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Modulating Inhibitors of Transthyretin Fibrillogenesis via Sulfation: Polychlorinated Biphenyl Sulfates as Models¹

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Abstract

Small molecules that bind with high affinity to thyroxine (T₄) binding sites on transthyretin (TTR) kinetically stabilize the protein's tetrameric structure, thereby efficiently decreasing the rate of tetramer dissociation in TTR related amyloidoses. Current research efforts aim to optimize the amyloid inhibiting properties of known inhibitors, such as derivatives of biphenyls, dibenzofurans and benzoxazoles, by chemical modification. In order to test the hypothesis that sulfate group substituents can improve the efficiencies of such inhibitors, we evaluated the potential of six polychlorinated biphenyl sulfates to inhibit TTR amyloid fibril formation *in vitro*. In addition, we determined their binding orientations and molecular interactions within the T₄ binding site by molecular docking simulations. Utilizing this combined experimental and computational approach, we demonstrated that sulfation significantly improves the amyloid inhibiting properties as compared to both parent and hydroxylated PCBs. Importantly, several PCB sulfates were of equal or higher potency than some of the most effective previously described inhibitors.

Keywords

amyloidosis; polychlorinated biphenyls; PCBs; sulfation; transthyretin; TTR

¹Abbreviations: ANS, 8-anilinoanthracene-1-sulfonate; FAC, familial amyloid cardiomyopathy; FAP, familial amyloid polyneuropathy; HBP, halogen binding pocket; OHPCB, hydroxyl-PCB; PCB, polychlorinated biphenyl; SSA, senile systemic amyloidosis; T₄, thyroxine; TTR, transthyretin

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1. Introduction

Besides its physiological function as a principal transporter of T₄ and the complex of retinol and retinol-binding protein, TTR is known as one of more than 30 amyloidogenic proteins.^{1, 2} *In vivo*, TTR exists in equilibrium between its monomeric and tetrameric forms, with the tetramer being the biologically active and predominant form.^{3, 4} The hallmark of TTR amyloidosis is an imbalance in this equilibrium towards the formation of the amyloidogenic monomer.^{5, 6} Misfolding of the monomeric subunits triggers their aggregation into prefibrillar, cytotoxic oligomers and large fibrils that deposit at various sites in the human body.^{2, 7} The most prevalent form of TTR amyloidosis, senile systemic amyloidosis (SSA), is caused by wild-type TTR (wtTTR) and has an incidence of 10–25 % in the elderly population.^{5, 8} SSA is characterized by the accumulation of TTR amyloid fibrils in the heart and leads to progressive heart failure.⁹ Hereditary TTR amyloidoses like familial amyloid cardiomyopathy (FAC) and familial amyloid polyneuropathy (FAP) have a lower incidence but are more severe than those caused by wtTTR due to the increased amyloidogenicity associated with TTR variants such as V122I (FAC) and V30M (FAP).^{10, 11} Although success has been achieved with liver transplantation in treatment of some patients diagnosed with severe transthyretin-related amyloidosis, there is an ongoing search for effective drug therapies.^{12, 13}

While TTR monomers comprise 127 amino acids that are predominantly arranged in β -sheets, the quaternary structure of the 55 kDa homotetramer is arranged as a dimer of dimers with a twofold axis of symmetry.^{14, 15} The interface between both dimers forms two identical T₄ binding sites that exhibit negative cooperativity (Figure 1A).^{15, 16} Both T₄ binding sites contain an inner and an outer binding cavity (Figure 1B) and are lined by symmetrical sets of amino acids that form three hydrophobic halogen-binding pockets (HBPs).¹⁷ The first HBP is located in the outer binding cavity, the second one is located between the outer and inner binding cavities, and the third HBP is within the inner cavity. The outer part of the T₄ binding site (i.e., Glu-54 and Lys-15 at the edge of the outer cavity) forms a polar binding region.

