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# In Vitro and in Silico Competitive Binding of Brominated Polyphenyl Ether Contaminants with Human and Gull Thyroid Hormone Transport Proteins

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**Abstract**
Tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz) is a highly brominated additive flame retardant (FR). Debrominated photodegradates of TeDBDiPhOBz are hydroxylated in vitro in liver microsomal assays based on herring gulls (Larus argentatus), including one metabolite identified as 4″-OH-2,2′,2″,4-tetrabromo-Di- PhOBz. Chemically related methoxylated tetra- to hexabromo- DiPhOBzs are known contaminants in herring gulls. Collectively, nothing is currently known about biological effects of these polybrominated (PB) DiPhOBz-based compounds. The present study investigated the potential thyroidogenicity of 2,2′,2″,4-tetrabromo-(TB)-DiPhOBz along with its para-methoxy (MeO)- and hydroxy-(OH)-analogues, using an in vitro competitive protein binding assay with the human thyroid hormone (TH) transport proteins transthyretin (hTTR) and albumin (hALB). This model para-OH-TB-DiPhOBz was found to be capable of competing with thyroxine (T4) for the binding site on hTTR and hALB. In silico analyses were also conducted using a 3D homology model for gull TTR, to predict whether these TB-DiPhOBz-based compounds may also act as ligands for an avian TH transport protein despite evolutionary differences with hTTR. This analysis found all three TB-DiPhOBz analogues to be potential ligands for gull TTR and have similar binding efficacies to THs. Results indicate structure-related differences in binding affinities of these ligands and suggest there is potential for these contaminants to interact with both mammalian and avian thyroid function.

**Introduction**
Polybrominated diphenyl ethers (PBDEs) were until recently some of the most widely used brominated flame retardants (BFRs) worldwide.1,2 Concerns of persistence, long-range transport, and exposure of humans3,4 and wildlife, e.g., fish and herring gulls from the Great Lakes5,6 and even more remote regions such as the Arctic,7 as well as toxicological effects, led to a worldwide phase-out of all three commercial mixtures (penta-, octa-, and deca-BDE) from commerce over the last several years.2 In 2009, penta- and octa-BDE the deca-BDE replacement tetradecabromo-1,4-diphenoxyben- zene (TeDB-DiPhOBz; CAS 58965-66-5). TeDB-DiPhOBz is a highly brominated additive flame retardant applied to polyesters, resins, wires, and cables.9 There are several Asian suppliers of TeDB-DiPhOBz-containing products and commercial formulations;10,11 however, no regulations exist for TeDB- DiPhOBz production, and little information is available on the magnitude and global range of its use.

TeDB-DiPhOBz has been found to undergo photolytic debromination including to the degradation products of Br4- to formulations were added to Annex A of the Stockholm convention on persistent organic pollutants (POPs), and deca-BDE was added in early 2017.8 With the phase-out and regulation of the various PBDE formulations, several replacement flame retardants are now being produced including Br7-DiPhOBzs.12−14 Recently, methoxylated (MeO) Br4- to Br6-DiPhOBz congeners were identified for the first time in herring gull (Larus argentatus) eggs, and upon retrospective analysis, these compounds were shown to be present in gull eggs for at least the last 30 years and in concentrations up to 100 ng/g ww.15,16 An in vitro metabolism study using herring gull and rat microsomes, that had been administered UV- degraded TeDB-DiPhOBz fractions containing Br4- to Br8-PB-DiPhOBzs, confirmed the formation of several Phase I biotransformed Br3- to Br5-OH-PB-DiPhOBz metabolites.17 In the same study, two hydroxylated tetra-brominated congeners were synthesized for the purpose of identifying specific metabolite products, i.e., 4″-OH-2,2′,2″,4-tetrabromo- DiPhOBz and 4″-OH-2,2″,3′,4-tetrabromo-DiPhOBz, and these

congeners were identified as metabolites formed in vitro in both the gull and rat microsomal assays.17 The MeO-Br4- to Br6-DiPhOBz contaminants that were found in Great Lakes herring gulls and eggs are thus suspected degradation products of TeDB-DiPhOBz, where the MeO-PB-DiPhOBzs are potentially phase II conjugation products of OH-PB-DiPhOBz metabolites.

Virtually no toxicological data exist for TeDB-DiPhOBz or its potential degradation products, apart from one in vitro study that identified alterations in CYP1A4 expression of chicken embryonic hepatocytes.13 Effects are expected to be similar to structurally analogous PBDEs including lower brominated hydroxylated (OH) PBDE metabolites, which are capable of perturbing thyroid homeostasis. Thyroid hormones (THs) are involved in several important functions including the regulation of metabolism in adult organisms and tissue differentiation during fetal and embryonic development.18 One potential mechanism of action of thyroid disruption is competition for TH binding sites on serum transport proteins, as THs triiodo-L- thyronine (T3) and its precursor thyroxine (T4) rely on binding to TH transport proteins to be delivered to target tissues. There are three major TH transport proteins in vertebrates including transthyretin (TTR), albumin (ALB), and thyroid binding globulin (TBG), where TTR and ALB are consistently important in mammalian and avian species.19,20

The objectives of the present study were to investigate the interactions between selected and model tetrabrominated (TB) DiPhOBz congeners with human and avian TH transport proteins as per the following approaches. First, in vitro competitive binding interactions of the test set of TB-DiPhOBz congeners with T4 and human TTR and ALB (hTTR and hALB) were studied using a recently optimized radioligand binding assay.21 Second, we used in silico molecular homology modeling of glaucous gull (Larus hyperboreus) TTR (gTTR) developed by Mortensen,22 to investigate competitive binding interactions of the test set of the TB-DiPhOBz congeners with T4 and T3. As we reported in Ucań-Marińet al.,23 glaucous gull and herring gull TTR were shown to have identical amino acid and nucleotide sequences, and thus, the protein structure and TH binding pocket of TTR are likely to be the same in both species.

