1 **Effect of triclosan, triclocarban, 2,2',4,4'-tetrabromodiphenyl ether, and bisphenol A**
2 **on the iodide uptake, thyroid peroxidase activity, and expression of genes involved in**
3 **thyroid hormone synthesis**
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Abstract

Triclosan, triclocarban, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), and bisphenol A (BPA) have been reported to disturb thyroid hormone (TH) homeostasis. We have examined the effects of these chemicals on sodium/iodide symporter (NIS)-mediated iodide uptake and the expression of genes involved in TH synthesis in rat thyroid follicular FRTL-5 cells, and on the activity of thyroid peroxidase (TPO) using rat thyroid microsomes. All four chemicals inhibited NIS-mediated iodide uptake in a concentration-dependent manner. A decrease in the iodide uptake was also observed in the absence of sodium iodide. Kinetic studies showed that all four chemicals were non-competitive inhibitors of NIS, with the order of K_i values being triclosan < triclocarban < BDE-47 < BPA. The transcriptional expression of three genes involved in TH synthesis, *Slc5a5*, *Tpo*, and *Tgo*, and three thyroid transcription factor genes, *Pax8*, *Foxe1*, and *Nkx2-1*, was examined using quantitative real-time PCR. No significant changes in the expression of any genes were observed with triclosan or triclocarban. BDE-47 decreased the level of *Tpo*, while BPA altered the expression of all six genes. Triclosan and triclocarban inhibited the activity of TPO at 166 and > 300 μ M, respectively. Neither BDE-47 nor BPA affected TPO activity. In conclusion, triclosan, triclocarban, BDE-47, and BPA inhibited iodide uptake, but had differential effects on the expression of TH synthesis-related genes and the activity of TPO.

Keywords

Thyroid disruptors; iodide uptake; thyroid hormone synthesis; thyroid peroxidase

Introduction

Thyroid hormones (THs) regulate a number of physiological processes and are essential for the metabolism of proteins, fats, and carbohydrates, brain development, and body growth (Mullur *et al.*, 2014). The maintenance of TH homeostasis and the proper execution of the biological functions of THs are critical for normal physical and mental development.

Extensive research has identified a broad range of chemicals that disrupt thyroid function (Boas *et al.*, 2006; Patrick, 2009). For instance, triclosan, triclocarban, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), and bisphenol A (BPA) have been reported to be thyroid disruptors (Boas *et al.*, 2006; Patrick, 2009). The fact that these four chemicals are commonly used and widely distributed in the environment warrants a deeper understanding of their thyroid disrupting effects and the underlying mechanisms.

Triclosan and triclocarban are two broad-spectrum anti-bacterial agents widely used in a variety of personal care products (Fang *et al.*, 2010; Witorsch and Thomas, 2010). There is increasing evidence that triclosan disturbs the thyroid system in different experimental organisms. For instance, triclosan has been shown to alter postembryonic development in frogs (Marlatt *et al.*, 2013) and to interfere with the thyroid axis in zebrafish (Pinto *et al.*, 2013). A significant decrease in serum thyroxine (T4) levels was observed in rats orally exposed to triclosan at ≥ 100 mg/kg body weight for 4 days (Crofton *et al.*, 2007; Paul *et al.*, 2010) and in mice dermally administered triclosan at ≥ 27 mg/kg body weight for 90 days (Fang *et al.*, 2015). Perinatal exposure to 300 mg triclosan/kg body weight decreased both maternal and neonatal serum T4 levels in rats (Paul *et al.*, 2012). Triclocarban has also been reported to alter the expression levels of TH responsive genes in rat pituitary cells (Hinther *et al.*, 2011).

Polybrominated diphenyl ethers (PBDEs) are used commercially as fire retardants in a variety of consumer products. Many PBDE congeners have been detected in environmental samples and human tissues, with the predominant form being BDE-47 (Hites, 2004). A number of studies have demonstrated that short-term oral exposure to BDE-47 or DE-71, a commercial PBDE mixture containing BDE-47, at ≥ 10 mg/kg body weight decreased serum T4 levels in

1 rats and mice (Hallgren *et al.*, 2001; Richardson *et al.*, 2008; Zhou *et al.*, 2001). Exposing
2 rats to BDE-47 or DE-71 during pregnancy and lactation also significantly reduced T4 levels
3 in their offspring (Ellis-Hutchings *et al.*, 2006; Szabo *et al.*, 2009).
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8 BPA is a high-production-volume chemical that is widely used as a monomer in the
9 production of polycarbonate plastics and epoxy resins (Rubin, 2011). Perinatal exposure to
10 BPA impaired the learning and memory ability of male offspring in mice and rats (Xu *et al.*,
11 2010; Xu *et al.*, 2007). Concomitantly, rat pups exhibited mild hyperthyroidism, as
12 evidenced by an increase in the circulating concentration of T3 or T4 (Delclos *et al.*, 2014;
13 Xu *et al.*, 2007; Zoeller *et al.*, 2005). In addition, there is evidence that BPA acts as an
14 antagonist of the human TH receptor and suppresses TH receptor-mediated transcription of
15 target genes *in vitro* (Moriyama *et al.*, 2002; Sheng *et al.*, 2012; Zoeller *et al.*, 2005).
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27 Thyroid disruptors may act through several pathways, including altering TH synthesis,
28 metabolism, and/or transport (Boas *et al.*, 2006; Patrick, 2009). Efforts have been made to
29 understand the mechanisms by which these chemicals interfere with the thyroid system in
30 animals, with most attention being paid to TH metabolism and transport. The
31 glucuronidation of TH, which is catalyzed by UDP glucuronosyltransferases, is one of the
32 major metabolic pathways of TH (Wu *et al.*, 2005). Triclosan, BDE-47, and PBDE mixtures
33 have been reported to induce hepatic microsomal T4-glucuronidation activity and elevate the
34 expression levels of UDP glucuronosyltransferases, which are believed to be responsible for
35 their T4 decreasing effects in rodents (Paul *et al.*, 2010; Richardson *et al.*, 2008; Szabo *et al.*,
36 2009; Zhou *et al.*, 2001). Hydroxylated metabolites of BDE-47 have also been shown to
37 compete with T4 for binding to the TH transport protein transthyretin and T4 binding
38 globulin (Marchesini *et al.*, 2008; Meerts *et al.*, 2000). The hyperthyroidism induced by
39 BPA in rodents has been hypothesized to result from the antagonist effect of BPA on the TH
40 receptor, which leads to the loss of negative feedback regulating TH synthesis (Zoeller *et al.*,
41 2005).
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60 Previous studies with triclosan, triclocarban, BDE-47, and BPA have focused mainly on the
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1 metabolism and transport of TH; however, the effects of these chemicals on TH synthesis
2 remain largely unknown. TH is produced by the follicular cells of the thyroid gland.
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4 Iodide uptake is a key step during TH synthesis. Specifically, iodide is actively transported
5 into thyroid follicular cells via the sodium/iodide symporter (NIS, encoded by the gene
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8 *SLC5A5*), translocated to the follicle colloid through chloride/iodide transporter pendrin,
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10 oxidized by thyroid peroxidase (TPO), and covalently bound to the tyrosine residues of
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12 thyroglobulin (TG). TSH produced by the pituitary gland binds to the TSH receptor in the
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14 thyroid follicular cells and stimulates endocytosis. The T4 is released from the
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16 thyroglobulin by proteases in lysosomes (Vitale *et al.*, 2013).
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21 Two critical molecules, NIS and TPO, involved in TH synthesis are targets of many thyroid
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23 disruptors. For instance, anions like perchlorate and thiocyanate are well-known competitive
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25 inhibitors of human NIS (Tonacchera *et al.*, 2004). Genistein, one of the major isoflavones in
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27 soybeans, inactivates rat TPO activity both *in vitro* and *in vivo* (Doerge and Chang, 2002);
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29 however, there is a lack of data regarding the effects of triclosan, triclocarban, BDE-47, and
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31 BPA on TH synthesis. In this study, we evaluated the influence of these chemicals on iodide
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33 uptake and the expression of TH synthesis-related genes in FRTL-5 cells, a well-
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35 characterized and widely used rat thyroid follicular cell line. In addition, the effects on TPO
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37 activity were assessed using rat thyroid microsomes.
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44 **Materials and Methods**

