Cell Biol Toxicol 2006; **22:** 339–349.
DOI: 10.1007/s10565-006-0079-z

Identification of reactive toxicants: Structure–activity relationships for amides

T.W. Schultz, J.W. Yarbrough and S.K. Koss
Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee, USA

Received 23 December 2005; accepted 10 May 2006; Published online 16 July 2006

Keywords: amides, *Tetrahymena pyriformis* toxicity, glutathione reactivity

Abstract

A diverse series of amides were evaluated for aquatic toxicity (IGC_{50}) assessed in the *Tetrahymena pyriformis* population growth impairment assay and for reactivity (EC_{50}) with the model soft nucleophile thiol in the form of the cysteine residue of the tripeptide glutathione. All alkylamides along with some halo-substituted amides are well predicted by the simple hydrophobicity ($\log K_{ow}$)–electrophilicity (E_{lumo}) response-surface model [$\log(IGC_{50}^{-1}) = 0.45(\log K_{ow}) - 0.342(E_{lumo}) - 1.11$]. However, 2-halo amides with the halogen at the end of the molecule and α,β-unsaturated primary amides are among those derivatives identified as being more toxic than predicted by the model. Amides, which exhibit excess toxicity, were capable of forming covalent bonds through an S_N2 displacement or a Michael addition. Moreover, only those amides exhibiting excess toxicity were reactive with thiol, suggesting that the reactivity with model nucleophiles such as the thiol group may provide a means of accurately defining reactive toxicants.

Abbreviations: DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); E_{lumo}, energy of the lowest unoccupied molecular orbital; GSH, glutathione; IGC_{50}, 50% growth inhibitory concentrations; $\log K_{ow}$, 1-octanol–water partition coefficients; QSAR, quantitative structure–activity relationship; REACH, Registration, Evaluation, and Authorization of Chemicals

Introduction

Because of the expected high costs of programs such as REACH, the Registration, Evaluation, and Authorization of CHemicals (Anon., 2001), the use of microscale surrogate test systems such as TETRATOX (Schultz, 1997) and structure–activity relationships are being evaluated as methods of filling in data gaps (Pavan et al., 2005). One of the more critical issues in predicting toxicity of industrial organic chemicals in a transparent manner is determining whether a toxicant is reactive and, especially in the case of electrophiles, the correct mechanism of that reactivity (Veith, 2004). Previous analyses, including those of Karabunarliev et al. (1996a) and Harder et al. (2003), have shown that *a priori* selection of the correct mode and in some cases the mechanism is essential to an accurate toxicity prediction. This selection is predicated on knowing the domain of applicability for each particular mode and mechanism of toxicity.

Cell-based aquatic test systems allow a mechanism of toxic action to be defined as what happens at the molecular/biochemical level, while a mode of toxic action is defined as what happens at the cellular/physiological level. Previous investigations (Seward et al., 2000; Schultz et al., 2004) revealed that the results from a simple cell population growth kinetics assay could segregate chemicals by mode of action. Specifically, such results segregate the reversible nonreactive mode of action from those acting by the irreversible reactive mode. Specifically, an early reduction in viable cells followed by population growth rates similar to controls is an indication of irreversible or reactivity toxicity; whereas an initial lag in population growth without loss of viability is an indication of a reversible or nonreactive toxicity.

The limitation of the applicability of organic chemistry to toxicology is that organic chemical reactions are often explained on the basis of experimental evidence acquired in environments (e.g., temperature, solvents) very different from those found in live biological systems. Nevertheless, the general rules of chemical reactivity are a good starting point for defining reactivity toxicity. The net result is that there are several molecular mechanisms of action (Jacobs, 1997). Included in the reactive molecular mechanisms is S_N2 displacement, which is associated with a halo-substituted methylene group activated by a carbonyl, such as in the Br–C–C(=O)–R structure. A second reactive molecular mechanism is Michael addition, which is associated with a polarized α,β-unsaturated group, such as the C=C–C(=O)–R structure. Michael addition is a nucleophilic addition in which a particular moiety such as a thiol group is "added" at the outer carbon atom of the carbon–carbon double bond.

While the acute aquatic toxicities of the majority of aliphatic industrial organic compounds, including simple amides, fit a generic, response-surface, quantitative structure–activity relationship (QSAR) (Schultz et al., 2002), carbonyl-containing compounds with an α-halo group (Schultz et al., 2002) or an α-carbon–carbon double bond (Schultz et al., 2005a) are considered to be reactive toxicants and are more potent than predicted by such a model.