**Materials and Methods**

Standards and Chemicals. Tris-EDTA, thyroxine (T4; ≥98% purity), and the lyophilized plasma proteins human transthyretin (hTTR; ≥95% purity) and albumin (hALB; ≥99% purity) were obtained from Sigma-Aldrich (Oakville, ON, Canada). The radioligand 125I-labeled T4 (125I-T4; 50 µCi) was obtained from MP Biomedicals (Solon, OH, USA). The PBDE metabolite 4′-hydroxy-2,2′,4,5′-tetrabromo diphenyl ether (4-OH-BDE-49; 97.8% purity) was obtained from Chromatographic Specialties Inc. (Brockville, ON, Canada). Pure standards of the diphenoxybenzene-based chemicals of interest for this study were developed and provided by Daniel Teclechiel at AccuStandard Inc. (New Haven, CT, USA). These included a TB DiPhOBz as well as para-OH- and MeOTB- DiPhOBz associated congeners with the same Br-positioning: 2,2′,2″,4-tetrabromo-diphenoxybenzene (BDPB-402; 98.1% purity), 4″-OH-2,2′,2″,4-tetrabromo-diphenoxybenzene (HBDPB-401; 100% purity), and 4″-MeO-2,2′,2″,4-tetrabromo- diphenoxybenzene (MOBDPB-401; 99.5% purity) (Figure 1).

Competitive Thyroid Hormone Binding Assay. A full and highly detailed account of the procedure, including reagent preparation, sample setup, and separation are described in Hill et al.21 The assay is based on previously described methods23−27 with some important modifications, that is, a reduction in sample volume, a reduction in the amount of radioisotope required, and a streamlined sample processing procedure. Briefly, the 100 μL incubation mixtures were prepared in triplicate and comprised of 55.5 nM T4/125I-T4 (ratio of 99.5:0.5, in Tris-EDTA), 30 nM hTTR (or 600 nM hALB), and a competitor (in a 2-fold serial dilution series). Negative controls were included for each competitor ligand series using DMSO in place of the competitor. Calibrations were conducted in duplicate using 5 to 5120 nM T4 as a competitor ligand in a homologous assay, as well as a negative control (DMSO) sample and a positive control sample (the IC50 of 4-OH-BDE-49, which is a known TTR competitor ligand). The PBDE metabolite 4-OH-BDE-49 has been previously identified as having a higher affinity for both human and recombinant gull TTR and ALB over T3 and T4 using a similar assay.20 Samples were prepared in polypropylene test tubes with caps. The initial radioactivity of each sample was recorded, followed by overnight incubation at 4 °C to allow mixtures to reach binding equilibrium. Protein-bound and free ligands were then separated by size exclusion chromatography using Bio-Rad Bio-Spin P-6 gel columns as per Bio-Rad instructions. Samples were centrifuged at 4 °C for 4 min at 1000 × g, and the eluate (containing only protein-bound ligand) was measured for radioactivity. The residual radioactivity remaining in the incubation tube and transfer pipette was also measured to account for nonspecific binding and transfer loss.

Analysis of in Vitro Data. The percent of 125I-T4-hTTR or -hALB protein binding was calculated for each sample by dividing the radioactivity of eluate by the initial radioactivity, minus transfer loss. Results were expressed by plotting the logarithmic concentration of the competitor ligand on the X-axis and mean ± standard deviation of the percent binding compared to controls on the Y-axis. The 50% inhibition concentration (IC50) was calculated for each competitor in GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA, USA), using the sigmoidal dose−response equation “log- [inhibitor] vs. response − variable slope”, defined by eq 1:

Equation (1)

The inhibition constant (Ki) for each competitor was determined on the basis of Cheng and Prusoff28 and following GraphPad guidance.29 In a homologous assay (i.e., with T4 as the competitor), the cold ligand and radioligand can be assumed to have the same binding affinities. As such, the Ki and the equilibrium dissociation constant (Kd) for T4 was determined with the IC50 of T4 and the concentration of radioligand (denoted as T4*) in samples using eq 2:

Equation (2)

With these defined parameters, the Ki of other competitors was determined using eq 3.

Equation (3)

Note that, at low concentrations of radioligand, the Ki for a competitor approximates the corresponding IC50, which is the case herein.

For competitors that did not exhibit a full in vitro dose−response competition curve, analysis of variance (ANOVA) was conducted in Graphpad based on percent of specific binding and followed by Dunnett's test to determine which concentration(s) of a competitor ligand resulted in significant differences from controls.

In Silico Methodology. Full details on the development of the in silico 3D homology model for gTTR is described in Mortensen.22 Homology modeling was performed with the internal coordinate mechanics (ICM) software v3.7 (www.molsoft.com; a well-established, efficient, and user-friendly program), using the amino acid sequence for herring and glaucous gull determined by Ucán-Marín et al.,23 and three selected template models in the protein data bank (PDB). Templates were selected on the basis of preference for high resolution of the tetramer crystal structure and high homology to the glaucous gull TTR sequence and having a ligand bound to allow for definition of the binding pocket. Three models were applied herein, of which two were developed from mammalian templates (rat, Rattus norvegicus; PDB IDs 1KGI and 1KGJ; 81% amino acid sequence identity to gTTR; Models 1 and 2) and one from a fish template (sea bream; Sparus aurata; PDB ID 1SNO; 65% amino acid sequence identity to gTTR Model 3). Each model was constructed by (a) generating the backbone of the structurally conserved regions using the rigid body homology modeling method, (b) assembling the nonconserved regions (i.e., loops), and (c) placing the side chains. The models were then refined using energy minimization and Monte Carlo simulations and evaluated by docking known binders and expected nonbinders (decoys, constructed in the database DUD·E, http://dude.docking.org/). The homology models were previously evaluated on their ability to separate ligands from decoys in a test set of 668 contaminants including suspected endocrine disrupting compounds. 22 Receiver operator characteristics (ROC) curves were generated from the results, whereby ligands were labeled 1, decoys were labeled 0, and the scoring values from the docking exercise were plotted as the number of ligands predicted as binders (true positives) against the decoys predicted to bind (false positives). The area under the curve (AUC) was then calculated from each model's ROC curve, with a positive correlation between the AUC and accuracy of distinguishing between potential binders and nonbinders. Resulting AUCs of 96%, 95%, and 88% were generated for Models 1, 2, and 3, respectively.22