45 **Chemicals and reagents**

46 Triclosan [5-chloro-2-(2, 4-dichlorophenoxy) phenol, purity 99%] was obtained from Alfa
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48 Aesar (Ward Hill, MA). BDE-47 (purity 99.5%) was purchased from Chem Service (West
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50 Chester, PA). Triclocarban [3,4,4'-trichlorocarbanilide, purity 99%], BPA (purity $\geq$ 99%),
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52 Coon's modified Ham's F12 medium, insulin, hydrocortisone, Gly-His-Lys acetate,
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54 somatostatin, transferrin, TSH, ammonium cerium(IV) sulfate dihydrate, arsenic(III) oxide,
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56 sodium iodide (NaI), sodium perchlorate (NaClO₄, purity $\geq$ 98%), potassium
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58 hexafluorophosphate (KPF₆, purity $\geq$ 98%), dimethyl sulfoxide (DMSO), thiazolyl blue
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tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO).

Penicillin-streptomycin solutions and 2.5% trypsin were purchased from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS) was acquired from Atlanta Biologicals (Lawrenceville, GA).

Cell culture

Rat thyroid follicular FRTL-5 cells were obtained from Sigma-Aldrich and cultured in Coon's modified Ham's F12 medium supplemented with 10 µg/ml insulin, 10 nM hydrocortisone, 10 ng/ml Gly-His-Lys acetate, 10 ng/ml somatostatin, 5 µg/ml transferrin, 10 mU/ml TSH, 5% FBS, and penicillin-streptomycin solution. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂.

Iodide uptake assay

Iodide uptake was determined using a non-radioactive Sandell-Kolthoff reaction-based colorimetric assay as described by Waltz et al. (Waltz et al., 2010) with minor modifications. FRTL-5 cells were treated in two ways. To determine the short-term effects on iodide uptake, FRTL-5 cells cultured in clear 96-well plates were washed with iodide uptake buffer (Hank's balanced salt solution supplemented with 10 mM HEPES) and then incubated with various concentrations of the test chemicals in the presence of 10 µM NaI for 1 h or 5 – 40 µM NaI for 5 min. A series of chemical solutions with concentrations that were 100X of the final concentrations in the iodide uptake assay were prepared in DMSO (triclosan, triclocarban, BDE-47, and BPA) or water (NaClO₄ and KPF₆), and stored at -20°C. For each well of the assay plate, 1 µl of the 100X chemical solution was diluted in 89 µl of iodide uptake buffer and was added to the assay plate using a 12-channel reservoir (USA Scientific, Orlando, FL). Immediately after that, 10 µl of NaI (50 – 400 µM) was added to each well using a 12-channel reservoir. To determine the long-term effects on iodide uptake, FRTL-5 cells were treated with various concentrations of the test chemicals for 24 or 48 h. At the end of the treatment, the culture medium containing the test chemicals was removed, and the cells were washed with uptake buffer and then incubated with 10 µM NaI for 1 h. After the NaI incubation, cells were washed twice with ice cold uptake buffer and lysed in 100 µl water. A

100 µl aliquot of 10.5 mM ammonium cerium (IV) sulfate was added to each well, followed by the addition of 100 µl of 24 mM sodium arsenite (III). The plates were left to stand at room temperature for 10 min, and the absorbance was then determined at 420 nm (A_{420}) using a BioTek Synergy 2 Plate Reader (BioTek, Winooski, VT). A set of wells containing known concentrations of NaI was included. The logarithm of A_{420} was plotted against the iodide content, and linear regression analysis was performed to obtain a standard curve.

Cytotoxicity determination

The effects of triclosan, triclocarban, BDE-47, and BPA on the growth of FRTL-5 cells were assessed using an MTT assay. Briefly, cells were plated into clear 96-well plates at cell densities of 5×10^4 cells/well for 48 h incubations and 3×10^4 cells/well for 72 h incubations. The cells were allowed to attach to the bottom of the wells for 24 h before being treated with 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, and 300 µM test chemical for 24 or 48 h. Control cells were fed with complete culture medium containing 0.1% (v/v) DMSO, which had no effect on cell growth. At the end of the treatment, MTT assays were performed as previously described (Wu *et al.*, 2015).

RNA isolation and real-time PCR

FRTL-5 cells were treated with various concentrations of triclosan, triclocarban, BDE-47, or BPA for 6, 24, or 48 h. At the end of the treatment, total RNA was isolated from the FRTL-5 cells using an RNeasy Mini kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using a SuperScript III first-strand synthesis system (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) on a Bio-Rad CFX96 real-time PCR system. Pre-designed primers targeting rat *Slc5a5* (encodes NIS, QT00183995), *Tpo* (encodes TPO, QT00194327), and *Tg* (encodes thyroglobulin, QT00182959), thyroid transcription factor genes *Pax8* (QT00182945), *Foxe1* (QT00395346), and *Nkx2-1* (QT01599752), and the endogenous control *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*, QT00199633) were obtained from Qiagen. All samples were run in triplicate. Data were normalized for *Gapdh* and analyzed using the $\Delta\Delta C_t$

method.