Amides are carbonyl-containing compounds in which the hydroxyl group of the carboxylic acid has been replaced with an amino group. Compared to corresponding carboxylic acids, amides have higher boiling and melting points owing to their ability to form strong intermolecular hydrogen bonds. Amides can be primary, secondary, or tertiary in structure. The presence of the carbonyl group in amides means that they are subject to nucleophilic addition, especially if substituted with a β-unsaturated group, as in the case of acrylamides, or a leaving group, as in the case of 2-haloamides. Therefore, amides have the potential to elicit toxicity by several mechanism of toxic action. It is hypothesized that while alkylamides are nonreactive toxicants, α-halo-substituted and α,β-unsaturated amides are reactive toxicants.

The purpose of this study was to examine the aquatic toxicity and abiotic thiol reactivity of selected amides. The specific aims were: (1) to determine reactive toxicants by examining the effects on *Tetrahymena pyriformis* population growth of short-term exposure to representatives of each structural group; (2) to determine the toxic potency for larger series of amides in the *T. pyriformis* population growth impairment assay; (3) to compare observed toxicity with that predicted by the general aliphatic response-surfaces model; and (4) to explain the fit or lack of fit to the model in terms of the ability of selected amides to react abiotically with the model nucleophile thiol.

Materials and methods

Test amides

Twenty-nine amides were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) or Lancaster Synthesis Inc. (Windham, NH, USA) in the highest purity available (95% minimum) and were

not further purified. Stock solutions of the amides to be tested were prepared by dissolving the agents in sterile water or dimethyl sulfoxide just prior to use.

Time course toxicity testing

Impairment of *Tetrahymena* population growth may be the result of effects on either reproduction or survival; noncovalent interactions affect reproduction, while covalent interactions affect survival (Schultz et al., 2004). In an effort to determine which mode of action was the result of short-term exposure to amides, the kinetic effects on population density were examined for model compounds following the protocol of Schultz et al. (2004).

Based on the results of standard population growth impairment assays (Schultz, 1997), four concentrations (representing low, medium, high, and total growth impairment at 40 h) of propionamide, *N*-phenylacrylamide, and 2-bromoacetamide were evaluated. In addition, a control containing no test substance was evaluated. All such experiments were performed with 50 ml of peptone-supplemented organic medium in 250 ml foam-stoppered Erlenmeyer flasks. At time zero, the appropriate amount of toxicant was added to the medium, and the flask was swirled to evenly disperse the toxicant. Immediately after swirling, 300 μl of 40 h *T. pyriformis* culture was inoculated. After toxicant and ciliates were mixed, a sample was taken without delay ($t = 0$). Additional samples were taken at 30, 60, 120, 180, 300, and 420 min. At each time point, the flask was gently swirled (one rotation of the wrist) and a 300 μl aliquot was transferred from each initial test flask to a sterile 250 ml foam-stoppered Erlenmeyer flask containing 50 ml of medium. After the sampling period, all flasks were incubated at 27° ± 1°C until the subcultured control flasks had an absorbance of approximately 0.50 (50–54 h). Following incubation, population density was quantified spectrophotometrically by absorbance at 540 nm.

TETRATOX testing

Typical *T. pyriformis* population growth impairment (TETRATOX) testing was executed using the protocol described by Schultz (1997). This static 40-hour assay used population density measured spectrophotometrically at 540 nm as its endpoint. Test conditions allow for 8–9 cell cycles in control cultures; concentrations of DMSO <400 mg/L have no effect on cell growth.

Each amide was tested in a range finder prior to testing in definitive testing in duplicate for three separate tests each with a freshly prepared stock solution of the toxicant. Two controls, one with no test material but inoculated with *T. pyriformis* and the other a blank (having neither toxicant nor ciliates), were included with each replicate. Each definitive test replicate consisted of six to eight different concentrations with duplicate flasks of each concentration. Only replicates with control-absorbency values >0.6 but <0.8 were used in the analyses. The effect levels are based on nominal concentrations. The 50% growth inhibitory concentrations, IGC_{50}, were determined by Probit Analysis of Statistical Analysis System (SAS) software with absorbance normalized to control as the dependent variable and toxicant concentration as the independent variable.

