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Understanding the microscopic binding mechanism of hydroxylated and sulfated polybrominated diphenyl ethers with transthyretin by molecular docking, molecular dynamics simulations and binding free energy calculations†

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Polybrominated diphenyl ethers (PBDEs), one typical type of persistent environmental contaminant, have toxicological effects such as disrupting thyroid homeostasis in the human body. The high binding affinities of hydroxylated metabolites of PBDEs (OH-PBDEs) with transthyretin (TTR) were considered to be one major reason for their extraordinary capacity of passing through the blood–brain barrier via competitive thyroid hormone (T4) transport protein binding. Recent findings showed that sulfated PBDEs can be formed in human liver cytosol as phase-II metabolites. However, experimentally determined data for the TTR binding potential of the sulfated PBDEs are still not available. Therefore, molecular docking and molecular dynamics (MD) simulations were employed in the present study to probe the molecular basis of TTR interacting with hydroxylated and sulfated PBDEs at the atomic level. The docking scores of LeDock were used to construct the structure-based predictive model. The calculated results showed that the sulfated PBDEs have stronger affinity for TTR than the corresponding OH-PBDEs. Further analysis of structural characteristics based on MD simulations indicated that upon the binding of PBDE metabolites, the stability of TTR was enhanced and the dissociation rate of the tetrameric protein structure was potentially decreased. Subsequent binding free energy calculations implied that van der Waals interactions are the dominant forces for the binding of these metabolites of PBDEs at the T4 site of TTR. The residues Ser117/Ser117' and Lys15/Lys15' were identified, by both residue energy decomposition and computational alanine-scanning mutagenesis methods, as key residues which play an important role in determining the binding orientations of the $-\text{OSO}_3^-$ group of sulfated PBDEs by formation of either hydrogen bonds or electrostatic interactions, respectively. In general, the combination of docking calculations with MD simulations provided a theoretically toxicological assessment for the metabolites of PBDEs, deep insight into the recognition mechanism of TTR for these compounds, and thus more comprehensive understanding of the thyroid-related toxic effects of PBDEs as well.

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Introduction

Polybrominated diphenyl ethers (PBDEs), as flame retardants added to various consumer products such as plastics and construction materials, comprise 209 possible congeners with

different bromination degrees. Owing to their highly structural stability and long distance transport, PBDEs have been frequently detected in most environmental media including soil, air, sediments, house dust, and blood and urine samples of humans and wildlife.^{1,2} As PBDEs might be released from consumer products, increasing public health attention has focused on their accumulation, metabolism and related toxicity in the human body during the last few decades.

Hydroxylated metabolites (OH-PBDEs) are generated from the oxidative metabolism of PBDEs in humans,^{3,4} catalyzed by the cytochrome P450 (CYP450) enzymes in hepatocytes tissue.^{5,6} Both *in vivo* and *in vitro* studies have proved that BDE-47 (2,2',4,4'-tetrabromodiphenyl ether) as a major PBDE

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congener could form several hydroxylated metabolites found in human serum and plasma. Though OH-PBDEs were detected in human blood at a lower concentration than PBDEs, it is necessary to assess the toxicological effects and molecular targets of these compounds and their metabolites as well. Further metabolism of OH-PBDEs catalyzed by cytosolic sulfotransferases (SULTs) produces the sulfate conjugate *via* the substitution of an –OH group with an –OSO₃[−] group. Specifically, 3-OH-BDE-47 was confirmed as a substrate of estrogen sulfotransferase (SULT1E1) by crystallographic evidence.⁷ Erratico *et al.*⁸ also reported that for phase-II metabolism, 3 sulfated compounds (2'-Sulf-BDE-28, 3-Sulf-BDE-47 and 4-Sulf-BDE-42) were formed by pooled human liver cytosol as the major sulfated metabolite of BDE-47. However, the toxicity of the sulfated PBDEs (sulfated-PBDEs) is still not available despite the *in vitro* evidence of the sulfation of certain OH-PBDEs.

Due to the structural similarity of PBDEs to the thyroid hormones (thyroxine, T4) and triiodothyronine (T3), the thyroid gland is considered as a primary toxicity target for them. Several reports^{9–11} have shown that PBDE exposure in experimental rodents could lead to a decreased T4 concentration in serum. As an important moderator of cellular metabolism and the development of brain tissues, T3 and T4 play an essential role in the normal physiological function. Thus, the PBDE-induced interference of thyroid homeostasis is proposed as an indirect development disorder mechanism of brain tissues. Epidemiological reports have revealed a relationship between the PBDE exposure and their developmental neurotoxicity for brain tissues, including decreased cognitive function and anxious behavior. The detailed neurotoxicity information on PBDEs was highlighted in several reviews.^{12–14} Besides, the relevant interference has mainly been ascribed to their extraordinary capacity of passing through the blood–brain barrier in the cerebrospinal fluid and fetal tissues^{15,16} *via* competitive thyroid hormone (T4) transport protein binding, especially transthyretin (TTR). Particularly, various experimental evidence^{17–22} based on quantitative analysis methods has demonstrated the higher TTR-binding affinities of hydroxylated metabolites (OH-PBDEs) than those of corresponding parent PBDEs. Moreover, 3-OH-BDE-47, 4-OH-BDE-42 and 5-OH-BDE-47 even showed TTR binding affinities that were significantly stronger than that of T4, indicating that OH-PBDEs may present similar but more severe thyroid toxic effects than the parent PBDEs by disrupting the delivery of thyroid hormones to the brain and fetus. Furthermore, the structural similarity between T4 and the sulfated PBDEs, the sulfated metabolism products of OH-PBDEs, implies that sulfated PBDEs may act as highly potent ligands binding with TTR. Therefore, the TTR binding affinity of PBDE metabolites is very important for evaluating the thyroid toxic effects of PBDEs and their risks to public health.

Considerable quantitative structure–activity relationship (QSAR) models^{23–27} have been developed to screen active compounds with TTR binding potency. Unfortunately, limited to the applicability domain defined by certain model structure features, the TTR binding capacity of the sulfated PBDEs cannot be obtained by the extrapolation of the reported QSAR

models, and neither can the atomic-level recognition mechanism for the TTR interaction with ligands. It is necessary to adopt molecular simulations, coupling the docking technique with the molecular dynamics (MD) method, to elucidate the TTR binding capacity of sulfated PBDEs since experimental data are still unavailable. Therefore, the molecular docking method was utilized here to construct the structure-based predictive model and to determine the potential molecular interactions between these metabolites and the T4 binding site of TTR. Subsequently, for further elaborating the binding modes of PBDE metabolites at the T4 site of TTR, MD simulations were used to explore the underlying molecular recognition mechanism for the dynamic binding process during complex formation. Such dynamics investigations can shed light on the atomic-level interactions involved in ligand recognition. Based on the *in silico* predictions, the representative docking poses of hydroxylated and sulfated PBDEs were subjected to MD simulations and subsequent binding free energy calculations. This computational strategy has been successfully applied in analyzing conformational dynamics of different ligand–protein systems, such as environmental pollutants binding with estrogen receptors (ERs),²⁸ androgen receptors (ARs)²⁹ and human serum albumin (HSA).³⁰

In the current work, we first assessed the performance of different docking programs by calculating the linear correlation between docking scores and experimental affinity data of TTR interaction with OH-PBDEs. The best docking program was then utilized to predict the binding pose and affinities of the corresponding sulfated PBDEs. The representative structures of TTR–ligand complexes were further investigated by MD simulations to probe the ligand recognition mechanism at the atomic level. Both docking and MD results demonstrated that the sulfated PBDE compounds were stronger TTR-binding ligands when compared with parent OH-PBDEs. Subsequent analyses of structural characteristics and binding free energy calculations revealed that the key residue recognitions for Ser117/Ser117' and Lys15/Lys15' by the PBDE metabolites mainly contribute to the enhanced binding potencies of sulfated PBDEs. The findings provide insight into the underlying molecular basis of the PBDE metabolites binding to TTR and thus emphasize the important role of sulfated PBDEs in the biological effect and health risk of PBDEs.