Isolation of rat thyroid microsomes

Rat thyroid microsomes were isolated as described by Paul *et al.* with minor modifications (Paul *et al.*, 2014). Briefly, frozen thyroid glands, pooled from 40 normal healthy Wistar male rats, were homogenized in 2 ml of ice-cold buffer (5 mM potassium phosphate, 200 mM sucrose, 1 mM EDTA, and 500 U/ml catalase) per 10 mg of thyroid tissue. The homogenate was centrifuged at 800 g for 10 min at 4 °C to remove large debris and then ultracentrifuged at 100,000 g for 60 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 250 µl of homogenization buffer (without catalase) per 10 mg of thyroid tissue. Glycerol (5%) was added to the final microsomal preparation, and aliquots were stored at –80 °C. The protein concentration of the microsomal preparation was determined using a BCA Protein Assay kit (Thermo Scientific, Boston, MA).

TPO activity assay

The activity of TPO in rat thyroid microsomes was determined using Amplex UltraRed (AUR, Life Technologies) as described by Paul *et al.* with some modifications (Paul *et al.*, 2014). The assay system contained 200 mM potassium phosphate buffer, 12.5 µg rat thyroid microsomal protein, 25 µM AUR, 300 µM H₂O₂, and various concentrations of the test chemicals in a total volume of 200 µl. First, 150 µl of potassium phosphate buffer containing thyroid microsomal protein and AUR was added to each well of a black 96-well plate, followed by the addition of 2 µl of 100X test compound solutions (dissolved in DMSO). The reaction was initiated by adding 48 µl of potassium phosphate buffer containing the desired concentration of H₂O₂. The test plates were shaken on a BioTek Synergy 2 Plate Reader for 30 min, and the fluorescence was measured using 530/25 nm as the excitation filter and 590/35 nm as the emission filter.

Data analysis

Data are presented as the mean ± standard deviation of three independent experiments. Comparisons amongst concentrations were conducted by one-way analysis of variance, with

multiple comparisons versus the control group being performed by Dunnett's method. When necessary, the data were log transformed to maintain an equal variance or normal distribution. The results were considered significant at $P < 0.05$.

Results

Short-term effects of triclosan, triclocarban, BDE-47, and BPA on the iodide uptake in FRTL-5 cells

The effects of triclosan, triclocarban, BDE-47, and BPA on the iodide uptake in FRTL-5 cells were determined in two different manners. The first was to examine whether or not these chemicals affected sodium/iodide symporter (NIS)-mediated iodide uptake by interacting with NIS. This was achieved by performing competition experiments, in which FRTL-5 cells were co-incubated with the test chemicals and NaI, the substrate of NIS, for a short period (5 min – 1 h). Iodide uptake was determined using a non-radioactive assay, as described by Waltz *et al.* (Waltz *et al.*, 2010). Following incubation with 10 μ M NaI for 1 h, iodide uptake was readily detected in the FRTL-5 cells (approximate 50 pmol iodide per well). The IC_{50} values of $NaClO_4$ and KPF_6 , two well-known NIS inhibitors, were tested as positive control and gave values of 0.277 and 0.018 μ M, respectively (Fig. 1), which were in agreement with the values reported by Waltz *et al.* (2010). The highest concentration of $NaClO_4$ completely blocked iodide uptake in the FRTL-5 cells, suggesting that the observed iodide uptake was mainly mediated by NIS (Fig. 1). There was a concentration-dependent decrease in the iodide uptake by FRTL-5 cells when they were incubated with NaI in the presence of triclosan, triclocarban, BDE-47, or BPA. The inhibitory effects on iodide uptake were not believed due to cytotoxicity, because exposure to these concentrations of the test chemicals and NaI for such a short time (1 h) did not affect cell viability.

To examine further the kinetics of these NIS inhibitors, the non-radioactive iodide uptake assay was validated for its application in kinetic studies. As shown in Fig. 2A, an inverse linear correlation was observed between the logarithm of A_{420} and the iodide content from 0 –

200 pmol. All the iodide levels obtained in the present study fell within this range. The time-dependent iodide uptake was examined by incubating FRTL-5 cells with 10 μ M NaI for 2 – 120 min. Iodide uptake in the FRTL-5 cells increased in a linear manner during the first 10 min, and tended to saturate after 30 min of incubation (Fig. 2B). Based on these data, the initial velocity of iodide uptake was determined by incubating FRTL-5 cells with 5 – 320 μ M NaI for 5 min (Fig. 2C). A Lineweaver-Burk double-reciprocal plot revealed that it followed the Michaelis-Menten equation (Fig. 2D). The apparent K_m for sodium iodide was 38.4 μ M (Fig. 2D), which is consistent with values previously reported in FRTL-5 cells (Kogai *et al.*, 2000; Kosugi *et al.*, 1996). These data demonstrated that the non-radioactive iodide uptake assay was appropriate for use in kinetic studies.

Next we examined the effect of triclosan, triclocarban, BDE-47, and BPA on the kinetics of iodide uptake in FRTL-5 cells. As shown in Fig. 3A, triclosan decreased the V_{max} in a concentration-dependent manner, whereas it had no significant effect on the K_m , indicating that triclosan inhibited NIS-mediated iodide uptake in a non-competitive manner. The slope of each line on the Lineweaver-Burk plot was plotted against the concentration of triclosan, which gave an inhibition constant (K_i) of 21.3 μ M for triclosan (Fig. 3E). Similar effects were observed with triclocarban, BDE-47, and BPA, all of which inhibited iodide uptake in a non-competitive manner (Fig. 3B – 4D), with the K_i values being 69.0, 77.8, and 112.3 μ M (Fig. 3F – 4H), respectively.

Long-term effects of triclosan, triclocarban, BDE-47, and BPA on the iodide uptake in FRTL-5 cells

The long-term effects of triclosan, triclocarban, BDE-47, and BPA on the iodide uptake in FRTL-5 cells were determined by exposing FRTL-5 cells to the test chemicals for 24 and 48 h in the absence of NaI. Iodide uptake was determined at the end of the treatment. To minimize the potential effect of cytotoxicity on the iodide uptake, an MTT assay was performed to monitor the viability of FRTL-5 cells following exposure to triclosan, triclocarban, BDE-47, and BPA. No significant decrease in viability was observed in cells exposed to triclosan at ≤ 10 μ M, triclocarban at ≤ 3 μ M, BDE-47 at ≤ 10 μ M, or BPA at $\leq$

30 μ M for up to 48 h (Fig. 4A). The highest tested concentrations in the long-term iodide uptake assay were selected in such a way to ensure that the cell viability was at least 70% throughout the experiment. As shown in Fig. 4B, there was a significant decrease in iodide uptake in FRTL-5 cells exposed to triclosan, triclocarban, BDE-47, or BPA, with the decrease occurring at non-cytotoxic concentrations.