Structure–toxicity modeling

The observed toxicities of all the amides were compared to potency estimated based on a previously developed (Schultz et al., 2002) model for nonspecific aliphatic toxicity predicted with the hydrophobicity- and electrophilicity-dependent linear regression equation

$$\begin{aligned}\log(IGC_{50}^{-1}) = {} & 0.45(0.014)(\log K_{ow}) \\ & - 0.342(0.035)(E_{lumo}) \\ & - 1.11(0.05) \end{aligned} \quad (1)$$

with $n = 353$, r^2 (adjusted) $= 0.859$, $s = 0.353$, r^2 (predicted) $= 0.857$. Logarithms of the 1-octanol–water partition coefficients ($\log K_{ow}$)

values were secured as measured or estimated values from ClogP for Windows software (BIOBYTE Corp., Claremont, CA, USA). The quantum-chemical term energy of the lowest unoccupied molecular orbital (E_{lumo}) was calculated by the AM1 method, as implemented in TSAR version 3.3 (Accelrys Inc, Oxford, UK).

Thiol reactivity

The thiol group of glutathione (GSH) was used as a model nucleophile, and chemical reactivity assessments were conducted in an abiotic concentration–response method for selected amides with free thiol being quantified spectrophotometrically at 412 nm (Schultz et al., 2005b).

Briefly, GSH was prepared fresh by dissolving 0.042 g of reduced glutathione in 100 ml of phosphate buffer at pH 7.4. Stock solutions of the amides were prepared by dissolving them in 5 ml of DMSO. Subsequently, phosphate buffer was added to the amide–DMSO solution so that the concentration of DMSO in the final solution was less than 5%, a concentration that has no effect of reactivity. Initial range-finding experiments were followed by definitive experiments with the concentrations adjusted so that there were no fewer than three partial effects and one partial effect on each side of the 50% effect concentration. Experiments were repeated with fresh GSH and toxicant solutions and two vials of buffer, one with GSH (the control) and the other without GSH (the blank) were included in each assay.

To each vial, 1 ml of GSH solution was added, followed by an aliquot of amide stock solution and an appropriate amount of phosphate buffer to bring the final volume to 10 ml. The net result was a final thiol concentration of 0.1375 mmol/L. Vials were shaken gently and let stand for 120 min prior to the addition of 200 μl of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and immediately reading of absorbance at 412 nm. The effect levels were determined from nominal toxicant concentrations, with the EC_{50} values being determined by Probit Analysis of SAS software with absorbance normalized to control as the dependent variable and toxicant concentration as the independent variable.

Results

Time course toxicity testing

The effect of the length on population density of exposure to the saturated alkyl amide propionamide is shown in Table 1. Regardless of length of exposure up to 7 h, no significant effect is observed with concentrations that elicit an effect less than total inhibition. As seen by the standard deviation values, these effects are highly reproducible. These results suggest that, like saturated alkyl alcohols (Schultz et al., 2004), saturated alkyl amides act via a reversible mode of toxic action.

The effects on population density of the length of exposure to 2-bromoacetamide are shown in Table 2. This compound elicits

Table 1. Effect of short-term exposure to propionamide (mean (absorbance at 412 nm × 100) ± SD of 3 replicates)

		Exposure time (min)				
Concentration	50 h absorbance	0	30	60	120	240
0	83.3 ± 2.89	48.3 ± 2.08	49.0 ± 2.65	48.7 ± 1.15	48.3 ± 3.06	48.0 ± 2.00
5 000	65.7 ± 5.13	48.3 ± 1.53	49.3 ± 2.08	48.3 ± 2.08	48.7 ± 3.06	49.0 ± 1.73
10 000	50.7 ± 5.03	49.0 ± 2.00	48.0 ± 2.00	50.0 ± 1.00	48.7 ± 1.15	47.3 ± 1.15
12 500	20.3 ± 1.53	50.0 ± 2.65	49.3 ± 1.53	48.7 ± 1.53	48.0 ± 1.73	47.7 ± 1.15
15 000	8.00 ± 2.00	49.3 ± 3.06	50.0 ± 2.00	49.0 ± 2.65	48.0 ± 3.61	47.0 ± 3.46

Table 2. Effect of short-term exposure to 2-bromoacetamide (mean (absorbance at 412 nm × 100) ± SD of 3 replicates)

		Exposure time (min)				
Concentration	50 h absorbance	0	30	60	120	240
0	80.7 ± 1.15	50.3 ± 2.52	49.0 ± 2.00	50.3 ± 2.08	47.3 ± 3.51	47.7 ± 3.51
50	64.7 ± 3.06	48.3 ± 1.53	42.7 ± 3.06	37.7 ± 3.79	27.7 ± 5.51	25.0 ± 5.00
100	39.0 ± 3.06	48.0 ± 1.00	34.0 ± 3.61	23.0 ± 5.29	13.7 ± 7.64	9.33 ± 5.03
200	8.70 ± 1.15	46.0 ± 2.00	25.7 ± 3.21	15.0 ± 4.00	4.3 ± 5.13	3.33 ± 3.06
250	0.00 ± 0.00	46.3 ± 1.15	11.7 ± 2.08	4.0 ± 3.46	0.00 ± 0.00	0.00 ± 0.00

significant reductions in population levels in both a concentration-dependent and length of exposure-dependent fashion. While qualitatively these relationships are consistent, the standard deviations reveal that quantitatively they vary significantly, especially for intermediate concentrations. The results for 2-bromoacetamide are consistent with compounds thought to act via an irreversible covalent mode of action as direct-acting electrophiles and are very similar to those reported by Seward et al. (2000) for other compounds with strong leaving groups and considered to act by the S_N2 displacement mechanism (Jacobs, 1997).