A promising approach for the treatment of TTR amyloidoses involves the use of small molecules that bind specifically and with high affinity to the thyroid hormone binding sites on the protein.^{5, 17} Binding of such molecules results in kinetic stabilization of the tetramer and slows TTR disassembly, the rate-limiting step in the formation of amyloid fibrils. Typically, TTR amyloid inhibitors consist of two or more aromatic rings with different linkers and ring substitutions.⁵ Among the most efficient inhibitors discovered to date are derivatives of biphenyls, dibenzofurans and benzoxazoles.^{5, 18–20} Recent pre-clinical studies have also revealed the synthetic compound CLR01²¹ and the natural product caffeic acid phenyl ester²² as highly effective inhibitors of TTR fibrillogenesis. In addition to identifying novel classes of inhibitors, current research focuses on the optimization of inhibitors by chemical modification.²³

The small molecule approach for treating transthyretin-related amyloidosis has produced a limited number of promising drug candidates, the most prominent of which is the drug tafamidis, which has been approved in Europe. In a recently published phase II clinical trial,

tafamidis reportedly stabilized a variety of non-V30M amyloidogenic transthyretin variants in approximately 95 % of the treated patients and was generally concluded as being safe, a finding that correlates with previously published clinical data.^{24, 25} In a similar trial, diflunisal was recently demonstrated to significantly reduce the progression of FAP in 130 patients studied over a period of two years.²⁶ These representative studies clearly indicate the importance and relevance of small molecule drugs for the management of transthyretin amyloid disease progression. In addition to small molecule inhibition of TTR amyloidosis, RNA interference is also currently being investigated as a potential treatment option.^{27–29}

In 2004, Purkey and coworkers conclusively demonstrated that certain polychlorinated biphenyls (PCBs), ubiquitous and toxic environmental contaminants, and their hydroxylated metabolites (OHPCBs) act as transthyretin amyloid inhibitors *in vitro*.¹⁹ These findings suggested a potential protective effect in PCB-exposed, susceptible populations, and further established biphenyl derivatives as promising lead molecules for the development of anti-amyloid therapeutics. We recently reported that sulfate ester metabolites of polychlorinated biphenyls (PCB sulfates) represent a class of high-affinity ligands for human TTR.³⁰ Importantly, these PCB sulfates bound with affinities similar to, or higher than, either their hydroxylated precursors (OHPCBs) or the natural ligand, T₄. While these findings may have implications for thyroid disruption in PCB exposed populations, they also prompted the hypothesis that these PCB sulfates have the ability to act as inhibitors of TTR fibrillogenesis that are potentially more effective than OHPCBs and parent PCBs. However, to date neither the amyloid inhibiting potential of PCB sulfates, nor the utilization of sulfate groups to improve the effectiveness of TTR anti-amyloid therapeutics has been evaluated. In the present study, we found that PCB sulfates inhibited the acid-induced formation of TTR amyloid fibrils *in vitro*. In addition, we developed a molecular docking model that was successfully applied to provide a structural explanation for the *in vitro* data.

2. Materials and methods

2.1 Chemicals

All PCBs and PCB metabolites (4-chlorobiphenyl (PCB 3), 3,3'-dichlorobiphenyl (PCB 11), 4'-chloro-biphenyl-3-ol (3'-OHPCB 3), 4'-chloro-biphenyl-4-ol (4'-OHPCB 3), 3,3'-dichloro-biphenyl-4-ol (4-OHPCB 11), 3,3',4',5-tetrachloro-biphenyl-4-ol (4'-OHPCB 79) and ammonium salts of 4-chloro-3'-sulfooxy-biphenyl (3'-PCB 3 sulfate), 4-chloro-4'-sulfooxy-biphenyl (4'-PCB 3 sulfate), 3,3'-dichloro-4-sulfooxy-biphenyl (4-PCB 11 sulfate), 2,4'-dichloro-4-sulfooxy-biphenyl (4-PCB 8 sulfate), 2,3',5-trichloro-4'-sulfooxy-biphenyl (4'-PCB 26 sulfate) and 2,3',4'-trichloro-4-sulfooxy-biphenyl (4-PCB 33 sulfate)) used in this study were provided by the Synthesis Core of the University of Iowa Superfund Research Program and synthesized and characterized as described elsewhere (Figure 2).^{31, 32} PCB sulfates were synthesized as the ammonium salts.³² Flufenamic acid, 8-anilinonaphthalene-1-sulfonic acid (ANS), and transthyretin purified from human plasma (> 95%) were all acquired from Sigma Aldrich (St. Louis, MO). The purity of TTR was routinely confirmed by SDS-PAGE.