Effects of triclosan, triclocarban, BDE-47, and BPA on the expression of genes involved in TH synthesis

The transcriptional expression levels of three genes involved in TH synthesis (*Slc5a5*, *Tpo*, and *Tg*) were examined in FRTL-5 cells exposed to the test chemicals for 6, 24, and 48 h. No significant changes in the expression of any genes were observed in cells exposed to triclosan or triclocarban (Fig. 5A). The levels of *Tpo* were reduced in cells exposed to 30 μ M of BDE-47 for 24 h or 48 h. Exposure to BPA down-regulated the expression of *Slc5a5* and *Tpo* only at the highest concentration of 100 μ M, while the expression of *Tg* was up-regulated in a concentration-dependent manner (Fig. 5A). A shorter exposure (6 h) did not cause any changes in the expression in any of the genes.

The transcriptional expression of *Slc5a5*, *Tpo*, and *Tg* is regulated by several thyroid transcription factors, including Pax8, FoxE1, and Nkx2-1, which are critical for thyroid function (Kimura, 2011). The effect of the test chemicals on the expression of these transcription factors was examined. Triclosan, triclocarban, and BDE-47 did not significantly affect the expression levels of *Pax8*, *Foxe1*, or *Nkx2-1* (Fig. 5B). In contrast, there was a concentration-dependent increase in the levels of *Pax8* and *Foxe1* in cells following exposure to BPA, with the increase being significant at the highest concentration of 100 μ M.

Effects of triclosan, triclocarban, BDE-47, and BPA on the activity of TPO

TPO is a protein critical for TH synthesis by catalyzing the oxidation of iodide and the addition of iodide to the tyrosine residues of thyroglobulin (Taurog *et al.*, 1996). A variety of compounds have been reported to target TPO and inhibit TH synthesis (Doerge and Chang,

2002; Paul *et al.*, 2014; Schmutzler *et al.*, 2007a). Triclosan, triclocarban, BDE-47, and BPA were evaluated for their effects on the activity of TPO in isolated rat thyroid microsomes. Triclosan inhibited TPO activity in a concentration-dependent manner, with the IC₅₀ value being 165.8 µM (Fig. 6). Although triclocarban also displayed a concentration-dependent inhibitory effect on TPO activity, the potency was rather low with the IC₅₀ value being > 300 µM. Neither BDE-47 nor BPA affected TPO activity (Fig. 6).

Discussion

Triclosan, triclocarban, BDE-47, and BPA are well-known endocrine disruptors. Although previous studies have reported the alteration of TH metabolism and transport, their effects on TH synthesis remain poorly understood. In this study we examined both the short- and long-term effects on iodide uptake and demonstrated that all four chemicals inhibited NIS-mediated iodide uptake in FRTL-5 cells. NIS is a key transmembrane glycoprotein that mediates transport of iodide into thyroid follicular cells. Anions, including perchlorate and thiocyanate, are well-known competitive inhibitors of human NIS (Tonacchera *et al.*, 2004), while hydrogen peroxide has been reported to inhibit NIS-mediated iodide uptake in a non-competitive manner (Fukayama *et al.*, 1991). In the current study, kinetics experiments showed that triclosan, triclocarban, BDE-47, and BPA were non-competitive inhibitors of NIS, with the K_i value of triclosan being the smallest. One common mechanism of non-competitive inhibition is the binding of the inhibitor to the enzyme at an allosteric site, which does not exclude the binding of native substrate at the active site but reduces the enzymatic activity. It is possible that the four chemicals tested here inhibited iodide uptake through a similar mechanism, but further studies are necessary regarding this matter because of the limited information of allosteric site of NIS.

NIS is regulated at both the transcriptional and post-translational levels, especially in relation to its subcellular localization (Kogai and Brent, 2012). Under physiological conditions, NIS is located at the basolateral membrane of the thyroid follicular cells, which is required for its functionality in mediating iodide uptake. Removal of TSH suppresses iodide uptake *via* both

down-regulating the transcriptional expression of *Slc5a5* and preventing NIS from translocating to plasma membrane (Riedel *et al.*, 2001). The observations that exposure to triclosan, triclocarban, BDE-47, and BPA inhibited NIS-mediated iodide uptake (Fig. 4B), but did not influence the transcriptional expression levels of *Slc5a5* (Fig. 5A) suggest that the four chemicals may affect the NIS at the post-translational level. Furthermore, when FRTL-5 cells were incubated with triclocarban, BDE-47, or BPA in the absence of NaI, inhibition of iodide uptake was observed at as low as 0.3, 10, and 10 μ M, respectively (Fig. 4B). These concentrations were much lower than the IC₅₀ values for iodide uptake (17.2, 41.5, and 91.7 μ M) when FRTL-5 cells were exposed to the test chemicals and NaI simultaneously (Fig. 1). These data suggest the possibility that the inhibition of iodide uptake by triclocarban, BDE-47, and BPA is mediated primarily through regulating NIS at the post-translational level. In contrast, triclosan exhibited inhibitory effect on the iodide uptake at comparable concentrations (6.6 versus 10 μ M), in the presence or absence of NaI. Thus, triclosan may acts as an inhibitor of iodide uptake *via* both binding to NIS and regulating NIS at the post-translational level. It is also likely that triclosan binds to NIS at an allosteric site, without effecting the post-translational regulation of NIS.

The expression levels of *Slc5a5*, *Tpo*, *Tg*, *Pax8*, *Foxe1*, and *Nkx2-1* were not affected by triclosan, triclocarban, or BDE-47, the only exception being the effect of 30 μ M BDE-47 on *Tpo*. However, exposure to BPA induced alterations in the levels of these genes. There was an increased trend in the levels of *Tg*, *Pax8*, and *Foxe1* in cells following BPA exposure, although the changes with regard to *Pax8* and *Foxe1* were only significant at 100 μ M BPA. Therefore, it is possible that the up-regulation of *Tg* by BPA was mediated through thyroid transcription factors Pax8 and FoxE1. The TPO inhibitor propylthiouracil has been shown to increase the expression level of *Slc5a5* and iodide uptake in FRTL-5 cells, whereas another TPO inhibitor methimazole did not exhibit a similar effect (Sue *et al.*, 2012). In addition, there were no significant changes in the levels of *Tpo*, *Tg*, *Nkx2-1*, *Foxe1*, and *Pax8* following exposure to either chemical. Soy extracts have been reported to inhibit iodide uptake, but increase the protein level of non-glycosylated NIS and a 40 kDa Tg fragment in FRTL-5 cells (Tran *et al.*, 2013). Previous reports and the data presented in this study

