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Research Article

In Vitro, *Ex Vivo*, and *In Vivo* Determination of Thyroid Hormone Modulating Activity of Benzothiazoles

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ABSTRACT

As *in vitro* assays are increasingly used to screen chemicals for their potential to produce endocrine disrupting adverse effects, it is important to understand their predictive capacity. The potential for a set of 6 benzothiazoles to affect endpoints related to thyroid hormone synthesis inhibition were assessed using *in vitro*, *ex vivo*, and *in vivo* assays. Inhibition of thyroid peroxidase (TPO) derived from pig thyroid glands was determined for benzothiazole (BTZ), 2-mercaptobenzothiazole (MBT), 5-chloro-2-mercaptobenzothiazole (CMBT), 2-aminobenzothiazole (ABT), 2-hydroxybenzothiazole (HBT), and 2-methylthiobenzothiazole (MTBT). Their rank order potency for TPO inhibition was MBT=CMBT>ABT>BTZ, whereas HBT and MTBT exhibited no inhibitory activity. The benzothiazoles were tested further in a *Xenopus laevis* thyroid gland explant culture assay in which inhibition of thyroxine (T4) release was the measured endpoint. In this assay all 6 benzothiazoles inhibited T4 release. The activity of the benzothiazoles for disrupting thyroid hormone activity was verified *in vivo* using *X. laevis* tadpoles in a 7-day assay. The 2 most potent chemicals for TPO inhibition, MBT and CMBT, produced responses *in vivo* indicative of T4 synthesis inhibition including induction of sodium iodide symporter mRNA and decreases in glandular and circulating thyroid hormones. The capability to measure thyroid hormone levels in the glands and blood by ultrahigh performance LC-MS/MS methods optimized for small tissue samples was critical for effects interpretation. These results indicate that inhibition of TPO activity *in vitro* was a good indicator of a chemical's potential for thyroid hormone disruption *in vivo* and may be useful for prioritizing chemicals for further investigation.

Key words: thyroid; endocrine disruption; *in vitro-in vivo* extrapolation; Xenopus; UHPLC-MS/MS

The potential disruption of endocrine hormone activity in humans and wildlife by exposure to chemicals in the environment continues to be an issue of ongoing concern. There is a need to characterize the endocrine disrupting activity of chemicals in current use, in an efficient systematic manner, and to incorporate more mechanistic-based *in vitro* assays into the hazard evaluation process (NRC, 2007). One of the endpoints of concern included in the discussions for development of testing programs for endocrine disrupting chemicals was the disruption of thyroid hormone signaling pathways (DeVito et al., 1999; U.S. EPA, 1998). This led in part to the development of the amphibian metamorphosis assay used by U.S. EPA and OECD for the evaluation of chemicals specifically for their potential to alter normal thyroid hormone homeostasis and signaling (U.S. EPA, 2009; OECD, 2009). This assay was designed for use as a diagnostic tool for identifying chemicals that can disrupt the hypothalamus-pituitary-thyroid (HPT) axis, but it does lack the higher-throughput characteristics desired for a more rapid evaluation of large chemical inventories.

There are a number of chemicals in the environment that have been found to disrupt the HPT axis via known or proposed mechanisms (Brucker-Davis, 1998; Boas et al. 2006; Hurley et al. 1998). Although the mechanisms by which all of these disrupt the HPT-axis are not fully understood, it is clear that exposure

to structurally diverse chemical groups can have impacts on thyroid hormone status. It has been recognized for some time that the diverse array of chemicals that can impact thyroid hormone status may be a result of the multiple biochemical and physiological processes both in the thyroid gland and in peripheral tissues, that when impacted can lead to decreased thyroid hormone levels (Capen, 1997). These include the steps in the regulation of thyroid hormone synthesis, hormone release from the gland, and the metabolism and elimination of hormone.

The role of thyroid peroxidase (TPO) in thyroid hormone production is well-documented. This enzyme catalyzes both the iodination of tyrosines of the thyroglobulin protein within the follicular colloid, and the coupling of iodinated tyrosines to produce tri- and tetra-iodothyronines (Taurog et al., 1996). Thus TPO regulates critical steps in the synthesis of thyroid hormone. TPO is the target of the pharmaceutical drugs methimazole and propylthiouracil that have been in use for decades to block thyroid hormone production in the treatment of hyperthyroid diseases such as Graves' disease (Haynes and Murad, 1980). It has previously been established that this enzyme can also be inhibited *in vitro* by various chemical classes including the plant flavonoids (Chang and Doerge, 2000; Divi and Doerge, 1996), benzophenones (Schmutzler et al. 2007), thiocarbamate pesticides (Marinovich et al., 1997), as well as resorcinol and related chemicals (Divi and Doerge, 1994).

The goal of the work presented here was to expand upon our previous finding that mercaptobenzothiazole (MBT) is a potent inhibitor of TPO activity *in vitro* and the most potent chemical we had tested to date in the amphibian metamorphosis assay (Tietge et al. 2013). In the present study a series of benzothiazoles with slight structural differences were tested. Benzothiazoles are a class of chemicals that have various uses including vulcanization agents in rubber manufacturing, antifreezes, and have been detected in the environment (Brownlee et al., 1992; Kloepfer et al., 2005). These specific chemicals were selected in part to expand our knowledge of the structural components of MBT that may be necessary for inhibiting thyroid hormone synthesis. Furthermore, these chemicals were used in this study to assess the concordance among responses in the *in vitro* inhibition of TPO activity, the *X. laevis* thyroid gland explant culture assay that measures the effect of chemicals on thyroid hormone release from the gland, and a 7-day assay with pro-metamorphic *X. laevis* tadpoles for detecting potential thyroid hormone disrupting chemicals *in vivo*. To assess thyroid hormone synthesis inhibition in the *X. laevis* tadpole assay it was necessary to measure changes in glandular and circulating thyroid hormones. A method was optimized for small samples, using ultrahigh performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) that is capable of measuring pmole amounts of iodotyrosines and iodothyronines in thyroid glands and pM concentrations of iodothyronines in plasma or serum.

MATERIALS AND METHODS

Chemicals. Methimazole (CAS No. 60-56-0; purity 98%), 2-mercaptobenzothiazole (MBT; CAS No. 149-30-4; purity 97%), 2-aminobenzothiazole (ABT; CAS No. 136-95-8; purity 97%), benzothiazole (BTZ; CAS No. 95-16-9; purity 96%), and 2-hydroxybenzothiazole (HBT; CAS No. 934-34-9; purity 98%) were purchased from Sigma Chemical (St Louis, Missouri). 5-Chloro-2-mercaptobenzothiazole (CMBT; CAS No. 5331-91-9; purity not reported) and 2-methylthio-benzothiazole (MTBT; CAS No. 615-22-5; purity 98%) were purchased from TCI America (Portland, Oregon). Stock solutions of benzothiazoles and methimazole for use in the *in vitro* TPO inhibition assay and gland explant culture assay were prepared in dimethylsulfoxide (DMSO). Chemical reagents used in the TPO and explant culture assays are as described previously (Hornung et al. 2010; Paul et al., 2013). Preparation of exposure solutions for *in vivo* assays is described below.