The ICM software v3.7 was also used for docking and scoring. Ligands were constructed in ICM from Simplified Molecular-Input Line-Entry System (SMILES) codes, and the formal charges of the compounds were set to a pKa corresponding to pH 7.4 to represent the pH of blood. To construct the receptor grid for docking, the binding pocket was defined to include all amino acids within 3.0 Å sphere radius from the template ligand. During docking, the protein and binding pocket remained rigid on the grid while the ligand being docked was considered to be flexible. The ligand was put through random conformational changes to fit into the defined binding pocket, and the 10 ligand positions with the lowest energy conformation were selected, which returned a scoring value to evaluate the interaction between the ligand and the protein model. The ICM virtual ligand screening (VLS) scoring function was used for scoring. Docking was performed three times in each model, and the strongest scores (i.e., higher binding affinities) were selected in each case. In the previously

completed model evaluation exercises, threshold values for each model were determined which distinguish between predicted binders and nonbinders. The threshold values for Models 1, 2, and 3 were −18, −17, and −16, respectively, whereby a scoring value greater than the threshold indicates a nonbinder and a lesser (i.e., more negative) scoring value indicates the compound is a potential ligand for gTTR.

**Results and Discussion**

In Vitro Interactions with Human TTR and ALB. An IC50 of 91.5 nM was calculated for the natural ligand T4 and hTTR (Table 1), which is comparable to hTTR-T4 IC50s published previously using similar assays (ranging from 50.9 to 88.3 nM).23−27 The positive control 4-OH-BDE-49 was a potent competitor ligand as expected, with an IC50 of 12.2 nM for hTTR, which was lower than the natural ligand T4. The IC50 calculated for 4-OH-BDE-49 herein is similar to the IC50 of 10 nM reported in Cao et al.30 using a fluorescence assay with hTTR and T4. The para-OH-TB-DiPhOBz congener HBDPB-401 also exhibited concentration-dependent competition with T4 for hTTR binding, with an IC50 of 359 nM, which was higher and less potent than the natural ligand T4 (with an IC50 of 91.5 nM). Conversely, the para-MeO-TB-DiPhOBz and TB-DiPhOBz congeners, MOBDPB-401 and BDPB-402, did not show strong affinity for binding to hTTR, with maximum inhibition of T4-hTTR binding of 15.4% and 1.46%, respectively, at concentrations in vitro up to 45 000 nM (Table 1, Figure 2). No significant reduction in T4-hTTR binding was observed for BDPB-402. Significant reduction in T4-hTTR binding by MOBDPB-401 was found at 512 and 45 000 nM; however, results were not significant in between these study concentrations at 1024 or 2048 nM (Figure 2). The trend in results for the TB-DiPhOBz compounds tested herein are similar to those reported on some PBDE conjugates by Ućan- Marin et al.20 based on interactions with recombinant gTTR and gALB and with T3 and T4. Ućan-Marin et al.20 identified lower binding potencies for the tetra-BDE BDE-49 and an associated 6-MeO-BDE-47 congener as compared to the 4-OHBDE- 49, where the calculated IC50s ranged from 89.1 to 529 nM, 42.2 to 234 nM, and 0.79 to 7.68 nM, respectively.

On the basis of the TB-DiPhOBz results with hTTR, the assays conducted with hALB focused on interactions with HBDPB-401 only, which also showed competition for the T4- hALB binding in a concentration-dependent manner (Figure 3). The IC50 value for T4-hALB binding inhibition by HBDPB-401 was 144 nM (Table 1), which was less potent relative to the natural ligand T4 with hALB with an IC50 of 4.80 nM. This is close to the T4-hALB IC50 for T4 of 7.1 nM was also reported by Ućan-Marin et al.20 Similar to T4-hTTR competitive binding, the positive control 4-OH-BDE-49 was a more potent competitor for hALB than the natural ligand T4 with an IC50 of 1.15 nM (Table 1).

Relative potencies were calculated by dividing the IC50 of T4 as a competitor ligand by the IC50 for each exogenous ligand for hTTR and hALB. For the positive control 4-OH-BDE-49, relative potencies of 7.49 and 4.18 were calculated for hTTR and hALB, respectively, indicating that this chemical is a more potent ligand than T4 for both proteins in vitro (Table 1). Relative potencies of 0.255 and 0.0333 were calculated for interactions of HBDPB-401 with hTTR and hALB against T4 (Table 1). The relative potencies of HBDPB-401 were much lower than those of 4-OH-BDE-49 and below one, indicating that HBDPB-401 is not a stronger competitor than T4 for either protein. Interestingly, relative potencies for both 4-OHBDE- 49 and HBDPB-401 were higher for hTTR than for hALB (Table 1). As such, these

exogenous ligands are expected to be stronger competitors for T4 at the TH binding site on hTTR than hALB.

These results indicate that the presence of a third phenyl ring substituent on a brominated polyphenol ether contaminant, such as HBDPB-401 as compared to 4-OH-BDE-49, does not necessarily hinder binding to hTTR or hALB (Figure 1, Table 1). The OH-TB-DiPhOBz congener under present study was capable of competing with T4 for hTTR and hALB in vitro. However, this was at a reduced potency relative to T4 or 4-OHBDE- 49, which is likely due to steric hindrance as a result of the increased molecular size with the presence of the third phenyl moiety. Conversely, the para-MeO-TB-DiPhOBz and TBDiPhOBz chemicals were not found to be competitive ligands for T4 on hTTR at concentrations of up to 45 000 nM in vitro. Overall, the findings herein provide further evidence that the presence of a para-OH group on an exogenous halogenated phenyl ring-based chemical is an important contributor to binding potency on TH transport proteins.