The effect on population density of the length of exposure to the polarized α,β-unsaturated *N*-phenylacrylamide is shown in Table 3. This compound also elicits significant reductions in population levels in both a concentration-dependent and length of exposure-dependent fashion. As with 2-bromoacetamide, qualitatively these relationships are consistent, but quantitatively they also vary, especially for the intermediate concentrations. These results are consistent with those observed by Schultz et al. (2004) for other polarized α,β-unsaturates considered to act via a irreversible covalent mode of action as direct-acting electrophiles, specifically as Michael-type acceptors (Jacobs, 1997).

The results of the time course growth kinetics experiments taken collectively support the proposition that amides act by different modes of action, which are structure-dependent.

TETRATOX testing

A summary of the Chemical Abstract Service registry number, structure, toxicity (mmol/L), and selected molecular descriptor values is given in Table 4. An examination of toxicity data reveals several trends. Generally, the potency of the alkyl-substituted amides is inversely related to molecular size and independent of whether the amide is primary, secondary or tertiary. The halogen-substituted analogues are more toxic than their alkyl-substituted counterparts, and bromo-substituted derivatives are more toxic than chloro-substituted ones. Furthermore, with the exception of the fluoro-derivative, 2-halo-substituted derivatives are more toxic than other halo-substituted

Table 3. Effect of short-term exposure to *N*-phenylacrylamide (mean (absorbance at 412 nm × 100) ± SD of 3 replicates)

		Exposure time (min)				
Concentration	50 h absorbance	0	30	60	120	240
0	82.3 ± 2.52	49.7 ± 3.21	49.3 ± 2.08	47.7 ± 4.04	49.0 ± 1.00	48.0 ± 2.00
200	65.3 ± 3.06	47.0 ± 1.73	44.7 ± 2.52	38.7 ± 3.21	30.0 ± 4.00	28.7 ± 3.21
500	37.3 ± 4.04	48.3 ± 2.08	40.3 ± 3.51	26.3 ± 4.51	17.3 ± 3.51	14.0 ± 3.00
700	14.0 ± 3.61	46.0 ± 1.73	34.7 ± 4.16	25.7 ± 2.08	3.3 ± 3.06	4.3 ± 5.13
900	0.00 ± 0.00	46.0 ± 1.00	11.0 ± 3.00	5.70 ± 4.93	0.70 ± 1.15	0.00 ± 0.00