2.2 Amyloid Inhibition Assay

Assays were conducted with minor modifications of a previously described procedure.¹⁹ Briefly, aliquots (5 μ l) of PCB metabolites, prepared as 720 μ M working solutions in either 33 % or 100 % (v/v) acetonitrile, were combined with 495 μ l of 7.2 μ M TTR (tetrameric concentration) in phosphate buffer (25 mM sodium phosphate; 100 mM sodium chloride (NaCl); 1 mM EDTA; pH 7.5) in disposable cuvettes. Following a 30 minute pre-incubation at 37°C, 500 μ l acetate buffer (200 mM sodium acetate; 100 mM NaCl; 1 mM EDTA; pH 4.2) were added, thereby decreasing the pH of the solution to 4.4 and yielding equimolar concentrations of TTR and potential inhibitor (3.6 μ M). Following 72 hours of incubation at 37°C in the dark, the cuvettes were vortexed and sample turbidities were determined by measuring their optical densities at 400 nm (OD_{400nm}). TTR incubated in the presence of 5 μ l acetonitrile (no inhibitor) served as the negative control and the determined OD_{400nm} value was normalized to 100 % fibril formation. OD_{400nm} values determined for samples incubated in the presence of potential inhibitors were evaluated as % fibril formation relative to that determined for the negative control. At least three separate determinations were made for each potential inhibitor. Correlations between amyloid inhibition and dissociation constants were determined using Sigma Plot 11.0 (Systat Software Inc., San Jose, CA).

2.3 Molecular Docking Simulations

Chemical structures (Figure 2) were created in ChemBioDraw Ultra 12.0 (Perkin Elmer, Waltham, MA) and imported into a database in the SYBYL X docking software (Tripos, St.Louis, MO). Ligands were then energy-minimized using the Ligand Preparation Tool applying the Tripos force field with default parameters. We prepared the TTR receptor structures (PDB no. 2F7I, 2G9K, 2G5U, 2GAB) in the SYBYL X Structure Preparation Tool by setting protein chain termini into their charged states and adding hydrogen atoms (H-bonding orientation). Subsequently, staged energy minimizations using the default setting were carried out applying the Powell method (no initial optimization; Termination: Gradient 0.5 kcal/(mol*Å); max iterations: 100) and the MMFF94s force field (Dielectric Function: Constant; Dielectric Constant: 1.0). The binding site (protomol) was defined following the extraction of co-crystallized ligands with the respective extracted ligand as a template (Threshold: 0.5; Bloat: 0.0). Once the receptor structures were prepared, energy-minimized PCB metabolites were docked into the binding site using the Geom algorithm in the SYBYL X Docking Suite as previously described for 3'-PCB 3 sulfate, 4'-PCB 3 sulfate and 4-PCB 11 sulfate.³⁰ Twenty different poses were generated per compound and ranked according to their binding energies. In order to evaluate the binding simulations in a consistent manner, only the lowest energy binding poses were used for the interpretation of the results.

2.4 ANS displacement assay

Determination of equilibrium dissociation constants for 4-PCB 8 sulfate was conducted as previously reported.³⁰ Briefly, a solution containing 0.5 μ M TTR and 5 μ M ANS was titrated with increasing concentrations of 4-PCB 8 sulfate and the decrease in fluorescence at 470 nm (ex. = 410 nm) was monitored. Fluorescence data in the concentration range between 0 and 100 nM were then applied to a one site plus nonspecific binding equation

$((y=(B_{\max 1} * x)/(K_{d1}+x)+N_s))$ to yield the high-affinity dissociation constant K_{d1} . K_{d2} was determined by fitting data in the 0–2000 nM range to a two-site binding equation $(y=(B_{\max 1} * x)/(K_{d1}+x)+(B_{\max 2} * x)/(K_{d2}+x))$. $B_{\max 1}$ and $B_{\max 2}$ are defined as the relative changes in fluorescence that indicate saturation of the high and low affinity binding sites and N_s is a term describing all non-specific, low affinity interactions between ligand and TTR. Ligand concentration and absolute fluorescence, are represented by x and y , respectively. The data were fit by non-linear regression to the equations shown above using Sigmaplot 11.0 (Systat Software, San Jose, CA).

