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Structure-Toxicity Relationships for the Effects of N- and N,N′-Alkyl Thioureas to *Tetrahymena pyriformis*

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Ecotoxicity testing with the freshwater ciliate *Tetrahymena pyriformis* allows for the examination of a sub-acute endpoint, population growth impairment, with a eucaryotic organism representative of the detritus food web (Larsen et al. 1997). Over the past two decades, *Tetrahymena* toxicity data have been used for the development and validation of structure-toxicity relationships for a variety of chemical classes (Schultz 1999; Cronin et al. 2001).

Thioureas are sulfur-containing aliphatic diamines of the general formula R-NH-C(=S)-NH-R. Despite their use as herbicides (Schelenz and Kramer 1985), little is known about their ecotoxicity to non-target species. The presence of two nitrogen atoms in a thiourea more than counters the hydrophobic nature of the sulfur atom; the net result is a relatively hydrophilic compound. This property results in a propensity for the compound to reside in the aqueous phase. In theory, this hydrophilicity should limit the toxic potency of thioureas. Initial evaluation of 1-methyl-2-thiourea and 1-phenyl-2-thiourea revealed them to have population growth impairment to *T. pyriformis* in excess of that expected if the chemicals act as baseline toxicants. These observations spurred evaluation of a congeneric series of alkyl-thioureas.

The purpose of this study was to examine the aquatic toxicity of selected thioureas. The specific aims were: (1) to determine the toxic potency for each thiourea in the *T. pyriformis* population growth impairment assay; (2) to develop a hydrophobic-dependent structure-toxicity model; (3) to compare this model to the baseline narcosis and amine narcosis models, and (4) to examine the *T. pyriformis* population growth kinetics of 1-methyl-2-thiourea.

METHODS AND MATERIALS

Fifteen thioureas were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA), MRM Research Chemicals Lancaster Synthesis Inc. (Windham, NH, USA), or Trans World Chemicals Inc. (Rockville, MD, USA). Each had purity of 95% or better and none were repurified prior to use. Stock solutions of each thiourea were prepared in dimethyl sulfoxide.

T. pyriformis population growth impairment testing was executed using the protocol described by Schultz (1997). This static 40-hr assay used population

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density measured spectrophotometrically at 540 nm as its endpoint. Test conditions allow for 8-9 cell cycles in control cultures.

Each thiourea was tested in a range finder prior to testing in duplicate for three additional replicates. Two controls, one with no test material but inoculated with *T. pyriformis*, and the other, a blank, which had neither toxicant nor ciliates were used to provide a measure of the acceptability of the test and a basis for interpreting treatment data. 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 (SAS Institute Inc. 1989).

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 structure-toxicity relationship was examined using the log of the inverse of the IGC_{50} (log (IGC_{50}^{-1})) in mM as the dependent variable, and log K_{ow} as the independent variable. Data were modeled using the linear regression procedure of MINITAB™ release 13 statistical software. Model fit was quantified with the coefficient of determination (r^2 value) and the root of the mean square for error (s value). In addition, the Fisher statistic (F value), and the probability greater than the F value (Pr > F) were noted.

Population growth kinetics was examined following the protocol described by Bearden et al. (1997). *Tetrahymena* was grown in a proteose peptone supplemental medium (see above). All cell growth and experiments were performed with 50 mL of media in 250-mL foam-stoppered Erlenmeyer flasks. Flasks were equilibrated to 27 ± 1°C prior to use and at time zero, 3 X 10^3 log growth cell/mL were inoculated. A control containing no 1-methyl-2-thiourea and 5 step concentration series was tested in duplicate. At T_0, samples were taken immediately after 1-methyl-2-thiourea and *T. pyriformis* had been added. Addition samples were taken every other hour for 8 h. Between samplings, experimental flasks were returned to an incubator maintained at 27° ± 1°C. Samples consisted of diluting 2 mL of ciliate culture in 50 mL of 10% Isoton. Five electronic counts using the Coulter Counter Model Zm were taken of each sample. The mean count for duplicates was used for plots. For verification each experiment was conducted twice.