Table 4. Toxicity and molecular descriptor values for selected amides

Name	CAS number[a]	SMILES[b]	IGC_{50} (mmol/L)	log K_{ow}^{c}		E_{lumo} (eV)[d]
Acetamide	60-35-5	CC(O)N	210	−1.26	M	3.2748
Propionamide	79-05-0	CCC(=O)N	123	−0.66	M	1.5499
n-Butyramide	541-35-5	CCCC(=O)N	61.0	−0.21	M	1.5509
2-Methylpropionamide	563-83-7	CC(C)C(=O)N	75.8	−0.25	E	1.6112
Trimethylacetamide	754-10-9	CC(C)(C)C(=O)N	30.3	0.20	E	1.6382
n-Hexanoamide	628-02-4	CCCCCC(=O)N	8.10	0.80	E	1.5499
N-Methylpropionamide	1187-58-2	CCC(=O)NC	33.6	−0.21	E	1.5132
N-Ethylacetamide	625-50-3	CC(=O)NCC	50.2	−0.21	E	1.5504
N-Propylacetamide	5331-48-6	CC(=O)NCCC	31.5	0.29	E	1.5500
N-(*tert*)Butylacetamide	762-84-5	CC(=O)NC(C)(C)C	14.4	0.67	E	1.5500
N,*N*-Dimethylacetamide	127-19-5	CC(=O)N(C)C	88.9	−0.77	M	1.4311
N,*N*-Diethylacetamide	685-91-6	CC(=O)N(CC)CC	34.8	0.34	M	1.4997
N,*N*-Dimethylpropionamide	758-96-3	CCC(=O)N(C)C	37.5	−0.11	M	1.4311
2-Fluoroacetamide	640-19-7	C(F)C(=O)N	>150	−1.05	M	0.9396
2-Chloroacetamide	79-07-2	C(Cl)C(=O)N	0.924	−0.62	M	0.6784
2,2-Dichloroacetamide	683-72-7	C(Cl)(Cl)C(=O)N	9.49	−0.19	M	0.0732
2,2,2-Trichloroacetamide	594-65-0	C(Cl)(Cl)(Cl)C(=O)N	1.94	1.04	M	−0.6363
2-Bromoacetamide	683-57-8	C(Br)C(=O)N	0.030	−0.52	M	0.1896
2-Chloropropionamide	27816-36-0	CC(Cl)C(=O)N	27.7	−0.16	E	0.6416
2-Bromopropionamide	5875-25-2	CC(Br)C(=O)N	0.993	−0.07	E	0.0574
3-Chloropriopionamide	5875-24-1	C(Cl)CC(=O)N	39.1	−0.42	E	1.2411
2,3-Dibromopropionamide	15102-42-8	C(Br)C(Br)C(=O)N	0.012	0.27	E	−0.2387
2-Chlorobutyramide	7462-73-9	CCC(Cl)C(=O)N	16.5	0.33	E	0.6718
2-Chloro-*N*,*N*-diethylacetamide	2315-36-8	C(Cl)C(=O)N(CC)CC	0.301	1.08	E	0.9670
Acrylamide	79-06-1	C=CC(=O)N	6.41	−0.78	M	0.3336
N-Isopropylacrylamide	2210-25-5	C=CC(=O)NC(C)C	20.3	0.57	E	0.3340
N-phenylacrylamide	2210-24-4	C1CCCCC1NC(=O)C=C	0.765	1.45	E	0.3340
N,*N*-Dimethylacrylamide	2680-03-7	C=CC(=O)N(C)C	17.2	−0.13	E	0.3348
N,*N*′-Methylenebisacrylamide	110-26-9	C=C(=O)NCNC(=O)C=C	3.27	−1.52	E	0.3336

[a]Chemical Abstract Services registry number.
[b]Simplified Molecular Input Entry System.
[c]1-Octanol–water partition coefficient; E = estimated value; M = measured value.
[d]Energy of the lowest unoccupied molecular orbital.

amides. Lastly, α,β-unsaturated primary amides are more toxic than the saturated amides.

Structure–toxicity modeling

In Table 5, the experimental or observed toxicity is compared with that predicted by the general aliphatic response-surface QSAR, equation (1). Toxicities of all 13 of the alkylamides, regardless of whether they are primary, secondary, or tertiary amides were well predicted by the hydrophobicity- and electrophilicity-dependent response-surface model.

Results for the halogen-substituted amides are less uniform. Both the 2-chloro- and 2-bromoacetamide (2-halo derivatives with the halogen at the end of the molecule) are more toxic than predicted by equation (1); similarly, the observed toxic potency of the 2-halo-tertiary amine 2-chloro-*N*,*N*-diethylacetamide is in excess of that

Table 5. Observed versus predicted toxicity for selected amides

Amide	$\log(IGC_{50}^{-1})$ Observed	$\log(IGC_{50}{}^{-1})$ Predicted by equation (1)
Acetamide	−2.32	−2.80
Propionamide	−2.09	−1.94
n-Butyramide	−1.79	−1.73
2-Methylpropionamide	−1.85	−1.77
Trimethylacetamide	−1.48	−1.58
n-Hexanoamide	−0.91	−1.28
N-Methylpropionamide	−1.53	−1.72
N-Ethylacetamide	−1.70	−1.73
N-Propylacetamide	−1.50	−1.51
N-(*tert*)Butylacetamide	−1.16	−1.34
N,*N*-Dimethylacetamide	−1.95	−1.95
N,*N*-Diethylacetamide	−1.54	−1.47
N,*N*-Dimethylpropionamide	−1.50	−1.37
2-Fluoroacetamide	< −2.17	−1.90
2-Chloroacetamide	*0.03*	−1.58
2,2-Dichloroacetamide	−0.98	−1.05
2,2,2-Trichloroacetamide	−0.29	−0.42
2-Bromoacetamide	*1.52*	−1.41
2-Chloropropionamide	−1.44	−1.40
2-Bromopropionamide	*0.00*	−1.16
3-Chloropriopionamide	−1.59	−1.72
2,3-Dibromopropionamide	*1.91*	−0.91
2-Chlorobutyramide	−1.22	−1.19
2-Chloro-*N*,*N*-diethylacetamide	*0.52*	−0.95
Acrylamide	*−0.81*	−1.58
N-Isopropylacrylamide	−1.31	−0.97
N-Phenylacrylamide	*0.12*	−0.57
N,*N*-Dimethylacrylamide	−1.24	−1.28
N,*N*′-Methylenebisacrylamide	*−0.51*	−1.91