3. Results

3.1 Amyloid inhibition assay

To demonstrate the amyloid inhibiting potential of PCB sulfates, we initially selected matching sets of PCB sulfates, OHPCBs and their parent PCBs which were derived from PCBs 3 and 11 (Figure 2). In addition, we included sulfate ester metabolites of PCBs 8, 26 and 33 in order to determine the effect of the chlorine substitution pattern on the amyloid inhibiting properties of PCB sulfates. All compounds were synthesized and characterized as published previously.^{30–33} We evaluated the amyloid inhibiting potential of all compounds by their ability to inhibit the acid-induced formation of TTR amyloid fibrils *in vitro*.³⁴ This assay method has been extensively used both for elucidation of small molecule inhibitors of TTR fibril formation and for mechanistic studies on the biochemical unfolding of the protein under conditions that mimic the acidic conditions in the lysosome.³⁴

To facilitate comparison with other studies on TTR amyloid fibrils, we adapted the protocol published by Purkey et al¹⁹ and performed appropriate control experiments to ensure the validity of the assay (Figure A.1). Flufenamic acid and 4'-OHPCB 79 served as positive controls, and their percentages of fibril formation ($22 \pm 8 \%$ and $32 \pm 5 \%$, respectively, normalized to transthyretin) were in agreement with values previously reported in the literature (Table 1).¹⁹ Incubation of all assay components in the absence of transthyretin did not result in increased optical density after 72 hours of incubation (Figure A.1), thereby clearly demonstrating that the observed turbidity was indeed due to transthyretin fibrillogenesis. Moreover, neither any of the buffer components, nor any of the inhibitor molecules exhibited significant light absorption at 400 nm, the wavelength at which fibrils were detected in this study (Figure A.1).

The screening of PCB metabolites revealed a structure-activity relationship whereby PCB sulfates were generally more efficient amyloid inhibitors than OHPCBs and PCBs (Table 1). Di- and tri-chlorinated PCB sulfates comprised a particularly effective group (7 – 13 % fibril formation) whose amyloid inhibitory potential was comparable to that reported for some of the most effective inhibitors known in the literature.^{17, 35, 36} Monochlorinated PCB sulfates and OHPCBs exhibited reduced effectiveness (31 – 66 % fibril formation), and parent PCBs 3 and 11 were the least effective inhibitors ($81 \pm 5 \%$ and $71 \pm 6 \%$ fibril formation). Moreover, fibril formation was significantly decreased upon incubation with 3'-PCB 3 sulfate and 4'-PCB 3 sulfate as compared to 3'-OHPCB 3 ($p=0.016$; $p=0.040$), 4'-OHPCB 3 ($p<0.001$; $p<0.001$) and PCB 3 ($p<0.001$; $p<0.001$) (Table 1). By contrast, 4-OHPCB 11 and

4-PCB 11 sulfate showed similar potencies and were, alongside 4-PCB 8 sulfate, the most effective inhibitors in this study (Table 1).