For analytical determination of iodotyrosines and iodothyronines water (LC-MS grade), acetonitrile (LC-MS grade) and methanol (LC-MS grade) were purchased from Honeywell Burdick & Jackson (Muskegon, Michigan). Isopropanol (HPLC grade, 99.9%) was purchased from ACROS Organics (Fisher Scientific, Pittsburgh, Pennsylvania). Formic acid (reagent grade, $\geq$ 95%), glacial acetic acid (99+ %) and ammonium hydroxide ($\geq$ 99.99%) were purchased from Sigma Chemical Co. (St. Louis). Hydrochloric acid (Optima grade) was purchased from Fisher Scientific. The iodotyrosines, 3-monoiodotyrosine (MIT; 99.4%) and 3,5-diiodotyronsine (DIT; $\geq$99%), and iodothyronines 3,5-diiodothyronine (3,5-T2; $\geq$ 99%), 3,3′,5-triiodothyronine (T3; 98%), 3,3′,5′-triiodothyronine (rT3; 97%), and L-thyroxine (T4; 99.4%) were from Sigma Chemical Co. The 3,3′-diiodothyronine (3,3′-T2; 98.4%) and the stable isotope-labeled iodothyronines 3,3′-diiodothyronine-$^{13}C_6$ ($^{13}C_6$-3,3′-T2; > 98.3%; 99% isotope incorporation), 3,3′,5-triiodothyronine-$^{13}C_6$ ($^{13}C_6$-T3; 98.8%; 99% isotope incorporation), and L-thyroxine-$^{13}C_6$ ($^{13}C_6$-T4; >99%; 99% isotope incorporation) were purchased from IsoSciences (King of Prussia, Pennsylvania).

Animals. *X. laevis* tadpoles from an in-house culture at US EPA, Mid-Continent Ecology Division, Duluth, MN were the source for thyroid glands for the thyroid gland explant culture experiments and tadpoles that were used in the 7-day *in vivo* exposure studies described below. Animals were used according to the Animal Care and Use Guidelines of the USEPA Mid-Continent Ecology Division's Animal Care and Use Committee.

Thyroid Peroxidase Inhibition In Vitro

Inhibition of TPO obtained from porcine thyroid glands was determined in a guaiacol oxidation assay as described previously (Tietge et al., 2013; Paul et al. 2013).

A full dose-response curve for methimazole (MMZ) was generated in parallel with each chemical as a positive control for inhibition. The IC50 for MMZ and benzothiazoles were calculated using GraphPad Prism software (v5.0, LaJolla, California). Each chemical that inhibited TPO activity was assessed in at least 2 different batches of microsomes. Additional information on the TPO assay method is provided in Supplementary Materials.

Ex Vivo *Inhibition of T4 Release from Thyroid Glands*

Thyroid glands were dissected from prometamorphic tadpoles at NF stage 59 (Nieuwkoop and Faber, 1994) and cultured as described previously in 96-well plates with 10 glands per treatment level in the presence of 4000 ng bTSH/ml (TSH activity = 7.2 mU/ ml) to stimulate T4 release (Hornung et al. 2010). Glands were exposed to vehicle alone, a single concentration of methimazole at 30 μM as a positive control, or graded concentrations of chemicals spiked into the culture media with a final DMSO concentration of 0.5%. Glands were transferred to a new culture plate containing fresh treatment media every 48 h for a total of 8 days in culture. The media in which the glands were cultured over the last 48 h of the experiment (day 6 to day 8) was collected for measurement of total T4 by RIA (Canine

Coat-A-Count Total T4 RIA Kit from Siemens Medical Solutions; Los Angeles, CA) as described in Hornung et al. (2010). Toxicity of the chemical to the thyroid glands was assessed on day 8 at the completion of the exposure by quantification of the ATP present in the glands (ATPlite assay kit; Perkin Elmer, Waltham, MA). Significant decrease in glandular ATP in treated versus controls was determined on log transformed luminescence data by ANOVA and Dunnett's multiple comparison test ($P < .05$). Additional information on gland culture and ATP assessment is provided in Supplementary Material available online.

In Vivo X. laevis *Tadpole 7 d Exposures*

Animals. *X. laevis* tadpoles were collected at NF stage 54 and randomly distributed at 8 tadpoles per tank into glass aquaria (22.5 × 14.0 × 16.5 cm deep) that contained 3.5 L of Lake Superior water (LSW), or exposure solution (see below). Fluorescent lamps provided a 12:12 h light:dark photoperiod. Tadpoles were fed each morning with a diet containing Silver Cup Trout Starter (Nelson and Sons, Murray, Utah), Spirulina algae discs (Wardley, Secaucus, New Jersey) and TetraFin flakes (Tetra Sales, Blacksburg, Virginia) mixed together in culture water. Tadpoles were also fed live 24-h old brine shrimp (Bio-Marine Brand, Bio-Marine, Hawthorne, California).

Exposure design and water quality parameters. The benzothiazoles were tested across 4 separate experimental runs separated by several weeks or months between experiments. Thus, tadpoles from different spawns were used for each experimental run and controls were included with each experimental run. For the purposes of reporting within this document they are labeled as experiments A, B, C, and D. Control tadpoles in Lake Superior water, MBT at 0.35 ppm (2.1 μM), and ABT at 10 ppm (67 μM) were tested in experiment A; experiment B included controls and CMBT at 1 ppm (5.0 μM); experiment C included controls, BTZ at 30 ppm (222 μM), and HBT at 10 ppm (66 μM); and experiment D had controls and MTBT at 2 ppm (11 μM). Detailed description of the preparation of exposure solutions and selection of test concentrations are provided in the Supplementary Material available online.

There were 3 exposure tanks per treatment level with 8 animals per tank at the start of all experiments. Exposures were conducted in a 24 h static renewal format with 90% solution renewal, and exposure tanks were replaced with cleaned tanks every 48 h. Dissolved oxygen in newly prepared exposure solutions (time = 0 h) were 80–91% saturation. This was measured again just prior to renewal of the exposure solution and in that 24 h interval had dropped to 65–70% saturation. Water temperature (range of means: 21.1–21.6°C) was achieved and maintained by partial immersion of the exposure tanks in a temperature controlled, common water bath in which the tanks were randomly distributed. Tank temperature means within a given experiment were within 0.1°C of each other. Conductivity (range of means: 107–115 μS/cm) and pH (range of means: 7.3–7.6) were also monitored daily and were within expected ranges.

Exposure water analysis. During the 7-day *in vivo* exposures, water samples (i.e., 10 ml) were collected daily from the controls and test chemical exposure solutions. On days 1, 4, and 7 a subset of samples were collected from the exposure tanks just prior to stock renewal to monitor the potential chemical loss over the 24 h static renewal period. LSW control samples were collected on days 0, 1, and 6. All samples were placed into precleaned 20-ml glass vials. All water samples, except those from the ABT exposure, were supplemented with acetonitrile (ACN) to yield a final composition of 10% ACN in water. Water samples from the ABT exposure were diluted with 10% ACN: 90% water with 0.1% formic acid. All samples were stored at 2°C prior to instrumental analysis. Initial measured concentrations were 93% or greater of nominal for each of the benzothiazoles. Analytical methods for water analysis for benzothiazoles and measured concentrations are provided in the Supplementary Materials.

