The interaction between organic phosphate ester and p53: An integrated experimental and in silico approach

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Abstract

Concerns have been raised in regards to the environmental impact of the more used organophosphate flame retardants (OPFRs). In this study, to better understand the relationship between molecular structural features of OPFRs and binding affinity for the tumor suppressor p53, an integrated experimental and in silico approach was used. From docking analysis, hydrogen bonding and hydrophobic interactions were found to be the dominant interactions, which implied the binding affinities of the compounds. The binding constants of 5 OPFRs were determined by surface plasmon resonance technology (SPR). Based on the observed interactions, appropriate molecular structural parameters were adopted to develop a quantitative structure-activity relationship (QSAR) model. The developed QSAR model had good robustness, predictive ability and mechanism interpretability. The interactions between the OPFRs and p53 (\(F_{\text{binding}}\)) and the partition ability of the OPFRs into the bio-phase are main factors governing the binding affinities.

1. Introduction

Owing to the phase-out of the commercial penta-BDE mixture in 2004, an increasing number of organophosphate flame retardants (OPFRs) are being used around the world and are consequently being introduced into the environment (Dishaw et al., 2011). Since OPFRs are not covalently bound to host materials, these compounds could diffuse out into surroundings relatively easily by volatilization, leaching or abrasion (Marklund et al., 2003; Reemtsma et al., 2008). OPFRs are widely distributed owing to their extensive use, and volatility, and are considered to be persistent in water and air (Reemtsma et al., 2008).

The chlorinated alkylphosphates such as tris-(2-chloro-, 1-methyl-ethyl)-phosphate (TCPP) and tris-(2-chloroethyl)-phosphate (TCEP) are mostly used as flame retardants in polyurethane foam (Andresen et al., 2004). The non-derivatised alkylphosphates such as tri-n-butylphosphate (TnBP), tri-iso-butylphosphate (TIBP), triphenylphosphate (TPP) and tris-(butoxyethyl)-phosphate (TBEQ) are predominantly utilised as plasticisers, lubricants and to regulated pore sizes, e.g., in concrete, though in some cases, they are also used as flame retardants. Many of these flame retardants and plasticizers have been frequently detected in various environments, including indoor and outdoor air (Hartmann et al., 2004), indoor dust (Marklund et al., 2003; Stapleton et al., 2009; Takigami et al., 2009), water (Bacaloni et al., 2008; Regnery and Puttmann, 2010), sediments (Garica-Lopez et al., 2009), soils (David and Seiber, 1999), landfill leachates (Yasuhrara et al., 1999) and even in the aquatic organisms and in human breast milk (Liu et al., 2012).

OPFRs are structurally similar to neurotoxic organophosphate pesticides, raising concerns about exposure and toxicity to humans (Dishaw et al., 2011). Previous studies have demonstrated that some OPFRs, especially chlorinated OPFRs, are highly persistent in the environment (Meyer and Bester, 2004; Regnery and Puttmann, 2010), and they can even persist in drinking water after conventional treatment, indicating that these compounds can reach the human body via drinking water (Stackelberg et al., 2004; Andresen and Bester, 2006). However, little is known about the toxicity of OPFRs, although some studies indicate that TIP and TnBP are suspected to be neurotoxic (Martinez-Carbello et al., 2007), while others like TCEP, TDCP and TCPP are carcinogenic for animals (Matthews et al., 1990; Commission, 2001).

TIP has been shown to be a potent human blood monocyte carboxylesterase inhibitor (Matthews et al., 1990; Commission, 2001).
2. Materials and methods

2.1. Total RNA extraction

Total RNA was extracted from zebrafish using the TRIzol Reagent (Invitrogen, USA) following the manufacturer’s protocol. The extracted RNA was then treated with RNase-Free DNase (Promega, USA) to remove DNA contamination.