3.2 Molecular docking simulations

Encouraged by these findings, we utilized computational docking studies in order to predict the lowest-energy binding orientations and potential molecular interactions of the PCB metabolites within the T₄ binding site. In order to validate the virtual screening settings, we initially chose the crystal structures of human TTR with bound 2',6'-difluorobiphenyl-4-carboxylic acid (PDB no. 2F7I), 4-hydroxy-2',3,3',4',5-pentachlorobiphenyl (PDB no. 2G9K), 4,4'-dihydroxy, 3,3',5,5'-tetrachlorobiphenyl (PDB no. 2G5U) and 4-hydroxy-3,3',5,4'-tetrachlorobiphenyl (PDB no. 2GAB) as receptor models for TTR. Following the extraction of the co-crystallized ligands, we attempted to dock the extracted ligands back into the T₄ binding site (Figure 3). A comparison between redocked ligands and their respective position in the original crystal structures revealed an overall high degree of similarity for crystal structures 2F7I, 2G9K and 2G5U. In the case of 4-hydroxy-3,3',5,4'-tetrachlorobiphenyl (PDB no. 2GAB), the docked ligand exhibited highly similar positioning of the biphenyl core, but was situated in an antiparallel pose with its hydroxyl group pointing towards the outer part of the binding site. It is known from crystal structure and docking analyses that binding of certain compounds to the T₄ binding site on TTR can occur in both a forward and reverse direction.³⁷ In order to further evaluate the potential of these four receptor structures to simulate the binding of PCB sulfates, we docked 2',6'-difluorobiphenyl-4-carboxylic acid, the ligand bearing the highest degree of structural resemblance to PCB sulfates and originally present in PDB no. 2F7I, into the T₄ binding sites (Figure A.2). While crystal structures 2G5U and 2GAB did not provide sufficient similarities between crystalized and docked ligands, docking of 2',6'-difluorobiphenyl-4-carboxylic acid into PDB no. 2F7I and 2G9K indicated overall great similarity as compared to the ligand positioning in the original crystal structure. However, as a result of slightly varied positioning of the Lys-15 in crystal structure 2G9K, electrostatic interactions between the carboxyl group of 2',6'-difluorobiphenyl-4-carboxylic acid and Lys-15 were only correctly indicated when using PDB no. 2F7I. Based on these results, we chose crystal structure 2F7I to evaluate the binding of PCB metabolites in this study. The lowest energy binding orientations for PCB sulfates and most OHPCBs were dictated by electrostatic interactions between sulfates or phenols with Lys-15, with the biphenyl cores being positioned in the outer and inner binding cavities through hydrophobic interactions (Figure 4, Figure A.3, Figure A.4). Due to the presence of the bulkier sulfate group, the biphenyl cores of 3'-PCB 3 sulfate and 4'-PCB 3 sulfate were positioned deeper within the inner binding cavity than their respective OHPCBs, thereby maximizing their hydrophobic interactions and providing a structural explanation for their increased amyloid inhibiting efficacies (Figure 4). An exception to this observation was 4-OHPCB 11. Its lowest energy binding mode suggested a reverse binding orientation that enabled hydrogen bonding interactions between its phenol and Ser-117 as well as anchorage of the ortho-chlorine within the innermost HBP of the neighboring monomer (Figure 4B). Due to this binding orientation, the biphenyl core of 4-OHPCB 11 was shifted farther into the inner binding cavity, similar to 4-PCB 11 sulfate, which explains their comparable amyloid inhibiting

potencies. The poor amyloid inhibiting potential of parent PCBs 3 and 11 was consistent with their lack of polar substituents.

3.3 Correlation between affinity and amyloid inhibiting potential

We further hypothesized that ligand binding affinities determined for TTR have predictive potential with respect to their amyloid inhibiting properties. We selected six PCB metabolites that were each used in this study and whose equilibrium dissociation constants (K_d values) were determined previously.³⁰ In addition, we determined K_d values for 4-PCB 8 sulfate ($K_{d1} = 4.3 \pm 3.4$ nM; $K_{d2} = 570 \pm 105$ nM), and included these data in our analysis (Figure A.5). A plot of fibril formation versus the high affinity dissociation constant (K_{d1}) (Figure 5) indicated a linear relationship with an r^2 value of 0.93 for all compounds with a similar predicted binding mode: 4-PCB 8 sulfate, 4-PCB 11 sulfate, 3'-PCB 3 sulfate, 4'-PCB 3 sulfate, 3'-OHPCB 3 and 4'-OHPCB 3. However, 4-OHPCB 11 deviated from this trend and its inclusion in the statistical evaluation resulted in a decrease in the observed linearity, with an adjusted r^2 value of 0.61.