Methods

Ligand and receptor preparation

3D structures of OH-PBDEs and sulfated PBDEs (Fig. 1) were sketched using the SyblyX1.2 program. The constructed structures were then optimized using the standard Tripos force field with an energy convergence gradient value of 0.001 kcal mol^{−1}. Eventual geometry optimizations were performed by Gaussian09³¹ at the B3LYP/cc-pVTZ level. The tetramer crystal structure of the TTR–T4 complex (PDB id: 1ICT)³² was employed as the receptor structure model. Swiss PDB viewer³³ was used to deal with the tetramer protein structure by adding missing hydrogen atoms and repairing incomplete residues.

Fig. 1 Structures of the tested OH-PBDEs and sulfated PBDEs.

Molecular docking study

Before the construction of a predictive model based on docking simulations, we first evaluated the prediction accuracy of different docking methods by calculating linear correlation between the docking scores and experimental data of TTR binding affinities. The TTR binding capacities of 20 mono-hydroxylated PBDEs with different degrees of bromination and substitutional positions of the –OH group were determined by different experimental assays.^{17–22} The concentrations of the ligand required to displace half of the probe from the TTR (IC_{50}) were extracted from previous studies. The logarithm of the relative binding affinity (log RBA) of a compound compared with T4 was employed to decrease the numerical biases of the IC_{50} values from different experimental measures (Table S1, ESI†). For certain compounds, which had different RBA values from different literature studies, the average value of RBA values was calculated and applied. The log RBA was used as the dependent variable to calculate their linear correlation with the docking scores.

During the docking process, the protein structure was rigid while the tested ligands were flexible for conformation searching. The crystal structure of TTR in complex with T4 was used to define the binding site of ligands, formed by chains A and C of 1ICT. The key settings of parameters for each docking program are described in the ESI.†

Molecular dynamics simulations

The 6 docked structures of TTR with OH-PBDEs and sulfated PBDEs were used as the initial structures for the MD simulations. The AMBER14SB force field³⁴ was used to establish the topology parameters of TTR, and the general AMBER force field (GAFF)³⁵ was adopted for the ligands. The partial charges of ligands were generated by fitting the electrostatic potentials calculated at the B3LYP/cc-pVTZ level, using the RESP fitting

technique³⁶ in the Antechamber module of AmberTools13.³⁷ Hydrogen atoms were added to the simulation systems using the tleap module. To simulate the physiological condition (pH = 7.4), the networks of hydrogen bonds and the protonation states of ionizable residues were constructed referring to the neutron diffraction studies of the human TTR structure.³⁸ An Apo TTR structure and TTR–ligand complexes were immersed, respectively, in a cubic periodic box of explicit TIP3P³⁹ water model, more than 10 Å away from the protein–ligand complex surface. All simulation systems were neutralized by randomly replacing water molecules with Na^+ . The particle mesh Ewald (PME)⁴⁰ method for the simulation of periodic boundaries was used to estimate the long-range electrostatic interactions with a cutoff of 10 Å. All R–H bond lengths were constrained using the SHAKE⁴¹ algorithm, and the integration time step was set to 2 fs using the Verlet leapfrog algorithm. To eliminate possible crash between the solute and the solvent, the entire system was minimized in two steps. In the first stage, the solute complex was restricted with a force constant $k = 100 \text{ kcal mol}^{-1} \text{ Å}^{-2}$. The positions of water molecules and Na^+ were optimized using the steepest descent method of 1000 steps, followed by 4000-step conjugate gradient calculations. Subsequently, the entire system was optimized by the first step method without any constraint. The two minimization steps were then followed by a simulated annealing phase with a weak restraint ($k = 10 \text{ kcal mol}^{-1} \text{ Å}^{-2}$) for the complex. The entire system was heated gradually in the *NVT* ensemble from 0 to 300 K over 500 ps, and then relaxed by 500 ps MD simulations in the *NPT* ensemble for density equilibrium. Finally, a 30 ns MD production simulation was performed for each system under 1 atm and 300 K conditions (*NPT* ensemble). Constant temperature was maintained using the Langevin thermostat with a collision frequency of 2 ps^{-1} , and constant pressure was maintained by employing an isotropic position scaling algorithm with a relaxation time of 2 ps. The MD simulations of the production

stage were implemented in the GPU accelerated PMEMD module⁴² (pmemd.cuda) using the hybrid SPFP model on a single Nvidia Tesla K20 card. Trajectory coordinates were saved every 1 ps for subsequent structure analyses and binding free energy calculations.

Binding free energy calculations

Binding free energy calculations of the end-state method were applied here using the MMPBSA.py⁴³ module of AmberTools13. The Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) algorithm was employed to calculate the relative binding free energy.^{44,45} The total binding free energy (ΔG_{cal}) can be calculated using the following equations:

$$\Delta G_{\text{cal}} = \Delta H - T\Delta S = \Delta E_{\text{MM}} + \Delta G_{\text{sol}} - T\Delta S \quad (1)$$

$$\Delta E_{\text{MM}} = \Delta E_{\text{vdw}} + \Delta E_{\text{ele}} + \Delta E_{\text{int}} \quad (2)$$

$$\Delta G_{\text{sol}} = \Delta G_{\text{GB}} + \Delta G_{\text{SA}} \quad (3)$$

ΔE_{MM} is the molecular mechanical (MM) energy, which includes ΔE_{vdw} (van der Waals), ΔE_{ele} (electrostatic) and ΔE_{int} (the internal energy), while ΔG_{sol} (the solvation free energy) includes ΔG_{SA} (nonpolar contribution) and ΔG_{GB} (polar contribution). ΔG_{GB} can be calculated using the GB models developed and implemented in Amber12, such as the Onufriev–Bashford–Case model and the modified Onufriev–Bashford–Case model (termed as GB^{OB1} (igb = 2) and GB^{OB2} (igb = 5), respectively).^{46,47} ΔG_{SA} is evaluated by solvent-accessible surface area (SASA) in equation $\Delta G_{\text{SA}} = \gamma \text{SASA} + \beta$ with correction constants γ and β (0.0072 and 0 kcal mol^{−1}, respectively) using the LCPO method.⁴⁸ The above energy terms stand for enthalpy contributions (ΔH) of total binding free energy. The contribution of ΔE_{int} is cancelled for complex systems to reduce the noise in a single trajectory approach.⁴⁹ The ionic strength (istrng) was set to 0.15, and the external and internal dielectric constants were set to 80 and 1, separately. All energy components were calculated using 2000 snapshots extracted from 10 to 30 ns MD trajectories of the production stage. The total enthalpy contributions (ΔH) were decomposed into each residue by means of an energy decomposition function of the MMPBSA.py module. According to the systematic research of Hou *et al.*, the inclusion of the conformational entropy may be crucial for the prediction of absolute binding free energies but not for ranking the binding affinities of similar ligands.⁴⁴ Therefore, the change of conformational entropy upon ligand binding has been ignored in our calculation because of expensive computational cost and low prediction accuracy. The same strategy has been successfully applied not only in analyzing a series of compounds of high structural similarity, triazine derivatives as ROCK1 inhibitors⁵⁰ but also in modeling the binding mechanisms of piperidine carboxamides as Type 11/2 ALK inhibitors⁵¹ and a thienopyridine JAK2 selective mechanism as well.⁵² Moreover, the comparison between the relative binding free energies computed without inclusion of conformational entropy and the experimentally known binding affinities for the OH-PBDEs included in the MD study has also been carried out.