RESULTS AND DISCUSSION

There are a number of categories of ecotoxicity effects that a chemical may elicit (Schultz et al. 1997, Bearden and Schultz 1998). Baseline toxicity is the minimal ecotoxic effect. It is characterized by the nonpolar narcosis mechanism of action (McKim et al. 1987) and is deemed the retardation of general cytoplasmic activity

(Veith and Broderius 1990). This mechanism of action has been examined several times, including the review of van Wezel and Opperhuizen (1995).

In contrast, reactive chemicals are more toxic than predicted as if they acted by the baseline narcosis mechanism of toxicity (Lipnick et al. 1987). This type of toxicity, especially evident for hydrophilic compounds, can be the result of either covalent or non-covalent interactions (Schultz et al. 1997). While non-covalent interactions are reversible, covalent interactions are irreversible. Ecotoxicological, non-covalent interactions may be referred to as narcoses (Russom et al. 1997). From the ecotoxicity standpoint, covalent interactions are primarily electrophilic in character (Hermens 1990). While great strides have been made in predicting mode of toxic action from molecular structure (Bradbury 1995), due to the complexity of the interaction (Nendza and Russom 1991) this is still a difficult process. A summation of the Chemical Abstract Service registry numbers, toxicity, and hydrophobicity for the chemicals considered in this study are given in Table 1. While toxicity varies over about 3 orders of magnitude, hydrophobicity varies over more than 5 orders. Due to low aqueous solubility, one compound, the diphenyl-derivative, was not toxic at saturation.

Table 1. Toxicity and molecular descriptor values for selected thioureas.

Derivative	CAS number[a]	Log $1/IGC_{50}$[b]	Log K_{ow}[c]
2-thiourea	62-56-6	-1.32	-0.99m
1-methyl	598-52-7	-1.37	-0.68m
1-ethyl	625-53-6	-1.13	-0.21m
1-n-propyl	927-67-3	-0.69	0.36e
1-n-butyl	1516-32-1	-0.19	0.89e
1-n-hexyl	21071-27-2	0.86	1.95e
1-n-octyl	13281-03-3	1.51	3.01e
1-n-decyl	24827-74-5	1.94	4.07e
1-phenyl	103-85-5	0.61	0.72m
1-benzyl	621-83-0	0.66	1.07e
1,3-dimethyl	534-13-4	-1.11	-0.24m
1,3-diethyl	105-55-5	-0.76	0.57m
1,3-dibutyl	109-46-6	0.86	2.75m
1,3-diphenyl	102-08-9	NTAS[d]	2.17m
1,1,3,3-tetramethyl	2782-91-4	-0.85	0.49m

[a] Chemical Abstract Services registry number
[b] *T. pyriformis* toxicity as log (1/50% inhibitory growth concentration) in mM
[c] hydrophobicity as log (1-octanol/water partition coefficient)
[d] not toxic at saturation
e = estimated value
m = measured value

Regression analysis yielded the hydrophobic-dependent toxicity model

$$\log (IGC_{50}^{-1}) = 0.77 (\log K_{ow}) + 0.71;$$

$$n = 14, r^2 = 0.88, s = 0.40, F = 90, Pr > F = 0.0001 \quad \text{Eq. [1].}$$

An examination of residual values for Eq. [1] revealed one compound, 1-phenyl-2-thiourea, had a large standardized residual value. Deletion of this compound with subsequent reanalysis resulted in the model:

$$\log (IGC_{50}^{-1}) = 0.85 (\log K_{ow}) + 0.72;$$

$$n = 13, r^2 = 0.93, s = 0.32, F = 147, Pr > F = 0.0001 \quad \text{Eq. [2].}$$

A comparison of Eq. [2] with the *T. pyriformis* baseline narcosis model,

$$\log (IGC_{50}^{-1}) = 0.74 (\log K_{ow}) - 1.86;$$

$$n = 148, r^2 = 0.96, s = 0.21, F = 3341, Pr > F = 0.0001 \quad \text{Eq. [3].}$$

developed by Schultz et al. (1997) from data for neutral organic chemicals reveals the thioureas exhibit an ecotoxicity well in excess of baseline narcosis.