Biological endpoints. At termination of the 7-day experiment the tanks were randomized to distribute the collection of tadpoles from treatment groups across the duration of the collection period. Tadpoles were euthanized with buffered MS222 (150 mg/l MS222 with 300 mg/l sodium bicarbonate) in LSW and wet weight and snout-vent length measurements were taken. Tadpoles were staged according to NF staging criteria. Blood was collected by snipping the aorta and collecting from this vessel into microcapillary tubes. For CMBT, HBT, BTZ, and MTBT the blood was collected in nonheparinized 25-μl microcapillary tubes (VWR) and samples were allowed to clot prior to centrifugation to collect serum. In Experiment "A" in which MBT and ABT were tested, the blood was collected in heparinized tubes (BecktonDickinson, No. 361027) and spun to collect plasma. There were no differences in detection of thyroid hormones whether collected as serum or plasma (data not shown). Analysis of serum and plasma samples for thyroid hormones T4, T3, and rT3 is described below. The paired thyroid glands from the tadpoles were removed after blood was collected. One gland was flash frozen for sodium iodide symporter (NIS) mRNA expression analysis by quantitative rtPCR as described previously (Tietge et al., 2013) and the other gland was frozen in buffer for later processing for determination of glandular hormones (MIT, DIT, T2, T3, rT3, T4). Glands from the tadpoles from a given tank were pooled so each tank was the experimental replicate with $n = 3$ tanks per treatment group.

Analytical Determination of Iodotyrosines and Iodothyronines in Glands and Blood

Stocks and standards. Stock solutions of MIT, DIT, 3,5-T2, T3, rT3, T4, and $^{13}C_6$-T4 were prepared by dissolving the chemicals in methanol with 5% formic acid. Stock solutions of 3,3′-T2, $^{13}C_6$-3,3′-T2, and $^{13}C_6$-T3 were purchased as solutions of 100 μg/ml in 0.1 N NH_3 in methanol directly from IsoSciences. Working stock solutions were prepared in methanol and stored at −20°C. For gland analyses, calibration standards of MIT, DIT, 3,5-T2, 3,3′-T2, T3, rT3, and T4 were prepared in 95% H_2O:5% acetonitrile (v/v) with 0.1% formic acid. Calibration standards ranged from 0.010 to 10 ng/ml. An internal standard (IS) solution of $^{13}C_6$-3,3′-T2, $^{13}C_6$-T3, and $^{13}C_6$-T4 was added to each calibration standard for a final concentration of 5 ng/ml for each stable isotope-labeled compound. For plasma or serum analyses, calibration standards of T3, rT3 and T4 were prepared in 75% H_2O:25% acetonitrile (v/v) with 0.1% formic acid. Calibration standards ranged from 0.010 to 1 ng/ ml. An IS solution of $^{13}C_6$-T3 and $^{13}C_6$-T4 was added to each calibration standard for a final concentration of 5 ng/ml for each stable isotope-labeled compound.

Plasma/serum. Plasma and serum samples from *X. laevis* were prepared similar to a sample preparation procedure by Luna et al. (2013). Twenty microliters of pooled plasma or serum was placed into an amber glass microsampling vial (Agilent Technologies, Santa Clara, California). To the same vial the following were added: 20 μl 1 N HCl, 1 μl of mixed stable isotope internal standard (IS) solution ($^{13}C_6$-T3 and $^{13}C_6$-T4 at 500 ng/ml in methanol), 100 μl H_2O, and 60 μl of 50%H_2O:50%acetronitrile

(v/v) with 0.1% formic acid. The samples were vortex-mixed for 20 s and then incubated at 37°C for 2 h. Following incubation the samples were cooled to room temperature, diluted with 300 µl of 0.1% acetic acid in H_2O (final volume,500 µl) and vortex-mixed for 20 s prior to solid phase extraction (SPE).

Thyroid glands. The procedure for proteolytic digestion of the thyroid glands was modified from our earlier method (Tietge et al., 2013). The gland pools containing 0.1 ml of 0.1 M potassium phosphate, pH 7.4, were removed from storage at −80°C and thawed. Four volumes of pronase digestion buffer (0.1 M Tris-HCl, pH 8.4, 2.5 mM propylthiouracil, 1% Triton X-100) were added and the samples homogenized by sonication for approximately 20 s at 20% power. To 100 µl of the homogenized pool, an equal volume of 20 mg/ml pronase (5 U/mg; Sigma, p8811) in pronase digestion buffer was added and incubated at 37°C with occasional vortex mixing. After 24 h, the digest was centrifuged for 10 min at 20 800× g and the supernatant was removed and stored at −80°C.

Gland digests were thawed at room temperature for 15 min and centrifuged at 20 800× g for 10 min. Twenty microliters of gland digests (20 µl represents 0.16 × of a gland) were placed into amber glass microsampling vials (Agilent Technologies). To each vial the following were added: 20 µl 1 N HCl, 1 µl mixed stable isotope IS solution ($^{13}C_6$-3,3′-T2, $^{13}C_6$-T3 and $^{13}C_6$- T4 at 500 ng/ml in methanol), 100 µl H_2O, 60 µl of 50%H_2O:50%acetronitrile (v/v) with 0.1% formic acid, and 300 µl of 0.1% acetic acid in H_2O. The diluted sample digests (final volume, 500 µl) were vortex-mixed for 20 s prior to SPE.

Diluted plasma or serum samples were immediately processed through SPE using a vacuum manifold. One microliter (10 mg) of Evolute strong cation exchange (CX) SPE columns (Biotage, Charlotte, North Carolina) was activated with 400 µl of methanol and then equilibrated with 400 µl of 0.1% acetic acid in H_2O. The samples were quantitatively transferred to SPE columns and pulled through the sorbent bed under vacuum (1 mm Hg). Following sample addition, the columns were rinsed with 400 µl of 0.1% acetic acid in H_2O and then 400 µl of methanol. The analytes were eluted from the columns with 2 × 250 µl of freshly prepared 2.5% NH_4OH in methanol and collected in disposable glass culture tubes, 12 × 75 mm (Fisher Scientific). The SPE extracts were vortex-mixed and then evaporated to dryness using nitrogen and a water bath heated to 40°C. Upon reaching dryness the culture tubes were immediately removed from the water bath and cooled to room temperature. The residues were reconstituted with 100 µl of 75% H_2O:25% acetonitrile (v/v) with 0.1% formic acid. The reconstituted extracts were vortex-mixed for 20 s and then transferred to amber microsampling vials.

Diluted gland digests were immediately processed through SPE using the same procedure as the plasma samples with the following exceptions. Slightly larger mass, 25 mg, 1 ml Evolute CX SPE columns were used and all reagent volumes were increased to 1 ml. Also the SPE residues were reconstituted with a 100 µl of 95% H_2O:5% acetonitrile (v/v) with 0.1% formic acid. This higher aqueous mixture provided better chromatography for MIT and DIT during the high pressure liquid chromatography gradient program.

Instrumental analysis. Samples were analyzed using ultra high pressure liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS). An Agilent 1290 UHPLC with an Agilent 6490 triple quadrupole mass spectrometer (6490 LC-MS/MS system) was used for the analysis of MIT; DIT; 3,5-T2; 3,3′-T2; T3; rT3, and T4 in *X. laevis* gland digests and T3, rT3, and T4 in serum samples.

For LC separation, 20 µl of sample was injected into an Agilent Zorbax SB-C18 RRHT analytical column (2.1 mm diameter × 50 mm length, 1.8 µm particle size) that was maintained at 30°C. Mobile phase A was 95% H_2O:5% acetonitrile (v/v) with 0.1% formic acid. Mobile phase B was 10% H_2O:90% acetonitrile (v/v) with 0.1% formic acid. The column was eluted under gradient conditions at a flow rate of 0.4 ml /min. At time 0 min the mobile phase was 100% A and over the next 5.75 min, the mobile phase was increased to 72% B. The mobile phase was then increased to 100% B in the next 0.25 min and held at 100% B for 2.5 min. Finally the mobile phase was returned to its initial composition of 100% A in 2 min. A 4 min post time was included at the end of each run. For each injection, the auto sampler flush port was used for 30 s and flushed with a mixture of 30% acetronitrile:30% methanol:30% isopropanol:10% H_2O.