The Ki values calculated for each competitor are almost identical to the corresponding IC50 values, due to the low concentration of 125I-T4 used in the assay (Table 1). Hill Slope values generated in Graphpad for the competitive ligand interaction with hTTR ranged from −1.08 for T4 to −1.89 for 4-OH-BDE-49. For hALB, Hill Slope values ranged from −0.984 for HBDPB-401 to −1.72 for 4-OH-BDE-49 (Table 1). A slope of −1 or steeper indicates that a ligand is binding to a single site on the substrate, whereas a slope shallower than −1 indicates potential for binding to multiple sites. The results found herein thus suggest a single binding site for T4 and other competitors on hTTR and hALB, which is consistent with previous findings of negative binding cooperativity.31,32

In Silico Interactions with Gull TTR. All compounds included in the present in silico modeling exercise (HBDPB- 401, MOBDPB-401, and BPDB-402; Figure 1) were predicted ligands in the three gTTR models (Table 2). The scoring values for T3 and T4 were calculated to be −23.31 ± 6.45 and −22.83 ± 8.36, respectively (presented as mean ± standard deviation of the three models). A mean scoring value of −25.09 ± 0.163 was calculated for the known competitor 4-OH-BDE- 49, and scoring values for the three PB-DiPhOBz compounds were calculated to be −29.39 ± 2.58 for HBDPB-401, −25.50 ± 4.18 for MOBDPB-401, and −26.15 ± 1.27 for BDPB-402 (Table 2). Standard deviations were lower for the exogenous ligands than the THs indicating less variability between modelpredicted scoring values for the exogenous ligands than for the THs.

The gTTR models were designed to differentiate between binders and nonbinders. In the docking exercise, the ligand is brought to the binding site without considering the entropy and enthalpy changes that may occur due to, e.g., competition with water or a TH molecule at the binding site. Comparing scoring values of the various exogenous ligands to those of the natural ligands allows for an analysis of the relative ligand binding strength; however, this does not necessarily represent a competitive advantage at the gTTR TH binding pocket. Accordingly, TH-relative scores were calculated by dividing the competitor ligand scoring value to the scoring value for T3 or T4. The comparative scoring values between T3 and T4 were very similar, with a mean T4-relative scoring value of 1.04 indicating T3 is a slightly stronger ligand for gTTR than T4. While the scoring values are not substantially different, this result is consistent with the literature as T3 is the major circulating TH in avian species.20

The positive control 4-OHBDE- 49 as well as the three PB-DiPhOBz congeners were predicted to be slightly stronger ligands for gTTR than both T3 and T4. Mean T3-relative scoring values of 1.13, 1.34, 1.17, and 1.18 were calculated for 4-OH-BDE-49, HBDPB-401, MOBDPB-401, and BDPB-402, respectively. Mean T4-relative scoring values for the same ligands were calculated to be 1.19, 1.42, 1.24, and 1.24 (Table 2).

In vertebrate species, TTR is a tetramer of two identical subunits with two TH binding pockets at the interface of these dimers. Negative binding cooperativity is commonly observed, whereby only one binding pocket is occupied at a time with a much lower TH binding affinity in the second pocket.32 As shown in the in silico Model 3 (Figure 4), the gTTR binding pocket is very hydrophobic, with only certain amino acid residues offering hydrogen donor and acceptor potential. The natural ligands T3 and T4 are most likely to be oriented within the TTR binding pocket such that the hydroxyl group faces the inner pocket (forward orientation); however, it may also bind with reverse orientation.27 In the forward orientation, the carboxyl group of T4 is predicted to form a hydrogen bond with Lys-15 near the outer edge of the binding site, whereas the hydroxyl group of T3 is predicted to form a hydrogen bond with Ser-117 at the inner binding site.22 Similar to T3, the hydroxyl group on HBDPB-401 is predicted to form a hydrogen bond between the oxygen atom and the polar side chain of the amino acid residue Ser-117 (Figure 5). Hydrogen bonding was not anticipated for MOBDPB-401 or BDPB-402 in the gTTR binding pocket as there is no hydroxyl group on these ligands. Several other amino acid residues in the gTTR binding pocket were predicted to form interactions with the TH and TB-DiPhOBz ligands, however, including: Met-13, Leu-17, Phe-52, Glu-54, Thr-106, Ala-108, Ala-109, Leu-110, Thr-118, Thr-119, and Val-121 (Figure 5). These residues align with many of those found to interact with ligands (e.g., OHBDE congeners) in the hTTR binding pocket.30,33

Implications of in Vitro and in Silico Results. In healthy vertebrate organisms, circulating levels of THs are kept within a specific and narrow range by the hypothalamic-pituitary thyroid axis.34 Disruption of the level of circulating THs as a result of displacement from transport proteins by exogenous ligands could thus potentially lead to alterations in processes driven by thyroid function.35 Furthermore, displacement of THs from transport proteins such as TTR and ALB may lead to increased excretion of THs and result in dysregulation of the thyroid system. Thyroid disruption can be particularly detrimental in early life stages; for example, hypothyroidism in avian embryos has been linked to delayed hatching.36 THs are necessary to stimulate pipping, and a chick that hatches late or at a less developed stage may be abandoned or otherwise unable to compete for resources.36

The amino acid sequence of TTR has been highly conserved throughout vertebrate evolution.32 The differences between TTR in mammals and birds are largely due to changes to the amino acid residues in the N-terminal region, including shortening of the sequence in eutherian mammals leading to increased hydrophilicity of this region.32,37 The width of the central channel is slightly wider in mammalian TTR compared to avian TTR, and the electrostatic surface potential differs between species.38,39 These changes are expected to be driving factors for the variation in ligand binding affinity, whereby mammalian TTR preferentially binds T4 over T3 while TTR in other vertebrates has a higher affinity for T3 over T4.32 Speciesspecific differences in binding affinity of TTR have also been

observed for other ligands including xenobiotic pollutants.23,40 The observed differences in binding affinity for the TBDiPhOBz compounds included in the present study could thus be due to these structural and physicochemical differences between human and gull TTR.