Computational alanine scanning

In order to verify the key residues identified by the energy decomposition of individual residues, computational alanine-scanning mutagenesis was carried out. The binding free energy difference between the wild type and the corresponding alanine mutants was calculated using the following equation:

$$\Delta\Delta G_{\text{cal}} = \Delta G_{\text{cal,wild}} - \Delta G_{\text{cal,mutant}} \quad (4)$$

Positive and negative values of $\Delta\Delta G_{\text{cal}}$ indicate unfavorable and favorable contributions, respectively. Each alanine mutant structure was generated by replacing the sidechain of the targeted residue at the C γ position with a hydrogen atom. Based on the assumption that the local perturbation of the alanine mutant induces no significant structural changes of the protein, such mutagenesis effect on the binding free energy could be calculated according to the corresponding snapshots extracted from the last 20 ns MD trajectories of the wild type.

Results and discussion

1. The predicted binding affinity by docking simulations

To better predict the binding affinities of TTR with both hydroxylated and sulfated metabolites of PBDEs, we first compared the performances of 8 docking programs used widely in the different complex systems. Two admitted evaluation criteria,⁵³ scoring power and ranking power, were adopted in this study to assess the prediction accuracy of each scoring function of each docking program. Scoring power is defined as the capability to correlate the docking scores with the experimental binding affinities of ligands, which is measured by the Pearson correlation coefficient (R_p) between the docking scores and the logRBA values of OH-PBDEs. Ranking power is evaluated by means of the Spearman ranking coefficient (R_s) to examine whether the rank of binding affinities of a series of TTR–OH–PBDE complexes calculated by docking programs was consistent with logRBA determined by experimental methods. The larger the absolute values of R_p and R_s become, the stronger the prediction accuracy of the scoring function is.

R_p and R_s for 20 TTR–OH–PBDE complexes are summarized Table 1 for each docking program. By comparing the R_p values, we found that the scoring powers of the 8 tested docking programs can be ranked as: LeDock (−0.88) > idock (0.81) > CDock (0.71) > AutoDock (LGA) (−0.68) > Surflex-Dock (−0.44) > AutoDock Vina (−0.41) > Libdock (0.13) > iGEMdock (−0.10). This indicates that the LeDock docking program exhibits the best scoring power in predicting the binding affinities of OH-PBDEs with TTR. The comparison of the R_s values also illustrates the top ranking power (−0.81) of LeDock among 8 docking programs. However, unexpectedly, some docking programs display low or no correlation ($|R_p|$ or $|R_s|$ cutoff ≤ 0.6) between their docking scores and experimental logRBA (Table 1). For example, Surflex-Dock has inferior capability to predict and rank the binding affinity of targeted OH-PBDEs with TTR ($R_p = -0.44$ and $R_s = -0.31$) though this docking program was previously used to successfully predict the binding affinity

Table 1 Overall ranking and scoring power of 8 tested docking programs in evaluating TTR–OH–PBDE complex stability

Docking program	R_p^a	R_s^a
AutoDock (LGA)	−0.68	−0.57
AutoDock Vina	−0.41	−0.52
idock	0.81	0.60
Cdock	0.71	0.66
Libdock	0.13	0.20
LeDock	−0.88	−0.81
Surflex-Dock	−0.44	−0.31
iGEMdock	−0.10	−0.13

^a R_p and R_s represent the Pearson correlation coefficient and the Spearman ranking coefficient, respectively.

of OH-PBDEs^{54,55} and OH-PCBs⁵⁶ with nuclear receptors. Considering that OH-PBDEs probably form halogen-bonding with TTR, we inferred that the current scoring functions of some docking programs might be insufficient to estimate the effect of a positive surface region involved in the σ -hole^{57,58} of bromide atoms on the interaction calculations.

Furthermore, comparing the obtained result with the previous prediction of binding potencies for 14 TTR–OH–PBDE complexes,²⁶ we found that the performance of LeDock is superior to that of the adjusted QM/MM score based on pK_a correction ($R_p = -0.82$). Moreover, ranking performance comparison between LeDock and Fitted, a docking program outperforming the other 7 programs in an exhaustive comparison study of 8 docking programs (Autodock Vina, Fitted, FlexX, Fred, Glide, GOLD, LibDock, MolDock) for their ability in predicting the rank-order activity of different ligand series for six pharmacologically important protein and enzyme targets (Factor Xa, Cdk2, Aurora A kinase, COX-2, pla2g2a, β estrogen receptor),⁵⁹ was made. According to the correlations between docking scores and experimentally determined binding affinity or biological activity given by the 16 scoring functions, the best prediction of $R_p = 0.86/R_s = 0.91$ was achieved by Fitted for 24 CDK2 kinase inhibitors with structural similarity. The R_p and R_s value obtained by LeDock for the same set of chemicals is 0.87 and 0.87 (Table S2, ESI[†]), respectively, showing that LeDock has comparable ranking ability to Fitted in the same case. Hence, on the basis of the above comprehensive evaluation of docking programs, we believed that the prediction accuracy of LeDock was quite satisfactory for the current study. Subsequently, a linear predictive model $-0.672 \times \text{docking score} - 3.591$ has been utilized to convert the docking scores of LeDock to their log RBA values. The predicted log RBA values are statistically significantly correlated with the experimental data ($p < 0.00001$), and therefore the constructed model based on the docking calculations can be adopted to estimate the logRBA values of OH-PBDEs with TTR (Fig. 2).

For the sulfated metabolites of PBDEs without experimental activity data, their potential binding affinities were calculated using the above predictive model due to their structural similarity with OH-PBDEs. Table 2 shows that each sulfated-PBDE compound gains better docking scores than their parent OH-PBDEs. The predicted log RBA values further imply that all tested sulfated-PBDEs have stronger binding affinity than

Fig. 2 Linear correlation between the experimental logRBA and the predicted logRBA based on the docking results of LeDock for 20 OH-PBDEs.

the native ligand T4. Similar conclusions could be reached for the previously reported sulfated metabolites of polychlorinated biphenyl (PCB),⁶⁰ and the fluorescence displacement studies showed that sulfated PCBs possess high binding abilities toward human TTR. According to the above evidence from both computational and experimental studies on sulfated PBDEs and PCBs, we speculated that the introduction of an $-\text{OSO}_3^-$ group facilitates ligand binding at the T4 site of TTR. Considering their strong competitive binding with TTR, attention should be paid on the thyroid homeostasis disrupting capacity of sulfated-PBDEs for toxicity assessment of PBDEs.