The LC column effluent was introduced into the mass spectrometer equipped with an electrospray ionization source (ESI) with a heated jet spray interface. The ESI source was operated in positive ion mode $[M+H]^+$ with multiple reaction monitoring (MRM). MS instrument parameters were similar to those listed in Doyle and McCann (2013). and were set as follows: gas temperature (125°C), gas flow (16 L/min), nebulizer pressure (45 psi), sheath gas heater (225°C), sheath gas flow (11 L/min), capillary voltage (4000 V),and V charging (2000 V). Nitrogen was used as the nebulizer, sheath and collision gas. The fragmentation voltage was set at 380 V and delta EMV was set at 500 V. MRM precursor $[M+H]^+$ to product ion transitions, collision energies (CE) and compound retention times are listed in (Table 1). Instrument control and data processing were performed with Agilent MassHunter software, version B.06.00. Analyte concentrations were determined using internal standard (IS) method of quantitation. Stable isotope $^{13}C_6$-3,3′-T2 was used as the IS for MIT, DIT, 3,5-T2, and 3,3′-T2. Stable isotope $^{13}C_6$-T3 was used as

TABLE 1. Tandem Mass Spectrometry (MS/MS) Parameters and Multiple Reaction Monitoring (MRM) Transitions in Positive Ion Mode

Compound	Retention time (min)	MRM Transitions (*m*/*z*)	CE (V)
MIT	1.383	307.98–262 (Q)	14
		307.98–135.1(C)	30
DIT	1.974	433.88–387.9 (Q)	18
		433.88–261(C)	34
3,5-T2	3.088	525.9–479.8 (Q)	20
		525.9–353 (C)	32
3,3′-T2	3.402	525.9–479.8 (Q)	18
		525.9–466.9 (C)	26
$^{13}C_6$-3,3′-T2	3.402	531.9–485.8 (Q)	18
T3	3.672	651.8–605.7 (Q)	22
		651.8–478.8 (C)	34
rT3	3.836	651.8–605.7 (Q)	22
		651.8–507.8 (C)	21
$^{13}C_6$-T3	3.672	657.8–611.7 (Q)	22
T4	4.078	777.7–731.6 (Q)	22
		777.7–350.9 (C)	46
$^{13}C_6$-T4	4.078	783.5–737.7 (Q)	22

Abbreviations: CE, collision energy; MIT, 3-monoiodotyrosine; DIT, 3,5-diiodotyrosine; 3,5-T2, 3,5-diiodothyronine; 3,3′-T2, 3,3′-diiodothyronine; $^{13}C_6$-3,3′-T2, 3,3′-diiodothyronine-$^{13}C_6$; T3, 3,3′,5-triiodothyronine; $^{13}C_6$-T3, 3,3′,5-triiodothyronine-$^{13}C_6$; rT3, 3,3′,5′-triiodothyronine; T4, L-thyroxine; $^{13}C_6$-T4, L-thyroxine-$^{13}C_6$; Q, quantitation ions; C, confirmation ions.

IS for T3 and rT3 and stable isotope $^{13}C_6$-T4 was used as the IS for T4. For each analyte, the most dominant product ion was used for quantitation (Q) whereas the second most dominant product ion was used for confirmation (C), (See Table 1). For gland sample analyses, analyte calibration curves (i.e., MIT, DIT, 2,5-T2, 3,3′-T2, T3, rT3, and T4) ranged from 0.010 to 10 ng/ ml. Analyte calibration curves (i.e., T3, rT3, and T4) ranged from 0.010 to 1 ng/ml for plasma or serum sample analyses. Concentrations of the stable isotope internal standards in all samples and standards were 5 ng/ml.

For the gland method the lowest limits of quantitation (LLOQ) were 0.020 pmole/gland (MIT), 0.014 pmole/gland (DIT), 0.012 pmole/gland (3,5-T2 & 3,3′-T2), 0.010 pmole/gland (T3 & rT3) and 0.01 pmole/gland (T4). For the plasma or serum method LLOQs were 77 pM for T3 and rT3 and 64 pM for T4.

RESULTS

The results from the experiments with the 6 benzothiazoles are presented below in order of decreasing potency in the TPO inhibition assay. MBT and CMBT were the most potent and are presented first, followed by ABT and BTZ which gave less response in the TPO assay, and the 2 chemicals which did not inhibit TPO are presented last.

2-Mercaptobenzothiazole

MBT was a potent inhibitor of TPO *in vitro* with an IC50 of 12 μM (Fig. 1A). This was approximately 1/10th the potency of methimazole in this assay which had an IC50 of 1 μM. MBT was also the most potent of the group in the thyroid explant culture assay (Fig. 1B). Thyroid hormone release from the cultured glands was inhibited by 50% at 3 μM, with no reduction of glandular ATP compared with controls when tested up to 100 μM. For methimazole, the IC50 for T4 inhibition was near 10 μM and no decrease in ATP was detected in explant thyroid glands exposed to methimazole for 8 days at concentrations up to 1000 μM (data not shown). The difference in activity between decreased thyroid hormone release by the glands and overt toxicity was greater than 100-fold for this model T4 synthesis inhibitor.

MBT was also a very potent thyroid hormone disruptor *in vivo*. Following waterborne exposure of tadpoles for 7 days to 2.1 μM (0.350 mg/l) MBT, the thyroid hormone levels in the thyroid gland itself were significantly reduced compared with controls (Table 2). Whereas T3 only exhibited a modest decrease to 71% of control levels, the T4 in glands from MBT exposed tadpoles was 4% of that in the controls. MIT and DIT were decreased to 65% and 18%, respectively, compared with that of controls. Circulating levels of hormone were also decreased with serum concentration of T4 reduced to 33% of control levels. NIS mRNA expression in the thyroid glands from the MBT exposed tadpoles was significantly increased to 225% of that in control tadpoles, indicative of a compensatory response of increased TSH to the decreased circulating TH (Fig. 2).

5-Chloro-2-mercaptobenzothiazole

CMBT showed similar potency to MBT in the TPO inhibition assay with an IC50 of 13 μm (Fig. 1C). This chemical reduced T4