During docking in the gTTR model, the ligand is positioned directly into the model binding pocket. The scoring calculations take into consideration the amino acids across the entire model protein; however, conformational changes only occur to the amino acids within the grid maps of the binding pocket. Any potential conformational changes that may occur outside the grid, such as the N-terminal region, are not considered, although this could potentially affect whether the ligand binds to TTR. Also not considered during docking are any potential structural changes in the central channel from interactions with the ligand, which may affect ligand orientation during the binding interaction. This is a common weakness in the molecular modeling of a ligand binding to a target protein. The N-terminal in gTTR, however, is considered when calculating the scoring value, which is related to the prediction of whether ligands are poor or good binders to the protein.

TeDB-DiPhOBz is the suspected precursor of methoxylated Br4- to Br6 -DiPhOBz contaminants reported in herring gulls from the Great Lakes of North America.15,16 To the best of our knowledge, these MeO-containing compounds are the only PBDiPhOBz- based contaminants identified to-date in environmental samples in the Great Lakes and worldwide. A recent effort was made to characterize and quantify TeDB-DiPhOBz and MeO-PB-DiPhOBz contaminants in surficial bottom sediments collected in three sites of the Great Lakes (Lakes Huron and Erie), including sites in Saginaw Bay, which receives river in-flow from the Saginaw River upon which is located a historical manufacturing site for TeDB-DiPhOBz flame retardant, but PB-DiPhOBzs were not detectable in any sediment sample.9 It is currently unknown whether TeDBDiPhOBz and/or PB-DiPhOBz degradation products are present in sediments elsewhere in the Great Lakes or in any other environmental compartments including other wildlife.

The extent of exposure of humans to this flame retardant and potential degradation products is also currently unknown. The present study provides in vitro and in silico evidence that known degradation products of TeDB-DiPhOBz are potential ligands for human and gull TH transport proteins. The test set in the current study focused on three TB-DiPhOBz congeners with varying substitution; however, it is hypothesized that several lower brominated (e.g., Br4- to Br6-) DiPhOBz congeners as well as their para-OH- and para-MeO-substituted analogues are competitive TH ligands for gTTR. Efforts by industry to reduce bioavailability of flame retardants by increasing molecular size, as is the case with TeDB-DiPhOBz compared to PBDEs, do not provide protection from these contaminants due to environmental and biological degradation. It is imperative that chemicals introduced to commerce are assessed not only for the toxicological and physicochemical properties of the parent product but also for the degradation products that may be formed.

**Supporting information**
The Supporting Information is available free of charge on the ACS Publications website.

**Notes**
The authors declare no competing financial interest.

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## Equations

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{(\log EC50 - X) \times \text{Hillslope}})} \quad (1)$$

$$(K_i)_{T4 \text{ and } T4^*} = (K_d)_{T4 \text{ and } T4^*} = (IC50)_{T4} - [T4^*] \quad (2)$$

$$(K_i)_{\text{competitor}} = \frac{(IC50)_{\text{competitor}}}{\frac{1 + [T4^*]}{(K_d)_{T4^*}}} \quad (3)$$

**Figures**

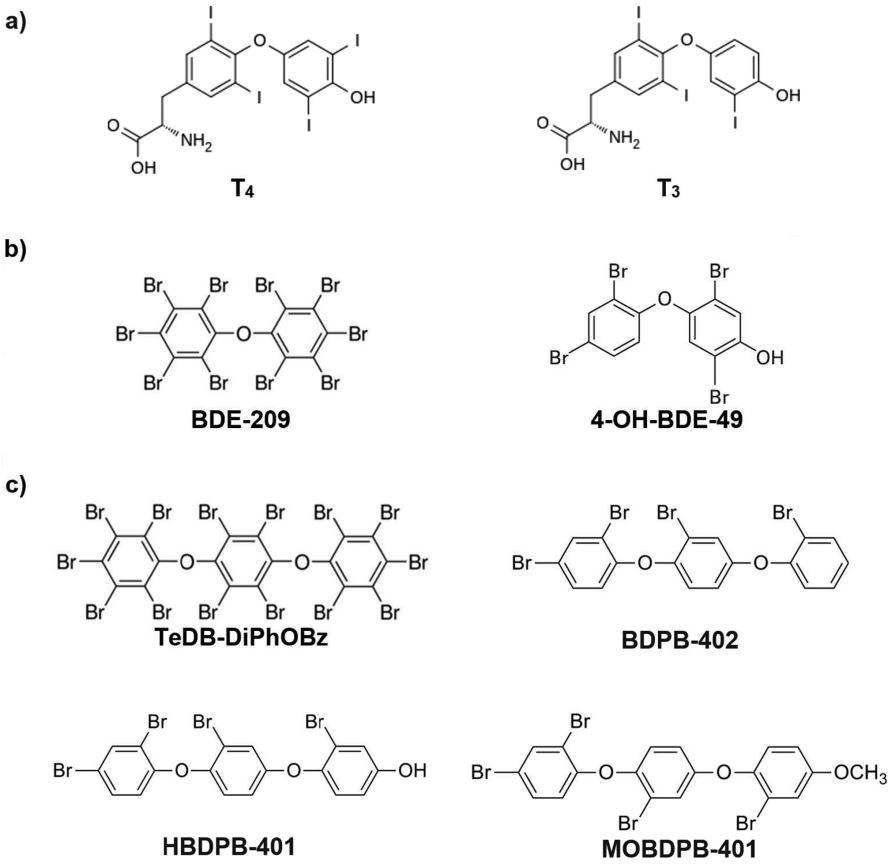


Figure 1. Chemical structures of (a) thyroid hormones thyroxine (T4) and triiodo-L-thyronine (T3); (b) decabromo-diphenyl ether (BDE-209) and the para-hydroxy tetrabrominated metabolite 4-OH-BDE-49; (c) tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz) and its hydroxyl- and methoxy-substituted analogues BDPB-402, HBDPB-401, and MOBDPB-401, respectively. The hydrogen atoms have been omitted for clarity.