2. Structural characteristics of the PBDE metabolites bound to TTR

To explore the difference of PBDE metabolites in TTR binding modes, we overlapped final docked poses based on the best docking score of LeDock. Conformational comparison, illustrated in Fig. 3, presents significant pose difference between some OH-PBDEs and their corresponding sulfated-PBDEs in the channel pocket of TTR, suggesting that the substitution of an $-\text{OH}$ group with an $-\text{OSO}_3^-$ group may exert some effect on the binding conformations of these molecules. In order to understand the potential structural basis of such differences, we first analyzed the known crystal structures^{61,62} in which the bound ligands have molecule structure features similar to our tested compounds. The scrutiny of these crystal structures of TTR–ligand complexes indicates that the binding sites contain two funnel-shaped pockets (Fig. S1, ESI[†]), which locate in the channel interface of the TTR tetramer structure. In the inner part of the channel-type pocket, the polar group of ligands can form a hydrogen bond with the hydroxyl group on the sidechain of Ser117/Ser117' lying at the pocket bottom. The channel entrance mainly consists of charged amino acids Lys15/Lys15' and Glu54/Glu54', which can stabilize the ionizable group of ligands by means of electrostatic interactions. Such structural peculiarity of the binding pocket leads to two opposite recognition orientations for the transthyretin amyloidogenesis inhibitors binding into the channel pocket of TTR (Fig. S1, ESI[†]). For example, the phenol compounds **2c** ((E)-2,6-dimethyl-4-styrylphenol) and **4f**

Table 2 Predicted logarithm of the relative binding affinity of OH-PBDEs and sulfated-PBDEs based on the best docking scores from LeDock

Compound	Experimental log RBA	Docking score	Predicted log RBA	Compound	Docking score	Predicted log RBA
2'-OH-BDE-03	-0.96	-4.19	-0.78	2'-Sulf-BDE-03	-5.37	0.02
2'-OH-BDE-07	-0.52	-4.82	-0.35	2'-Sulf-BDE-07	-5.82	0.32
2'-OH-BDE-28	0.22	-5.27	-0.05	2'-Sulf-BDE-28	-6.49	0.77
2'-OH-BDE-68	0.57	-6.04	0.47	2'-Sulf-BDE-68	-6.07	0.49
3-OH-BDE-154	0.33	-5.99	0.43	3-Sulf-BDE-154	-6.4	0.71
3-OH-BDE-47	0.55	-5.78	0.29	3-Sulf-BDE-47	-6.78	0.97
3'-OH-BDE-07	-0.19	-4.96	-0.26	3'-Sulf-BDE-07	-5.73	0.26
3'-OH-BDE-28	-0.07	-5.47	0.08	3'-Sulf-BDE-28	-6.5	0.78
4-OH-BDE-42	0.41	-6.28	0.63	4-Sulf-BDE-42	-6.49	0.77
4'-OH-BDE-17	0.18	-5.53	0.13	4'-Sulf-BDE-17	-6.14	0.54
4'-OH-BDE-49	0.51	-5.9	0.37	4'-Sulf-BDE-49	-6.52	0.79
5-OH-BDE-47	0.59	-6.1	0.51	5-Sulf-BDE-47	-6.62	0.86
6-OH-BDE-47	-0.11	-5.47	0.08	6-Sulf-BDE-47	-6	0.44
6-OH-BDE-85	0.63	-5.73	0.26	6-Sulf-BDE-85	-6.48	0.76
3-OH-BDE-100	0.08	-5.75	0.27	3-Sulf-BDE-100	-6.51	0.78
4-OH-BDE-188	0.14	-5.79	0.30	4-Sulf-BDE-188	-6.81	0.99
2'-OH-BDE-66	-0.05	-5.71	0.25	2'-Sulf-BDE-66	-6.2	0.58
4'-OH-BDE-30	-0.39	-4.59	-0.51	4'-Sulf-BDE-30	-5.62	0.19
4'-OH-BDE-69	0.09	-5.12	-0.15	4'-Sulf-BDE-69	-6.08	0.49
4'-OH-BDE-121	0.15	-5.67	0.22	4'-Sulf-BDE-121	-6.02	0.45

Fig. 3 The binding models of OH-PBDEs and sulfated-PBDEs in the channel-type pocket of TTR. OH-PBDEs were colored by atom type, depicting carbon atoms in green, bromine in pink, oxygen in red and sulfur in yellow. Sulfated-PBDEs were also colored by atom type, demonstrating carbon atoms in orange, bromine in pink, oxygen in red and sulfur in yellow. Key contact residues are colored by atom type with carbon atoms in white, nitrogen in blue, and oxygen in red. Hydrogen bond interactions of OH-PBDEs and sulfated-PBDEs with surrounding residues were plotted as green and orange dotted lines, respectively.

(2-(3,5-dimethyl-4-hydroxyphenyl)benzoxazole) display the so-called reverse binding mode where the *para*-position -OH groups of the ligands bridge residues Ser117/Ser117'. In contrast, the -OH groups

of compounds **2d** ((*E*)-2,6-dibromo-4-styrylphenol), **3d** (3,5-dibromo-[1,1'-biphenyl]-4-ol), and **4d** (2-(3,5-dibromo-4-hydroxyphenyl)benzoxazole) embed themselves deeply into the TTR

pocket in the forward binding mode, pointing toward the entrance of the binding pocket *via* electrostatic interactions with residues Lys15/Lys15'.

Subsequently, the effect of Ser117/Ser117' and Lys15/Lys15' on the molecular recognition and binding orientation of hydroxylated and sulfated PBDEs was analyzed. As for the TTR–OH–PBDE complexes, all 20 ligands exhibit various and inconsistent binding orientations in the docking results of TTR (Fig. 3). The hydrophilic –OH groups of 8 OH–PBDE compounds (2'-OH-BDE-03, 3-OH-BDE-100, 3'-OH-BDE-07, 3'-OH-BDE-28, 4-OH-BDE-42, 4'-OH-BDE-17, 4'-OH-BDE-69, and 5-OH-BDE-47) prefer to interact with the O atom of the backbone or sidechain –OH group of Ser117/117'. Meanwhile, the hydroxyl groups in 2'-OH-BDE-07, 2'-OH-BDE-66 and 2'-OH-BDE-68 are close to the middle of the binding site, exhibiting hydrogen bonding interactions with the sidechain –OH group of Thr119'. In contrast, only two OH–PBDE compounds (4'-OH-BDE-30 and 4'-OH-BDE-49) bind to TTR by forming hydrogen bonds between their –OH groups and the residues Lys15/Lys15' or Glu54/Glu54' lying at the cavity entrance. Moreover, we found that 7 OH–PBDE compounds (2'-OH-BDE-28, 3-OH-BDE-47, 3-OH-BDE-154, 4-OH-BDE-188, 4'-OH-BDE-121, 6-OH-BDE-47 and 6-OH-BDE-85) have no obvious hydrogen bonding interactions with the aforesaid residues Ser117/117', Thr119/Thr119', Lys15/Lys15' and Glu54/Glu54'. Interestingly, however, the structural analysis of TTR–sulfated–PBDE interactions indicates that all sulfated–PBDE compounds allow the formation of hydrogen bonding with Ser117/Ser117' or electrostatic interactions with Lys15/Lys15' at the binding site. Among these sulfated–PBDEs, *ortho*-position –OSO₃[−] groups all adopt the forward binding mode by establishing electrostatic interactions with Lys15/Lys15'. For the sulfated–PBDEs with *meta*-position or *para*-position –OSO₃[−] groups, there is no consistent conclusion about the orientation preference of binding for the ionizable group of the ligands. For instance, the binding mode of 3-Sulf-BDE-47 is referred to as the reverse mode (see red dashed box in Fig. 3), where the *meta*-position –OSO₃[−] group immerses into the inner cavity and forms hydrogen bonding with the sidechain –OH group of Ser117 and Ser117' (distance is 3.4, 3.2 and 2.9 Å, respectively), while the phenyl ring with 2,4-bromine substitution is placed within the outer binding cavity close to residues Lys15 and Lys15'. 4-Sulf-BDE-42 exhibits the opposite binding orientation as the forward binding mode instead (see blue dashed box in Fig. 3), whose *para*-position –OSO₃[−] group interacts with the positively charged –NH₃⁺ group of Lys15' at the outer binding cavity. The distance between the O atom on the –OSO₃[−] group and the N atom on the –NH₃⁺ group is 3.4 Å, which favors the criterion of electrostatic interactions (< 5 Å).⁶³

