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Quantitative Structure-Activity Relationships for the Toxicity to the Tadpole *Rana japonica* of Selected Phenols

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It is infeasible to test the toxicity of every chemical to all species due to the constraints of time and money. Under these circumstances, Quantitative Structure-Activity Relationships (QSARs), which can provide a useful tool to predict the hazards of untested chemicals, have proved to be attractive and have been shown to be credible. Toxicants have been divided into many subsets according to their mode of toxic action and separate QSARs were constructed by mechanism-based QSAR methods (Schultz et al 1990a, Cronin and Dearden 1995). Interspecies relationships were also studied to seek the possibility of predicting toxicity to higher organisms following the development of fast, sensitive, easy and low-cost toxicity test systems utilizing microorganisms.

Phenol and its derivatives are widely used industrial chemicals, and consequently have a high potential for environmental pollution. Comparative studies were performed on the mechanisms of toxicity of phenols to *Pimephales promelas* and to *Tetrahymena pyriformis* by Schultz et al (1986) and Bearden et al (1997). Polar narcosis and/or uncoupling of weak acid respiratory mechanisms were found to be main mechanisms for most phenols. Similar mechanisms were observed in these two systems and strong interspecies correlation was obtained (Schultz et al 1986, Cronin and Schultz 1996).

Frogs are common amphibious animals bridging aquatic organisms and terrestrial animals. The larvae of frogs, tadpoles, proved to be more sensitive to hazards than adult frogs and there is similarity between tadpoles and fishes in physiology and morphology (Gong 1994). It is thus interesting and valuable to investigate the adverse effect of industrial materials on tadpoles; however, studies on the toxicity of phenols to frogs and mechanisms of toxic action were not readily available in literature. The objective of this paper is to study the mechanism of acute toxicity of phenols to frog tadpoles, to investigate interspecies relationship between *Rana japonica* (tadpole) and *Tetrahymena pyriformis*, and to further develop surrogate organisms for the prediction of the acute toxicity of phenols to tadpoles.

MATERIALS AND METHODS

24 substituted phenol derivatives were provided by the Department of Chemistry

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at Nanjing University. They were all of sufficient purity (analytical purity) and further purification was not necessary.

Rana japonica tadpoles were employed for toxicity test because *Rana japonica* are very common in China and green frog tadpoles proved to be more sensitive to hazards than other tadpoles (Berrill et al 1995). *Rana japonica eggs* were collected from a single frog-pond in Nanjing where *Rana japonica* frogs are exclusively bred. All eggs had been laid no more than 2 days prior to collection. Eggs and the resulting tadpoles were maintained at room temperature (22-25°C) in aerated stream water in the laboratory. Tadpoles were fed with an excess of rinsed, salt free canned spinach daily, and the water was renewed every other day up to 30 days. Feeding was halted 24 hours before each test. Healthy individuals with a similar size and weight were selected for testing. The average body length and body weight were 2.5±0.1. lcm and 0.09±0.01g, respectively.

Stock solutions of chemicals to be tested were prepared with aerated water after range finding experiments. 10 tadpoles were moved into a 500ml solution-filled glass beaker. Each assay was set up in triplicates and consisted of a six-step concentration series to cover the range from no effect to 100% lethal concentration. Solutions were renewed every 6 hours to achieve semi-static exposure. Aerated water without test compounds served as blank. Lethality percentage was recorded for each concentration after 24hr exposure. Logarithms of the inverse median lethal concentration, expressed as 24hr-LC_{50}, were calculated using linear regression analysis in *STATISTICA* software (version 5.0) for each chemical as its toxic potency. Logarithms of the inverse 48hr cell population growth half impairment concentration, expressed as log (BR), were cited from literature (Cronin and Schultz 1996).

Logarithms of the 1 -octanol/water coefficient ($\log K_{ow}$) were computer estimated or retrieved as measured values from SRC-WSKOW for Microsoft Windows (version 1.26). Dissociation constants *(pKa)* were retrieved from literature (Dean 1985). Multiple regression analysis was performed using the *STATISTICA* for QSAR analysis. The toxic potency (24hr-LC_{50}) was employed as the dependent variable, and the physio-chemical descriptors as the independent variables in QSAR study. Quality of the model is characterized by the number of observations *(n),* the square of correlation coefficient (r^2), the standard error of estimate (SE), the Fisher criterion (*F*) and the significance level (*P*),

RESULTS AND DISCUSSION

The toxicity of the 24 phenol derivatives to *Rana japonica* tadpoles and to *Tetrahymena pyriformis* are listed in Table 1. A summary of the calculated physio-chemical parameters used for QSAR analysis is also presented. Chemical persistence studies was not undertaken, as Schultz et al (1989) demonstrated that abiotic loss of phenols did not affect the toxicity tests.