predicted by this QSAR. However, the potencies of 2,2-dichloro- and 2,2,2-trichloroacetamide are both well predicted by this model. Similarly, the toxicities of 2-chloropropionamide, 3-chloropropionamide, and 2-chlorobutyramide are near the values predicted with the generic response-surface model. However, the observed toxicities of both 2-bromopropionamide and 2,3-dibromopropionamide are far in excess of those predicted by equation (1).

Results for the α,β-unsaturated amides, while more limited in number, are more directly interpreted. The observed toxicity for α,β-unsaturated primary amides is in excess of that predicted with the general response-surface QSAR (note that despite what may be surmised from its name, *N*,*N*′-methylenebisacrylamide is really a di-α,β-unsaturated primary amide). The toxicities of the aliphatic α,β-unsaturated secondary and tertiary amides are well-predicted by this equation. However, the toxicity of the aromatic secondary amide *N*-phenylacrylamide is well in excess of that predicted.

Table 6. Abiotic thiol reactivity for selected amides

Amide	EC_{50} (mmol/L)[a]
Acetamide	NR at 1200
Propionamide	NR at 1000
2-Fluoroacetamide	NR at 100
2-Chloroacetamide	16.9
2,2-Dichloroacetamide	NR at 500
2-Bromoacetamide	0.263
2-Chloropropionamide	NR at 900
2-Bromopropionamide	24.5
3-Chloropriopionamide	NR at 900
2-Chloro-*N*,*N*-diethylacetamide	1.50
Acrylamide	14.3
N-Isopropylacrylamide	NR at 100
N-Phenylacrylamide	7.93
N,*N*′-Methylenebisacrylamide	9.71

[a]NR = not reactive.

Thiol reactivity

In an effort to make more sense of the modeling results, abiotic thiol reactivity experiments were run on selected amides; the reactivities (mmol/L) of these amides are shown in Table 6. Thiol reactivity is indicative of the ability to elicit an irreversible toxicity (Schultz et al. 2005b). Only those amides exhibiting toxic potency in excess of that predicted by equation (1) were reactive with the thiol in GSH.

Discussion

The toxicant-biological interactions cover a range from reversible interactions that cause narcosis to irreversible interactions that cause reactive toxicity. In general, modeling of reversible interactions is limited only by the availability of data for the toxicity endpoint in question. A greater challenge shown by these results is the development of models for the irreversible reactions, in particular those where covalent bonds are formed between the toxicant and cellular molecules.

Impairment of *Tetrahymena* population growth may be the result of effects on either reproduction or survival (Schultz, 2004). Specifically, noncovalent interactions affect reproduction without causing a reduction in inocula (i.e., initial cell counts); in contrast, covalent interactions affect survival, causing an immediate reduction in cell counts. The results of the time course cell population growth assays (see Table 1) reveal that alkylamides, which exhibit an initial lag in population growth without loss of cell viability, act in a manner consistent with the reversible narcotic mode of action (Schultz et al., 2004). In contrast, the results in Tables 2 and 3 reveal that amides with a leaving group (e.g., 2-chloroacetamide) or a β-unsaturated group (e.g., acrylamides)—compounds that exhibit a rapid reduction in initial cell viability—act in a manner consistent with the universal concept of irreversible covalent binding producing toxicity by altering the protein(s) and/or biomolecules such that normal function cannot be maintained (Hinson and Roberts, 1992), and the irreversible reactive mode of action (Schultz et al., 2004). Such irreversible interactions between reactive toxicants and cellular components are not specific like many receptor-binding interactions but rather are able to disrupt a wide assortment of cellular processes.

Efforts to develop quantitative structure–activity relationship (QSAR) models of acute aquatic toxicity of industrial organic compounds that take the form of unambiguous, easily applicable, mechanism-based algorithms (Jaworska et al., 2003) have focused on regression-analysis using physicochemical and quantum-chemical descriptors, especially ones for hydrophobicity and electro(nucleo)philicity, to model effects. While several specific electro(nucleo)philic mechanism with differing domains have been identified (see Karabunarliev et al., 1996a), as demonstrated by Karabunarliev et al. (1996b) and Schultz et al. (2002), among others, the toxic potencies of the majority of industrial chemicals are well-fitted to and predicted by simple two-descriptor response-surface models. This

reflects the fact that the majority of industrial organic chemicals react noncovalently, resulting in common hydrophobicity-dependent nonspecific electrophilic-dependent toxicity to aquatic organisms.

