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Toxicological Evaluation and QSAR Modelling of Aromatic Amines to *Chlorella vulgaris*

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The European Commission's proposed legislation for the Registration, Evaluation, and Assessment of CHemicals (the REACH initiative) requires the development of rapid, cost-effective, reliable and validated alternative methods to generate toxicological and eco-toxicological information. Within the application of this legislation, computational prediction models will undoubtedly have an important role in the process of reduction and replacement of animal testing (Combes et al. 2003). Key amongst these is the use of quantitative structure-activity relationships (QSARs) and extrapolations of toxicity from one species to another (quantitative activity-activity relationships, QAARs). It was noted recently that there are relatively good QSARs for the toxicity of chemicals to fish, while the predictive capability for invertebrates and algae is poor (Cronin et al. 2003). In addition, the need for the further development of new, rapid and sensitive *in vitro* tests for eco-toxicity testing has been emphasized recently by Combes et al. (2002).

The aromatic amines are commonly used in the chemical manufacture of dyes, rubber and textiles and can also originate from gasoline and coal combustion (Palmiotto et al. 2001). As such they are frequently found to be environmental pollutants. Sewage treatment units and agriculture are also responsible for the presence of a large variety of nitrogenous compounds in the environment, including anilines (Oliviero et al. 2003). The aims of this study were 1) to evaluate a series of aromatic amines in a novel rapid toxicity test utilizing the alga *Chlorella vulgaris*; 2) to investigate the interspecies relationships of toxicity with other aquatic species (the luminescent bacterium *Vibrio fischeri* and the ciliate *Tetrahymena pyriformis)*; and 3) to develop mechanistically sound QSAR models for the anilines in the algal toxicity test.

MATERIALS AND METHODS

A total of 14 chemicals were selected for toxicological assessment and these are listed in Table 1. An effort was made to select anilines incorporating narcotic, as well as other mechanisms of toxic action. A number of other criteria were applied to select the chemicals: the chemicals were required to span a sufficient range of

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hydrophobicity to make the QSAR meaningful; there should be comparative toxicological information available for the majority of chemicals tested, and finally that they should be easily and cheaply available. All chemicals were purchased from the Aldrich Chemical Company, Poole, Dorset, England, with chemical purity more than 95%. Chemicals were not re-purified prior to use.

Table 1. Chemical Abstracts Service (CAS) number, chemical name, acute algal and other toxicities and calculated descriptors for the aromatic amines studied.

CAS	Name	*C. v.*[a] (mM)	*V. f.*[b] (mM)	*T. p.*[c] (mM)	log K_{ow}	E_{LUMO} (eV)
62-53-3	Aniline	-1.34	0.12	-0.23	0.90[d]	0.639
87-62-7	2,6-Dimethylaniline	-0.87	0.66	-0.43	1.84[d]	0.595
97-02-9	2,4-Dinitroaniline	-0.36	0.58	0.53	1.72[d]	-1.474
99-30-9	2,6-Dichloro-4-nitro aniline	0.64	1.75	-	2.80[d]	-1.097
106-40-1	4-Bromoaniline	-0.33	-	1.01	2.26[d]	0.218
108-42-9	3-Chloroaniline	-0.31	0.96	0.22	1.88[d]	0.263
348-54-9	2-Fluoroaniline	-1.05	-	-0.37	1.26[d]	0.266
608-31-1	2,6-Dichloroaniline	0.26	1.97	0.33	2.82[d]	-0.006
618-87-1	3,5-Dinitroaniline	0.03	0.98	0.80	1.89[d]	-1.781
626-43-7	3,5-Dichloroaniline	0.24	1.19	0.71	2.90[d]	-0.042
634-93-5	2,4,6-Trichloroaniline	1.11	1.63	1.01	3.69[d]	-0.240
3481-20-7	2,3,5,6-Tetrachloro aniline	1.48	2.16	1.76	4.47[d]	-0.507
3531-19-9	6-Chloro-2,4-dinitro aniline	0.80	1.03	1.12	2.46[e]	-1.666
5388-62-5	4-Chloro-2,6-dinitro aniline	1.19	1.76	-	2.46[e]	-1.877