4. Discussion

In this study, we demonstrate that PCB sulfates are excellent inhibitors of TTR amyloid fibril formation with potencies that are similar to some of the most effective inhibitors previously reported in the literature.^{18, 19, 36} The fact that, with the exception of 4-OHPCB 11, PCB sulfates were more efficient than OHPCBs and their parent PCBs clearly indicates that sulfate group substituents represent a promising chemical tool for the improvement of TTR amyloid inhibitors. In order to provide a structural explanation for the observed *in vitro* amyloid-inhibiting properties of the compounds used in this study, we applied *in silico* modeling of TTR-ligand interactions. Our initial evaluation of four TTR crystal structures with co-crystallized biphenyl derivatives (PDB no. 2F7I, 2G9K, 2G5U, 2GAB) confirmed that the virtual screening settings we applied provided highly accurate positioning of docked ligands as compared to the original crystal structures (Figure 3). However, docking of 2',6'-difluorobiphenyl-4-carboxylic acid, the ligand most closely resembling PCB sulfates, into all four crystal structures revealed that only two of the four crystal structures could successfully reproduce its binding orientation and positioning within the T_4 binding site, and only PDB no. 2F7I could also reliably predict hydrogen bonding interactions between the ligand's carboxylic acid and Lys-15 (Figure A.2). This observation may be a result of minor conformational variations (i.e. Lys-15) in the TTR crystal structures due to the presence of the co-crystallized ligands. Logically, PDB no. 2F7I, which was based on TTR co-crystallized with 2',6'-difluorobiphenyl-4-carboxylic acid, provided the best receptor model to simulate PCB sulfate binding to TTR.

Following the validation of the docking parameters and the selection of the receptor structure, we docked all test compounds (Figure 2) into the T_4 binding site on TTR in order to simulate the steric, hydrophobic, and electrostatic interactions that may represent the structural basis for their amyloid inhibiting potential. Overall, the docking results demonstrated that electrostatic interactions between phenol groups and anionic sulfate groups with Lys-15, or with Ser117 for 4-OHPCB 11, are key determinants for the positioning and binding orientations of the test compounds within the T_4 binding site of

TTR. This finding correlates well with previous crystal structure analyses that demonstrated the importance of electrostatic interactions between carboxyl groups of T4, tafamidis and related compounds.^{15, 35, 38} In addition, the *in silico* modeling analysis was in good agreement with the *in vitro* dataset indicating that the increased efficiencies observed for PCB sulfates, as compared to their corresponding OHPCBs, are a result of a steric shift of the biphenyl core towards the inner binding cavity (Figure 4). This shift can be directly related to the sterically bulkier sulfate group. As compared to hydroxylated PCB 3 metabolites, the dichlorinated 4-OHPCB 11 was found to be bound in a reverse orientation that induced hydrogen bonding with Ser-117 and therefore resembled the binding modes previously determined for some higher-chlorinated OHPCBs.¹⁹ While we cannot completely rule out a lower affinity interaction due to interaction of the phenol with Lys-15, the increased ability to inhibit fibril formation is consistent with the opposite orientation seen in the docking experiments. Additionally, the higher fibrillogenesis-inhibiting efficacy seen with 4-OHPCB 11 suggests that the positioning of chlorine substituents, particularly in the meta position adjacent to the hydroxyl group, is a more important determinant for tetramer stabilization than the absolute number of chlorines present. This finding is in agreement with previous reports and was further supported by the observation in the current study that dichlorinated PCB sulfates were equally or more effective than the trichlorinated ones.^{39, 40}

Even though already implicated by the results of the *in vitro* screening and molecular docking analyses, a plot of fibril formation percentages versus equilibrium dissociation constants showed that fibrillogenesis-inhibiting properties of small molecules cannot be predicted solely based on their affinities to the protein. However, the fact that PCBs and metabolites with similar binding characteristics, i.e. orientation, positioning and molecular interactions, were linearly correlated with an r^2 value of 0.93, indicates that binding affinities are a primary determining factor for the amyloid inhibiting potential of a small molecule inhibitor. Additional important determinants include the location of the (thyroid hormone- or non-thyroid hormone) binding site on transthyretin, as well as positioning and orientation of the ligand within the site(s). This conclusion is based on the determined characteristics of 4-OHPCB 11, the PCB metabolite that surprisingly revealed a different binding orientation and different molecular interactions within the T4 binding site, a finding that was concordant between *in vitro* and *in silico* analyses. In the case of this metabolite, the different binding mode has been found to be a major determinant for the inhibition of transthyretin fibrillogenesis, resulting in a deviation of the linear correlation and a decrease of the r^2 value from 0.93 to 0.61.