2.2. Cloning of the full-length cDNAs for p53

Tumor suppressor protein p53 cDNA fragments were amplified and cloned from the total RNAs by reverse transcription and polymerase chain reaction method (RT-PCR) using p53-specific primers 5'-GAATTCGCGGATCCATGGATT-3' and 5'-TTTCTGAGCTTGCTCCTCAC-3’. Single-stranded cDNAs were synthesized from one microgram of the total RNA using M-MLV reverse transcriptase (Promega, USA) according to the manufacturer’s protocol.

2.3. Induction and expression for p53

The final amplified product was cloned in the pET28a expression vector (Novagen). The recombinant plasmids were prepared by an alkaline lysis procedure and sequenced to confirm the size and orientation of the insert. The core DNA-binding domain was overproduced in Escherichia coli BL21 (DE3). The cells were incubated at 37°C until an OD of 0.5~0.8 had been attained. Then, 0.6 mM isopropyl β-D-thiogalactoside was added to induce the expression of the recombinant protein, and the incubation was continued at 25°C for 6 h. All subsequent procedures were performed at 4°C. The cells were harvested by centrifugation and were washed and purified as previously described (Xue et al., 2009). Then, the purified p53DBD was checked on an SDS-polyacrylamide gel for purity.

2.4. Binding constants for OPFRs using biacore analyses

The interaction between p53 protein and OPFRs was studied by surface plasmon resonance (SPR) on Biacore T100 (GE Healthcare) using CM5 sensor chip at 25°C. Research grade CM5 sensor chips were employed. The CM5 sensor chip was activated using the recommended protocol (BIAapplications Handbook, BIAcore, Uppsala, Sweden). The zebrafish p53 DBD was immobilized on a CM5 chip. The immobilization levels were selected for binding and kinetic assays, respectively. The running buffer was filtered (pore size 0.22 mM) and degassed using a filtration apparatus immediately before use.

The CM5 sensor chip surface was activated by injection of EDC/NHS (1:1) at 10 μL/min for 7 min, then injection of 50 μg/mL p53 in 10 mM sodium acetate pH 5.0 at 10 μL/min for 7 min to immobilize p53 on the CM5 sensor chip surface, deactivate excessive reactive groups using ethanamine. The binding of ligands to the p53 immobilized on the sensor chip was monitored in real time, using HBS with 0.05% P20 and 5% DMSO as the running buffer (10 mM HEPES, 0.15 M NaCl, pH 7.4) at the flow rate of 30 μL/min. The sensorgrams were fitted with a 1:1 binding model using BIA T100 evaluation software (GE Healthcare).

In the standard SPR analysis, the association rate constant (k+a) and dissociation rate constant (k-d) are obtained individually, and the dissociation constant (Kd) value can be determined by the quotient of the rate constants (k-a/k-d). More details could be found in the previous study (Drake et al., 2012; Katayama et al., 2012).

2.5. Simulation for binding interaction by molecular docking

Homologous 3D model of p53DBD was built on SWISS-MODEL net server. The binding interactions were studied by CDOCKER, which has been incorporated into Discovery Studio 2.5 (Accelrys Software Inc.) through the Dock Lipids protocol. CDOCKER is an implementation of the docking tool based on the CHARMM force field that has been proven to be viable (Wu et al., 2003). In CDOCKER, random ligand conformations are generated through molecular dynamics, and a variable number of rigid-body rotations/translations are applied to each conformation to generate the initial ligand poses. The conformations are further refined by grid-based simulated annealing in the receptor active site, which makes the results accurate. From the docking analysis, insights into the interactions between the ligands and the receptor were gained. Additionally, the binding free energies (E(binding)) of the 5 OPFRs were obtained.