1 suggest that effects on iodide uptake, TPO activity, and the expression of thyroid specific
2 genes are independent, and each of the chemicals may have different effects on these
3 endpoints.
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8 Although triclosan and triclocarban inhibited the TPO activity in a concentration-dependent
9 manner, TPO seemed not a primary target of these two chemicals, because the IC₅₀ values
10 for TPO activity inhibition were much higher than those for iodide uptake. A low
11 inhibitory potency of triclosan on TPO activity has also been reported in the same assay in
12 a high-throughput screening mode, with the IC₅₀ value being 184 µM (Paul *et al.*, 2014),
13 which is comparable to the value (166 µM) shown in the current study. The performance
14 of four known TPO inhibitors, including [methimazole, propylthiouracil, 2-
15 mercaptobenzothiazole (MBT), and ethylene thiourea (ETU)] was also tested in the current
16 study, with their IC₅₀ values being 3.1, 27.9, 1.2, and > 300 µM, respectively (Suppl. Fig.
17 1). The potency of these chemicals was consistent with previous reports using
18 conventional assays (Freyberger and Ahr, 2006; Schmutzler *et al.*, 2007b; Tietge *et al.*,
19 2013; Vickers *et al.*, 2012), but lower than that reported by Paul *et al.* using a high-
20 throughput screening mode, which could due to the higher sensitivity of the assay
21 performed in a high-throughput screening mode (Paul *et al.*, 2014).
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40 Due to the extensive use of triclosan, the general population is exposed to triclosan through
41 the ingestion or dermal contact with daily hygiene products and the consumption of food
42 and drinking water contaminated with triclosan. The blood levels of total triclosan in
43 humans following use of either mouth rinses or dentifrices vary among different
44 individuals and can be as high as 1.4 µM (DeSalva *et al.*, 1989; Sandborgh-Englund *et al.*,
45 2006). We have previously found that mice dermally treated with 100 mg triclosan/kg
46 body weight have plasma levels of triclosan and its metabolites of approximately 150 µM
47 (Fang *et al.*, 2014), and the topical application of similar level of triclosan resulted in a
48 decrease in the serum T4 levels in mice (Fang *et al.*, 2015). In the present study, inhibition
49 of iodide uptake by triclosan was observed under concentrations that were comparable to
50 the plasma levels in mice and approaching human relevant levels. Nonetheless, triclosan is
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1 extensively metabolized in mice following dermal application, and the extent of its
2 metabolites varies among tissues and body fluids, with the parent compound being only 1%
3 in the plasma (Fang *et al.*, 2014). Since we did not examine the effects of triclosan
4 metabolites on the iodide uptake, caution should be exercised in extrapolating the findings
5 in this *in vitro* study to potential effects in humans. In addition, the test models used in the
6 current study were a rat-derived cell line and microsomes isolated from rat thyroid, and
7 there may be species difference in both the toxicokinetics of triclosan and the
8 pharmacokinetics of thyroid functions between humans and rats should be considered.
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11 Triclosan, BDE-47, and BPA have been demonstrated to disrupt TH homeostasis in rodents,
12 and epidemiological studies also suggest potential adverse effects in humans (Koeppe *et al.*,
13 2013; Stapleton *et al.*, 2011; Wang *et al.*, 2013). Although there is a lack of rodent studies
14 investigating the influence of triclocarban on the thyroid system, triclocarban has been
15 reported to alter the expression levels of TH responsive genes *in vitro*. Several studies
16 have reported that serum concentrations of triclocarban and BPA in humans are in the nM
17 range for triclocarban (Ye *et al.*, 2011) and the pM range for BPA (Teeguarden *et al.*, 2013);
18 PBDEs are in the range of ng/g lipid (Sjödin *et al.*, 2008). The lowest observed
19 concentration of triclocarban that inhibited iodide uptake (300 nM) in the present study was
20 considered to be relevant to human exposure levels, whereas BPA and BDE-47 exhibited
21 inhibitory effects on iodide uptake only at 10 µM, concentrations much higher than human
22 blood levels. Therefore, although the concentrations of BPA and BDE-47 in human thyroid
23 are currently unknown, the inhibition of iodide uptake by BPA and BDE-47 seems not to be
24 a primary mechanism underlying their disturbing effects on TH homeostasis.
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28 In conclusion, as summarized in Table 1, triclosan, triclocarban, BDE-47, and BPA acted as
29 non-competitive inhibitors of NIS and inhibited NIS-mediated iodide uptake in FRTL-5 cells.
30 Transcriptional expression levels of TH synthesis-related genes, including *Slc5a5*, *Tpo*, *Tg*,
31 *Pax8*, *Foxe1*, and *Nkx2-1*, were not significant changed in cells following exposure to
32 triclosan, triclocarban, or BDE-47 (with the exception of *Tpo* being affected by BDE-47),
33 whereas BPA induced alterations in the levels of each of these genes. Compared to iodide
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uptake, TPO seemed not a primary target of the test chemicals.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

- Boas, M., Feldt-Rasmussen, U., Skakkebaek, N. E., and Main, K. M. (2006). Environmental chemicals and thyroid function. *Eur. J. Endocrinol.* **154**, 599-611.
- Crofton, K. M., Paul, K. B., DeVito, M. J., and Hedge, J. M. (2007). Short-term *in vivo* exposure to the water contaminant triclosan: evidence for disruption of thyroxine. *Environ. Toxicol. Pharmacol.* **24**, 194-197.
- Delclos, K. B., Camacho, L., Lewis, S. M., Vanlandingham, M. M., Latendresse, J. R., Olson, G. R., Davis, K. J., Patton, R. E., Gamboa da Costa, G., Woodling, K. A., Bryant, M. S., Chidambaram, M., Trbojevich, R., Juliar, B. E., Felton, R. P., and Thorn, B. T. (2014). Toxicity evaluation of bisphenol A administered by gavage to Sprague Dawley rats from gestation day 6 through postnatal day 90. *Toxicol. Sci.* **139**, 174-197.
- DeSalva, S. J., Kong, B. M., and Lin, Y. J. (1989). Triclosan: a safety profile. *Am. J. Dent.* **2 Spec No**, 185-196.
- Doerge, D. R., and Chang, H. C. (2002). Inactivation of thyroid peroxidase by soy isoflavones, *in vitro* and *in vivo*. *J Chromatogr B* **777**, 269-279.
- Ellis-Hutchings, R. G., Cherr, G. N., Hanna, L. A., and Keen, C. L. (2006). Polybrominated diphenyl ether (PBDE)-induced alterations in vitamin A and thyroid hormone concentrations in the rat during lactation and early postnatal development. *Toxicol. Appl. Pharmacol.* **215**, 135-145.
- Fang, J.-L., Stingley, R. L., Beland, F. A., Harrouk, W., Lumpkins, D. L., and Howard, P. (2010). Occurrence, efficacy, metabolism, and toxicity of triclosan. *J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev.* **28**, 147-171.
- Fang, J.-L., Vanlandingham, M., Gamboa da Costa, G., and Beland, F. A. (2014). Absorption and metabolism of triclosan after application to the skin of B6C3F1 mice. *Environ. Toxicol.* doi: 10.1002/tox.22074.

