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Development of a Thyroperoxidase Inhibition Assay for High-Throughput Screening

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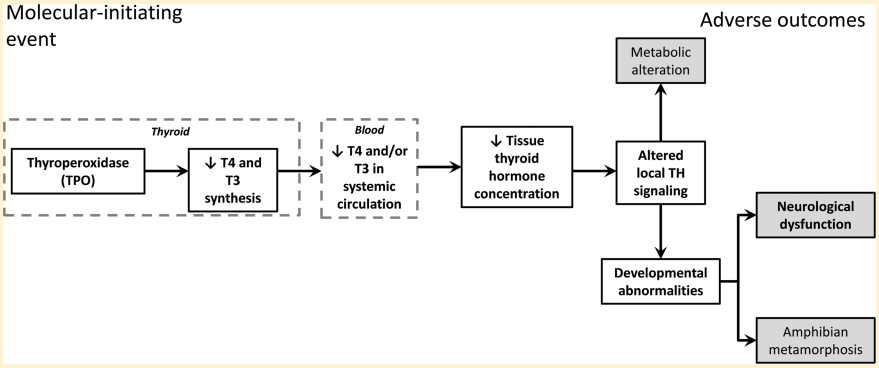
S *Supporting Information*



ABSTRACT: High-throughput screening (HTPS) assays to detect inhibitors of thyroperoxidase (TPO), the enzymatic catalyst for thyroid hormone (TH) synthesis, are not currently available. Herein, we describe the development of a HTPS TPO inhibition assay. Rat thyroid microsomes and a fluorescent peroxidase substrate, Amplex UltraRed (AUR), were employed in an end-point assay for comparison to the existing kinetic guaiacol (GUA) oxidation assay. Following optimization of assay metrics, including Z', dynamic range, and activity, using methimazole (MMI), the assay was tested with a 21-chemical training set. The potency of MMI-induced TPO inhibition was greater with AUR compared to GUA. The dynamic range and Z' score with MMI were as follows: 127-fold and 0.62 for the GUA assay, 18-fold and 0.86 for the 96-well AUR assay, and 11.5-fold and 0.93 for the 384-well AUR assay. The 384-well AUR assay drastically reduced animal use, requiring one-tenth of the rat thyroid microsomal protein needed for the GUA 96-well format assay. Fourteen chemicals inhibited TPO, with a relative potency ranking of MMI > ethylene thiourea > 6-propylthiouracil > 2,2′,4,4′-tetrahydroxy-benzophenone > 2-mercaptobenzothiazole > 3-amino-1,2,4-triazole > genistein > 4-propoxyphenol > sulfamethazine > daidzein > 4-nonylphenol > triclosan > iopanoic acid > resorcinol. These data demonstrate the capacity of this assay to detect diverse TPO inhibitors. Seven chemicals acted as negatives: 2-hydroxy-4-methoxybenzophenone, dibutylphthalate, diethylhexylphthalate, diethylphthalate, 3,5-dimethylpyrazole-1-methanol, methyl 2-methyl-benzoate, and sodium perchlorate. This assay could be used to screen large numbers of chemicals as an integral component of a tiered TH-disruptor screening approach.

INTRODUCTION

Thyroid hormones (THs) modulate myriad processes in adult and developing organisms in many target tissues, including brain, pituitary, heart, fat, liver, and bone, to maintain diverse functions ranging from metabolic and cardiac output to neurodevelopment.[1−3] Disruption of THs during neurodevelopment results in permanent changes, whereas the effects during adulthood are generally reversible with a return to euthyroid status.[4] Severe TH deficiencies during pregnancy result in neurological cretinism, indicated by sensory deficits and mental retardation.[1,5,6] Furthermore, the correlation between moderate TH disruption (i.e., maternal hypothyroxinemia) and irreversible neurodevelopmental brain abnormalities is well-established.[7−12] Even relatively small decreases in maternal thyroxine (T4) during pregnancy affect the IQ of children[13] as well as other measures of cognition, socialization, and motor function.[8,14−17]

Detection of possible thyroid-disrupting activity by environmental chemicals represents a salient need for a chemical-screening program designed to identify potential developmental neurotoxicants. Thyroid-disrupting chemicals (TDCs) include xenobiotics that may impact the structure or function of the

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thyroid gland, perturb enzyme functions that maintain euthyroid hormone concentrations, or disrupt systemic or tissue thyroid hormone concentrations.[18−20] TDCs may act via one or more of several molecular-initiating events, including inhibition of TH synthesis by disruption of iodide uptake or thyroperoxidase (TPO), binding to thyroid hormone transport proteins, interference with thyroid receptors systemically, inhibition of iodothyronine deiodinases at sites of thyroid action, and increased rates of metabolic clearance of THs.[20−22] Screening to identify TDCs will require a multiassay approach, as maintenance of euthyroid status involves a complicated system of regulatory processes in multiple organs.[23]

