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Evaluation and Structure-Activity Relationship Study of Acute Toxicity of Naphthoquinones to *Photobacterium phosphoreum*, *Photobacterium* T3B

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Abstract The acute toxicities of five naphthoquinone compounds to *Photobacterium phosphoreum* were determined. We evaluated the mechanism of toxicity using the structure-activity relationship technique. The results showed that some factors, including the species of substituents, shape/size of molecule and oil–water partition coefficient (log *P*) played the important roles in the interaction between the naphthoquinones and the target. Among of these, the toxicities of Atovaquone and Buparvaquone were lower than the other naphthoquinones we tested because of the alkyl-substitution with the bigger volume and strong hydrophobicity. Conversely, Menadione had the highest toxicity because of the appropriate log *P* and shape/size of molecule resulting in the easier interaction with the target.

Keywords *Photobacterium phosphoreum* · Naphthoquinones · Structure-activity relationship · Mechanism of toxicity

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Naphthoquinones are widely used in the production of medicines. Buparvaquone is very effective in curing cattle artificially infected with *Theileria parva* and *Theileria annulata* (Mchardy et al. 1985). A relatively new drug, marketed under the trade name of Malarone, is a combination of Atovaquone and Proguanil that has blood-stage and liver-stage activity against *Plasmodium falciparum*. Malarone has proved effective both for treatment and prophylaxis of chloroquine-resistant malaria in adult and pediatric populations (Boggild et al. 2007). Parvaquone and Buparvaquone (Dolan et al. 1992) were both successful in curing clinical East Coast fever on farms in Kenya. Diosquinone, a common ingredient in several folk medicines and foods, has been shown to have broad-spectrum antibacterial activity (Lajubutu et al. 2006).

Generally speaking, the naphthoquinones may enter the environment as a result of research, production, transportation, use or waste treatment. Even, these compounds may be residual in the surface or underground runoff by rainfall. Atovaquone can reversibly combine with 11,500 Da molecular group. Dihydroorate dehydrogenase plays an important part in biosynthesis of pyridine. Electron transport will occur when coenzyme Q connect with mitochondria. Atovaquone can prevent the synthesis of pyridine by inhibiting the electron transport. Some metabolic enzymes can partake the electron transport occurred in the mitochondria via the coenzymes. So, Atovaquone can inhibit the activities of the metabolic enzymes by inhibiting the electron transport. Naphthoquinone compounds can be transported into the water supply via atmospheric and terrestrial processes. It can bring some threats to environment. Thus, the enough attention must be paid to avoid a threat to the ecological environment.

Bioluminescent bacteria are used as a bioluminescent indicator of pollutants to monitor the water quality with

high sensitivity and rapid response (Kuznetsov et al. 1998). The bioluminescent bacterial test utilizes a standardized culture of a selected strain of a marine bacterium, *Photobacterium phosphoreum*, the light output of which can be measured. The addition of a toxic substance to the bacterial suspension in most cases results in a rapid decrease of its light emission, and the toxicity is recorded as the percent decrease in luminescence after a certain time (Brenner et al. 1993). As a simple, fast and comparatively inexpensive alternative to in vivo bioassay with higher organisms, the bioluminescent bacteria-test has been widely applied.

System, in-depth study on the environmental impact of these compounds, especially on the aquatic eco-toxicity and mechanism of toxicity, is an urgent need. To achieve this, *P. phosphoreum* was used as the candidate to investigate the acute toxicity of naphthoquinone compounds. In addition, we evaluated the structure-activity relationship (SAR) for naphthoquinone compounds to conjecture the mechanism of toxicity for naphthoquinones to *P. phosphoreum*.

Materials and Methods

The naphthoquinones tested here are listed in Table 1. Acetone was purchased from the Tianjin standard Chemical Reagent Company (Tianjin, China). $HgCl_2$ was purchased from the Tianjin Xigui Company (Tianjin, China).

The test procedures followed the national standard method of China, GB/T 15441-1995. Stock solutions of the naphthoquinones were prepared by ultrasonic dispersion in acetone because this carrier has low toxicity to *P. phosphoreum*. Prior to each experiment, the stock solutions were freshly prepared, diluted to the desired concentrations in medium. The concentrations of samples are listed in Table 2.

P. phosphoreum (T3 mutation) was supplied in the form of freeze-dried powder by the Institute of Soil Science, Chinese Academy of Sciences, Nanjing, China. The MICROTOX test instrument (DXY-2, the Institute of Soil Science, Chinese Academy of Sciences China) was used. A working solution of luminescent bacteria was prepared by reconstituting a vial of freeze-dried *P. phosphoreum*, using 1 mL of 2.5% NaCl aqueous solution at 2–5°C and fully homogenized. Toxicity was measured with the DXY-2 by quantifying the decrease in light emission from the bacteria as a result of exposure to the tested chemicals in 3% NaCl solution for 15 min in a test tube at 21–23°C. The decrease in light emission was measured at five different concentrations and the control group (three treatment groups per concentration) (Chu et al. 1997). Based on the decrease in light emission, the half effective concentration (EC_{50}) was calculated. The suppression ratio was used to statistically estimate the EC_{50} after exposure using the probit method in SPSS software (SPSS Inc., Chicago, USA) (Wang et al. 2007). The square of correlation coefficient (r^2) was used to describe the remarkable correlation between the suppression ratios and concentrations tested.