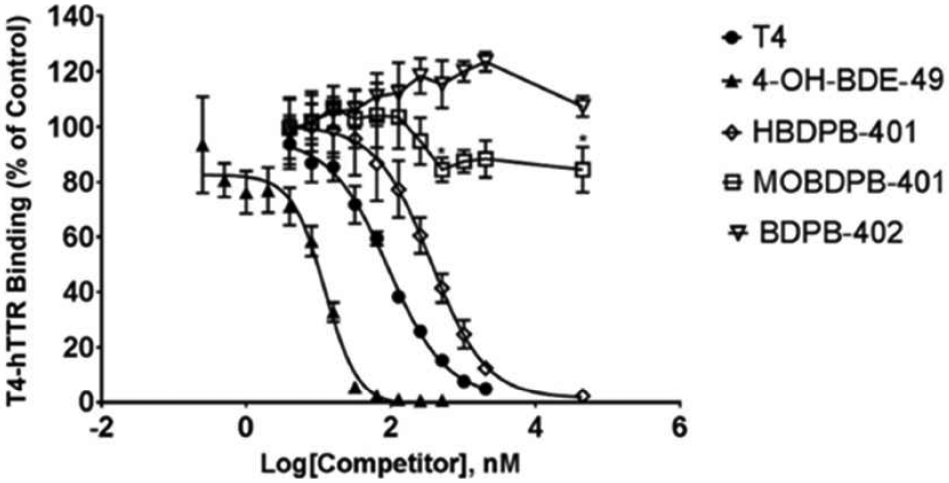


Figure 2. Competitive ligand binding curves generated in Graphpad Prism for thyroxine (T4) interactions with human transthyretin (hTTR) with competitors including the natural ligand T4, the positive control, and known potent ligand 4-OH-BDE-49 and the three PB- DiPhOBz congeners HBDPB-401, MOBDPB-401, and BDPB-402 (see Figure 1). Results are presented as means ± standard deviations, with six replicates for each concentration tested. Significant reductions in T4-hTTR binding compared to controls are indicated by asterisks ($p < 0.05$).

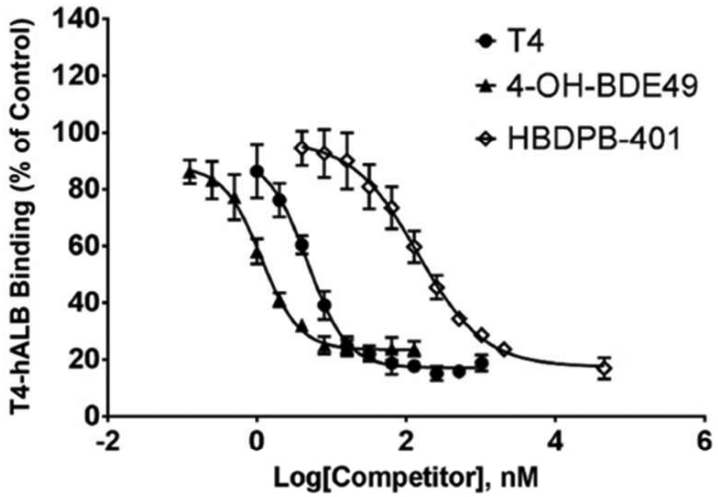


Figure 3. Competitive ligand binding curves generated in Graphpad Prism for thyroxine (T4) interactions with human albumin (hALB) with competitors including the natural ligand T4, the positive control, and known potent ligand 4-OH-BDE-49 and the para-hydroxy PB-DiPhOBz congener HBDPB-401 (see Figure 1). Results are presented as means ± standard deviations, with six replicates for each concentration tested.

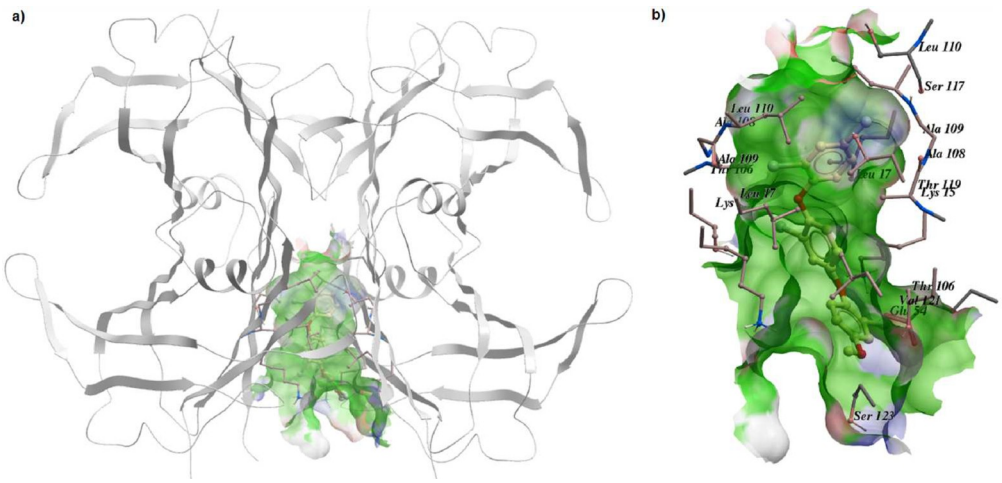


Figure 4. In silico Model 3 depictions of the binding pocket of gull transthyretin (gTTR), where green represents the hydrophobic surface; the hydrogen bond acceptor potential is displayed in blue, and hydrogen bond donor potential is displayed in red; with the exogenous ligands (a) tetrabromo-diphenoxybenzene (TB-DiPhOBz) congener BDPB-402 and (b) methoxy-TB-DiPhOBz congener MOBDPB-401. Interactions with specific amino acid residues are indicated in (b) and labeled in accordance to human TTR sequence.