[a]Toxicity to *C. vulgaris* [log ($1/EC_{50}$)]
[b]Toxicity to *V. fischeri* [log ($1/EC_{50}$)]
[c]Toxicity to *T. pyriformis* [log ($1/IGC_{50}$)]
[d]Measured log K_{ow}
[e]Calculated log K_{ow}

Toxicity data [log ($1/EC_{50}$)] were determined in a biochemical assay utilizing the unicellular alga *C. vulgaris*. Algae in the logarithmic phase of their growth cycle were used. All toxicological analyses were performed in a buffer solution with a pH of 6.9 and temperature of between 25°C and 30°C. Assays were conducted following the protocol described by Worgan et al. (2003) with a 15-minute static design. The disappearance of fluorescein diacetate (FDA) was accounted for by spectrofluorimetrical measurement of fluorescein (the product of hydrolysis) at an excitation wavelength set to 465 nm and an emission wavelength to 515 nm. Range-finding experiments were performed in order to determine the highest and lowest concentrations required to produce a dose-response relationship ranging from 100% inhibition of enzyme activity to no observed toxicological effect. Blank buffer solution was utilized as a control and the relative responses to it were used to generate the dose-response curve. The 50% effective concentration

was estimated by Probit analysis using the SPSS software. Between six to ten concentrations, were evaluated per test. The average EC_{50} was taken from a minimum of three analyses with at least one test being performed afresh.

Toxicity data to *Vibrio fischeri* [log $(1/EC_{50})$] were determined in a 30-minute static assay. The toxicity values were taken from the compilation of Kaiser and Palabrica (1991). Toxicity data to *Tetrahymena pyriformis* [log $(1/IGC_{50})$] were determined in a 40-hour static population growth impairment assay utilizing strain GL-C. Large data sets of aromatic compounds, tested for acute toxicity to *T. pyriformis*, can be found in the papers of Schultz (1999) and Schultz et al. (2003).

Hydrophobicity was quantified by the logarithm of the 1-octanol/ water partition coefficient (log K_{ow}). The hydrophobicity values were measured or estimated (the measured value was preferred when available) by the ClogP for Windows ver. 1.0.0 software (BIOBYTE Corp., Claremont, CA, USA). The energy of the lowest unoccupied molecular orbital (E_{LUMO}) was obtained from the VAMP module of the TSAR ver. 3.3 molecular spreadsheet (Accelrys, Oxford, England) using the AM1 Hamiltonian. Initially SMILES strings were converted into 3-D structures by the CORINA conformation analysis software, as implemented in TSAR. The 3D structures underwent energy minimization utilizing full geometry optimization with AM1 in the VAMP module of TSAR ver. 3.3. The inter-species relationships and QSARs were investigated using regression analysis as implemented in MINITAB ver. 13.1 (MINITAB Inc., State College PA, USA).

RESULTS AND DISCUSSION

The anilines form an important group of environmental pollutants for which toxicity data are required. This study has provided a novel set of algal toxicity values and a number of computational models for predicting toxicity from structure alone and from toxicity to other species.

Toxicity data to *C. vulgaris* for a total of 14 aromatic amines, including aniline and 13 halo- and nitro-substituted derivatives are presented in Table 1. Compounds exhibited a reasonably wide range of algal toxicity (from –1.34 to 1.48). Hydrophobicity, quantified by log K_{ow}, ranged from 0.90 to 4.47 and the reactivity, quantified by E_{LUMO}, ranged from –1.877 to 0.639.

Analysis of the relationship between toxicity from the novel algal assay and data from other assays is illustrated below. The relationship between the *V. fischeri* [log $(1/EC_{50})_{V.f.}$] toxicities and those measured with *C. vulgaris* [log $(1/EC_{50})_{C.v.}$] is:

$$\log (1/EC_{50})_{V.f.} = 0.628\ (0.115) \log (1/EC_{50})_{C.v.} + 1.083\ (0.099) \quad (1)$$
$$n = 12,\ R^2 = 0.748,\ R^2_{CV} = 0.677,\ s = 0.328,\ F = 29.7$$

