INHIBITION OF THYROID HORMONE SULFOTRANSFERASES BY BROMINATED FLAME RETARDANTS AND HALOGENATED PHENOLICS

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Introduction

The maintenance of thyroid hormone (TH) homeostasis is complex, but critical for normal physical and mental development. The thyroid gland primarily secretes thyroxine (3,5,3',5'-tetraiodothyronine, T4), a prohormone that is known to have negligible biological activity. The biologically active hormone is 3,5,5'-triiodothyronine (T3) which is formed through the reductive deiodination of the T4 phenolic ring ("outer-ring" deiodination, ORD) via deiodinase enzymes (DI). Deiodination can also occur on the tyrosyl ring ("inner-ring" deiodination, IRD) resulting in the formation of 3,3',5'-triiodothyronine (rT3). Both T3 and rT3 are further deiodinated, via IRD and ORD respectively, to yield 3,3'-diiodothyronine (3,3'-T2). The rT3 and 3,3'-T2 are thought to be biologically inactive. In addition to deiodination, THs undergo phase II metabolism via conjugation of the hydroxyl group with glucuronic acid or sulfate. Interestingly, the sulfation of some THs increases their deiodination efficiency as compared to non-sulfated analogues [1].

Previous work from our group has shown, through *in vitro* experiments with human liver microsomes, that several classes of halogenated phenolic compounds inhibit deiodinase enzyme activity [2]. Specifically, we found that triclosan, trihalogenated phenols, halogenated analogues of bisphenol A and hydroxylated polybrominated diphenyl ethers (PBDEs) inhibited the deiodination of T4 to T3, rT3 and 3,3'-T2. Structure-activity relationships generally showed increasing inhibition potency with increasing halogen size (i.e. greater similarity to the endogenous TH). Similarly, previous work by Schuur et al. [3, 4] and Visser et al. [5] 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. Further, Szabo et al. [6] showed increased Sult1b1 mRNA expression in male rat pups that were maternally exposed to a PentaBDE commercial mixture.

The objective of this study was to further expand our knowledge of TH sulfotransferase inhibition by halogenated phenolic compounds using *in vitro* assays. We used 3,3'-T2 as the substrate since it has been shown that this compound is the most efficient TH compound for sulfation [5]. Our model system was human liver cytosol to allow comparison to our previous DI inhibition experiments. We tested several brominated flame retardants and their metabolites as potential TH sulfation competitors. Further, we explored structure-activity relationships by investigating TH sulfation inhibition by fluorinated, chlorinated and iodinated analogues. A novel aspect of this work was the analysis of THs, and their sulfated metabolites, by LC-MS/MS.

Materials and methods

Sulfation inhibition assays were modified from methods described by Visser et al. [5]. The primary modification in our methods was clean-up by solid phase extraction (SPE) and the use of LC-MS/MS for thyroid hormones and sulfated metabolite analysis. Briefly, cytosol was isolated from pooled human liver S9 (Invitrogen, Carlsbad, California) by centrifuging at 100,000g (4^{o}C) for 1 hour and collecting the supernatant. Protein content was determined using the Bradford assay. Cytosol was diluted to 0.25 mg protein/ml in 0.1 M potassium phosphate buffer (pH 7.2) with 50 μM 3'-phosphoadenosine-5'-phosphate (PAPS) as a cofactor with 1 μM 3,3'-T2 as the substrate (total volume = 200 μl). Stock solutions of competitors were prepared in acetone and were added such that solvent volume was 0.5% of the incubation volume. "Active" control samples were prepared by spiking with clean acetone. Buffer blanks were prepared by incubating without the addition of cytosol. Substrate deiodination was not observed in buffer blanks, nor was the production of the 3,3'-T2 sulfate conjugate (3,3'-T2S); thus blank correction was not necessary. Vials were incubated at 37^{o}C for 30 min in a shaking water bath, reactions were stopped by the addition of 0.1 M HCl (800 μl) and spiked with $^{13}C_6$-3,3'-T2 as the internal standard. Extracts were cleaned using SampliQ OPT SPE cartridges (Agilent Technologies).

Analysis was performed by LC-MS/MS using similar techniques as described by Butt et al. [2]. Monitored analytes included 3,3'-T2, 3,3'-T2S and 3'-monoiodothyronine (3'-T1). MS/MS parameters for 3,3'-T2 and 3'-T1 were optimized using authentic standards, whereas 3,3'-T2S parameters were adapted from those obtained from the T3 sulfate conjugate which was synthesized in our lab. All analyte responses were normalized to the response of the $^{13}C_6$-3,3'-T2. Sulfotransferase inhibition was calculated by comparing the relative response of the 3,3'-T2S in the dosed treatments to that of the "active" control (acetone only).

