COMPETITVE BINDING OF THYROXINE TO HUMAN THYROXINE-BINDING GLOBULIN BY BROMINATED FLAME RETARDANTS, METABOLITES AND HALOGENATED PHENOLICS

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Introduction

Thyroid hormones (THs) are essential for normal physical and mental development in humans. THs are produced in the thyroid gland, primarily as the pro-hormone thyroxine (T4) and the biologically active 3,3',5'-triiodothyronine (T3). Delivery of THs to the cells throughout the body is necessary for maintaining TH homeostasis. Only a small fraction (<1%) of circulating THs are "free" or unbound, and the vast majority of circulating THs are bound to plasma proteins. The plasma proteins include serum albumin, transthyretin (TTR) and thyroxine-binding globulin (TBG). TBG has the highest affinity for T4 and binds approximately 75% of serum T4. In contrast, TTR and serum albumin bind 20% and 5% of circulating T4, respectively. TBG is a 54 kDa protein that contains 1 T4 binding site per molecule.

It is well known that halogenated phenolic compounds may disrupt TH normal homeostasis. Presumably this is due to their structural similarity to the endogenous THs. For example, *in vitro* experiments with human liver microsomes have shown that several classes of halogenated phenolic compounds inhibit deiodinase enzyme activity [1]. Further, previous studies have shown that hydroxylated polychlorinated biphenyls (OH-PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and several halogenated phenols inhibit *in vitro* 3,3'-T2 sulfotransferase activity [2, 3]. In addition, it has been shown that some halogenated phenolics can competitively bind with the TBG and TTR transport proteins, thus displacing T4. Whereas, the competitive binding to TTR is fairly well studied, there is comparatively less known about the potential for halogenated phenolics to competitively bind to TBG. For example, Lans et al. [4] examined TTR and TBG competitive binding by OH-PCBs, PCDDs and PCDFs using an *in vitro* assay. Cao et al. [5] used a fluorescence displacement assay to investigate the competitive binding of hydroxylated polybrominated diphenyl esters (OH-PBDEs) to TTR and TBG. Finally, Marchesini et al. [6] examined the TBG and TTR competitive binding using a T4-coated biosensor chip. These authors tested a suite of 62 halogenated and non-halogenated phenols, including several brominated flame retardants and their metabolites (i.e. OH-PBDEs).

The objective of this study was to utilize *in vitro* assay techniques to investigate the competitive binding of T4 to TBG, by several halogenated phenolic compounds. Previously developed radioisotope methods[7] were modified such that the ^{13}C stable-isotope T4 compound was analyzed by LC-MS/MS. We used human TBG since TBG is the primary transporter of THs in humans. We tested several brominated flame retardants and their metabolites as potential TBG competitive binders, and structure-activity relationships were explored using several fluorinated, chlorinated and iodinated analogues.

Materials and methods

The TBG competitive binding assays were adapted from methods previously described by Lans et al.[7]. The primary differences in our methods were the use of $^{13}C_{12}$-T4 (Isotec, Miamisburg, OH) as the substrate and instrumental analysis by LC-MS/MS. Briefly, human TBG (Calbiochem, EMD Chemicals, Gibbstown, NJ) was diluted to 50 nM (0.1 M Tris-HCl buffer, 0.1 M NaCl and 1 mM EDTA, pH 8.0) and combined with 90 nM $^{13}C_{12}$-T4. The incubation mixture (195 µl) was spiked with the competitors (5 µl), at varying concentrations, in 1.5 ml microcentrifuge tubes and incubated overnight, in darkness, at 4°C. Stock solutions of the competitors were prepared in DMSO. Competitors included tetrabromobisphenol A (TBBPA), 2,4,6-tribromophenol (2,4,6-TBP) and triclosan. T4 was used as the reference compound. Control samples consisted of clean DMSO. All treatments were performed in triplicate. After overnight incubation, TGB-bound $^{13}C_{12}$-T4 was separated from "free" $^{13}C_{12}$-T4 using mini-size exclusion chromatography columns as previously described[7]. Briefly, the samples were loaded onto 1ml gel columns (Bio-Gel P-6DG, Bio-Rad, Hercules, CA), spun for 1 min at 100g

(centrifuge pre-cooled to 4°C), eluted with 200 µl of Tris buffer and spun again for 1 min at 100g. The TGB-bound $^{13}C_{12}$-T4 was eluted in the first two fractions, whereas the "free" $^{13}C_{12}$-T4 was retained on the column. Samples were spiked with internal standard ($^{13}C_6$-T4, Cambridge Isotope Laboratories, Andover, MA) and the TGB-bound $^{13}C_{12}$-T4 was analyzed by LC-MS/MS using methods previously developed by our laboratory [1]. Analyte responses were normalized to the response of the internal standard. Competitive binding ratios were calculated as the percent TGB-bound $^{13}C_{12}$-T4 relative to the controls. Concentrations that resulted in $^{13}C_{12}$-T4 binding inhibition by 50% (IC_{50} values) were calculated using the Regression Wizard in SigmaPlot (v. 12.0, Systat Software Inc., Chicago, IL) assuming a "one site competition" model. Relative potency was assessed by calculating the ratio of the T4 IC_{50} value to that of the competitor. Relative potency ratios <1 represent less potent TBG binders as compared to T4.