Fang, J.-L., Vanlandingham, M. M., Juliar, B. E., Olson, G. R., Patton, R. E., and Beland, F. A. (2015). Dose–response assessment of the dermal toxicity of triclosan in B6C3F1 mice. *Toxicol. Res.* **4**, 867-877.

Freyberger, A., and Ahr, H. J. (2006). Studies on the goitrogenic mechanism of action of N,N,N',N'-tetramethylthiourea. *Toxicology* **217**, 169-175.

Fukayama, H., Murakami, S., Nasu, M., and Sugawara, M. (1991). Hydrogen peroxide inhibits iodide uptake and iodine organification in cultured porcine thyroid follicles. *Thyroid* **1**, 267-271.

Hallgren, S., Sinjari, T., Hakansson, H., and Darnerud, P. O. (2001). Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. *Arch. Toxicol.* **75**, 200-208.

Hinther, A., Bromba, C. M., Wulff, J. E., and Helbing, C. C. (2011). Effects of triclocarban, triclosan, and methyl triclosan on thyroid hormone action and stress in frog and mammalian culture systems. *Environ. Sci. Technol.* **45**, 5395-5402.

Hites, R. A. (2004). Polybrominated diphenyl ethers in the environment and in people: a meta-analysis of concentrations. *Environ. Sci. Technol.* **38**, 945-956.

Kimura, S. (2011). Thyroid-specific transcription factors and their roles in thyroid cancer. *J. Thyroid Res.* **2011**, 710213.

Koeppe, E. S., Ferguson, K. K., Colacino, J. A., and Meeker, J. D. (2013). Relationship between urinary triclosan and paraben concentrations and serum thyroid measures in NHANES 2007-2008. *Sci. Total Environ.* **445-446**, 299-305.

Kogai, T., and Brent, G. A. (2012). The sodium iodide symporter (NIS): regulation and approaches to targeting for cancer therapeutics. *Pharmacol. Ther.* **135**, 355-370.

Kogai, T., Schultz, J. J., Johnson, L. S., Huang, M., and Brent, G. A. (2000). Retinoic acid induces sodium/iodide symporter gene expression and radioiodide uptake in the MCF-7 breast cancer cell line. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 8519-8524.

Kosugi, S., Sasaki, N., Hai, N., Sugawa, H., Aoki, N., Shigemasa, C., Mori, T., and Yoshida, A. (1996). Establishment and characterization of a Chinese hamster ovary cell line, CHO-4J, stably expressing a number of Na⁺/I⁻ symporters. *Biochem. Biophys. Res. Commun.* **227**, 94-101.

Marchesini, G. R., Meimaridou, A., Haasnoot, W., Meulenberg, E., Albertus, F., Mizuguchi, M., Takeuchi, M., Irth, H., and Murk, A. J. (2008). Biosensor discovery of thyroxine transport disrupting chemicals. *Toxicol. Appl. Pharmacol.* **232**, 150-160.

Marlatt, V. L., Veldhoen, N., Lo, B. P., Bakker, D., Rehaume, V., Vallee, K., Haberl, M., Shang, D., van Aggelen, G. C., Skirrow, R. C., Elphick, J. R., and Helbing, C. C. (2013). Triclosan exposure alters postembryonic development in a Pacific tree frog (*Pseudacris regilla*) Amphibian Metamorphosis Assay (TREEMA). *Aquat. Toxicol.* **126**, 85-94.

Meerts, I. A. T. M., van Zanden, J. J., Luijckx, E. A. C., van Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A., and Brouwer, A. (2000). Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. *Toxicol. Sci.* **56**, 95-104.

Moriyama, K., Tagami, T., Akamizu, T., Usui, T., Saijo, M., Kanamoto, N., Hataya, Y., Shimatsu, A., Kuzuya, H., and Nakao, K. (2002). Thyroid hormone action is disrupted by bisphenol A as an antagonist. *J. Clin. Endocrinol. Metab.* **87**, 5185-5190.

Mullur, R., Liu, Y.-Y., and Brent, G. A. (2014). Thyroid hormone regulation of metabolism. *Physiol. Rev.* **94**, 355-382.

Patrick, L. (2009). Thyroid disruption: mechanism and clinical implications in human health. *Altern. Med. Rev.* **14**, 326-346.

Paul, K. B., Hedge, J. M., Bansal, R., Zoeller, R. T., Peter, R., DeVito, M. J., and Crofton, K. M. (2012). Developmental triclosan exposure decreases maternal, fetal, and early neonatal thyroxine: a dynamic and kinetic evaluation of a putative mode-of-action. *Toxicology* **300**, 31-45.

Paul, K. B., Hedge, J. M., DeVito, M. J., and Crofton, K. M. (2010). Short-term exposure to triclosan decreases thyroxine *in vivo* via upregulation of hepatic catabolism in young Long-Evans rats. *Toxicol. Sci.* **113**, 367-379.

Paul, K. B., Hedge, J. M., Rotroff, D. M., Hornung, M. W., Crofton, K. M., and Simmons, S. O. (2014). Development of a thyroperoxidase inhibition assay for high-throughput screening. *Chem. Res. Toxicol.* **27**, 387-399.

Pinto, P. I. S., Guerreiro, E. M., and Power, D. M. (2013). Triclosan interferes with the thyroid axis in the zebrafish (*Danio rerio*). *Toxicol. Res.* **2**, 60-69.

Richardson, V. M., Staskal, D. F., Ross, D. G., Diliberto, J. J., DeVito, M. J., and Birnbaum, L. S. (2008). Possible mechanisms of thyroid hormone disruption in mice by BDE 47, a major polybrominated diphenyl ether congener. *Toxicol. Appl. Pharmacol.* **226**, 244-250.

Riedel, C., Levy, O., and Carrasco, N. (2001). Post-transcriptional regulation of the sodium/iodide symporter by thyrotropin. *J. Biol. Chem.* **276**, 21458-21463.

Rubin, B. S. (2011). Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. *J. Steroid Biochem. Mol. Biol.* **127**, 27-34.

Sandborgh-Englund, G., Adolfsson-Erici, M., Odham, G., and Ekstrand, J. (2006). Pharmacokinetics of triclosan following oral ingestion in humans. *J. Toxicol. Environ. Health A* **69**, 1861-1873.

Schmutzler, C., Bacinski, A., Gotthardt, I., Huhne, K., Ambrugger, P., Klammer, H., Schlecht, C., Hoang-Vu, C., Gruters, A., Wuttke, W., Jarry, H., and Kohrle, J. (2007a). The ultraviolet filter benzophenone 2 interferes with the thyroid hormone axis in rats and is a potent *in vitro* inhibitor of human recombinant thyroid peroxidase. *Endocrinology* **148**, 2835-2844.