Since the toxicity of amides represents at least two modes of action (nonreactive and reactive), it would not be expected that the toxic potency of all the amides would be predicted accurately by a single QSAR such as equation (1). This assumption is supported indirectly by the structural trends observed in Table 4, where the terminal 2-halogen-substituted analogues are observed to be more toxic than their alkyl-substituted counterparts and α,β-unsaturated primary amides are more toxic than their saturated counterparts.

The creation of a covalent bond, such as through a Michael addition or an S_N2 displacement, is the initiating step along the toxicity pathway that leads to excess toxicity. This must be understood *a priori* in order to predict the potential of chemicals to cause specific harmful effects. We know that many soft electrophiles involve organic chemicals that have unsaturated π-bonds polarized by neighboring substituents, as in acrylamide, *N*-phenylacrylamide, and *N*,*N*′-methylenebisacrylamide. With these compounds, toxicity is initiated by the soft nucleophilic addition of the sulfhydryl group to the outer or β-carbon atom of the C=C moiety (Karabunarliev et al., 1996a). This Michael-type addition is thought to be a two-step process that forms a covalent adduct at a soft electrophilic center without the presence of a leaving group in the molecule (March, 1992).

Another important mechanism involving organic chemicals is one having leaving groups such as halogens neighboring a π-bonded polarized substituent such as an amide (Karabunarliev et al., 1996a). In this case, the order of reactivity follows the trend for the lability of the leaving group, which is generally I > Br > Cl $\gg$ F. A halo-substituted methylene group conjugated with a carbonyl, as in an α-haloamide, is generally selective for an S_N2 displacement reaction with a soft nucleophile. In this reaction, the halogen acts as a nucleofugal group, taking a pair of electrons with it when it leaves.

Armed with such information, it is tempting to describe simple substructure characteristics to define mechanistic classes and then extrapolate by assuming that chemically similar substances have similar mechanisms of toxic action. Indeed, such a strategy is clearly successful for the alkylamides (see Table 5). Moreover, while the data is more limited, it appears also to be successful for Michael acceptor-acting amides.

The results in Table 5 suggest that in the case of the halo-substituted amides molecular similarity is less transparent. To briefly recap, 2-chloroacetamide, 2- bromoacetamide, and 2-chloro-*N*,*N*-diethylacetamide as well as 2- bromopropionamide and 2,3-dibromopropionamide exhibit experimental toxicity far in excess of that predicted by equation (1). However, the experimental potencies of 2,2-dichloroacetamide and 2,2,2-trichloroacetamide and those of 2-chloropropionamide, 3-chloropropionamide, and 2-chlorobutylamide closely fit the values predicted by this QSAR.

This pattern for the various haloacetamides is in good accordance with well-established relative reactivity trends for S_N2 reactions of alkyl halides. Specifically: (1) primary > secondary; so 2-chloroacetamide > 2-chlorepropionamide. (2) Br > Cl; so 2-bromoacetamide > 2-chloroacetamide, and 2-bromopropionamide is a reactive toxicant while 2-chloropropionamide is not. (3) Adding a second halogen on the same carbon atom deactivates very strongly (e.g., CH_2Cl_2, an inert solvent, with CH_3Cl being an industrial methylating agent); so 2,2-dichloroacetamide and 2,2,2-trichloroacetamide show no reactive toxicity.

Having a halogen (X) α to a carbonyl group activates strongly, so 2-chloroacetamide is much more reactive (and toxic) than 3-chloropropioamide, which shows no reactive toxicity. However, β-halocarbonyl compounds can quite easily lose HX to give α,β-unsaturated carbonyls. Presumably GSH is not

basic enough to cause this elimination. With 2,3-dibromopropionamide it is likely that HBr will be eliminated much more easily as the hydrogen on the α-carbon is made more acidic by the bromine. The net result is an α-bromo-α,β-unsaturated carbonyl, which is a strong Michael acceptor.

Accuracy in prediction of toxic potency is predicated on using the most appropriate model. This is most correctly done by *a priori* assignment of a chemical to its most likely mode or mechanism of action. Yet identifying the most probable mode or reaction mechanism for a heterogeneous database from simple chemical structure is still fraught with problems. Since misapplication of a model is frequently the cause of prediction errors, it has the potential to have a major impact on the implementation of REACH (Anon., 2001). Interestingly, the toxicity results in Table 5 parallel the reactivity results noted in Table 6. These results suggest that the reactivity with model nucleophiles such as the thiol group of GSH may provide a means of more accurately defining the applicability domain for mechanisms of reactive toxicity.