Clearly, the structural analysis results indicate that the substitution of the –OH group with the –OSO₃[−] group not only affects the shape and size of ligands, but also dominates the binding orientation of ligands in the channel pocket of TTR. Moreover, Ser117/Ser117' and Lys15/Lys15' may act as major recognition sites responsible for interacting with the hydrophilic groups of OH–PBDEs and sulfated–PBDEs, and result in the binding mode preference (forward or reverse) of ligands. However, the structural flexibility of the sidechain on key

residues also plays a role in ligand binding. Further comparison of hydroxylated PCBs (OH–PCBs) bound to TTR⁶⁴ revealed significant variations in the conformation of the sidechain of Ser117/117' upon binding the –OH group of the ligands (Fig. S2, ESI†). In addition, among 5 published TTR structures co-crystallized with T4,^{32,65–67} we also observed that the distances between the O atom of –OH groups from T4 and the O atom of the Ser117 sidechain varied greatly from 3.7 to 7.2 Å (Table S3, ESI†). Unfortunately, such flexibility of the Ser117 sidechain is not taken into consideration during the docking simulation. This is an inherent flaw in the traditional semi-flexible docking method when the receptor conformation is kept in a rigid form for fast and effective calculations. Likewise, the –NH₃⁺ group of Lys15 was reported to be flexible for the recognition of the –SO₃[−] group from 1-amino-5-naphthalene sulfonate, thereby inducing the multiple bound conformations of the ligand in the binding pocket of TTR (PDBid: 3CFT)⁶⁸ (Fig. S3, ESI†). The above analyses suggest that in the channel pocket of TTR, the binding mechanism of ligands comprising –OH and –SO₃[−] groups might be affected significantly by the residues Ser117/Ser117' and Lys15/Lys15'. Consequently, for deciphering the molecular recognition of OH–PBDEs and sulfated–PBDEs by TTR, their binding modes should be further explored based on MD simulations, a computational technique effectively adapting for structural plasticity.

3. Exploring the binding mechanism based on MD simulations

In order to clarify the structural characteristics of the TTR–sulfated–PBDE complexes, we further investigated and refined the initial complex structures obtained from the docking results of LeDock by performing explicit solvent MD simulations. 2'-Sulf-BDE-28, 3-Sulf-BDE-47 and 4-Sulf-BDE-42 were selected as the representative molecules of sulfated–PBDEs because they were detected as the prevailing sulfated metabolites of BDE-47 *in vitro*,⁸ and presented two opposite binding orientations (forward and reverse) in the predicted binding modes. The binding features of the corresponding hydroxylated metabolites of PBDEs (2'-OH-BDE-28, 3-OH-BDE-47 and 4-OH-BDE-42) with TTR were also evaluated by the same MD simulation method.

During 30 ns simulations, the root-mean-square deviation (RMSD) values of backbone C α atoms of TTR, referenced to the T4–TTR crystal structure (1ICT), were calculated to assess the stability of the simulation systems. All simulation systems reach equilibrium quickly at the beginning of the simulation process (0–2.5 ns) and then maintain stable fluctuation in subsequent simulation time (Fig. S4, ESI†). The average RMSD values of the whole simulation stage were marked for each system (Fig. S4, ESI†). These small RMSD values (ranging from 1.24 $\pm$ 0.13 to 1.45 $\pm$ 0.15 Å) suggest that upon ligand binding, TTR can retain a fairly rigid tetramer structure with little conformational change throughout the simulation time. In addition, we calculated the C α root-mean-square fluctuation (RMSF) of individual residues to estimate the flexibility of the TTR backbone structure over the course of the simulation. The region showing larger RMSF values demonstrates more

extensive motions in space. Clearly, the six TTR–ligand complexes exhibited a fluctuation trend of the backbone structure similar to that of apo TTR (Fig. S5, ESI†). By averaging the RMSF values of all residues, the apo TTR system was found to show an average value of 0.80 Å, larger than that of the TTR–ligand complexes (0.73–0.79 Å). Furthermore, the analyses of the radius of gyration (R_g) also indicate that the structures of all TTR–ligand complexes become more compact compared with the apo TTR due to the presence of OH-PBDEs and sulfated-PBDEs (Table S4, ESI†). Therefore, the binding of the 6 metabolites of PBDEs can stabilize the TTR's tetrameric structure, thereby potentially slowing down the dissociation rate of the protein, and inhibit the related amyloidoses.

Moreover, we depicted the RMSD values of the 6 ligands for the entire MD simulations. 2'-OH-BDE-28 suffers a low RMSD evolution (~ 0.4 Å) from 0–4.8 ns, followed by a rapid RMSD increase to 1.3 Å for the remaining course of the simulation, while 2'-Sulf-BDE-28 has relatively consistent RMSD values around 1.0 Å for most of the simulation time (Fig. S6, ESI†). Similarly, 3-OH-BDE-47 exhibits two dissimilar poses (~ 0.5 and 1.5 Å) distinguished from the initial docking result during the dynamic binding process, whereas the RMSD of 3-Sulf-BDE-47 consistently keeps below 1 Å within the binding site of TTR. In the case of 4-OH-BDE-42, the RMSD of the ligand has a relatively smaller value (~ 1 Å) in the first 18 ns MD and rose to ~ 2.0 Å in last 12 ns simulations. Differently, 4-Sulf-BDE-42 displays a RMSD value of ~ 1.0 Å throughout the whole simulation time. Besides, considering the pose fluctuation during the MD simulations, the representative conformation during the 30 ns MD simulations for each chemical was obtained by the average-linkage clustering algorithm using the cptraj module of AmberTools13. The clustering analysis was performed using RMSD as the pairwise metric, and all the sampled conformations of MD simulations were partitioned into 3 distinct clusters. The conformation of the largest cluster was then acquired as the representative MD conformation. Fig. S7 (ESI†) illustrates the alignment result for the best-scoring poses and the representative conformations. Clearly, the obtained representative MD conformations of sulfated-PBDEs exhibit smaller pose deviation from their best-scoring poses in terms of RMSD values when compared to that of OH-PBDEs, implying again that the binding modes of

sulfated-PBDEs in TTR are more stable than those of the corresponding parent OH-PBDEs.