It is generally accepted that the most successful QSARs have separated toxicants

into subsets according to their mechanism of toxic action and a separate QSAR can be derived for each mechanism of toxic action (Schultz et al 1990a, Cronin and Dearden 1995). LogK_{ow}, dependent QSAR models are often developed to study the different mechanisms of toxic action of chemicals (Schultz et al 1986, Bearden and Schultz 1997). Polar narcosis has proved to be the main mechanism for most substituted phenols, including alkyl and alkoxyl substituted, mono- and di- halogen substituted phenols, etc (Schultz et al 1989, 1992). This mode usually represents the majority of aromatic compounds with strong electron releasing amino or hydroxy1 moieties, resulting in greater dipolarity and /or hydrogen bond donor acidity than in non-polar narcotics (Russom et al 1996). Schultz et al (1986) studied the relative toxic response of selected phenols in 48hr chronic static *Tetrahymena pyriformis* (ciliate protozoan) test system, developing log K_{ow} dependent linear QSAR models for polar narcosis toxicity. These models were improved by adding *pKa*, a variable characterizing the dissociation behavior of polar chemicals. The log K_{ow}–dependent model and the improved *pKa*-containing model for the toxicity of phenols to *Tetrahymena pyriformis* are shown as follows:

Table 1 Name, Toxicity and Physio-chemical Parameters of substituted phenols

No	Chemical	CAS NO.[1]	24h- LC_{50}[2]	Log(BR)[3]	Log K_{OW}[4]	*Pka*[5]
1	2-Nitrophenol	88-75-5	3.686	3.67	1.91	7.22
2	3-Nitrophenol	554-84-7	3.482	3.506	1.91	8.36
3	4-Nitrophenol	123-30-8	4.052	4.42	1.91	7.15
4	4-Chloro-2-Nitrophenol	89-64-5	4.880	5.053	2.55	6.48
5	2-Chlorophenol	95-57-8	3.024	3.183	2.15	8.55
6	4-Chlorophenol	106-48-9	3.308	3.545	2.16	9.43
7	2,4-Dichlorophenol	121-83-2	3.996	4.036	2.8	7.85
8	4-Bromophenol	106-41-2	3.619	3.68	2.4	9.34
9	2-Bromo-4-methylphenol	-	3.753	3.599	2.95	8.67
10	4-Fluorophenol	371-41-5	2.670	3.017	1.71	9.89
11	4-Methoxylphenol	150-76-5	2.371	2.857	1.59	10.2
12	2-Methoxylphenol	90-5-1	2.500	2.49	1.34	9.99
13	4-Methylphenol	106-44-5	2.928	2.816	2.06	10.28
14	2-Methylphenol	95-48-7	2.819	2.705	2.06	10.33
15	2,6-Dimethylphenol	576-26-1	3.331	3.277	2.61	10.59
16	Salicylaldehyde	90-02-8	3.968	3.424	2.01	8.34
17	4-Hydroxylbenzaldehyde	123-08-0	2.804	3.266	1.23	7.62
18	Salicylic acid	69-72-7	2.841	2.488	2.24	2.98
19	4-Hydroxyl,methyl benzoate	99-76-3	3.163	3.084	2.0	-
20	Resorcinol	108-46-3	2.077	2.307	1.03	9.44
21	3-Aminophenol	591-27-5	2.064	2.476	0.24	9.83
22	4-Hydroxylacetophenone	99-93-4	2.509	2.698	1.19	8.05
23	4-Tert-Butyl, phenol	98-54-4	4.170	3.914	3.31	10.23
24	Phenol	108-95-2	2.804	2.792	1.51	9.99

[1] Chemical Abstract Services registry number

[2] logarithm of inverse 24hr median lethal toxicity to tadpole in $(mole/L)^{-1}$

[3] logarithm of inverse 48hr cell population growth impairment toxicity to *Tetrahymena pyriformis* in $(mole/L)^{-1}$, cited from Cronin and Schultz (1996)

[4] retrieved from SRC-WSKOW for Microsoft Windows (1.26)