A similar comparison of Eq. [2] with the *T. pyriformis* amine narcosis model,

$$\log (IGC_{50}^{-1}) = 0.78 (\log K_{ow}) - 1.42;$$

$$n = 20, r^2 = 0.93, s = 0.27, F = 254, Pr > F = 0.0001 \quad \text{Eq. [4].}$$

developed by Sinks et al. (1998) from data for aliphatic primary amines reveals the thioureas have ecotoxicity only in slight excess of aliphatic amines. These relationships are better demonstrated in Figure 1.

While a comparison of Eq. [2] with Eq. [4] shows a similar slope for the K_{ow} dependence but a significantly greater intercept for the thioureas, examination of Figure 2 reveals these two lines are very similar. At the pH of the test system, amines with an ionization constant (pK_a) greater than 9.0 are protonated. It is not yet possible to determine if separate mechanisms of action exists for the thioureas or amines, or whether their requirements for separate modeling is merely an artifact of poor quality log K_{ow} values. However, it is known that protonated amines have a higher affinity for membranes than non-ionized compounds (Austin et al. 1995); therefore, the toxicity of such chemicals would be enhanced over that expected from baseline narcosis.

As noted previously, enhanced ecotoxicity can be due to covalent or non-covalent interactions. Population growth kinetics for *T. pyriformis* cultures exposed to hydrophilic toxicants acting by non-covalent interactions exhibit a direct relationship between the concentration of the chemical and the generation time of the ciliates (Bearden et al. 1997). Moreover, there is no observed relationship

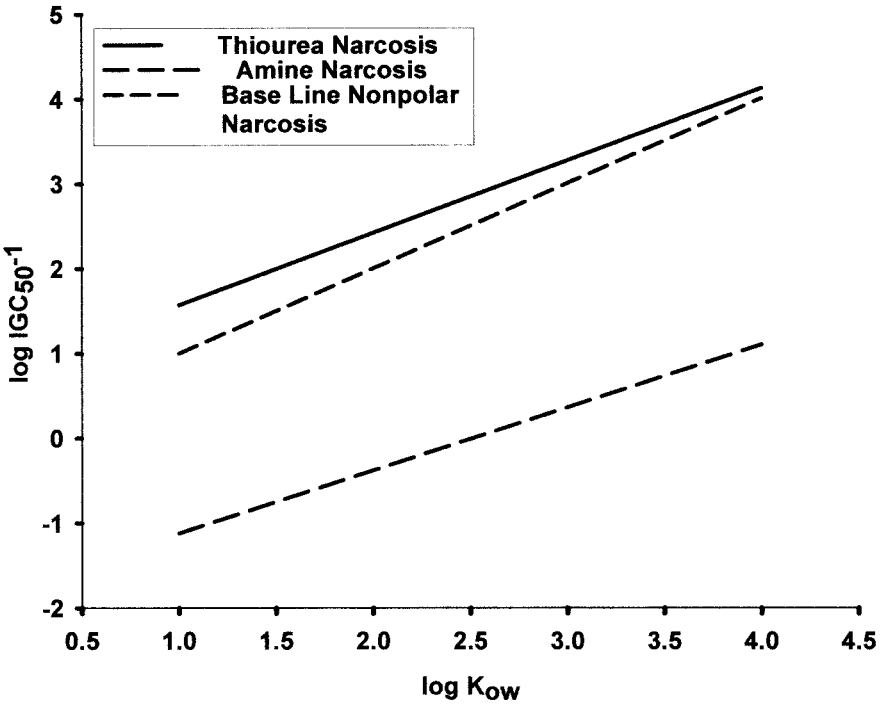

Figure 1. Comparisons of the log K_{ow} dependent *T. pyriformis* toxicity (log (IGC_{50}^{-1})) models for baseline narcotics, aliphatic amines, and thioureas.

between the y-intercepts and the endpoint; therefore, it is assumed that no lag phase in growth occurred (Bearden et al. 1997).