2.6. Molecular structural descriptor generation and QSAR development

It was hypothesized that the binding activity of OPFRs depended on the following two processes: (a) the penetration of OPFRs through bio-membrane and reaching the target site of action, and (b) the interactions between the OPFRs and p53. Thus, the logarithm of octanol/water partition coefficient (logKow) and E(binding) were selected to describe these processes and construct QSAR models. logKow was purposely selected to describe the partition process. E(binding) was also considered because binding to
the receptor was the initiating step in toxicity by chemicals. $\log K_{ow}$ values were computed by the EPI Suite 4.0™ (http://www.epa.gov/oppt/exposure). QSARs model were developed using MLR, an easily applicable and unambiguous algorithm suggested by OECD (2007). In MLR, multicollinearity among the input variables may result in wrong signs and magnitudes of regression coefficients. Thus the variance inflation factor ($VIF$) was adopted to evaluate multicollinearity among the input variables. $VIF$ values exceeding 10 are often regarded as serious multicollinearity. The robustness of the model was evaluated by internal validation, and the leave-one-out cross-validated coefficient ($Q_{loo}^2$) was calculated. The performance of external validation was characterized by the root mean square error (RMSE). These parameters are defined as follows (Schüürman et al., 2008):

\[
Q_{loo}^2 = 1 - \frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{n} (y_i - \bar{y})^2}
\]

\[
RMSE = \sqrt{\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n}}
\]

where $y_i$ and $\hat{y}_i$ are the observed and predicted values for the $i$-th compound, respectively. $\bar{y}$ is the average response value of the training set. $n$ stand for the number of compounds in the training sets.

3. Results and discussion

3.1. Expression and purification of tumor suppressor protein p53DBD

To investigate the binding reaction between p53DBD and OPFRs, a truncated form of p53DBD was expressed and purified. SDS-PAGE analysis showed that the 57 kDa p53DBD protein was the predominant polypeptide in the purified fraction (Fig. 1). The concentration of p53DBD protein in this fraction was estimated to be 0.83 mg/L.

3.2. Binding kinetic analysis between p53 and OPFRs

When OPFRs reacts with p53DBD to form complex, the complex formation depends on the free concentrations of OPFR, p53DBD and the stability of the complex, which can be described by the following equation:

\[
d[\text{OPFR} - \text{p53DBD}] = k_a[\text{OPFR}][\text{p53DBD}] - k_d[\text{OPFR} - \text{p53DBD}]
\]

where $k_a$ is the association rate constant and $k_d$ is the dissociation-rate constant. The p53DBD was immobilized on gold surface and OPFRs was injected into the flow system as the analyte. The kinetic constants of the binding process were given in Table 1.

3.3. Docking analysis

The docking view of three representative OPFRs (TCEP, TEP and TPrP) in the binding site of p53 was shown in Fig. 2. At the deep end of the pocket, His282 and Ala275 serve as anchoring points for the ligands, which explains the predominant contribution of the hydroxyl group to the binding affinity of chemicals. The ligands also interact with another polar region within the binding pocket, that is, with Val141.

As shown in Fig. 2, hydrogen bonding and hydrophobic interactions are characteristic interactions between the OPFRs and p53. As shown in Fig. 2, there are mainly three types of H-Bonds: (a) H-bonds formed between the oxygen of TCEP, TPrP and the hydrogen of Ala129 and His182, (b) H-bonds between the hydrogen of TCEP, TEP with the carbonyl oxygen of Val141, and (c) H-bonds between the chlorine of TCEP and phenyl hydrogen of His182 and Leu162. Acting as an ‘anchor’, the hydrogen-bonding intensely determines the 3D space position of the chemicals in the binding pocket, and facilitates the hydrophobic interaction of the OPFRs with the side chain of Val141. Arg181, Ile163, Ala129, His182, Ser183. Fig. 3 shows the electrostatic potential of the ligand binding site for p53. The binding site has positive potentials, from which it can also be concluded that the negative potentials of the OPFRs molecules facilitate them to bind with p53.

The binding free energies ($E_{binding}$) of the 5 OPFRs are listed in Table 1. A simple linear free energy relationship (LFER) between log $K_d$ and $E_{binding}$ was obtained (Fig. 4), which further proved that the binding to p53 was a key step for OPFRs to exert their toxicity. However $E_{binding}$ itself was not a good predictor for log $K_d$, as indicated by the big prediction residuals for some OPFRs (Fig. 4). Thus, it is necessary to develop poly-parameter QSAR models for log $K_d$ prediction.