[5] cited from Dean (1985)

$$\text{Log (BR)} = 0.51 \log(K_{ow}) - 0.69 \quad (1)$$
$$n = 28 \quad r^2 = 0.52 \quad SE = 0.45 \quad F = 27.8 \quad P = 0.0001$$

$$\text{Log (BR)} = 0.68 \log(K_{ow}) - 0.26\, pKa + 1.12 \quad (2)$$
$$n = 28 \quad r^2 = 0.90 \quad SE = 0.21 \quad F = 116.6 \quad P = 0.0001$$

Where

Log (BR) is the logarithm of inverse 48hr static cell population growth half impairment concentration to *Tetrahymena pyriformis.*

Linear regression analysis of the acute semi-static 24-hr toxicity of selected phenols to *Rana japonica* Tadpole with log K_{ow} was also performed:

$$\text{24hr-LC}_{50} = 0.74 \log K_{ow} + 0.50 \quad (3)$$
$$n = 24 \quad r^2 = 0.55 \quad SE = 0.50 \quad F = 27.0 \quad P = 0.00003$$

An improved model was also derived after the addition of *pKa* (note that the number of observations was 23 due to absence of the *pKa* value for 4-hydroxyl, methyl benzoate):

$$\text{24hr-LC}_{50} = 0.73 \log K_{ow} - 0.53\, pKa + 4.27 \quad (4)$$
$$n = 23 \quad r^2 = 0.83 \quad SE = 0.32 \quad F = 48.8 \quad P < 0.00000$$

Following the examination of the molecules tested and their residuals between the observed values and the predicted values by Eq. (4) 4 outliers were observed. These compounds could not be modeled by this QSAR because of their excess toxicity. These 4 toxic compounds were removed from the toxicity data set. 4-Cl-2-nitrophenol might act as a weak acid respiratory uncoupler as its molecular structure features similar to weak acid respiratory uncouplers: (1) there is a moderately weak acid moiety (hydroxy1 group) on a bulky and hydrophobic aromatic ring system; (2) with additional electron-withdrawing substituents: a chloro group and a nitro group; (3) with a *pKa* value (6.48) close to the intramitochondrial pH value (Schultz et al 1990a). 4-Nitrophenol has been regarded as a pro-electrophile in other test systems including *E. coli, Tetrahymena pyriformis* and *Pimephales promelas* (Jaworska and Schultz 1994; Cronin and Schultz et al 1996; Bearden and Schultz 1997). The molecular structure of salicylaldehyde is very similar to an α,β-unsaturated compound because its carbonyl group is conjugated with aromatic double bonds. Thus it may covalently react with macromolecular soft nucleophiles via nucleophilic Michael-type addition reaction and exhibit a highly bio-reactive toxicity. The excess toxicity of salicylaldehyde was also observed in *Pimephales promelas* assay system, and salicylaldehyde was classified to be a electrophile/ proelectrophile (Russom et al 1996). Amino-substituted phenols can be metabolized and activated to quinone-like compound (Dupuis and Benezra 1982) the greater toxicity of quinones and quinone-like compounds may also be involved in the nucleophilic Michael-type addition with macromolecular soft nucleophiles, as the targets attacked are similar to those attacked by α,β-unsaturated compounds (O'Brien 1991).

Reanalysis after the removal of the four bioreactive compounds resulted in another improvement in the model with no further outliers observed:

$$\text{24hr-LC}_{50} = 0.92 \log K_{ow} - 0.41\, pKa + 3.32 \quad (5)$$
$$n = 19 \quad r^2 = 0.90 \quad SE = 0.20 \quad F = 70.5 \quad P < 0.00000$$

A comparison of Eq.(1) and (3), (2) and (5) reveals that the two $\log K_{ow}$-dependent models and the pair of *pKa*-containing models show great statistical similarity. These findings may suggest that similar mechanisms exist in the two test systems and phenols modeled by the *pKa*-containing equation act as polar narcotics in the tadpole test system. The difference in slopes and intercepts of the $\log K_{ow}$ dependent models may be attributed to the difference in interspecies sensitivity to xenobiotics between the two test species and different test media, respectively.

