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The joint effects of sulfonamides and their potentiator on *Photobacterium phosphoreum*: Differences between the acute and chronic mixture toxicity mechanisms

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ABSTRACT

Organisms are typically exposed to mixtures of chemicals over long periods of time; thus, chronic mixture toxicity analysis is the best way to perform risk assessment in regards to organisms. However, most studies focus on the acute mixture toxicity. To investigate the difference between chronic mixture toxicity and acute mixture toxicity, *Photobacterium phosphoreum* were exposed to chronic (24 h exposure) and acute (15 min exposure) toxicity of single sulfonamide (SA) and their potentiator (trimethoprim, TMP), both individually and mixtures (SA with TMP). A comparison of chronic vs. acute mixture toxicity revealed the presence of an interesting phenomenon, that is, that the joint effects vary with the duration of exposure; the acute mixture toxicity was antagonistic, whereas the chronic mixture toxicity was synergistic. Based on the approach of Quantitative Structure Activity Relationships (QSARs) and molecular docking, this phenomenon was proved to be caused by the presence of two points of dissimilarity between the acute and chronic mixture toxicity mechanism: (1) the receptor protein of SAs in acute toxicity was Luc, while in chronic toxicity it was Dhps, and (2) there is a difference between actual concentration of binding-Luc in acute toxicity and individual binding-Dhps in chronic toxicity. This deep insight into the difference between chronic and acute mixture toxicity will benefit environmental science, medical science, and other disciplines. The existence of these differences poses a challenge for the assessment of routine combinations in medicine, risk assessment, and mixture pollutant control, in which, previously, only a synergistic effect has been observed between SA and their potentiator.

1. Introduction

Organisms are typically exposed to mixtures of chemicals over long periods of time (Yang et al., 1998), therefore, chronic mixture toxicity data are better determinants for risk assessment compared to any other toxicity data, such as acute mixture toxicity, acute single toxicity or chronic single toxicity (Cleuvers, 2008). Accordingly, it is urgent and necessary to study chronic mixture toxicity.

Until now, most studies have been performed on the chronic toxicity of single chemicals. Those studies have indicated that single chronic toxicity differs from acute toxicity. This difference depends on the chemical model of action (MOA) (Ahlers et al., 2006), which classifies the chemicals as narcotic substances or specific acting compounds (Verhaar et al., 1992). For narcotic chemicals, both single chronic toxicity and acute toxicity can be explained by the disruption of the proper function of the cell membrane and can be quantified by using log K_{ow} (octanol/water partition coefficient) (Verhaar et al., 1992). For example, Wei et al. (1999) investigated the acute and chronic toxicity of narcotic compounds to *Daphnia magna*, and the results indicated that both the acute and chronic toxicity were correlated with logK_{ow}. Furthermore, Blaschke et al. (2010) observed the acute and chronic toxicity of narcotic compounds with regards to *Vibrio fischeri* and found that they all had good a relationship with logK_{ow}, which was displayed by the squared correlation coefficient (r^2) of 0.95 and 0.94. However, the compound specific acting on the substrates may interfere with proteins in acute toxicity tests, while they may interfere with the biosynthesis in a chronic toxicity test

Abbreviations: Luc, luciferase; Dhps, dihydrofolate reductase; SA, sulfonamide; TMP, trimethoprim; Dhfr, dihydropteroate synthase; PABA, 4-aminobenzoic acid; TU, toxicity unit; QSARs, Quantitative Structure Activity Relationships.

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(Backhaus et al., 1997; Blaschke et al., 2010). Therefore, the chronic toxicity of these chemicals could be observed to be higher than in acute toxicity (Ahlers et al., 2006). For instance, Heckmann et al. (2007) performed the acute (48 h-immobilization) and chronic (14 d-reproduction) toxicity test of ibuprofen to *D. magna*, and the results showed that the inhibition of the eicosanoid biosynthesis by ibuprofen caused the most toxic effect (48 h-EC_{50} = 10–100 mg L^{-1}, 14 d-EC_{50} = 13.4 mg L^{-1}) in the chronic toxicity test rather than in the acute test. Furthermore, a bioluminescence inhibition study with triazine herbicides on photobacterium (Q67) was conducted by Zhu et al. (2009), in this study that the chronic toxicity of four chemicals (velpar, prometon, metribuzin, and aminotriazine) were determined to be higher than in the acute test, and the results were explained by their interference with the duplication and transcription of DNA in the chronic toxicity test.

However, in the field of mixture toxicity, little is known about the chronic effects on organisms because of the complex nature of the determination method and the high cost of these analyses (Lund et al., 1999; Cleuvers, 2008), although the pioneering report considering this topic was published almost 90 years ago (Hunt, 1928). There have been no studies on the chronic mixture toxicity mechanism. Consequently, it remains unknown whether there is a difference between acute and chronic mixture toxicity mechanisms. If there is a difference, the factors that cause these differences should be determined. The present study addresses this problem.