To interpret the binding mechanisms of the TTR–ligand complexes, the hydrogen bond interactions were calculated based on the entire MD simulations. The criteria of hydrogen bonds were defined by the distance (donator...acceptor ≤ 3.5 Å) and the angle ($\angle$ donator–H...acceptor $\geq 120^\circ$). In this study, hydrogen bonds showing more than 5% occupancy for the total simulation time, formed between the ligands and TTR, were recorded and summarized in Table 3. Different from the docking results shown in Fig. 3 illustrating no hydrogen bond between 2'-OH-BDE-28 and adjacent residues in TTR, the residues Ala108' and Thr119' of TTR solidly form hydrogen bonds with the –OH group of 2'-OH-BDE-28 with quite high occupancy (84.09 and 78.29%) in the whole simulation, respectively. In contrast, only an unstable hydrogen bond with 10.27% occupancy appears between the –OSO₃[–] group of 2'-Sulf-BDE-28 and the –NH₃⁺ group of Lys15. As for the 3-OH-BDE-47 system, the residue Ser117 interacts with the –OH group of the ligand with a low hydrogen bond occupancy of 18.44%, consistent with the feature of the initial docking structure showing no hydrogen bonds in Fig. 3. Interestingly, in the 3-Sulf-BDE-47 system, the –OH group of Ser117' forms hydrogen bonds with the alternate O atoms of the –SO₃[–] group of the ligand with moderate occupancies (40.33, 37.88 and 37.69%, respectively). In addition, weak hydrogen bonds also appear in the 3-Sulf-BDE-47 system for the –OH group of the Thr119' sidechain with low occupancies (27.23, 22.98 and 18.01% for three identical O atoms in the –SO₃[–] group, respectively). Meanwhile, the hydrogen bond occupancy between 4-OH-BDE-42 and the residue Ser117' is 87.24%, indicating the establishment of strong hydrogen bonds in the complex based on the MD assessment. A moderate hydrogen bond interaction of 4-OH-BDE-42 with the sidechain –OH group of Thr119' is also of importance for the 4-OH-BDE-42–TTR binding process. The hydrogen bond interaction formed between Lys15' and the –OSO₃[–] group of 4-Sulf-BDE-42 was observed with a low occupancy (5.5%), in accordance with that of the docking results. Obviously, the analysis of hydrogen bonds identified the key role of the sidechain –OH groups from Ser117' and Thr119' in anchoring the hydrophilic groups of all OH-PBDEs and the sulfated-PBDEs preferring the reverse mode in the initial pose.

Table 3 Hydrogen bonds of more than 5% occupancy formed between the tested compounds and TTR during MD simulations

System	Acceptor	Donor	Distance (Å)	Angle (°)	Occupancy (%)
2'-OH-BDE-28	Ala108'@O	Ligand@O2-H7	2.77	146.80	84.09
	Ligand@O2	Thr119'@OG1-HG1	3.00	156.27	78.29
2'-Sulf-BDE-28	Ligand@O3	Lys15@NZ-HZ3	2.92	147.86	10.27
3-OH-BDE-47	Ser117@OG	Ligand@O2-H6	2.89	140.76	18.44
3-Sulf-BDE-47	Ligand@O3	Ser117'@OG-HG	2.89	152.55	40.33
	Ligand@O5	Ser117'@OG-HG	2.91	150.88	37.88
	Ligand@O4	Ser117'@OG-HG	2.90	152.15	37.69
	Ligand@O5	Thr119'@OG1-HG1	2.77	161.63	27.23
	Ligand@O4	Thr119'@OG1-HG1	2.76	161.66	22.98
	Ligand@O3	Thr119'@OG1-HG1	2.77	161.50	18.01
	Ser117'@OG	Ligand@O2-H6	2.78	154.76	87.24
4-OH-BDE-42	Ligand@O2	Thr119'@OG1-HG1	2.97	153.85	36.87
4-Sulf-BDE-42	Ligand@O5	Lys15'@NZ-HZ3	2.95	146.36	5.50

To elucidate the recognition of Lys15/Lys15' involving the electrostatic interactions with 2'-Sulf-BDE-28 and 4-Sulf-BDE-42, we investigated the evolution of the distance (d_{O-N}) between the O atoms of the $-SO_3^-$ group from the two compounds and the N atoms of the $-NH_3^+$ group from Lys15/Lys15' during the MD simulations (Fig. S8, ESI†). The results show that both systems could maintain d_{O-N} below 5 Å, which is in accordance with the criterion of electrostatic interactions between two atoms with opposite charges.⁶³ Based on the above observations, we inferred that the residues Lys15/Lys15' have important effects on maintaining the forward binding mode by enhancing the electrostatic interactions with the $-SO_3^-$ group of the ligands.

4. Binding free energy calculations for the metabolites of PBDEs

To further dissect the protein-ligand interactions and identify the dominant forces of the binding affinity, MM/GBSA calculations of the binding free energies were performed. Table 4 shows that based on the calculations of the GB model⁴⁶ (GB^{OB1}, $igb = 2$), the sulfated PBDEs have lower binding free energies than their parent OH-PBDEs, suggesting that the sulfation of OH-PBDEs improves their binding affinities toward TTR. This trend is also detected in the docking results of LeDock showing higher absolute values of scores for sulfated PBDEs (Table 1). To consolidate and complement the prediction of MM/GBSA calculations, we also adopted the modified GB model⁴⁷ (GB^{OB2}, $igb = 5$) to calculate the binding free energies of the tested compounds. As shown in Table 4, similar conclusions could be obtained that the sulfated PBDEs have higher binding capacity than their corresponding OH-PBDEs. Therefore, it is emphasized from the energy perspective that sulfated PBDEs might disturb thyroid homeostasis by competitive TTR binding. Additionally, the averaged RBA values calculated from the reported experimental data (Table S1, ESI†) for 2'-OH-BDE-28, 3-OH-BDE-47 and 4-OH-BDE-42 are 1.65, 3.55 and 2.58, respectively. Obviously, both 3-OH-BDE-47 and 4-OH-BDE-42 show higher binding affinity than 2'-OH-BDE-28 does, while 3-OH-BDE-47 exhibits a binding potential comparable to 4-OH-BDE-42. As expected, the calculated relative binding free energies of the three OH-PBDEs display that 2'-OH-BDE-28 has the lowest binding affinity for TTR among the three OH-PBDEs.

In addition, HF/6-31G* has been used to produce partial charges of ligands in most cases because it can obtain overestimation of dipole moments in the gas phase and thus helps to counteract the effect of molecular polarization in the real

system in MD simulations. Therefore, the RESP charges were also calculated for 6 studied ligands using the HF/6-31G* basis set from the optimized geometry at the B3LYP/cc-pVTZ level and then compared with the ones calculated using B3LYP/cc-pVTZ. The largest difference in charge between two computation strategies is about 0.2 units and presents at the sulfur atoms in the sulfonated-PBDE molecules, while OH-PBDEs range in charge difference from 0.01 to 0.13 units (Tables S5–S7, ESI†). To evaluate the effect of the charge on the binding affinity of hydroxylated and sulfonated PBDEs, the MD simulation of 3-OH-BDE-47 and 3-Sulf-BDE-47 was repeated subsequently using the RESP charges of HF/6-31G* (Table S8, ESI†). The ΔG_{cal} value of 3-Sulf-BDE-47 increases by 7.39% if the RESP charges at the HF/6-31G* level are used, while little decrease in the ΔG_{cal} value can be observed for 3-OH-BDE-47 instead (0.16%). The TTR binding affinity calculation is quite sensitive to the effect of charge for ionic ligands. Nevertheless, the conclusion of the present study remains the same.

To understand the contribution of individual binding free energy components for complex stability, detailed information on the energy terms for each target compound was analyzed based on the calculated results of the GB^{OB1} model (Table 4). By comparing with the results of energy decompositions, we could clarify the predominant force of the binding process for the TTR recognition of 6 PBDE metabolites. The sum of ΔE_{vdw} and ΔG_{SA} , which represents total nonpolar contributions, is the most favorable component for binding free energy. It is considered to play a significant role in forming stable complexes because the negative values occur in all simulation systems (−40.45 to −53.35 kcal mol^{−1}). This result is consistent with the previously reported studies on protein-ligand complexes,^{69,70} identifying such nonpolar interactions as a favorable binding force for the PBDE metabolites in the channel pocket of TTR. Among all the contributors, the van der Waals interactions (ΔE_{vdw}) of the gas phase are identified as the dominant forces to stabilize the ligands at the binding site of TTR.