Much effort has been put into exploring interspecies correlations and finding alternatives to toxicity testing. Surrogate test, Such as Microtox have been developed to study hazards to higher animals. Interspecies correlations of toxicity and QSARs for many species are comparable for compounds acting non-polar narcosis (baseline toxicity) (Cronin and Dearden 1993, Schultz et al 1990b) with Microtox; however, when compounds acting as polar narcotics or more specific reactive mechanisms are considered, the Microtox test proves to be less suitable as a surrogate for higher species (Cronin and Dearden 1993). This may partly be due to the significant difference in length of exposure, dramatic difference in test media and great interspecies difference in physiology between microorganisms and higher species. Whereas other species such as *Tetrahymena pyriformis* give better an estimation of toxicity for *Pimephales promelas* for polar narcotic and reactive compounds (Cronin and Dearden 1993). Similar $\log K_{ow}$ dependent QSAR models were developed for the *Pimephales promelas* and *Tetrahymena pyriformis* systems, which demonstrated the possibility of establishing a surrogate relationship between the two test species and to predict *Pimephales promelas* toxicological data from that of *Tetrahymena pyriformis*. The toxicity of substituted phenols both determined in tadpole assay system and measured in the *Tetrahymena pyriformis* (Schultz et al 1996) assay system was compared, and a good interspecies correlation was obtained:

$$\text{LC}_{50} = 0.94 \log (\text{BR}) - 0.16 \quad (6)$$
$$n = 24 \quad r^2 = 0.89 \quad F = 130.3 \quad SE = 0.25 \quad P < 0.00000$$

Where log (BR) is the inverse of the logarithm of the 48-hr cell population growth impairment concentration of *Tetrahymena pyriformis.* The residuals showed salicylaldehyde to be an outlier and exhibited excess toxicity in tadpole system as compared to *Tetrahymena pyriformis.* The excess toxicity of salicylaldehyde was also observed in *Pimephales promelas* test system (Russom et al 1996). The above comparison results may suggest that salicylaldehyde exhibited different mechanisms in higher aquatic organisms and primitive organisms. The removal of salicylaldehyde resulted in the following equation, no more outlier were observed:

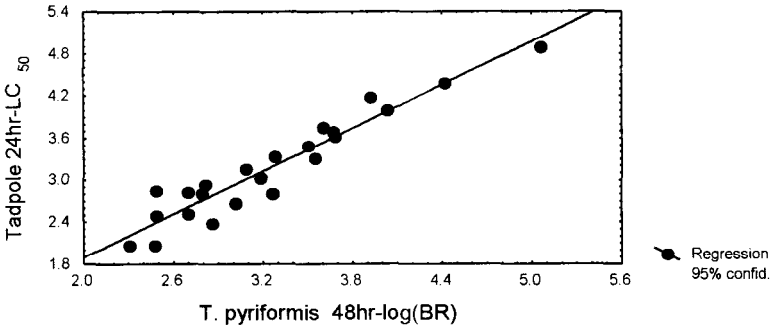

Figure 1. 24hr-LC_{50} for Tadpole vs 48hr-log(BR) for T. pyriformis

$$LC_{50} = 0.95 \log (BR) - 0.15 \quad (7)$$
$$n = 23 \quad r^2 = 0.91 \quad F = 211.4 \quad SE = 0.23 \quad P < 0.00000$$

Figure 1 illustrates the scatter plot of the 24hr-LC_{50} for *Rana japonica* versus48hr-log (BR) for *Tetrahymena pyriformis.* The good correlation suggests the phenols exhibit similar mechanisms in the two assay systems. Hence *Tetrahymena pyriformis* can serve as a surrogate organism for tadpole toxicity test. Since varied mechanisms, including polar narcosis, electrophilicity and pro-electrophilicity, have been observed in our investigation of the acute toxicity of phenols to tadpole, the good correlation between these two systems, regardless of the diversity of the toxic mechanisms, implies that surrogate relationship can also be established for polar narcotics and more specific mechanism of toxic action.

In conclusion, the 24hr semi-static acute mortality toxicity of 24 phenol derivatives to *Rana japonica* tadpoles was determined polar narcosis proved to be the main mechanism of toxic action for most of the substituted phenols and an excellent QSAR model was achieved with combination of $\log K_{ow}$, and pKa.

Electrophilicity and pro-electrophilicity were also observed in selected phenols. A comparative study was carried out between the relative toxicity of phenols to tadpole and to *Tetrahymena pyriformis* in which a strong interspecies correlation was found. This suggested a great similarity of the mechanisms that phenols exhibit in the two test systems, with salicylaldehyde being a exception.

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