or computational chemistry. Refinement of *in vivo* techniques has not been implemented in our studies on *S*-conjugate-induced toxicity, which depend on classical clinical chemical and morphological endpoints. Although animal studies will continue to be used in *S*-conjugate research for the foreseeable future, advances in the development of alternatives to animals will continue to reduce the number of animals used.

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