Here, n is the number of compounds, R^2 is the coefficient of determination, R^2_{CV} is the cross-validated (in a leave-one-out procedure) coefficient of determination, s is the standard error of the estimate, F is Fishers' criterion and the numbers in parentheses are the standard errors on the coefficients. It was found that that 2,6-dichloroaniline had a large standardized residual. Its exclusion improved the statistical fit of Eq. (1). The model without the outlier is present in Eq. (2).

$$\log (1/EC_{50})_{V.f.} = 0.626\ (0.083) \log (1/EC_{50})_{C.v.} + 1.017\ (0.074) \quad (2)$$
$$n = 11,\ R^2 = 0.863,\ R^2_{CV} = 0.807,\ s = 0.237,\ F = 56.7$$

The relationship between the *T. pyriformis* toxicities ($\log (1/IGC_{50})_{T.p.}$) and those measured to *C. vulgaris* is:

$$\log (1/IGC_{50})_{T.p.} = 0.681\ (0.115) \log (1/EC_{50})_{C.v.} + 0.558\ (0.095) \quad (3)$$
$$n = 12,\ R^2 = 0.778,\ R^2_{CV} = 0.697,\ s = 0.329,\ F = 35.0$$

A reasonably good statistical fit was obtained without statistically significant outliers. For comparative purposes the relationship between *V. fischeri* and *T. pyriformis* toxicity was also assessed:

$$\log (1/EC_{50})_{V.f.} = 0.663\ (0.253) \log (1/IGC_{50})_{T.p.} + 0.742\ (0.215) \quad (4)$$
$$n = 10,\ R^2 = 0.461,\ R^2_{CV} = 0.214,\ s = 0.494,\ F = 6.85$$

2,6-Dichloroaniline was found again to be significant outlier. Its exclusion improved the relationship to some extent:

$$\log (1/EC_{50})_{V.f.} = 0.739\ (0.175) \log (1/IGC_{50})_{T.p.} + 0.584\ (0.155) \quad (5)$$
$$n = 9,\ R^2 = 0.718,\ R^2_{CV} = 0.458,\ s = 0.338,\ F = 17.8$$

A good correlation was obtained between algal toxicity data and the values measured for the other two aquatic species. One outlier (2,6-dichloroaniline) was detected in the relationship between *Chlorella* and *Vibrio* toxicities. Since this compound also was an outlier in the *Vibrio* and *Tetrahymena* interspecies relationship, one can conclude that the EC_{50} value of this chemical to the bacterium was probably not determined correctly. With this exception, the algal toxicity data compare favourably with the toxicity to the other two organisms.

In order to develop QSARs, an empirical approach was applied to the selection of descriptors. This was to select descriptors that are known to model the two main features of toxicity, namely penetration through the cell membrane ($\log K_{ow}$) and reactivity (E_{LUMO}) inside the cell (McFarland 1970). The values of these two descriptors for the anilines studied are listed in Table 1.

Analysis of the relationship between algal toxicity and hydrophobicity resulted in a statistically significant equation, however, with a relatively low coefficient of determination ($R^2 = 0.763$). A plot of $\log (1/EC_{50})_{C.v.}$ versus $\log K_{ow}$ for all compounds is presented in Figure 1. Figure 1 also illustrates the QSAR for

baseline (non-polar narcosis) toxicity and polar narcosis toxicity in the same algal assay as described by Worgan et al. (2003). As it can be seen from Figure 1, almost all aromatic compounds are more toxic than baseline, and most of them have an excess toxicity compared to the polar narcosis model. It is evident from Figure 1 that hydrophobicity alone is not enough to describe accurately the toxicity of the anilines and an additional descriptor may be beneficial to improve statistical fit.

The statistical criteria of the model were considerably improved by including the reactivity term E_{LUMO}:

$$\log (1/EC_{50})_{C.v.} = 0.740\ (0.070) \log K_{ow} - 0.406\ (0.072)\ E_{LUMO} - 1.852\ (0.176) \quad (6)$$
$$n = 14,\ R^2 = 0.939,\ R^2_{CV} = 0.890,\ s = 0.233,\ F = 84.9$$

There were no statistical outliers to this relationship. Both descriptors are statistically significant ($p < 0.001$) and have a coefficient to error ratio (t-criterion) equal to 10.5 and -5.7 for $\log K_{ow}$ and E_{LUMO}, respectively.