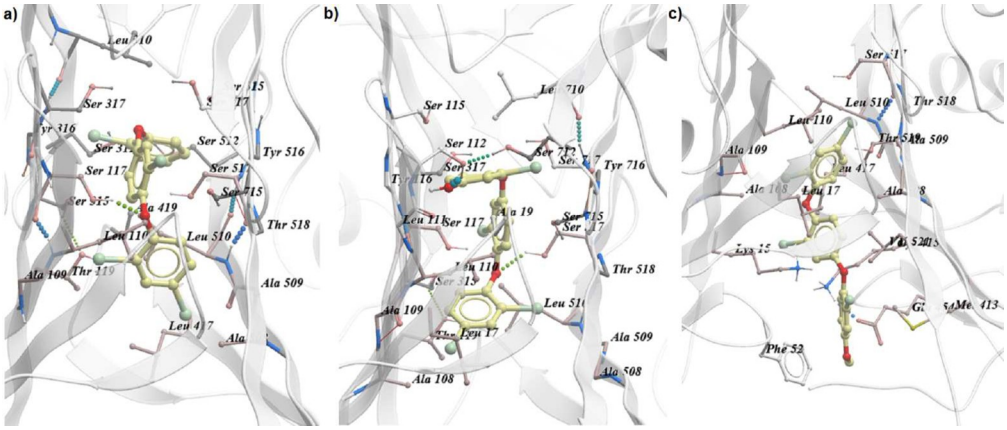


Figure 5. In silico Model 1 depictions of interactions between exogenous ligands (a) tetrabromo-diphenoxybenzene (TB-DiPhOBz) congener BDPB-402; (b) hydroxy-TB-DiPhOBz congener HBDPB-401; (c) methoxy-TB-DiPhOBz congener MOBDPB-401 with amino acids in the gull transthyretin (gTTR) binding pocket.

## Tabular material

Table 1. IC50 Values and Relative Potencies Calculated for Competitor Interactions with Human Transthyretin (hTTR) or Albumin (hALB) and the Natural Ligand Thyroxine (T4)[a]

| Competitor compound | IC50 (nM) | $R^2$ | relative potency[b] | $K_i$[c] (nM) | Hill slope | maximum % competition[d] | highest tested concentration (nM) |
|---|---|---|---|---|---|---|---|
| | | | | T4-hTTR Interactions | | | |
| T4 | 91.5 | 0.98 | 1 | 91.2 | −1.08 | 95.1 ± 0.5 | 2048 |
| 4-OH-BDE-49 | 12.2 | 0.95 | 7.49 | 12.2 | −1.89 | 99.0 ± 0.4 | 128 |
| HBDPB-401 | 359 | 0.94 | 0.255 | 358 | −1.15 | 97.8 ± 0.3 | 45 000 |
| MOBDPB-401 | >45 000 | ND | ND | ND | ND | 15.4 ± 8.3 | 45 000 |
| BDPB-402 | >45 000 | ND | ND | ND | ND | 1.46 ± 5.74 | 45 000 |
| | | | | T4-hALB Interactions | | | |
| T4 | 4.80 | 0.97 | 1 | 4.52 | −1.65 | 84.8 ± 2.5[e] | 1024 |
| 4-OH-BDE-49 | 1.15 | 0.98 | 4.18 | 1.08 | −1.72 | 77.7 ± 1.0[f] | 128 |
| HBDPB-401 | 144 | 0.96 | 0.0333 | 135 | −0.984 | 83.1 ± 3.6 | 45 000 |

[a]See Figure 1 for the chemical structures of the competitive ligands. ND = not determined; data not available due to lack of competition. [b]Calculated by dividing T4 IC50 by competitor IC50. [c]Calculated by dividing the Ki for T4 by the relative potency of the competitor. [d]Mean ± standard deviation (highest concentration tested unless otherwise noted). [e]Maximum % competition occurred at 256 nM; 81.2% competition at highest concentration tested. [f]Maximum % competition occurred at 32 nM; 76.2% competition at highest concentration tested.

Table 2. Scoring Values and Relative Scores Calculated for Competitor Interactions with Gull Transthyretin (gTTR) Using in Silico Molecular Homology Modeling[a]

| | | competitor compound | | | | | |
|---|---|---|---|---|---|---|---|
| parameter | model no. | T3 | T4 | 4-OH-BDE-49 | HBDPB-401 | MOBDPB-401 | BDPB-402 |
| scoring value | 1 | −30.75 | −32.44 | −25.02 | −26.52 | −21.72 | −25.65 |
| | 2 | −19.28 | −18.86 | −25.28 | −30.13 | −24.79 | −25.21 |
| | 3 | −19.89 | −17.19 | −24.98 | −31.51 | −29.99 | −27.60 |
| | mean ± SD | −23.31 ± 6.45 | −22.83 ± 8.36 | −25.09 ± 0.163 | −29.39 ± 2.58 | −25.50 ± 4.18 | −26.15 ± 1.27 |
| T3-relative score | 1 | NA | 1.05 | 0.814 | 0.862 | 0.706 | 0.834 |
| | 2 | NA | 0.978 | 1.31 | 1.56 | 1.29 | 1.31 |
| | 3 | NA | 0.864 | 1.26 | 1.58 | 1.51 | 1.39 |
| | mean | — | 0.966 | 1.13 | 1.34 | 1.17 | 1.18 |
| T4-relative score | 1 | 0.948 | NA | 0.771 | 0.818 | 0.670 | 0.791 |
| | 2 | 1.02 | NA | 1.34 | 1.60 | 1.31 | 1.34 |
| | 3 | 1.16 | NA | 1.45 | 1.83 | 1.74 | 1.61 |
| | mean | 1.04 | — | 1.19 | 1.42 | 1.24 | 1.24 |

[a]SD = standard deviation. NA = not applicable.