While there are exceptions, the majority of toxic effects ascribed to reactive toxicity are the result of the reaction between an electrophilic toxicant and a biological nucleophile. Because of the specificity of reactivity, no single binding assay will capture all aspects of reactivity. As a result, predicting the most likely molecular initiating event from the spectrum of electrophile–nucleophile interactions is the crux of modeling reactive toxicity. While a systematic description of the selectivity of reactive chemicals for cellular targets will be required to profile the reactivity of all chemicals, as demonstrated in this study, results from binding assays such as the GSH assay can be used to group chemicals on the basis of their ability to evoke a particular molecular initiating event.

Acknowledgments

This work is supported in part by the US Army Medical Research and Material Command under contract no. W81XWH-050C-0017. The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless otherwise so designated by other documentation. Ms Koss is supported on a Summer Research Internships sponsored by The University of Tennessee Center of Excellence in Livestock Disease and Human Health.

References

Anon. White Paper on a Strategy for a Future Chemical Policy. (COM(2001)88 final). Brussels Belgium: Commission of the European Communities; 2001: 32p.

Harder A, Escher BI, Schwarzenbach RP. Applicability and limitation of QSARs for the toxicity of electrophilic chemicals. Environ Sci Technol. 2003;37:4955–61.

Hinson JA, Roberts DW. Role of covalent and noncovalent interactions in cell toxicity: effects on proteins. Annu Rev Pharmacol Toxicol. 1992;32:471–510.

Jacobs A. Understanding organic reaction mechanisms. Cambridge: Cambridge University Press; 1997: 304.

Jaworska JS, Comber M, Auer C, Van Leeuwen CJ. Summary of a workshop on regulatory acceptance of (Q)SARs for human health and environmental endpoints. Environ Health Perspect. 2003;111:1358–60.

Karabunarliev S, Mekenyan OG, Karcher W, Russom CL, Bradbury SP. Quantum-chemical descriptors for estimating the acute toxicity of electrophiles to the fathead minnow (*Pimephales promelas*): an analysis based on molecular mechanisms. Quant Struct-Act Relat. 1996a;15:302–10.

Karabunarliev S, Mekenyan OG, Karcher W, Russom CL, Bradbury SP. Quantum-chemical descriptors for estimating the acute toxicity of substituted benzenes to the guppy (*Poecilia reticulata*) and fathead minnow (*Pimephales promelas*). Quant Struct-Act Relat. 1996b;15:311–20.

March J. Advanced organic chemistry reactions, mechanisms, and structure. 4th ed. New York: Wiley; 1992: 1495.

Pavan M, Worth AP, Netzeva TI. Comparative assessment of QSAR models for aquatic toxicity. Report EUR 21750 EN of 2005; 2005:122.

Schultz TW. TETRATOX: *Tetrahymena pyriformis* population growth impairment endpoint-A surrogate for fish lethality. Toxicol Methods 1997;7:289–309.

Schultz TW, Cronin, MTD, Netzeva TI, Aptula AO. Structure–toxicity relationships for aliphatic chemicals evaluated with *Tetrahymena pyriformis*. Chem Res Toxicol. 2002;15:1602–9.

Schultz TW, Seward-Nagel J, Foster KA, Tucker VA. Population growth impairment of aliphatic alcohols to *Tetrahymena*. Environ Toxicol. 2004;19:1–10.

Schultz TW, Netzeva TI, Roberts DW, Cronin MTD. Structure–toxicity relationships for carbonyl-containing α,β-unsaturated aliphatic chemicals evaluated with *Tetrahymena pyriformis*. Chem Res Toxicol. 2005a;18:330–41.

Schultz TW, Yarbrough JW, Johnson EL. Structure–activity relationships for glutathione reactivity of carbonyl-containing compounds. SAR QSAR Environ Res. 2005b;16:313–22.

Seward JR, Sinks GD, Schultz TW. Population growth kinetics of *Tetrahymena pyriformis* exposed to select pyridines. Eur J Protist. 2000;36:139–49.

Veith GD. On the nature, evolution and future of quantitative structure–activity relationships (QSAR) in toxicology. SAR QSAR Environ Res. 2004;15:323–30.

Address for correspondence: T.W. Schultz, Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, 2407 River Drive, Knoxville, TN 37996–4543 USA.
E-mail: tschultz@utk.edu