Schmutzler, C., Bacinski, A., Gotthardt, I., Huhne, K., Ambrugger, P., Klammer, H., Schlecht, C., Hoang-Vu, C., Gruters, A., Wuttke, W., Jarry, H., and Kohrle, J. (2007b). The ultraviolet filter benzophenone 2 interferes with the thyroid hormone axis in rats and is a potent *in vitro* inhibitor of human recombinant thyroid peroxidase. *Endocrinology* **148**, 2835-2844.

Sheng, Z.-G., Tang, Y., Liu, Y.-X., Yuan, Y., Zhao, B.-Q., Chao, X.-J., and Zhu, B.-Z. (2012). Low concentrations of bisphenol a suppress thyroid hormone receptor transcription through a nongenomic mechanism. *Toxicol. Appl. Pharmacol.* **259**, 133-142.

Sjödin, A., Wong, L.-Y., Jones, R. S., Park, A., Zhang, Y., Hodge, C., DiPietro, E., McClure, C., Turner, W., Needham, L. L., and Patterson, D. G., Jr. (2008). Serum concentrations of polybrominated diphenyl ethers (PBDEs) and polybrominated biphenyl (PBB) in the United States population: 2003-2004. *Environ. Sci. Technol.* **42**, 1377-1384.

Stapleton, H. M., Eagle, S., Anthopolos, R., Wolkin, A., and Miranda, M. L. (2011). Associations between polybrominated diphenyl ether (PBDE) flame retardants, phenolic metabolites, and thyroid hormones during pregnancy. *Environ. Health Perspect.* **119**, 1454-1459.

Sue, M., Akama, T., Kawashima, A., Nakamura, H., Hara, T., Tanigawa, K., Wu, H., Yoshihara, A., Ishido, Y., Hiroi, N., Yoshino, G., Kohn, L. D., Ishii, N., and Suzuki, K. (2012). Propylthiouracil increases sodium/iodide symporter gene expression and iodide uptake in rat thyroid cells in the absence of TSH. *Thyroid* **22**, 844-852.

Szabo, D. T., Richardson, V. M., Ross, D. G., Diliberto, J. J., Kodavanti, P. R. S., and Birnbaum, L. S. (2009). Effects of perinatal PBDE exposure on hepatic phase I, phase II, phase III, and deiodinase 1 gene expression

involved in thyroid hormone metabolism in male rat pups. *Toxicol. Sci.* **107**, 27-39.

1 Taurog, A., Dorris, M. L., and Doerge, D. R. (1996). Mechanism of simultaneous iodination and coupling
2 catalyzed by thyroid peroxidase. *Arch. Biochem. Biophys.* **330**, 24-32.

3 Teeguarden, J., Hanson-Drury, S., Fisher, J. W., and Doerge, D. R. (2013). Are typical human serum BPA
4 concentrations measurable and sufficient to be estrogenic in the general population? *Food Chem. Toxicol.* **62**,
5 949-963.

6 Tietge, J. E., Degitz, S. J., Haselman, J. T., Butterworth, B. C., Korte, J. J., Kosian, P. A., Lindberg-Livingston,
7 A. J., Burgess, E. M., Blackshear, P. E., and Hornung, M. W. (2013). Inhibition of the thyroid hormone
8 pathway in *Xenopus laevis* by 2-mercaptobenzothiazole. *Aquat. Toxicol.* **126**, 128-136.

9 Tonacchera, M., Pinchera, A., Dimida, A., Ferrarini, E., Agretti, P., Vitti, P., Santini, F., Crump, K., and Gibbs,
10 J. (2004). Relative potencies and additivity of perchlorate, thiocyanate, nitrate, and iodide on the inhibition of
11 radioactive iodide uptake by the human sodium iodide symporter. *Thyroid* **14**, 1012-1019.

12 Tran, L., Hammuda, M., Wood, C., and Xiao, C. W. (2013). Soy extracts suppressed iodine uptake and
13 stimulated the production of autoimmunogen in rat thyrocytes. *Exp. Biol. Med. (Maywood)* **238**, 623-630.

14 Vickers, A. E., Heale, J., Sinclair, J. R., Morris, S., Rowe, J. M., and Fisher, R. L. (2012). Thyroid
15 organotypic rat and human cultures used to investigate drug effects on thyroid function, hormone synthesis
16 and release pathways. *Toxicol. Appl. Pharmacol.* **260**, 81-88.

17 Vitale, G., Salvioli, S., and Franceschi, C. (2013). Oxidative stress and the ageing endocrine system. *Nat. Rev.*
18 *Endocrinol.* **9**, 228-240.

19 Waltz, F., Pillette, L., and Ambroise, Y. (2010). A nonradioactive iodide uptake assay for sodium iodide
20 symporter function. *Anal. Biochem.* **396**, 91-95.

21 Wang, T. G., Lu, J. L., Xu, M., Xu, Y., Li, M., Liu, Y., Tian, X. G., Chen, Y. H., Dai, M., Wang, W. Q., Lai, S.
22 H., Bi, Y. F., and Ning, G. (2013). Urinary Bisphenol A Concentration and Thyroid Function in Chinese
23 Adults. *Epidemiology* **24**, 295-302.

24 Witorsch, R. J., and Thomas, J. A. (2010). Personal care products and endocrine disruption: a critical review
25 of the literature. *Crit. Rev. Toxicol.* **40 Suppl 3**, 1-30.

26 Wu, S.-y., Green, W. L., Huang, W.-s., Hays, M. T., and Chopra, I. J. (2005). Alternate pathways of thyroid
27 hormone metabolism. *Thyroid* **15**, 943-958.

28 Wu, Y., Beland, F. A., Chen, S., and Fang, J.-L. (2015). Extracellular signal-regulated kinases 1/2 and Akt
29 contribute to triclosan-stimulated proliferation of JB6 Cl 41-5a cells. *Arch. Toxicol.* **89**, 1297-1311.

30 Xu, X.-h., Zhang, J., Wang, Y.-m., Ye, Y.-p., and Luo, Q.-q. (2010). Perinatal exposure to bisphenol-A impairs
31 learning-memory by concomitant down-regulation of *N*-methyl-*D*-aspartate receptors of hippocampus in male
32 offspring mice. *Horm. Behav.* **58**, 326-333.

33 Xu, X., Liu, Y., Sadamatsu, M., Tsutsumi, S., Akaike, M., Ushijima, H., and Kato, N. (2007). Perinatal
34 bisphenol A affects the behavior and SRC-1 expression of male pups but does not influence on the thyroid
35 hormone receptors and its responsive gene. *Neurosci. Res.* **58**, 149-155.