It is well known that the Quantitative Structure–Activity Relations (QSAR) approach has been determined to be a promising method for providing information on toxicological and ecotoxicological mechanisms over the last decade (Wang et al., 2001; Lin et al., 2003). Furthermore, studies on the QSAR-based mechanism have been further developed by the introduction of the protein-receptor interaction energy ($E_{binding}$). Recently, Li et al. (2010) revealed the QSAR-based mechanism of HO-PBDEs by using $E_{binding}$ between hydroxylated polybrominated diphenyl ethers (HO-PBDEs) and thyroid hormone receptors (TRβ).

It is necessary for researchers to study the chronic toxicity of antibiotic mixtures (Cleuvers, 2003; Crane et al., 2006) because of their widespread use and continuous emissions into the environment, they are categorized as persistent pollutants (Quinn et al., 2008; Kummerer, 2009a,b). Some antibiotics, such as sulfonamides (SAs), are widely used in combination therapy together with their potentiator (mostly TMP) in human and veterinary medicine(Sarmah et al., 2006); thus, the occurrence of TMP together with other antibiotics have been commonly detected (Kolpin et al., 2002). Eguchi et al. (2004) studied the mixture toxicity of SAs with TMP using microlage, and the synergistic effect of the mixture was observed. De Liguoro et al. (2009) evaluated the acute mixture toxicity of combining sulfamethazine with TMP towards *D. magna*, and only additivity effects were observed. However, these results cannot represent the mixture toxicity between the SAs and TMP in an actual environment because non-target organisms (microlage and *D. magna*) were used in these studies. Bacterium is typically the target-organism of an antibiotic, and thus, a bioassay with *Photobacterium phosphoreum* is a reliable tool to determine the toxicity of various antibiotics, which has been proved as early as Kavanagh (1947).

Therefore, the purpose of this study is as follows: (1) to determine the chronic (24 h exposure) and acute (15 min exposure) toxicity to *P. phosphoreum* for single SA and their potentiator, and for their mixtures (SA with TMP), (2) to evaluate the differences between chronic and acute mixture toxicity, and (3) to reveal the difference between their toxicity mechanisms by using QSAR models with $E_{binding}$.

2. Materials and methods

2.1. Instruments, chemicals, and toxicity test

The tested pharmaceuticals were trimethoprim (TMP) and seven sulfonamide antibiotics, that is, sulfamethazine (SM 2), sulfapyridine (SPY), sulfamethoxazole (SMZ), sulfadiazine (SD), sulfisoxazole (SSZ), sulfamonomethoxine (SMM), and sulfachloropyridazine (SCP). All test antibiotics were purchased from Sigma Co. Ltd and used without further purification (purity ⩾ 99%). The freeze-dried marine bacterium, *P. phosphoreum* (T3 mutation), was supplied by the Institute of Soil Science, Academia Sinica, Nanjing PRC. It was reconstituted and maintained on agar slants at 4 °C.

The media effective concentrations of acute and chronic toxicity were expressed as $-\log(EC_{50}^{15m})$ and $-\log(EC_{50}^{24h})$, respectively. The EC_{50M} and toxicity units (*TU*), parameters used to describe the mixture toxicity effect, were calculated by Eqs. (1) and (2), respectively.

$$EC_{50M} = \frac{C_M}{\frac{C_A}{EC_{50A}} + \frac{C_B}{EC_{50B}}} \quad (1)$$

$$\mathrm{TU} = \frac{C_A}{EC_{50A}} + \frac{C_B}{EC_{50B}} \quad (2)$$

where C_A and C_B are concentration of individual chemical in mixtures at median inhibition, it can be calculated according to the median effective concentration of mixture. EC_{50A} and EC_{50B} are the median effective inhibition concentration of component A and B, the unit of EC_{50} is M. According to Broderius et al. (1995), simple addition is characterized by 1.2 > TU > 0.8, where TU > 1.2 represents antagonism and TU < 0.8 indicates synergism. Details on the stability of the chemical test and toxicity measurements can be found in the Supplementary material.

2.2. Homology modeling and molecular docking

The crystal structures of luciferase (Luc, E.C.1.14.14.3, 1BRL.pdb) were obtained from the protein data bank (http://www.pdb.org). The homology models of Dhps and dihydropteroate synthase (Dhfr) were built using a template structure of 1AJ0 (PDB ID) and 3FRB (PDB ID), respectively. Detailed information is provided in the Supplementary material.

2.3. Statistics

The statistical analysis was performed using SPSS 13.0 software (SPSS Inc.). The developed models were evaluated by the coefficient of determination (R^2), the standard error of estimate (*SE*), the Fisher criterion (*F*), and a p-value test (*P*).

3. Results and discussion

3.1. Toxicity data

Before the determination of the toxicity of single compounds, the stability of seven SAs and TMP was tested, and the results are listed in Table 1. Table 1 shows that there was only a slight loss of the test antibiotics (<1.50%) after 24 h, and therefore the tested compounds were stable enough to obtain valid toxicity results.

The acute toxicity of the mixtures of individual antibiotics to *P. phosphoreum* was determined, and the results ($-\log(EC_{50}^{15m})$) are presented in Table 1. SCP ($-\log(EC_{50}^{15m})$ = 4.30 M) was more toxic than other SAs, whereas SPY ($-\log(EC_{50}^{15m})$ = 2.92 M) was relatively