3.4. Development and validation of the QSAR model

For the training compounds, MLR analysis with log $K_d$ as the dependent variable and the selected parameters as predictor variables resulted the following optimal QSAR model:

\[
\log K_d = -5.41 + 5.53 \times 10^{-3} V + 1.36 \times 10^{-2} E_{binding}
\]

$n = 5$, $R^2 = 0.990$, $Q_{loo}^2 = 0.968$, $RMSE = 0.078$, $p < 0.01$

where $p$ is the significance level.

The $VIF$ values of $V$ and $E_{binding}$ are 1.29 and 1.27, respectively. For the current QSAR model, all the predictor variables are statistically significant ($p < 0.005$), and all the $VIF$ values are <2, implying that the QSAR model overcomes the problem of multicollinearity and thus the values of the regression parameters are genuine. The $R^2$ value of the QSAR model is 0.990, indicating a good goodness-of-fit of the model. $Q_{loo}^2$ of the model is as high as 0.968, implying good robustness of the model. The differences between $R^2$ and $Q_{loo}^2$ do not exceed 0.3, indicating no over-fitting in the model (42). The model has acceptable predictability, as indicated by $RMSE = 0.078$.

In the developed model, the t statistics for $V$ and $E_{binding}$ are 6.83 ($p < 0.05$) and 14.3 ($p < 0.005$), respectively. Indicating that these parameters are statistically significant. log $K_d$ values of OPFRs increase with $V$ values. The parameters molecular volume ($V$) was selected to partly describe the partition ability of OPFRs. $V$ correlates with log $K_{ow}$ positively, implying that OPFRs with large $V$ values tend to partition into the biophase easily. Moreover, the
Table 1
The association rate constant ($k_a$), the dissociation rate constant ($k_d$), the binding constant ($K_D$) and the binding energy ($E_{binding}$) for 5 OPFRs.

| No. | Compound                  | Abb.   | $k_a$ (1/M·s) | $k_d$ (1/s) | $K_D$ (M) | $E_{binding}$ (kJ/mol) |
|-----|---------------------------|--------|---------------|-------------|-----------|-----------------------|
| 1   | Tris(2-chloroethyl)phosphate | TCEP   | $5.19 \times 10^3$ | $1.45 \times 10^{-2}$ | $2.79 \times 10^{-6}$ | $94.33$ |
| 2   | Tri-n-propylphosphate      | TPrP   | $1.44 \times 10^4$ | $4.07 \times 10^{-2}$ | $2.82 \times 10^{-6}$ | $-18.40$ |
| 3   | Di-n-buty lphosphate       | DnBP   | $1.16 \times 10^3$ | $4.39 \times 10^{-2}$ | $3.8 \times 10^{-5}$ | $-28.40$ |
| 4   | Triethylphosphate          | TEP    | $6.11 \times 10^3$ | $7.29 \times 10^{-2}$ | $1.19 \times 10^{-5}$ | $-7.20$  |
| 5   | Tributylphosphate          | TnBP   | $5.22 \times 10^3$ | $4.16 \times 10^{-2}$ | $7.97 \times 10^{-6}$ | $-62.42$ |

(a) Tris(2-chloroethyl)phosphate
(b) Tri-n-propylphosphate
(c) Tri-n-propylphosphate

Fig. 2. Hydrogen bondings (left) and hydrophobic interactions (right) between TCEP, TEP and TPrP in the binding site of p53.
involvement of $E_{\text{binding}}$ in the Q SAR model implies the effects of ligand-receptor interaction on the estrogenic activity.

4. Conclusions

The binding constants of 5 OPFRs were determined by surface plasmon resonance (SPR). Docking analysis showed that hydrogen bonding and hydrophobic interactions between the ligand molecules and p53 governed the binding activities of the OPFRs. Based on the mechanism of action, a Q SAR was developed to characterize the interactions and model the binding affinities of the OPFRs. The partition ability of the OPFRs into the bio-phase was significant on the mechanism of action, a Q SAR was developed to characterize the interactions and model the binding affinities of the OPFRs. The developed Q SAR model has good robustness, predictive ability, and mechanism interpretability.

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