36 Ye, X., Zhou, X., Furr, J., Ahn, K. C., Hammock, B. D., Gray, E. L., and Calafat, A. M. (2011). Biomarkers of
37 exposure to triclocarban in urine and serum. *Toxicology* **286**, 69-74.

38 Zhou, T., Ross, D. G., DeVito, M. J., and Crofton, K. M. (2001). Effects of short-term *in vivo* exposure to
39 polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicol.*
40 *Sci.* **61**, 76-82.

41 Zoeller, R. T., Bansal, R., and Parris, C. (2005). Bisphenol-A, an environmental contaminant that acts as a
42 thyroid hormone receptor antagonist *in vitro*, increases serum thyroxine, and alters RC3/neurogranin
43 expression in the developing rat brain. *Endocrinology* **146**, 607-612.

Figure legends

Fig. 1 Short-term effects of triclosan, triclocarban, BDE-47, and BPA on the iodide uptake in FRTL-5 cells. Iodide uptake curves of FRTL-5 cells co-incubated with various concentrations of the indicated chemical and 10 μ M NaI for 1 h. The results shown are the mean and standard deviation of three independent experiments.

Fig. 2 Validation of the non-radioactive iodide uptake assay in kinetic studies. (A) The standard curve of iodide content. A series of standard solutions (100 μ l) with known concentrations of NaI (0, 100, 200, 300, 400, 500, 600, 700, 1000, or 2000 nM) were added to each well of a 96-well plate. This is equivalent to iodide content of 0 – 200 pmol per well. The non-radioactive iodide uptake assay was carried out as described in Materials and Methods. The logarithm of A_{420} was plotted against the iodide content, and linear regression was performed using GraphPad Prism 6.0 to obtain the standard curve. (B) Time-dependent iodide uptake in FRTL-5 cells incubated with 10 μ M NaI for 2, 5, 10, 20, 30, 60 or 120 min. (C) Substrate concentration-dependent initial velocity of iodide uptake in FRTL-5 cells incubated with 5, 10, 20, 40, 80, 160, or 320 μ M NaI for 5 min. (D) Lineweaver-Burk plot of data shown in (C). Linear regression was performed by GraphPad Prism 6. The K_m value was determined as the reciprocal of the X-intercept of the linear regression line. Values shown in the parenthesis are the 95% confidence intervals. The results shown are the mean and standard deviation of three independent experiments.

Fig. 3 Kinetics of inhibition of iodide uptake by triclosan, triclocarban, BDE-47, and BPA. (A – D) Lineweaver-Burk plots showing the initial velocity of iodide uptake in FRTL-5 cells co-incubated with various concentrations of the indicated chemical and NaI (5, 10, 20, or 40 μ M) for 5 min. Linear regression was performed by GraphPad Prism 6. (E – H) Plots of slopes of linear regression lines shown in A – D against the concentrations of the test chemicals. Linear regression was performed by GraphPad Prism 6. The inhibition constant (K_i) values were determined as the X-intercepts of the linear regression lines shown in E – H. The results shown are the mean and standard deviation of three independent experiments.

Fig. 4 Long-term effects of triclosan, triclocarban, BDE-47, and BPA on the iodide uptake in FRTL-5 cells. (A) Representative cell growth curves of FRTL-5 cells treated with triclosan, triclocarban, BDE-47, or BPA (0.03 – 300 μ M) for 24 or 48 h. The results shown are the mean and standard deviation of three independent experiments. (B) Iodide uptake of FRTL-5 cells that were exposed to various concentrations of triclosan, triclocarban, BDE-47, or BPA for 24 or 48 h. At the end of the treatment, cells were washed with iodide uptake buffer and then incubated with 10 μ M NaI for 1 h. MTT assays were performed in parallel plates. Iodide uptake was normalized to the MTT assay data, and further normalized to the control. The results shown are the mean and standard deviation of three independent experiments. *, significantly ($p < 0.05$) different from the control group.

Fig. 5 Effects of triclosan, triclocarban, BDE-47, and BPA on the expression of genes involved in TH synthesis. FRTL-5 cells were exposed to the indicated concentration of triclosan, triclocarban, BDE-47, or BPA for 6, 24 or 48 h. Transcriptional expression levels of *Slc5a5*, *Tpo*, *Tg*, *Pax8*, *Foxe1*, and *Nkx2-1* were determined using quantitative real-time PCR. The results shown are the mean and standard deviation of three independent experiments. *, significantly ($p < 0.05$) different from the control group.

Fig. 6 Effects of triclosan, triclocarban, BDE-47, and BPA on the activity of TPO. Curves of activity of TPO in rat thyroid microsomes incubated with various concentrations of triclosan, triclocarban, BDE-47, or BPA. The results shown are the mean and standard deviation of three independent experiments. The IC_{50} values were obtained from the TPO activity curves using GraphPad Prism 6.0.

Table 1. Summary of the effects of triclosan, triclocarban, BDE-47, and BPA on iodide uptake, the expression levels of genes involved in TH synthesis in FRTL-5 cells, and the activity of TPO in rat thyroid microsomes.

			Triclosan	Triclocarban	BDE-47	BPA
Iodide uptake	Short-term effects	Inhibition type	Non-competitive	Non-competitive	Non-competitive	Non-competitive
		K_i (μM)	21.3	69.0	77.8	112.3
	Long-term effects		Decrease at $\geq 10 \mu\text{M}$	Decrease at $\geq 0.3 \mu\text{M}$	Decrease at $\geq 10 \mu\text{M}$	Decrease at $\geq 10 \mu\text{M}$
mRNA expression of TH synthesis related genes	<i>Slc5a5</i>		No effect	No effect	No effect	Decrease at 100 μM
	<i>Tpo</i>		No effect	No effect	Decrease at 100 μM	Decrease at 100 μM
	<i>Tg</i>		No effect	No effect	No effect	Increase at $\geq 30 \mu\text{M}$
	<i>Pax8</i>		No effect	No effect	No effect	Increase at 100 μM
	<i>Foxe1</i>		No effect	No effect	No effect	Increase at 100 μM
	<i>Nkx2-1</i>		No effect	No effect	No effect	No effect
TPO activity	IC_{50} (μM)		165.8	> 300	> 1000	> 1000

Figure(s)
Fig. 1

Fig. 2

Fig. 3

Fig. 4

A

B

Fig. 5

A

Fig. 5

B

Fig. 6

Highlights

Triclosan, triclocarban, BDE-47, and BPA inhibited iodide uptake in rat thyroid follicular cells.

All four chemicals acted as non-competitive inhibitors of the sodium/iodide symporter.

The chemicals exhibited differential effects on thyroid hormone synthesis gene expression.

Thyroid peroxidase was not a primary target of the four chemicals.