NOVEL BROMINATED POLYPHENYL ETHER CONTAMINANTS AND *IN VITRO* COMPETITIVE BINDING WITH THE THYROID HORMONE THYROXINE FOR HUMAN TRANSTHYRETIN AND ALBUMIN

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Introduction:

Thyroxine (T4) and 3,5,3-triiodothyronine (T3) are thyroid hormones (THs) involved in the regulation of many important physiological processes in the (human) body including neurological and behavioural development, growth, metabolism, and respiration.[1] The vast majority (>99%) of THs in (human) plasma are delivered to target tissues by binding to TH transport proteins, which include albumin (ALB), transthyretin (TTR), and thyroxine-binding globulin (TBG). Perturbation of TH transport is considered to be one mechanism of action that may affect thyroid function, which is a major concern for PBDE-like compounds of environmental concern and especially hydroxy- (OH)-containing metabolites.[2] A limited number of studies have investigated interactions of exogenous chemicals with THs and TTR using variations of an *in vitro* competitive binding assay technique.[3,4,5,6]

At least 75 different BFRs have been or are currently commercially produced.[7] However, environmental studies have been primarily focused on polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCDD), and tetrabromobisphenol A (TBBPA). Current knowledge of the other novel non-PBDE FRs remains relatively scarce including the highly brominated tetradecabromo-1,4-diphenoxybenzene (TeDB-DiPhOBz, CAS No: 58965-66-5).[8,9] Our group reported the detection of various novel methoxylated polybrominated diphenoxybenzene (MeO-PB-DiPhOBz) contaminants in herring gull (*Larus argentatus*) eggs from fourteen colony sites across the Laurentian Great Lakes of North America.[10] As of 2010 these newly discovered MeO-PB-DiPhOBz congeners (mainly tetra- to hexa-brominated) were found to be contaminants in herring gull eggs for the previous 30 years. TeDB-DiPhOBz has been hypothesized to be the precursor of MeO-PB-DiPhOBz contaminants reported in herring gulls and their eggs. Irradiating TeDB-DiPhOBz in solution or as a solid with natural sunlight or UV results in step-wise debromination that produces polybrominated diphenoxybenzenes (PB-DiPhOBzs).[11] The *in vitro* metabolism of TeDB-DiPhOBz and these PB-DiPhOBz-containing solutions was investigated using harvested wild herring gull and adult male Wister-Han rat liver microsomal assays.[11] For the Br_{4-6}-PB-DiPhOBz containing solution, in the gull and rat assays OH-PB-DiPhOBz metabolites were detectable. The present study compares the competitive *in vitro* binding of 2,2',2",4-Br_4-DiPhOBz and its methoxy- (MeO-) and OH-containing analogues with T4 on human TH transport proteins TTR and ALB.

Materials and Methods:

The test substances 2,2',2",4-tetrabromodiphenoxybenzene (BDPB-402), 4"-OH-2,2',2",4-tetrabromo-diphenoxybenzene (HBDPB-401) and 4"-methoxy-2,2',2",4-tetrabromodiphenoxybenzene (MOBDPB-401) were all synthesized at and provided by AccuStandard Inc. (New Haven, CT, U.S.A.). Recently we optimized, validated, and fully described the competitive *in vitro* protein binding assay that was presently used to investigate

thyroidogenicity of chemicals via interaction between T4 and human TTR and ALB.[12] Briefly, solutions of the proteins, THs and competitive ligands to be used in the *in vitro* assay were prepared. The positive control substrate was 4-OH-2,2',4,5'-Br_4-diphenyl ether (4-OH-BDE-49), which is often detected in human serum[13] and has previously been found to bind to human TTR isolated from human plasma with higher affinity than T4 *in vitro*.[5] Negative controls consisted of the same components except DMSO was used in place of a competitor ligand. All assays were done in triplicate and conducted twice on separate days to include inter-and intra-day replicates (n=6 total). Inter-laboratory reproducibility of this *in vitro* assay was confirmed by comparing T4-TTR calibration data produced in our National Wildlife Research Centre (Ottawa, ON, Canada) laboratory to those produced in the laboratory of Dr. Timo Hamers (Institute for Environmental Studies, Free University Amsterdam, Amsterdam, The Netherlands).[12]