Interestingly, we observed that the electrostatic interactions (ΔE_{ele}) of the gas phase and the polar contributions for solvation (ΔG_{GB}) were assigned positive values (90.71–103.96 kcal mol^{−1}) and negative values (−79.75 to −96.48) for the sulfated-PBDEs and negative values (−4.58 to −11.99 kcal mol^{−1}) and positive values (14.51 to 16.86) for the OH-PBDEs, respectively. One possible explanation is the strong desolvation effect and electrostatic repulsion for charged ligands upon complex formation with the protein with like charge. In fact, a negative ΔE_{ele} value is generally

Table 4 Binding free energy components^a of TTR–ligand complex simulation systems (unit: kcal mol^{−1})

Systems	ΔE_{vdw}	ΔG_{SA}	ΔE_{ele}	ΔG_{GB} ($igb = 2$)	ΔG_{GB} ($igb = 5$)	ΔG_{calc} ($igb = 2$)	ΔG_{calc} ($igb = 5$)
2'-OH-BDE-28	−36.50(2.13)	−3.95(0.18)	−10.82(2.37)	15.75(1.19)	15.50(1.24)	−35.52(2.02)	−35.77(2.14)
2'-Sulf-BDE-28	−43.45(2.53)	−5.29(0.21)	90.71(11.62)	−79.75(10.35)	−83.15(10.19)	−37.78(2.77)	−41.18(3.25)
3-OH-BDE-47	−42.63(2.78)	−4.28(0.15)	−4.58(4.20)	14.51(2.57)	13.55(2.36)	−36.99(2.33)	−37.95(2.45)
3-Sulf-BDE-47	−47.95(2.55)	−5.40(0.15)	103.96(6.48)	−96.48(5.90)	−101.91(5.95)	−45.88(3.01)	−51.31(3.40)
4-OH-BDE-42	−39.77(2.64)	−4.31(0.19)	−11.99(3.08)	16.86(1.94)	16.56(2.00)	−39.21(2.48)	−39.51(2.53)
4-Sulf-BDE-42	−44.69(3.29)	−5.07(0.32)	97.51(15.73)	−87.15(13.78)	−89.58(13.66)	−39.41(3.30)	−41.84(3.75)

^a $\Delta G_{cal} = \Delta E_{vdw} + \Delta G_{SA} + \Delta E_{ele} + \Delta G_{GB}$. Standard error of mean values is displayed in parenthesis.

expected for ligand–protein interaction since it symbolizes the electrostatic attraction between the ligand and the receptor. Both situations $\Delta E_{\text{ele}} < 0/\Delta G_{\text{GB}} > 0$ and $\Delta E_{\text{ele}} > 0/\Delta G_{\text{GB}} < 0$, may occur. Decisions have to be made on a case by case basis. Nevertheless, all we know is founded on its own rhythms: the ΔE_{ele} value may be increased from the negative one to the positive one with increasing electrostatic repulsion. As for ΔG_{GB} , the more soluble a molecule is, the more negative ΔG_{GB} becomes. The ΔG_{GB} values of the complex formation process, plus or minus, largely depend on whether the complex is more soluble than the free molecules. In addition, the protonation state of both the ligand and receptor is vital in binding free energy calculations *via* MD simulations. TTR is highly negatively charged with a net charge of $-16 e$, and the tested sulfated PBDEs are also present as anionic ligands under physiological conditions since they are strong acids. The positive ΔE_{ele} value of sulfated-PBDEs is mainly attributed to the electrostatic repulsion of the negatively charged TTR ($-16 e$) to the anionic sulfated PBDEs in the gas phase. In contrast, less TTR repulsion can be found for OH-PBDEs due to their neutral molecular forms adopted at current MD simulations, and prevailing speciations for the OH-PBDEs under physiological conditions because of their low dissociation constants ($\text{p}K_{\text{a}} > 8.5$).⁷¹ Nevertheless, solvation forces can mitigate the unfavorable electrostatic interaction of the sulfated PBDEs because solvation has a much larger effect on the protein binding of ionic ligands than on that of neutral ones. Specifically, ΔG_{GB} can offset ΔE_{ele} for ionic ligands. The negative ΔG_{GB} occurs in our study simply because the complex is more soluble than the separate TTR and sulfated PBDEs.⁷² Therefore, the total polar contribution ($\Delta E_{\text{ele}} + \Delta G_{\text{GB}}$) of sulfated-PBDEs (7.48 – $10.96 \text{ kcal mol}^{-1}$) is still comparable to that of OH-PBDEs (4.87 – $9.93 \text{ kcal mol}^{-1}$) due to the counteraction effect of ΔG_{GB} . Similar results can be found in the energy terms of MM/GBSA calculations for the ligand–protein interaction of some anionic chemicals containing a sulfonic or carboxylic group with the negatively charged protein.^{73,74} The binding free energy terms for the Bcl-xL/UBQF14 and Bcl-xL/UBQF17 complexes provided positive ΔE_{ele} ($113.7/30.4 \text{ kcal mol}^{-1}$) and negative ΔG_{GB} values ($-90.2/0.0 \text{ kcal mol}^{-1}$), respectively.⁷³ The MM/GBSA calculations for the β -lactoglobulin in complex with fatty acids presented the same trend.⁷⁴ In order to further verify the above deduction, the neutral form of 3-Sulf-BDE-47 and the anionic form of 3-OH-BDE-47 were subjected to the binding free energy calculations of the same strategy, respectively. The data revealed, as expected, a negative ΔE_{ele} and a positive ΔG_{GB} value for 3-Sulf-BDE-47 and a positive ΔE_{ele} and a negative ΔG_{GB} value for 3-OH-BDE-47 (Table S9, ESI†). It is worth mentioning that the ΔG_{cal} value of 3-Sulf-BDE-47 is still lower than that of 3-OH-BDE-47 when inadequate speciation was employed for MD calculations, indicating a higher TTR binding affinity of 3-Sulf-BDE-47. The only difference is the absolute value of the difference in ΔG_{cal} . Considering the true speciation of the PBDE metabolites, the choice of the neutral OH-PBDE molecule and sulfated PBDE anion is adequate for the simulation study. Fortunately, such speciation preference results in a large difference in ΔG_{cal} value ($8.89 \text{ kcal mol}^{-1}$) between 3-OH-BDE-47

and 3-Sulf-BDE-47 (Table 4). Therefore, the conclusions of the present study are adequate and remain the same.

Subsequently, the total binding free energies of the complex systems were further decomposed into individual residue contributions to recognize the key residues responsible for ligand–protein binding and gain further understanding of the binding mechanism. In the current study, a residue with energy contribution $\leq -1.0 \text{ kcal mol}^{-1}$ is defined as a key residue for ligand recognition. On the whole, Lys15/Lys15', Leu17/Leu17', Ala108/Ala108', Ala109', Leu110/Leu110', Ser117/Ser117', Thr118' and Thr119/Thr119' were identified as key residues for stabilizing the ligands in the TTR pocket (Fig. 4). Among these residues, Lys15/Lys15' located at the outer part of the channel-type pocket of TTR establish hydrogen bonding/electrostatic attraction and π -alkyl interactions with 2'-Sulf-BDE-28 for recognizing the $-\text{OSO}_3^-$ group and phenyl ring of the ligand, respectively (Fig. 5). Molecular recognition of the phenyl rings in 2'-Sulf-BDE-28 by Leu17' is also realized by π -alkyl interactions. However, in the binding mode of 2'-OH-BDE-28, less polar and

Fig. 4 Energy contributions of key residues for stabilizing TTR–ligand complex systems (unit: kcal mol^{-1}).