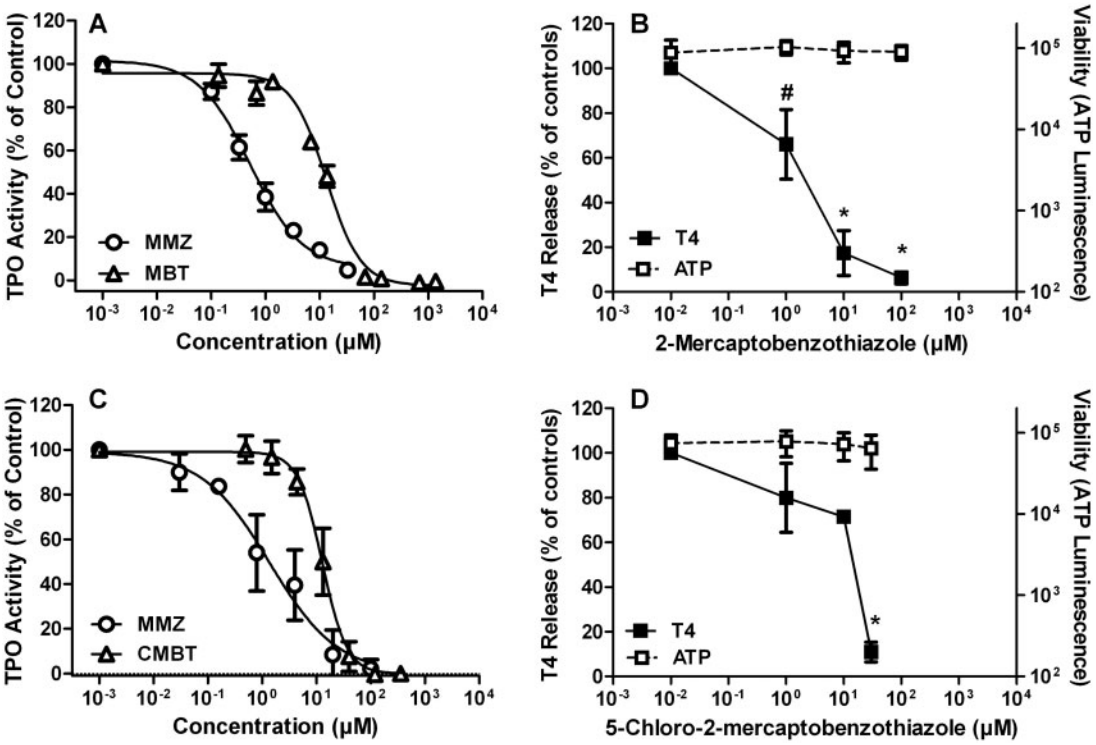

FIG. 1. *In vitro* TPO activity and *ex vivo* T4 release from thyroid gland explant cultures. TPO activity assays (A and C) include methimazole (MMZ; ○) inhibition curves that were included as a positive control in parallel with each test chemical run (△; 2-mercaptobenzothiazole; MBT; panel A, or in panel C 5-chloro-2-mercaptobenzothiazole; CMBT). Symbols represent mean of the 3 separate determinations of activity normalized to control activity as 100%, using 3 separate batches of microsomes. Panels B and D show inhibition of T4 release (filled symbols) in thyroid gland explants exposed for 8 days to graded concentrations of benzothiazoles and concurrent stimulation with bovine TSH. Glandular ATP (open symbols) was determined at the end of the 8 d test period. Symbols show means from at least 2 separate experiments. Asterisks (*) indicate where T4 release or ATP was significantly different from controls in both experimental runs, a pound sign (#) indicates where the response was lower than controls in only one of the experiments.

TABLE 2. *In Vivo* Thyroid Hormone Levels and NIS Expression Following Exposure to 2-Mercaptobenzothiazole (MBT) or 5-Chloro-2-mercapto-benzothiazole (CMBT)[a,b]

	Gland Hormones (pmole/gland)				Blood T4	NIS mRNA
	MIT	DIT	T3	T4	(pmole/l)[d]	% control
Control	1.27 (0.1)	7.17 (0.8)	0.049 (0.003)	6.17 (0.47)	1421 (332)	100 (10)
MBT	0.82 (0.12)*	1.26 (0.2)*	0.035 (0.005)*	0.26 (0.08)*	472 (117)*	225 (14)*
effect	↓	↓	↓	↓	↓	↑
Control	1.35 (0.44)	7.34 (2.3)	0.08 (0.03)	4.89 (1.1)	1098 (505)	100 (10)
CMBT	0.49 (0.09)*	0.66 (0.13)*	<LLOQ[c]	0.10 (0.04)*	107 (20)*	359 (35)*
effect	↓	↓	↓	↓	↓	↑

[a]Values are mean (SD) of $n = 3$ tanks per treatment group
[b]Asterisks indicate significant difference from controls ($P < .05$) and arrows indicate direction of change (increase or decrease) of significant differences from controls
[c]LLOQ is lower limit of quantitation. In gland samples LLOQ in pmole/gland for T3 was 0.01 pmole/gland
[d]For reference: 1000 pmole T4/l is equivalent to 0.78 ng T4/ml

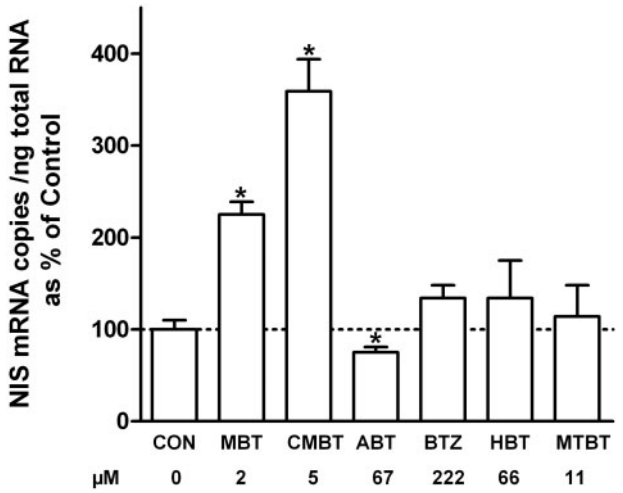

FIG. 2. Effects of benzothiazoles on NIS mRNA in thyroid glands of tadpoles at termination of the 7-day exposure. Bars show mean and standard deviation of $n = 3$ tanks per treatment level. Asterisks indicate responses significantly different from controls within the specific experiments by *t*-test at $P < .05$. Rather than show the controls from the 4 *in vivo* experiments, a single control is shown with 100% response and a SD of 10% around the control NIS mRNA measurement representing the variability around the control NIS mRNA response across the 4 separate *in vivo* experiments.

release from thyroid gland explants at 30 μM (Fig. 1D). Glandular ATP dropped to background levels at concentrations of CMBT 120 μM and above in an initial range finding study (data not shown).

Following waterborne exposure to 5 μM (1 mg/l) CMBT, all of the glandular thyroid hormones and iodotyrosines were significantly reduced compared with controls, as was found for MBT (Table 2). Thyroid hormone levels in serum were also significantly reduced to 10% of that of controls. The NIS mRNA response in the thyroid gland reflected the compensatory response to reduced circulating hormone with an increased expression to 359% of that of controls (Fig. 2). Thus addition of the chlorine atom to the benzene ring (CMBT) while keeping the exposed thiol group on the 2-carbon of the thiazole ring did not significantly affect the inhibitory activity compared with MBT (Fig. 4).

2-Aminobenzothiazole

The 2-amino substituted benzothiazole exhibited nearly full inhibition of TPO activity with an IC50 of 1200 μM (Fig. 3A), but was approximately 3-orders of magnitude less potent than methimazole. Changing the thiol group of MBT to an amine (Fig. 4) reduced the TPO inhibition potency by a factor of 100 (IC50 for MBT was 12 μM compared with 1200 μM for ABT), but there was still complete inhibition of the activity. In the explant culture assay ABT decreased T4 release at 100–300 μM (Fig. 3B). This occurred without any significant decrease in glandular ATP, until concentrations of chemical exceeded 950 μM (data not shown).

The response of the tadpoles exposed to 67 μM (10 mg/l) ABT *in vivo* for 7 days showed no effects on gland T4 levels. There were significant increases in gland MIT, DIT, and a slight but significant decrease in T3 (Table 3). Serum concentrations of T4 were not significantly different from controls, and as would be predicted in the absence of decreased circulating thyroid hormone the expression of NIS mRNA in the thyroid gland was not elevated above controls; to the contrary it was decreased to 75% of NIS mRNA abundance in the controls (Fig. 2).