Results and discussion:

All three competitors tested (TBBPA, 2,4,6-TBP and triclosan) showed the ability to competitively bind to TBG in a dose-response manner (Figure 1). These results indicate that the tested halogenated phenolic compounds can displace $^{13}C_{12}$-T4, and by extension presumably can also displace the endogenous circulating T4 from TBG in the body. Calculated IC_{50} values were 0.22 µM, 20 µM and 149 µM for T4, TBBPA and triclosan, respectively. Competitive binding for 2,4,6-TBP was only observed at the highest dose and regression parameters could not be calculated by SigmaPlot. The corresponding relative potency values for TBBPA and triclosan were 0.01 and 0.001, respectively. These trends indicate that TBBPA and triclosan are much less potent TBG binders, by approximately 2- and 3-orders of magnitude, respectively.

Lans et al. [4] showed that only very high concentrations of some OH-PCBs resulted in competitive binding to TBG. Further, PCDDs PCDFs did not show competitive binding TBG. In contrast, it was shown that several OH-PCBs had TTR binding potencies greater than T4. Using a T4-coated biosensor chip, Marchesini et al. [6] showed that 2,4,6-TBP and TBBPA were "non-binders", but triclosan was a "moderate binder" with a relative potency of 0.2. In contrast, our results showed that TBBPA was a more potent TBG binder as compared to triclosan.

The present study demonstrated that two brominated flame retardants as well as the anti-microbial, triclosan, can compete with T4 for binding to TBG. This study provides further evidence that some halogenated phenolics can competitively bind with TBG and thus may impact the circulation of THs, particularly T4, throughout the human body. Therefore, this study contributes to the growing body of literature demonstrating that halogenated phenolics may disrupt TH homeostasis. Future work will examine additional halogenated competitors including fluorinated, chlorinated and iodinated analogues of bisphenol A and trihalogenated phenol.

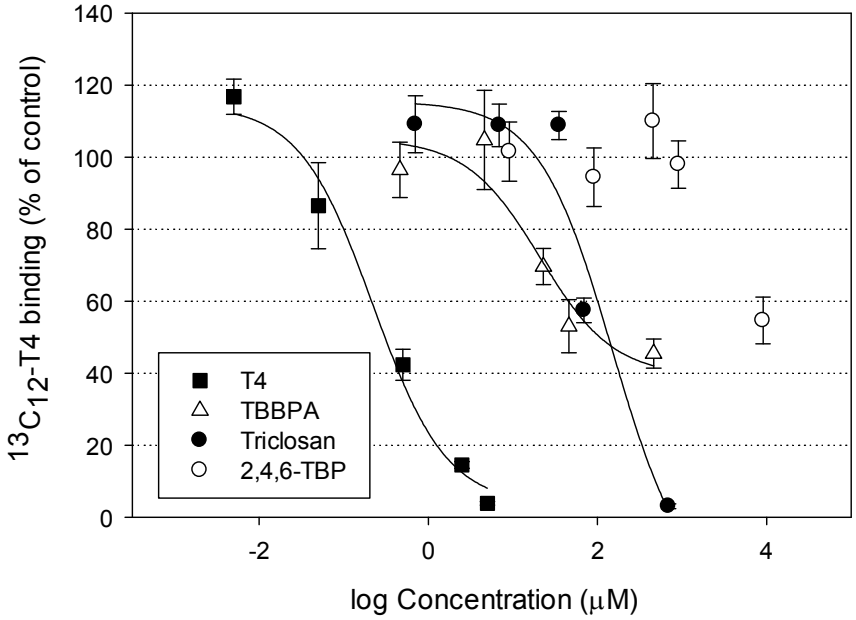

Figure 1. $^{13}C_{12}$-T4 binding (expressed as percent relative to control) to human TBG for T4, TBBPA, Triclosan and 2,4,6-TBP. Curves drawn using the Regression Wizard in SigmaPlot. Regression parameters could not be calculated for 2,4,6-TBP. Error bars represent the standard error of 3 replicates.

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