Fig. 5 2D representation of intermolecular interactions between the tested compounds and TTR based on the representative conformations of 30 ns MD simulations.

more hydrophobic contacts were formed. No cation- π can be found in the 2'-OH-BDE-28-TTR complex, and only hydrogen bonds between the ligand phenolic group and Ala108' and Thr119' are observed. But the more intensive π -alkyl interactions between the aryl ring and Ala108, Leu110/Leu110' are generated in the channel pocket of the TTR tetramer (Fig. 5). Therefore, based on the total binding free energy, 2'-Sulf-BDE-28 is slightly energetically more favorable than 2'-OH-BDE-28 for TTR binding (-37.78 versus -35.52 kcal mol $^{-1}$). Differently, as shown in Fig. 4 and 5, we found that in comparison with 3-OH-BDE-47, 3-Sulf-BDE-47 has higher TTR affinity mainly through its significantly enhanced polar interactions with key residues, including the cation- π interactions of its phenyl ring with Lys15' and the hydrogen bonding of its sulfate moiety with Ser117' and Thr119'. The large occupancy of the related hydrogen bonds gives further confirmation (Table 3). As for 4-OH-BDE-42 and 4-Sulf-BDE-42, hydrogen bonding between Ser117' and the phenolic group in 4-OH-BDE-42 provides additional stability for the complex of

OH-PBDEs. Dissimilarly, neither cation- π interaction nor hydrogen bonding is established for anchoring the sulfate group for 4-Sulf-BDE-42. The charged residue Lys15' only forms limited electrostatic contact and π -alkyl interaction with the $-\text{OSO}_3^-$ group and the phenyl ring in 4-Sulf-BDE-42, separately (Fig. 5). Therefore, 4-OH-BDE-42 exhibits a comparable binding affinity of TTR relative to the corresponding sulfate (-39.21 versus -39.41 kcal mol $^{-1}$) primarily due to the opposite change (enhanced vs. decreased) of two chemicals in the polar contribution.

On the basis of energy analysis of key residues and the binding modes of the representative complex conformations, we identified that relative to OH-PBDEs, the higher TTR binding affinities of the corresponding sulfated-PBDEs can be ascribed to the enhanced molecular recognition of the negatively charged sulfate group of the ligands by the hydrogen bonding of Ser117'/Ser117' or electrostatic interactions of Lys15'/Lys15'. Computational alanine-scanning was conducted subsequently to further

confirm the role of the individual residues in the binding free energy of the complexes. The ΔG_{cal} values of all three sulfated-PBDEs are reduced by more than 2.50 kcal mol⁻¹ when Lys15/Lys15' or Ser117/Ser117' are mutated to alanine. This identification of computational alanine scanning is consistent with that of energy decomposition of the individual residue, suggesting that Ser117/Ser117' and Lys15/Lys15' are responsible for the sulfated-PBDE binding with TTR (Table S10, ESI†). Still, the complex stability resulting from hydrophobic contacts must not be overlooked. As previously reported,²¹ the TTR binding potencies of OH-PBDEs, nonionic substances under physiological pH conditions, exhibited a parabolic relationship with the logarithm values of their *n*-octanol/water partition coefficients, indicating the vital role of hydrophobic contact in OH-PBDE-TTR interaction. Our analyses of binding energy and ligand conformation preference also point out that selected hydrophobic residues, such as Leu17/Leu17', Ala108/Ala108', Ala109' and Leu110/Leu110', offer necessary stabilization for the common structure (diphenyl ether scaffold) of both OH-PBDEs and sulfated-PBDEs *via* van der Waals interactions.

Finally, considering the limitations of the docking program and the fact that the stable binding of sulfonated-PBDEs cannot change its orientation during the present MD operation, it is necessary to evaluate the binding ability of the flipped conformation. Therefore, the docking poses of the sulfonated-PBDEs obtained by LeDock were clustered based on 1.0 Å RMSD. The first pose of each cluster is listed in Fig. S9–S11 (ESI†). Specifically, both 3-Sulf-BDE-47 and 4-Sulf-BDE-42 display flipped poses toward the best-scoring ones (Fig. S10 and S11, ESI†); 2'-Sulf-BDE-28, however, is an exception (Fig. S9, ESI†). Additional MD simulations using the first flipped binding poses of 3-Sulf-BDE-47 and 4-Sulf-BDE-42 as initial points were performed. Subsequent binding free energy calculations show that the TTR binding affinity of the flipped pose of 3-Sulf-BDE-47 (forward binding mode) is significantly reduced by 16.59% when compared to that of its best-scoring pose. Similarly, the binding affinity of the flipped pose (reverse binding mode) of 4-Sulf-BDE-42 is less favorable toward that of its best-scoring one (forward binding mode) (-35.60 ± 3.64 versus -39.41 ± 3.30 kcal mol⁻¹) (Table 4 and Table S11, ESI†). By comparing the energy decomposition of the individual residue, we observed that the increased stabilization provided by Ser117/Ser117', Ala109', Leu110', Thr118' and Thr119' makes the reverse binding pose energetically more favorable for 3-Sulf-BDE-47 (Fig. S12A, ESI†), while the energy contribution of Lys15' and Thr119/Thr119' decreases obviously in the reverse binding mode of 4-Sulf-BDE-42, thereby impairing its binding stability (Fig. S12B, ESI†). On the basis of the above results, it is revealed that the position of the $-\text{OSO}_3^-$ group on the phenyl ring plays an important role in the orientation of sulfonated-PBDEs in the TTR binding cavity.

Conclusions

In this study, molecular docking and MD simulations were employed to investigate the interactions of hydroxylated and

sulfated metabolites of PBDEs with TTR. The docking score of LeDock performed well to predict the binding affinity of the TTR-OH-PBDE complex. The obtained predictive mode based on the docking simulations was used to assess the binding affinities of the tested sulfated PBDEs with TTR. The results indicate that the sulfated PBDE compounds are high affinity ligands of TTR, the binding affinities of which are even stronger than that of endogenous ligand T4. Further structural analysis shows that the binding modes of OH-PBDEs and sulfated PBDEs exhibit two types of binding orientations for the recognition of the ionizable groups in the ligands. The two distinct binding modes, respectively termed as the forward and reverse modes, are further explored by MD simulations to probe the structural basis of molecular recognition. Subsequent investigations of RMSD, RMSF and R_g show that the representative compounds 2'-Sulf-BDE-28, 3-Sulf-BDE-47, 4-Sulf-BDE-42, 2'-OH-BDE-28, 3-OH-BDE-47 and 4-OH-BDE-42 can stabilize TTR's tetrameric structure and potentially reduce the dissociation rate of the protein involved in related amyloidoses.

By means of binding free energy calculations, van der Waals interactions (ΔE_{vdw}) are identified as the major contributors and dominant forces for the binding of the metabolites of PBDEs in the TTR channel-type pocket. Both computational alanine-scanning mutagenesis and the energy decomposition of residues combined with the analysis of hydrogen bond occupancy imply that the strong binding potencies of sulfated PBDEs, generally higher than those of corresponding OH-PBDEs, are mainly ascribed to the intensive polar interactions between key residues, such as polar residues Ser117/Ser117' and charged residues Lys15/Lys15', and the negatively charged groups of sulfated-PBDEs. These findings provide insight into the recognition mechanism for OH-PBDEs and sulfated-PBDEs in the active pocket of TTR. It can also help to identify the potential chemicals which may induce thyroid homeostatic disruption and provide a theoretical toxicity assessment for these hydroxylated and sulfated PBDE metabolites.

Conflict of interest statement

The authors declare no conflict of interest.

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