Benzothiazole

The unsubstituted benzothiazole inhibited TPO activity by 40% at the highest concentration tested as shown in Figure 3C. In the explant culture, BTZ produced significant inhibition of T4 release at 457 μM which was approximately 1/10th the concentration at which toxicity was detected (Fig. 3D). The response of tadpoles *in vivo* to exposure to 222 μM (30 mg/l) BTZ resulted in a decrease in glandular T4 and T3, but no effect on MIT or DIT compared with controls (Table 3). Although serum T4 was lower than that in controls it was not significantly lower by *t*-test. BTZ did not affect NIS mRNA expression in thyroid glands (Fig. 2).

2-Hydroxybenzothiazole

For HBT the thiol of MBT is replaced by a hydroxyl group (Fig. 4) which reduced the TPO inhibition activity such that HBT only gave slight inhibition of TPO prior to concentrations where precipitation was evident. HBT inhibited TPO activity by 16% from the control activity at the highest concentration of 600 μM tested in this assay (Fig. 5A). This chemical inhibited T4 release from the glands at concentrations above 67 μM in the absence of a reduction in glandular ATP (Fig. 5B). ATP was not reduced at HBT concentration of 637 μM, but was decreased at 1900 μM (data not shown). HBT was the third most potent in this group of chemicals for inhibiting T4 release from the glands, but as indicated above it produced minimal inhibition of TPO activity at the highest concentration tested. The effects of HBT on thyroid endpoints in tadpoles exposed to 66 μM (10 mg/l) HBT were mixed. There were slight effects on glandular hormones

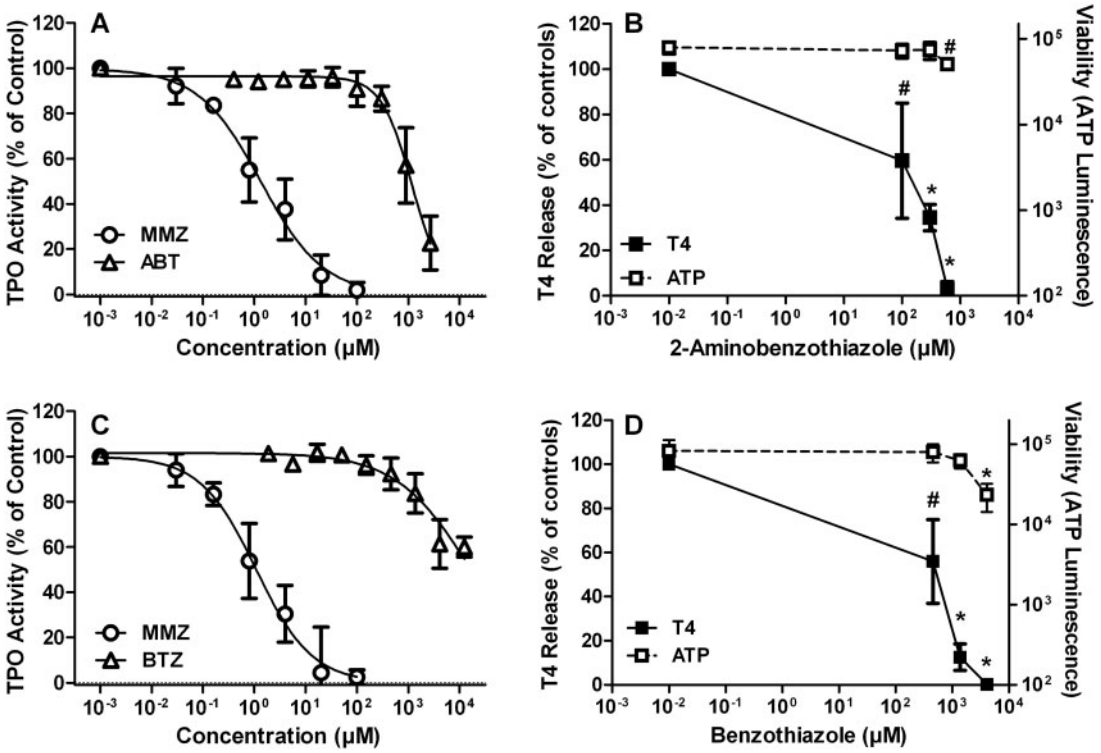

FIG. 3. *In vitro* TPO activity (Panels A and C) and *ex vivo* T4 release from thyroid gland explant cultures (Panels B and D) by 2-aminobenzothiazole (ABT) or benzothiazole (BTZ). Other information is the same as in the legend to Figure 1.

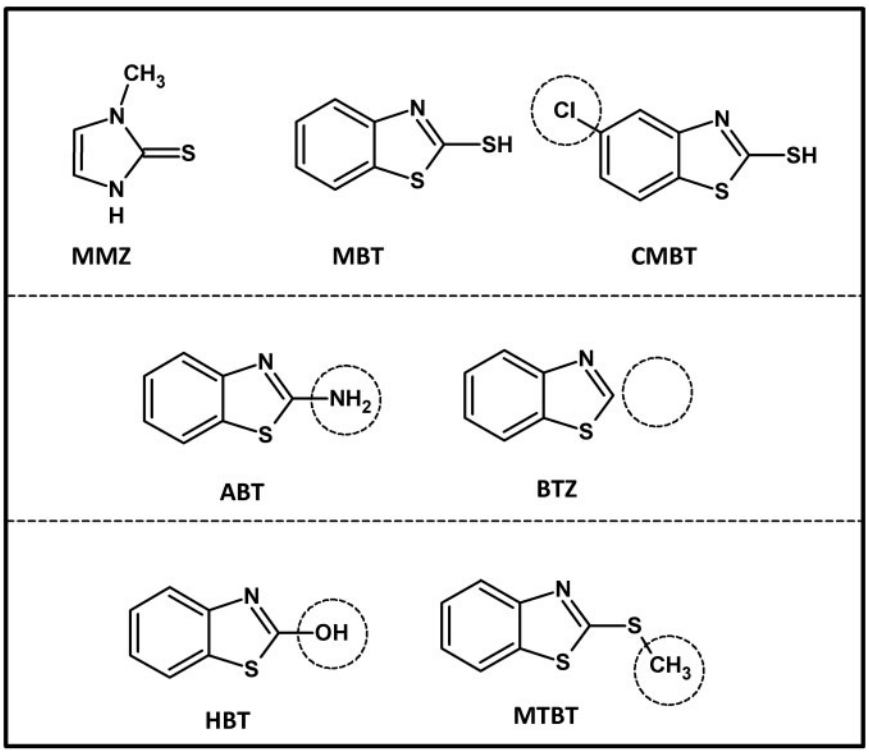

FIG. 4. Methimazole and the 6 benzothiazoles tested in the *in vitro*, *ex vivo*, and *in vivo* experiments. Acronyms: MMZ (methimazole), MBT (2-mercaptobenzothiazole), CMBT (5-chloro–mercaptobenzothiazole), MTBT (2-methylthio-bezothiazole), BTZ (benzothiazole), ABT (2-aminobenzothiazole), and HBT (2-hydroxybenzothiazole). Structural differences between MBT and the 5 other benzothiazoles are indicated by the dashed circles.