The calculations for SAR study were performed using the following three steps: Firstly, the structure of compound tested was obtained by Chemoffice software (CambridgeSoft Corporation, MA, USA). The logarithm of *n*-octanol/water partition coefficient (log *P*), molecular diameter (Dia) and molecular topological index (TIndx) were also calculated by Chemoffice. Secondly, the structure of naphthoquinone was optimized by Hartree–Fock method and 6-31G basis using Gaussian 03w package (Gaussian Inc., Pittsburgh, USA) until the stable point in the potential energy surface was found. Then the net charges of atoms and the energies of the frontier orbits, including the highest occupied molecular orbit (E_{HOMO}), the lowest unoccupied molecular orbit (E_{LUMO}) were calculate by Gaussian 03w (Gaussian Inc. 2008).

Results and Discussion

P. phosphoreum is suggested as specific spoiler organisms in modified atmospheres packed cod because the characteristics of the bacteria in pure cultures qualitatively and quantitatively match those of spoiled packed cod (Dalgaard 1995). The acute toxicities of naphthoquinones to *P. phosphoreum* (expressed as EC_{50}) were determined at 15 min post-exposure, and are listed in Table 2.

As shown in Table 2, the toxicities of five naphthoquinones to *P. phosphoreum* varied significantly. The toxicity of Buparvaquone ($EC_{50} > 80$ mg/L) was significantly lower than the other naphthoquinones we tested. The toxicity of Atovaquone ($EC_{50} > 40$ mg/L) was slightly lower than Buparvaquone. Therefore, Buparvaquone and Atovaquone had a very low toxicity to *P. phosphoreum*. Conversely, Menadione had the highest toxicity (EC_{50}: 0.226 mg/L). And 2-ethoxy-1,4-naphthoquinone was moderately toxic to *P. phosphoreum* ($p < 0.05$).

The molecular structure and the atomic serial numbers used for calculations are shown in Table 1. The results of quantitative calculation, including the net charges, the energies of the frontier orbits, log *P*, Dia and TIndx were listed in Table 3. SAR was analyzed based on these results.

Exogenous chemicals can arrive at the active site through the barriers of the cell wall and membrane, then act with the target. The process is influenced by the lipophilic property and stereoscopic effect of molecular structure. Studies showed that the toxicity of nitrogen heterocyclic

Table 1 Description of the structure, manufacturers and CAS of naphthoquinones tested in the current study (Tong et al. 2005)

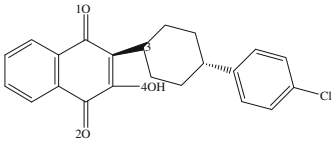
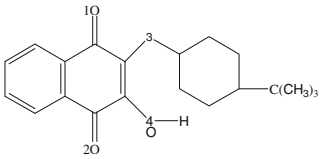
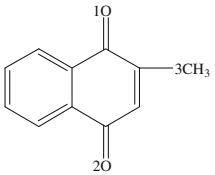
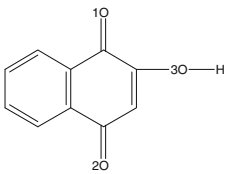
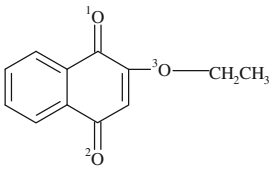
No.	Compound	CAS	Structural formula	Purity and manufacturer
1	Atovaquone	95233-18-4		99.2%, Beijing Intermediate Import and Export Company
2	Buparvaquone	88426-33-9		99.6%, Beijing Intermediate Import and Export Company
3	Menadione	58-27-5		99%, Aladdin Products Corporation
4	2-Hydroxy-1,4-naphthoquinone	83-72-7		99%, J&K Chemical Ltd
5	2-Ethoxy-1,4-naphthoquinone	–		99%, Synthesized according to the literature

Table 2 Concentrations and EC_{50} (15 min) of the acute toxicity of naphthoquinones to *P. phosphoreum*

No.	Concentration (mg/L)					EC_{50} (mg/L) (95% confidence)	n	r^2
1	40.000	35.000	30.000	25.000	20.000	$>40^{A}$	5	–
2	80.000	70.000	60.000	50.000	40.000	$>80^{B}$	5	–
3	0.400	0.300	0.200	0.100	0.050	0.226^{C} (0.184–0.267)	5	0.975
4	20.000	15.000	10.000	5.000	1.000	8.408^{D} (6.708–10.108)	5	0.987
5	20.000	15.000	10.000	5.000	1.000	7.627^{E} (6.892–8.363)	5	0.931