Results and discussion:
In the absence of competitors, the 3,3'-T2 was readily sulfated. No deiodination was observed, presumably because the assays were not optimized for DI activity (i.e. no dithiothreitol or NADPH). Measured as a loss of 3,3'-T2, the sulfation was 65% (standard deviation of 3 replicates = 3.5%) and 33% (8.1%) with 0.25 mg protein/ml and 0.10 mg protein/ml, respectively. If we assume all T2 lost was converted to the 3,3'-T2S, the sulfation activity was 1.0 and 1.3 pmol/mg protein.hr for the 0.25 mg protein/ml and 0.10 mg protein/ml treatments, respectively. Using similar experimental conditions (0.10 mg protein/ml), Visser et al. [5] observed a sulfation of ~60% (estimated from figure in original paper) for 3,3'-T2. The lower sulfation percentage in the current study may be because our cytosol was derived from a pool of multiple donors and thus may reflect inter-individual variability.

We investigated tetrabromophenol A (TBBPA), 2,4,6-tribromophenol (2,4,6-TBP), triclosan, 5'OH BDE 99 and 6'OH BDE 99 as potential inhibitors of 3,3'-T2 sulfotransferase activity. Two doses per inhibitor were tested as a range finding exercise. The results showed nearly complete inhibition of 3,3'-T2 sulfotransferase activity for the tested doses of TBBPA, 2,4,6-TBP and triclosan. The hydroxylated PBDE metabolites (5'OH BDE 99 and 6'OH BDE 99) showed 3,3'-T2 sulfation inhibition of ~20-40%. The results suggest that 6'OH BDE 99 may be a more potent inhibitor than 5'OH BDE 99, although this is unclear considering the relative large uncertainty. It should be noted that the dosing between inhibitors were at different concentrations and thus are not directly comparable. The previous work by Visser et al. [5] also showed that 2,4,6-TBP was a potent inhibitor of TH sulfation activity, however, to our knowledge, none of the other competitors have previously been tested. The inhibition of sulfotransferase activity has potential in vivo consequences with regards to TH metabolism. Sulfated conjugated are more polar and more readily excreted from the cell, reducing cellular TH levels. Further, sulfated analogues of some THs are more efficiently deiodinated and thus could be more readily converted to a deactivated or activated form.

The present study demonstrated that several brominated flame retardants and the hydroxylated metabolites of PBDEs (as well as the anti-microbial, triclosan) can inhibit TH sulfation. This potentially may impact TH metabolism and thus may influence circulating TH levels in the body. 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.

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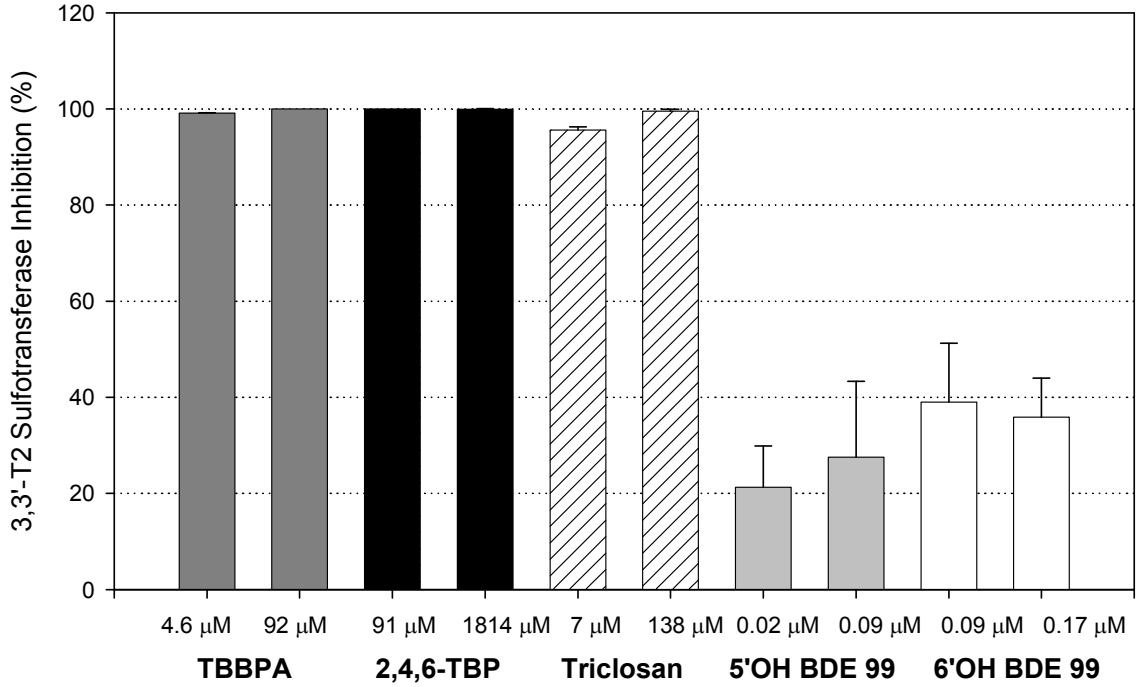

Figure 1. 3,3'-T2 sulfotransferase inhibition using tetrabromobisphenol A (TBBPA), 2,4,6-tribromophenol (2,4,6-TBP), triclosan, 5'OH BDE 99 and 6'OH BDE 99. Mean (n=3) and standard deviation of 3 replicates.

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