TABLE 3. *In Vivo* Thyroid Hormone Levels and NIS Expression Following Exposure to 2-Aminobenzothiazole (ABT) or Benzothiazole (BTZ)[a,b]

	Gland Hormones (pmole/gland)				Blood T4	NIS mRNA
	MIT	DIT	T3	T4	(pmole/l)	% control
Control	1.27 (0.1)	7.17 (0.8)	0.049 (0.003)	6.17 (0.47)	1421 (331)	100 (10)
ABT	1.98 (0.33)*	12.9 (1.7)*	0.042 (0.004)*	6.68 (0.71)	1455 (537)	75 (6)*
effect	↑	↑	↓	–	–	↓
Control	1.31 (0.22)	6.36 (1.22)	0.045 (0.003)	5.20 (1.00)	1352 (860)	100 (10)
BTZ	1.64 (0.36)	10.9 (2.71)	0.018 (0.002)*	1.20 (0.35)*	257 (39)	134 (14)
effect	–	–	↓	↓	–	–

[a]Values are mean (SD) of $n = 3$ tanks per treatment group
[b]Asterisks indicate significant difference from controls ($P < .05$) and arrows indicate direction of change (increase or decrease) of significant differences from controls

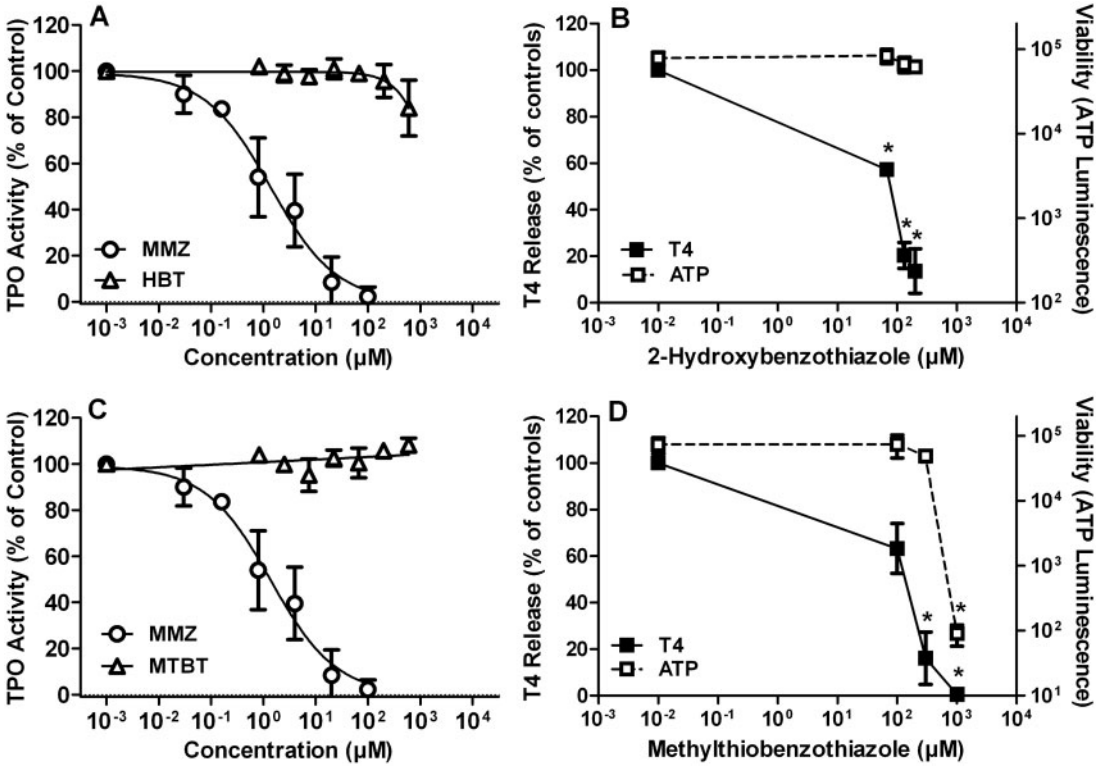

FIG. 5. *In vitro* TPO activity (A and C) and *ex vivo* T4 release from thyroid gland explant cultures (B and D) by 2-hydroxybenzothiazole (HBT) or 2-methylthio-benzothiazole (MTBT). Other information is the same as in the legend to Figure 1.

showing elevated MIT, DIT, and T3 levels and decreased T4 (Table 4). There was no significant difference in circulating T4 in HBT exposed versus controls tadpoles and NIS mRNA in the thyroid glands from these tadpoles was not significantly different from controls (Table 4; Fig. 2).

2-Methylthio-benzothiazole

MTBT showed no significant inhibition of TPO activity (Fig. 5C). This chemical inhibited T4 release from *X. laevis* thyroid gland explants at concentrations of 300 μM and above and toxicity as determined by decreased glandular ATP was detected at 1000 μM (Fig. 5D). In the 7-day *X. laevis* tadpole study MTBT was originally tested at 5 mg/l (28 μM) and produced some toxicity as determined by severely reduced growth and the observation that the serum appeared clear compared with the yellowish color of serum from both the controls and the CMBT-exposed tadpoles run in the same experiment. Therefore MTBT was retested at 11 μM (2 mg/l). At this lower concentration MTBT did not produce any changes in glandular thyroid hormone levels compared with controls and no significant decrease in circulating T4 as determined by *t*-test (Table 4). There was also no significant effect on glandular NIS mRNA compared with controls (Fig. 2). Thus the addition of a methyl group to the exposed thiol which is the only structural difference between MBT and MTBT (Fig. 4) completely eliminated the TPO inhibition activity.

Morphometric measurements

Tadpole body weight and snout-vent length were not significantly affected by exposure to these benzothiazoles in the *in vivo* 7-day experiments except for decreased wet weight of the CMBT and MTBT exposed tadpoles; but these showed no significant decrease in snout-vent length (Supplementary Materials, Table S3). The distribution of tadpoles across the 3 NF stages at the termination of the experiments was not

TABLE 4. *in vivo* Thyroid Hormone Levels and NIS Expression Following Exposure to 2-Hydroxybenzothiazole (HBT) or 2-Methylthio-Benzothiazole (MTBT)[a,b]

	Gland Hormones (pmole/gland)				Blood T4	NIS mRNA
	MIT	DIT	T3	T4	(pmole/l)	% control
Control	1.31 (0.22)	6.36 (1.22)	0.045 (0.003)	5.20 (1.00)	1352 (860)	100 (10)
HBT	2.71 (0.41)*	12.8 (2.0)*	0.070 (0.01)*	2.56 (0.53)*	796 (234)	134 (41)
effect	↑	↑	↑	↓	–	–
Control	1.24 (0.083)	7.16 (1.05)	0.033 (0.007)	5.86 (0.83)	1509 (887)	100 (10)
MTBT	1.32 (0.16)	8.43 (1.2)	0.046 (0.007)	4.49 (0.81)	693 (171)	114 (34)
effect	–	–	–	–	–	–

[a]Values are mean (SD) of $n = 3$ tanks per treatment group

[b]Asterisks indicate significant difference from controls ($P < .05$) and arrows indicate direction of change (increase or decrease) of significant differences from controls

necessarily diagnostic of thyroid effects in this short duration assay (Supplemental Materials, Table S4). MBT delayed development as might have been expected for this chemical, but CMBT which also affected all other thyroid endpoints (see above) did not affect developmental stage. ABT showed some indication of delaying development, but BTZ, HBT, and MTBT produced little effect on altering the developmental stage distribution in this short duration assay. Although rT3 and T3 were analyzed for in the serum samples from these experiments, the concentrations were generally near or below their LLOQ (see Methods section) so were not presented here. Similarly, 3,3′-T2 and 3,5-T2 were analyzed for in the thyroid glands but all determinations were below the LLOQ.