The same hydrophobicity/electrophilicity approach was applied to the other two organisms in this study. Statistically significant model with $\log K_{ow}$ and E_{LUMO} was developed for *T. pyriformis*:

$$\log (1/EC_{50})_{T.p.} = 0.478\ (0.097) \log K_{ow} - 0.329\ (0.113)\ E_{LUMO} - 0.683\ (0.243) \quad (7)$$
$$n = 12,\ R^2 = 0.810,\ R^2_{CV} = 0.717,\ s = 0.321,\ F = 19.1$$

The relationship for *V. fischeri*, however, demonstrated lower R^2 and R^2_{CV} values and insignificance of the molecular orbital term E_{LUMO}:

$$\log (1/EC_{50})_{V.f.} = 0.555\ (0.105) \log K_{ow} - 0.101\ (0.107)\ E_{LUMO} - 0.208\ (0.281) \quad (8)$$
$$n = 12,\ R^2 = 0.768,\ R^2_{CV} = 0.631,\ s = 0.332,\ F = 14.9$$

The development of QSARs for aquatic toxicity is related to the understanding that partitioning into non-aqueous phases, i.e. membranes, occurs with almost any chemical in all organisms (Nendza and Müller 2000). It is not surprising, therefore, that the lipophilicity term $\log K_{ow}$ is a highly successful descriptor in toxicological QSARs, especially when narcosis is anticipated to be one of the possible mechanisms of toxic action. Robust, lipophilicity-dependant QSARs were developed for anilines eliciting a narcotic effect to *Tetrahymena pyriformis* and *Pimephales promelas* (Dimitrov et al. 2003). The compilation of data sets of more diverse chemicals, acting by different mechanisms of toxic action, requires additional terms for successful QSAR modelling. E_{LUMO} reflects the overall ability of the molecule to accept an electron pair from a potential nucleophile. Thus, the electronic term in Eqs. (6–8) accounts for electrophilicity of the chemicals at the molecular level.

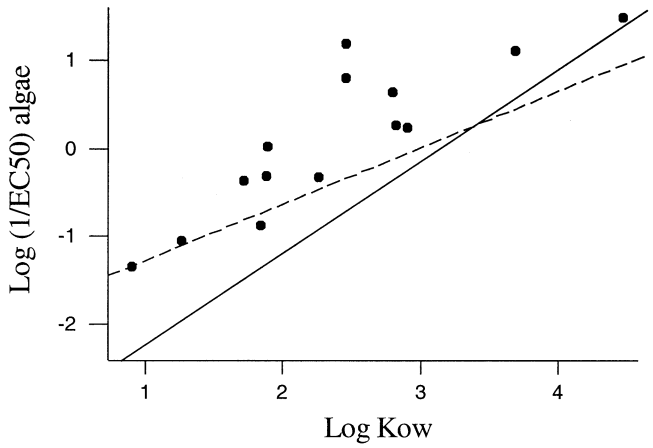

Figure 1. Plot of the observed toxicity to the alga *C. vulgaris* against log K_{ow}. The non-polar narcosis toxicity (solid line) is calculated by the equation log $(1/EC_{50})$ = 1.04 log K_{ow} – 3.28 and the polar narcosis toxicity (dashed line) is calculated by the equation log $(1/EC_{50})$ = 0.641 log K_{ow} – 1.91 (Worgan et al. 2003).

The general applicability of the hydrophobicity/electrophilicity approach to aquatic toxicity QSARs was demonstrated to both *C. vulgaris* and *T. pyriformis*. The consistency of the QSAR results was expected bearing in mind the mechanistic basis of the approach. The unsatisfactory performance in the modelling of *V. fischeri* toxicity could be explained with the higher possible error associated with the bacterial toxicity data (Cronin and Schultz 1997).

In conclusion, a rapid and economical 15-minute algal toxicity test was utilized to measure the toxicity of 14 anilines to the alga *Chlorella vulgaris*. The test was assessed by investigating the relationship with toxicity data to other species (*Vibrio fischeri* and *Tetrahymena pyriformis*). A successful two-descriptor QSAR model, accounting for hydrophobicity and electrophilicity, was developed. It can be used to predict toxicity of new, untested, aromatic amines to the alga *Chlorella vulgaris* from chemical structure alone.

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