In contrast, population growth kinetics for *T. pyriformis* cultures exposed to electrophiles revealed a threshold concentration where death of the initially exposed ciliates is observed (Bearden et al. 1999). Moreover, Bearden et al. (1999) noted that at concentrations below this threshold, population growth kinetics exhibit a short lag phase without any cell death. This lag phase was followed by growth at rates (i.e., slope) similar to control populations. At concentrations larger than the threshold, a concentration dependent death of the initially exposed cells occurred; surviving *Tetrahymena* grew at rates similar to control populations (Bearden et al. 1999).

Typical population growth kinetics for *T. pyriformis* exposed to 1-methyl-2-thiourea are shown in Figure 2. This figure reveals a direct relationship between the concentration of the urea and the generation time of the ciliates. This is consistent with the results reported by Bearden et al. (1997) for ciliates exposed to other hydrophilic narcotics, ethanol and acetone. This observation suggests that aliphatic thioureas act via a non-covalent mode of toxic action.

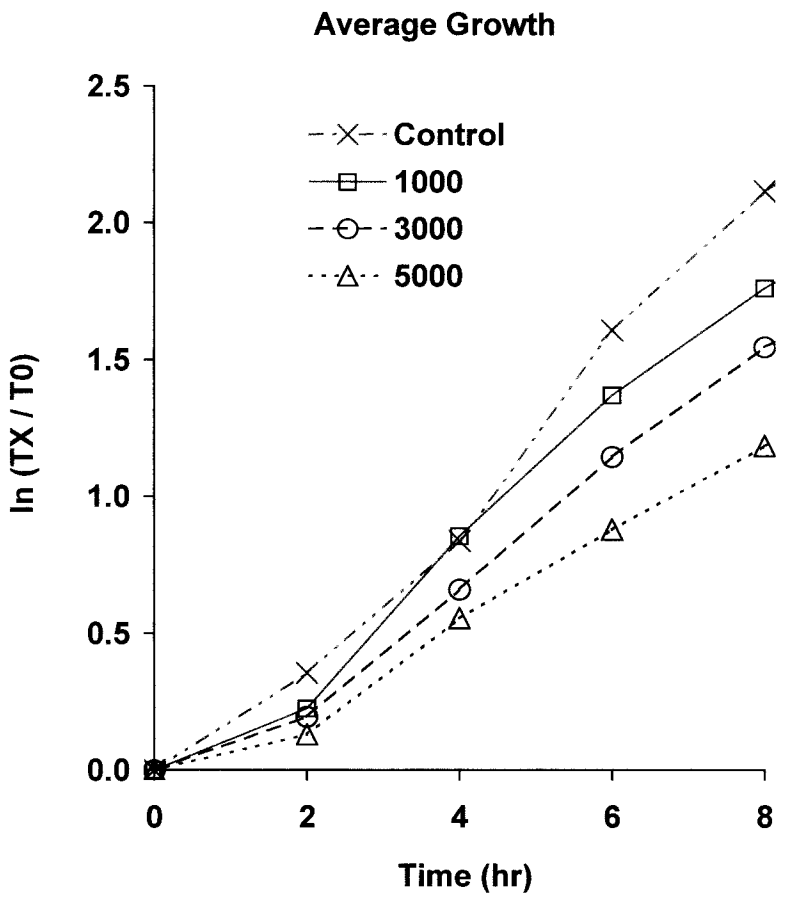

Figure 2. Population growth kinetics of *T. pyriformis* exposed to 1-methyl-2-thiourea.

The results of this investigation indicate that alkyl-chain length has an impact on thiourea toxicity by its alteration of hydrophobicity. While population growth kinetic experiments indicate thioureas act by a non-covalent mode of action, their toxicity is in excess of that predicted by baseline narcosis but is strikingly similar to amine narcotics.

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