DISCUSSION

Utilization of *in vitro* assays for assessing chemical activity toward thyroid axis endpoints has become a topic gaining international attention (Murk et al. 2013; OECD 2014). TPO is a critical enzyme necessary for maintaining adequate levels of thyroid hormone, but there have been limited systematic approaches toward understanding the number and types of chemicals that can inhibit this enzyme's activity. To address this information gap, methods are being developed that will lead to higher throughput testing of chemicals for TPO inhibiting activity. Use of cell lines to express recombinant enzyme may provide a reliable source of enzyme and some success with this approach has been reported using human TPO to investigate TPO inhibition by UV filtering chemicals (Schmutzler et al. 2007). Significant progress toward higher throughput screening has also been made recently in the development of a stable endpoint for TPO inhibition using a fluorescent substrate and adapting the assay to a 384-well plate format (Paul et al., 2014).

As *in vitro* assays are developed for chemical hazard assessment for thyroid endpoints, the predictive capacity of these assays also needs to be understood within the context of the toxicity pathway of concern. A research approach that uses an Adverse Outcome Pathway (AOP) framework can help to define and select the assays required to make strong predictive models of chemical action and downstream adverse effects (Ankley et al. 2010). Translating the activity of chemicals at the molecular initiating event (TPO enzyme inhibition) to the next key events in the AOP was explored in this study using a *X. laevis* thyroid explant assay with further verification in an *in vivo* assay.

The thyroid explant assay integrates the responses at the level of the gland, where decreased T4 synthesis via TPO inhibition would be expected to result in a decrease in the amount of T4 released to the media. However, all 6 of the benzothiazoles fully inhibited T4 release from the thyroid explant cultures, which was unexpected based on the differences in activity of the benzothiazoles in the *in vitro* TPO inhibition assay. Some benzothiazoles produced toxicity as indicated by decreased glandular ATP that might have contributed to the decreased hormone release. For others, ATP was not decreased, or ATP decreased at concentrations of chemical well above those that decreased T4 release. For chemicals of high potency for TPO inhibition such as the model TH synthesis inhibitors methimazole or propylthiouracil (Hornung et al. 2010), and MBT and CMBT in this study, the explant culture T4 release assay may provide an accurate indication of a specific effect on a thyroid hormone synthesis-to-release pathway. However, for chemicals of lower potency or no activity in the *in vitro* TPO inhibition assay, decreased T4 release from the explant cultured glands is not diagnostic of synthesis inhibition. In these cases, chemical concentrations that inhibit T4 release may also impact other cellular pathways (in a nonspecific manner) that affect the ability of the gland to release T4. It remains to be determined whether incorporating analysis of the suite of iodotyrosines and iodothyronines (MIT, DIT, T3, and T4) in the thyroid gland explant assay could be a more useful diagnostic and confirmatory endpoint of T4 synthesis inhibition than the release of T4 to the media.

Inhibition of mammalian (porcine) TPO in this study did identify chemicals with the potential to produce thyroid hormone disrupting effects in an amphibian *in vivo* model. The 2 most potent inhibitors of TPO activity *in vitro*, MBT and CMBT, also decreased gland hormone levels, decreased circulating thyroid hormone, and increased NIS mRNA in the thyroid gland, all *in vivo* responses consistent with T4 synthesis inhibition. The other benzothiazoles that showed low potency or no TPO inhibition *in vitro* either produced no effects *in vivo*, or the hormone and NIS mRNA responses did not clearly indicate a mechanism of thyroid hormone synthesis disruption. These results further support that TPO activity is a well-conserved mechanism and an *in vitro* inhibition assay using enzyme derived from one vertebrate class may be used to prioritize chemicals for further screening in other vertebrates.

In contrast to the high potency of MBT for thyroid disrupting effects in this amphibian model, MBT did not produce effects indicative of thyroid hormone disruption when tested in rats (U.S. NTP, 1988). Response to a chemical perturbation *in vitro* may indicate the potential for a chemical to impact a given pathway, but whether it actually has an impact *in vivo* in a particular species is dependent in large part upon absorption, distribution, metabolism, and elimination (ADME) factors. These

factors will influence whether sufficient dose of chemical gets to the site of action to produce an adverse effect. In the waterborne exposure of the *X. laevis* tadpoles to MBT, the majority of chemical uptake was most likely via the gills. When MBT was tested in rats they were exposed via oral dosing (U.S. NTP, 1988), so the route of exposure and first pass metabolism may significantly affect the amount of parent chemical that reaches the thyroid gland. Efforts to understand the ADME properties of diverse classes of chemicals and the conservation of these properties across vertebrate classes will be essential for taking *in vitro* assay results to applications beyond chemical prioritization.

The results from the thyroid explant culture demonstrated that it was not diagnostic of *in vivo* effects, which may be due in part to its dependence on detecting a decrease in signal. The integrated pathway in the thyroid gland leading to T4 release from these cultured glands is likely susceptible to general toxicity that may not be readily detected with a single, or multiple standard toxicity assays, such as ATP as measured in these experiments. Moreover, other off-pathway targets may be affected that impair the gland's response to TSH, in turn attenuating T4 release. Alternatively, the 7-day test in the tadpoles was more diagnostic of thyroid axis disruption than the explant culture; in terms of time, effort, and animal use, the 2 assays are similar suggesting that the 7-day *in vivo* tadpole test, at this time, is a better targeted follow-up assay to confirm *in vitro* results. However, because the majority of control tadpoles in this 7-day test only advance from NF stage 54 to stage 56, this endpoint is not robust enough to detect a developmental delay, an effect better detected in a longer duration test such as the 21-day AMA (OECD, 2009). Yet, the use of a well-designed and maintained static exposure system for the *in vivo* tests here was successfully demonstrated.

Finally, the analytical methods optimized using SPE for sample preparation and UHPLC-MS/MS with stable isotope-labeled internal standards to measure 2 iodotyrosines and 5 iodothyronines in gland samples, and 3 iodothyronines in plasma or serum samples, were not only critical for interpreting the *in vivo* responses to the benzothiazoles, but will be critical for diagnostic work going forward to investigate the effects of chemicals on the HPT-axis, whether in amphibians or other vertebrates. For example, this optimized method may provide the means to quantify circulating T4 in tadpoles as early as NF stage 54, which were at or below the limit of detection by HPLC/ICP-MS in a study by Sternberg et al. (2011). The use of SPE and UHPLC-MS/MS with multiple reaction monitoring eliminated complex sample matrix effects and produced high sensitivity and specificity. The method was capable of measuring low levels of iodotyrosines and iodothyronines in small gland samples with LLOQs of 0.01–0.02 pmole/gland and LLOQs of 64–80 pM for iodothyronines in 20 μl of plasma or serum which suggests this method should be adaptable to any small tissue samples where accurate determination of tissue hormone levels are required.

This study highlights several principles important for extrapolating *in vitro* assay data to *in vivo* effects and demonstrates conservation of responses starting at the molecular initiating event and extending across different levels of biological organization. The activity of the potent benzothiazoles toward inhibition of mammalian TPO *in vitro* did correspond to thyroid hormone disrupting effects in the *X. laevis* tadpoles *in vivo* as indicated by altered glandular and circulating thyroid hormones and increased NIS mRNA expression. This supports the use of *in vitro* TPO assays as a useful tool for prioritization of chemicals for further screening or testing within regulatory programs such as the US EPA's Endocrine Disruptor Screening Program.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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