While our results support a conclusion that sulfate esters may be useful for development of new agents to stabilize TTR, it should also be noted that we have focused exclusively on wtTTR, the causative agent of the most prevalent form of TTR amyloidosis, SSA. Future studies will be necessary to determine the potential of sulfated compounds to inhibit fibril formation caused by variant forms of TTR such as V122I or V30M.

More specific to the molecules investigated here, the *in vivo* toxicities of PCB sulfates have not been extensively evaluated, although it is clear that lower-chlorinated, volatile PCBs can be biotransformed to their sulfated metabolites.⁴¹ While exposed human populations may thus benefit from a protective effect, thyroid disrupting effects remain a major concern for a

therapeutic application of any potential TTR amyloid inhibitor. Small molecule inhibition of TTR amyloidosis and thyroid hormone displacement from binding sites on TTR, a potential key mechanism in xenobiotic mediated thyroid disruption, share the same molecular mechanism: ligand binding at the high affinity binding site on TTR. The most feasible lead molecules therefore may not be represented by the most effective amyloid inhibitors, but rather by those compounds that exhibit specific interactions with the thyroid hormone binding site with an affinity that is high, although insufficient to completely displace T4.

While PCB metabolites may not be direct drug candidates, our results (1) provide a model to explain the potential molecular mechanisms underlying their amyloid inhibiting properties, (2) indicate that the introduction of a sulfuric acid ester moiety may have beneficial effects on the ability of phenolic molecules to stabilize TTR amyloid fibril formation, (3) indicate a potential protective effect in PCB exposed populations and (4) further demonstrate the potential of certain PCB sulfates for PCB-mediated thyroid disruption. The extent to which these sulfated PCB metabolites may affect TTR amyloid fibril formation *in vivo* is currently unknown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- PCB sulfates are effective inhibitors of transthyretin amyloid fibril formation *in vitro*
- Sulfate groups represent chemical tools to improve amyloid inhibiting properties
- Docking studies provide a structural explanation for amyloid inhibiting properties

Figure 1.

Crystal structure of TTR in complex with T₄ (PDB no. 2ROX) indicating its twofold symmetrical axis (dotted lines) (A) and close-up view on the T₄ binding site (B).

Figure 2.
Chemical structures of PCBs, OHPCBs and PCB sulfates utilized in this study

Figure 3.

Validation of molecular docking simulations

Ligands (blue sticks) present in available TTR crystal structures (PDB no. 2F7I, 2GAB, 2G5U and 2G9K) were extracted from and subsequently redocked (grey sticks) into the T₄ binding site.

Figure 4.

Comparison of the predicted binding modes of PCB sulfates (blue), OHPCBs (cyan) and PCBs (red). PDB no. 2F7I was used as an *in silico* model to dock PCB 3, 4'-OHPCB 3, 4'-PCB 3 sulfate (**A**) and PCB 11, 4-OHPCB 11 and 4-PCB 11 sulfate (**B**) into the T₄ binding site.

Figure 5.

Correlation of K_d s and fibril formation percentages indicating a linear correlation between all metabolites with similar binding orientation ($r^2 = 0.93$, all data points, except 4-OHPCB 11, included). Inclusion of 4-OHPCB 11, which exhibits a different binding mode, decreases the linear correlation to an r^2 of 0.61. Error bars reflect standard deviations determined from at least three different experiments.

Table 1

Transthyretin amyloid fibril formation in the presence of PCBs and metabolites

	Fibril Formation (%)	Std. Dev. (%)
PCB 3	80.5	4.4
PCB 11	70.6	5.7
4'-OHPCB 3	65.6	2.7
3'-OHPCB 3	52.3	7.5
4'-PCB 3 sulfate	37.2	4.4
4'-OHPCB 79	32.2	5.1
3'-PCB 3 sulfate	30.7	5.5
Flufenamic Acid	22.3	8.0
4-PCB 33 sulfate	12.7	1.6
4'-PCB 26 sulfate	10.5	2.8
4-PCB 11 sulfate	8.3	2.6
4-OHPCB 11	7.5	1.2
4-PCB 8 sulfate	6.2	2.3