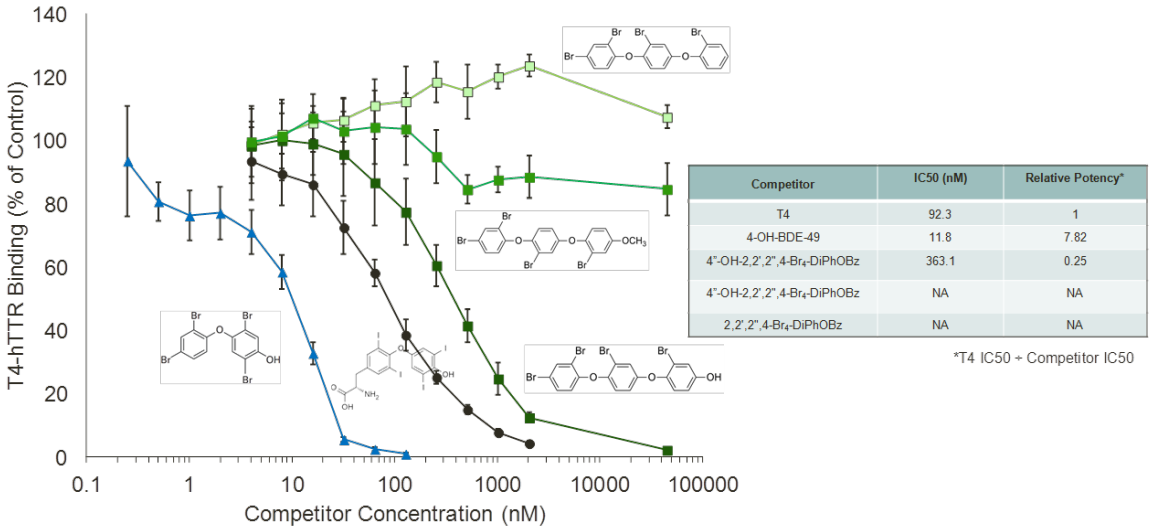
The percent of protein binding for each sample was determined by dividing the radioactivity of eluate by the initial radioactivity measured, minus any transfer loss (the incubation tube and pipette tip measurement). Results were expressed with the logarithmic competitor concentration on the X-axis, and mean ± standard deviation of percent binding compared to controls on the Y-axis. IC50 estimates were generated in GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA), using the one site-fit logIC50 equation: Y=Bottom + (Top-Bottom)/ (1+10^(X-LogIC50)).

Results and Discussion:

As mentioned, TeDB-DiPhOBz has been hypothesized to be the precursor of MeO-PB-DiPhOBz contaminants reported in herring gulls and their eggs from the Laurentian Great Lakes of North America.[9,10] Irradiating TeDB-DiPhOBz in solution or as a solid with natural sunlight or UV results in step-wise debromination that produces polybrominated diphenoxybenzenes (PB-DiPhOBzs) and including Br_4-Br_6-DiPhOBzs.[11] *In vitro* metabolism of these $Br_{4–6}$-PB-DiPhOBzs using harvested wild herring gull and adult male Wister-Han rat liver microsomal assays, resulted in the formation of OH-Br_{4-5}-DiPhOBz metabolites, and two were confirmed to be 4″-OH-2,2′,2″,4-Br_4-DiPhOBz and 4″-OH-2,2″,3′,4-Br_4-DiPhOBz.[11] Thus, it is possible that such OH-PB-DiPhOBz metabolites formed in animals and humans exposed to PB-DiPhOBzs, are thyroidogenic and compete for THs for TH transport proteins such as TTR. To our knowledge, this is the first study to examine the TH-related activity PB-DiPhOBzs and metabolites.

As shown in Figure 1, neither 2,2′,2″,4-Br_4-DiPhOBz nor 4″-MeO-2,2′,2″,4-Br_4-DiPhOBz (up to concentrations of 50,000 nM (or 50 μM)) were effective competitors with T4 for human TTR binding, as the IC50 and relative potency could not be determined. However, 4″-OH-2,2′,2″,4-Br_4-DiPhOBz was a potent ligand for TTR binding with an IC50 of 363.1 nM and relative potency to T4 of 0.25 (Figure 1). The positive control 4-OH-BDE-49 (also containing four bromine atoms) had an IC50 of 11.8 nM and with a relative potency of 7.82, and thus was a more potent than T4 for TTR binding. This result showed that the presence of third phenyl ring does not prevent *para*-OH-PB-DiPhOBzs from strongly competing with T4 for TTR.

The competition of 4″-OH-2,2′,2″,4-Br_4-DiPhOBz with T4 for binding with human ALB was less than what was found for TTR. The IC50 of 4″-OH-2,2′,2″,4-Br_4-DiPhOBz for ALB was 144.2 nM and the relative potency (to T4) was 0.020. The binding potencies for 4″-OH-2,2′,2″,4-Br_4-DiPhOBz and other OH-PB-DiPhOBzs are likely to be similar for other vertebrates (e.g. birds). These results emphasize the importance of elucidating the large data gaps that exist for PB-DiPhOBzs, OH-PB-DiPhOBzs and MeO-PB-DiPhOBzs with respect to sources, environmental and biological pathways and toxicological (endocrine) effects.



Competitor	IC50 (nM)	Relative Potency*
T4	92.3	1
4-OH-BDE-49	11.8	7.82
4"-OH-2,2',2",4-Br$_4$-DiPhOBz	363.1	0.25
4"-OH-2,2',2",4-Br$_4$-DiPhOBz	NA	NA
2,2',2",4-Br$_4$-DiPhOBz	NA	NA

*T4 IC50 ÷ Competitor IC50

Figure 1. Competitive ligand binding curves for T4-TTR interactions with T4, positive control competitor ligand 4-OH-BDE-49, or 2,2',2",4-Br$_4$-DiPhOBz, 4"-OH-2,2',2",4-Br$_4$-DiPhOBz and 4"-MeO-2,2',2",4-Br$_4$-DiPhOBz. Results are presented as means ± standard deviations, with 6 replicates for each competitor assay.

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