EC_{50} values sharing the same superscript letter are not significantly different ($p > 0.05$). n means the sample number. r means the correlation coefficient

Table 3 Results of quantitative calculation

No.	Net charge				E_{HOMO} (au)	E_{LUMO} (au)	log P	Dia	TIndx
	Atom 1	Atom 2	Atom 3	Atom 4					
1	−0.55	−0.58	0.03	−0.26	−0.3191	0.0242	4.0034	14	12,452
2	−0.56	−0.51	0.02	−0.24	−0.3477	0.0362	4.1402	12	10,384
3	−0.53	−0.54	0.09	–	−0.3532	0.0318	1.5780	6	1,694
4	−0.50	−0.56	−0.24	–	−0.3550	0.0321	0.1888	6	1,608
5	−0.53	−0.56	−0.63	–	−0.3477	0.0362	0.8883	8	2,568

pyridine compounds to aquatic organisms was mainly affected by the hydrophobicity and molecular shape/size (Moulton and Schlutz 1986). Among which the molecular topology index, representing the stereoscopic effect of molecule, can characterize the structural information, such as the order of interatomic combination, the number of branches, the molecular shape and the presences of heteroatoms. According to the information, the physical–chemical properties and biological activity of some organic chemicals can be effectively predicted.

Dia and TIndx are often used to characterize the shape and size of molecules. As showed in Table 3, Dia and TIndx values of Atovaquone and Buparvaquone were much bigger than the other three compounds because of the substituents with bigger volumes occupied in 2-position of Atovaquone and Buparvaquone. 2-Positions of Atovaquone and Buparvaquone were successively substituted by six-membered ring with bigger volume and steric hindrance than the others. The shape and size of molecule can influence the ability of pollutant penetrating the biofilm to arrive at the target. It is an important factor for the toxicity of pollutants to aquatic organisms. It is speculated that the type and shape/size of substituent may influence the action model of pollutant with target enzymes. Therefore, larger volumes of 2-subsitituent may help Atovaquone and Buparvaquone to reduce the opportunity to react with biological macromolecules of organisms and the toxicity to *P. phosphoreum*.

SAR analysis about the toxicity of chemicals towards *P. phosphoreum* is often performed employing log *P* as parameters (Deneer et al. 1989; Gough et al. 1994). It is found that the main factor affecting the acute toxicity of pollutants is the partition coefficient. The descriptor, log *P*, can characterize the distribution of pollutants in the water phase and the organism, thus affecting the toxic effects of pollutants. In this study, Atovaquone and Buparvaquone also had strong hydrophobicity because of six-membered ring substituted in 2-position. The hypothesis was in accordance with the results of calculation. As shown in Table 3, the log *P* values of Atovaquone and Buparvaquone (4.0034 and 4.1402, respectively) were much higher than the other compounds. The oil–water partition coefficient, often represented as log *P*, plays an important role in the toxicity of compounds. High value of log *P* means the pollutant had a low solubility in water and the concentration of pollutant in water phase leading to the lower toxicity to *P. phosphoreum*. The results of experiment indicated that Atovaquone and Buparvaquone had low toxicities to *P. phosphoreum*, which was consistent with the speculation. The log *P* of Menadione was higher than 2-hydroxy-1,4-naphthoquinone and 2-ethoxy-1,4-naphthoquinone. When they were dispersed in the water, Menadione was easier to penetrate the epicyte because of its better lipochicity. So, the toxicity would be the highest, which was proved by the experiments.

The energies of the frontier orbits, such as E_{HOMO} and E_{LUMO}, have a close connection with the abilities of receiving and donoring the electrons. But in this study, there was no obvious trend to influence the toxicity of naphthoquinone.

According to the literature (Ding et al. 2008), the active positions of these naphthoquinones acting with the biomacromolecule of *P. phosphoreum* probably were 1-carbonyl and 2-alkyl groups. When the pollutants interacted with the biomacromolecule, the oxygen atom of 1-carbonyl may form hydrogen bond with the biomacromolecule. Therefore, Menadione had a higher toxicity than any other compounds because of the methyl group with the appropriate steric hindrance. Atovaquone and Buparvaquone having the lower toxicities may be due to the too much steric hindrance of 2-subsitituent.

These results indicated that considering the various elements, including log *P*, steric field, donor and acceptor and hydrogen bond could improve the reliability of QSAR models when 3D-QSAR or hologram QSAR (HQSAR) study was conducted.

In sum, the toxicities of Buparvaquone and Menadione were the lowest and the highest to *P. phosphoreum*, respectively. Some factors, including the species of substituents, shape/size of molecule and the oil–water partition coefficient played the important roles in toxic effects of naphthoquinones to *P. phosphoreum.* This study provides a foundation for further investigation about 3D-QSAR and HQSAR study to evaluate the aquatic ecological risk and a more detailed mechanism of toxicity.

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