**Supporting Information**

**Manuscript Title:** *In Vitro* and *In Silico* Competitive Binding of Brominated Polyphenyl Ether Contaminants With Human and Gull Thyroid Hormone Transport Proteins

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**Number of figures:** 0
**Number of tables:** 3

**Table S1.** Model 1 predicted electrostatic interactions of natural ligands L-thyroxine (T4) and triiodo-L-thyronine (T3), as well as exogenous ligands tetrabromo-diphenoxybenzene (TB-DiPhOBz) BDPB-402, and hydroxy- and methoxy-TB-DiPhOBz congeners HBDPB-401 and MOBDPB-401, with gull transthyretin (gTTR) in the thyroid hormone binding pocket.

| Amino Acid No. | T3 | | T4 | | HBDPB-401 | | MOBDPB-401 | | BDPB-402 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Subunit | | Subunit | | Subunit | | Subunit | | Subunit | |
| | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| Met13 | X | | | | | | | | | X |
| Lys15 | X | X | ***X*** | X | X | X | X | X | X | X |
| Leu17 | X | X | X | X | X | X | X | X | X | X |
| Pro24 | | | | | | | | | | |
| Phe52 | | | | | | | | | X | |
| Glu54 | | | | | X | | | | | |
| Thr106 | X | | X | X | X | | | X | | X |
| Ala108 | X | X | X | X | X | X | X | X | X | X |
| Ala109 | X | X | X | X | | X | X | | X | X |
| Leu110 | X | X | X | X | X | X | X | | | X |
| Ser117 | ***X*** | X | X | | X | ***X*** | X | X | | X |
| Thr118 | X | | X | | | X | X | X | | X |
| Thr119 | X | X | X | X | X | X | X | X | | |
| Val121 | X | | | X | X | | | X | | X |
| Ser123 | | | | | | | | | | |

Subunits 1 and 2 indicate which of the two dimers of gTTR is interacting with the ligand.
"X" indicates predicted interaction between ligand and amino acid residue.
"*X*" indicates hydrogen bonds are predicted between ligand and amino acid residue.
Note: model configured at pH 7.4 to simulate blood.

**Table S2.** Model 2 predicted electrostatic interactions of natural ligands L-thyroxine (T4) and triiodo-L-thyronine (T3), as well as exogenous ligands tetrabromo-diphenoxybenzene (TB-DiPhOBz) BDPB-402, and hydroxy- and methoxy-TB-DiPhOBz congeners HBDPB-401 and MOBDPB-401, with gull transthyretin (gTTR) in the thyroid hormone binding pocket.

| Amino Acid No. | T3 | | T4 | | HBDPB-401 | | MOBDPB-401 | | BDPB-402 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Subunit | | Subunit | | Subunit | | Subunit | | Subunit | |
| | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| Met13 | | | | | | X | | X | | X |
| Lys15 | X | *X* | X | *X* | X | X | X | X | X | X |
| Leu17 | X | X | X | X | X | X | X | X | X | X |
| Pro24 | | | | | X | | X | | | |
| Phe52 | | | | | | | | | | |
| Glu54 | | | | | | X | | X | | |
| Thr106 | X | | X | | X | | X | X | X | X |
| Ala108 | X | X | X | X | X | X | X | X | X | X |
| Ala109 | | X | | X | X | X | X | X | X | X |
| Leu110 | X | X | X | X | X | X | X | X | X | X |
| Ser117 | *X* | *X* | *X* | *X* | | X | | X | | X |
| Thr118 | X | | X | | | X | | X | | X |
| Thr119 | X | | X | | X | ***X*** | | X | | X |
| Val121 | | | | | | X | | X | | X |
| Ser123 | | | | | | | | | | |

Subunits 1 and 2 indicate which of the two dimers of gTTR is interacting with the ligand.
"X" indicates predicted interaction between ligand and amino acid residue.
"*X*" indicates hydrogen bonds are predicted between ligand and amino acid residue.
Note: model configured at pH 7.4 to simulate blood.

**Table S3.** Model 3 predicted electrostatic interactions of natural ligands L-thyroxine (T4) and triiodo-L-thyronine (T3), as well as exogenous ligands tetrabromo-diphenoxybenzene (TB-DiPhOBz) BDPB-402, and hydroxy- and methoxy-TB-DiPhOBz congeners HBDPB-401 and MOBDPB-401, with gull transthyretin (gTTR) in the thyroid hormone binding pocket.

| Amino Acid No. | T3 | | T4 | | HBDPB-401 | | MOBDPB-401 | | BDPB-402 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Subunit | | Subunit | | Subunit | | Subunit | | Subunit | |
| | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| Met13 | | | | | X | | | | | |
| Lys15 | X | X | X | X | X | X | X | X | X | X |
| Leu17 | X | X | X | X | X | X | X | X | X | X |
| Pro24 | | | | | | | | | | |
| Phe52 | | | | | | | | | | |
| Glu54 | | *X* | | X | X | | | X | | |
| Thr106 | X | X | X | X | X | X | X | X | | X |
| Ala108 | X | X | X | X | X | X | X | X | X | X |
| Ala109 | X | | X | X | X | X | X | X | X | X |
| Leu110 | X | X | X | X | X | X | X | X | X | X |
| Ser117 | | | | | X | | | X | | X |
| Thr118 | | | | | | | | | | |
| Thr119 | | | | | ***X*** | | | X | X | X |
| Val121 | | X | | X | X | | | X | | |
| Ser123 | | | | | | | | X | | |

Subunits 1 and 2 indicate which of the two dimers of gTTR is interacting with the ligand.
"X" indicates predicted interaction between ligand and amino acid residue.
"*X*" indicates hydrogen bonds are predicted between ligand and amino acid residue.
Note: model configured at pH 7.4 to simulate blood.