Toxicants that inhibit TPO activity prevent iodothyronine production in the thyroid gland.[22,23] TPO is a heme-containing, multifunction enzyme critical to TH synthesis located at the apical membrane of follicular thyroid cells.[24,25] TPO catalyzes the oxidation of iodide to hypoiodate, the addition of hypoiodate to tyrosyl residues on thyroglobulin (Tg), and concurrent oxidative coupling of iodinated tyrosyl residues to form iodothyronine hormones triiodothyronine (T3) and T4.[26−28] The antithyroid medication methimazole (MMI) corrects hyperthyroidism by decreasing TH synthesis via TPO inhibition.[29] Many other compounds are known to inhibit TPO activity. The antithyroid drug 6-propylthiouracil (PTU),[29−31] the ultraviolet filter benzophenone-2 (BP2),[32] isoflavones,[33] the parasiticide malachite green,[34] and ethylene bisdithiocarbamate pesticides including mancozeb, ziram, and zineb[35,36] have all inhibited TPO. A lack of medium- and high-throughput screening assays for TPO inhibitors has limited large-scale screening efforts to identify additional chemicals with potential endocrine-disrupting activity through this specific mode of action.[23]

Previous efforts to identify xenobiotics that alter TPO activity have primarily used models including purified peroxidases,[37−39] thyroid follicles,[31] or microsomes in spectrophotometric assays to measure the TPO-catalyzed iodination of tyrosine to yield monoiodotyrosine[38] or the oxidation of guaiacol to dihydroxy-dimethoxy-diphenyl.[30,33] TPO has a C-terminal extension thought to be the transmembrane anchor of the protein that is notably absent from other human peroxidases, including lactoperoxidase (LPO), myeloperoxidase, and eosinophil peroxidase, making TPO more difficult to express and purify.[40] Due to the high sequence homology and conservation of catalytic activity among human peroxidases,[28,40,41] purified LPO activity has been utilized as a commercially available surrogate for TPO activity to identify TPO inhibitors successfully.[28,38,39] Cultured porcine thyroid follicles were previously used to demonstrate TPO inhibition by MMI using the guaiacol oxidation assay as well as simultaneous increases in TPO mRNA at high MMI concentrations.[31] More recently, guaiacol oxidation by TPO-containing thyroid microsomes has been used to identify the in vitro TPO inhibition activity of a plethora of chemicals.[33,35,42,43] The rubber vulcanization accelerant, 2-mercaptobenzothiazole (MBT), a high-production-volume chemical intermediate for benzothiazole derivatives, was identified as a porcine microsomal TPO inhibitor and as a thyrotoxicant using amphibian models.[44]

The U.S. Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP) is currently tasked with determining how to screen for potential endocrine effects of chemicals. Because of the multitarget nature of TH disruption, a battery of assays will be needed for screening and prioritization of potential TDCs. Currently, a high- or medium-throughput screening assay to detect TPO inhibitors is unavailable, owing largely to the dependence on low-throughput guaiacol oxidation chemistry. The guaiacol oxidation assay requires absorbance measurements during a 60 s kinetic reaction to monitor the development of an unstable diguaiacol product and requires the use of a large amount of thyroid microsomal protein per test.[43,44] The critical need for high-throughput assays to assess multiple molecular-initiating events for TH disruption is underscored by the lack of association between rodent thyrotoxicity and high-throughput screening (HTPS) assays for thyroid hormone receptor interactions.[45] In the present work, we propose to adapt an existing rat thyroid microsome assay to a high-throughput assay for screening that could be used to screen hundreds to thousands of chemicals for potential effects on TPO activity. This work tested the hypothesis that a commercial fluorescent horseradish-peroxidase substrate, Amplex UltraRed (AUR), could be used to detect TPO activity in thyroid microsomes obtained from rats to generate an in vitro 384-well format assay that can be used in a high-throughput screening application. Implementation of this assay drastically reduces the need for animal tissue and provides a valuable intermediary approach to anchor in vitro screening data from this model or from potential in vitro cell-based systems to effects on TH homeostasis in whole-animal models. As part of the optimization process, a comparison between the performance of a 96-well format guaiacol oxidation assay and 96-well as well as 384-well formats of the AUR-TPO assay are demonstrated. Importantly, we report a novel method for the AUR-TPO assay along with its associated assay metrics and a training-set-based assay verification. We propose that the AUR-TPO assay could be used for high-throughput screening to identify potential TPO inhibitors.

METHODS

Animals. Untreated male Long Evans rats (68−72 days old) were obtained from Charles River Laboratories Inc. (Raleigh, NC) in groups of 60 and acclimated 1−7 days in an American Association for Accreditation of Laboratory Animal Care International (AALAC)-approved animal facility. Rats were pair-housed in plastic hanging cages (45 × 24 × 20 cm^3), with heat-sterilized pine-shavings bedding (Northeastern Products Corp., Warranton, NC). Colony rooms were maintained at 21 ± 2 °C with 50 ± 10% humidity on a photoperiod of 12 h light/12 h dark. Food (Purina Rodent Chow no. 5001, Barnes Supply Co., Durham, NC) and water were provided ad libitum. Tap water (Durham, NC water) was filtered through sand and activated charcoal and rechlorinated to 4 to 5 ppm Cl^- before use in the animal facility. Rats were decapitated, and thyroid glands were removed from the trachea, weighed (approximately 10 mg per lobe), frozen in liquid nitrogen, and stored at −80 °C until use. To prevent loss of tissue, decapitation was performed with the shoulders of the animal approaching the guillotine, such that the thyroid glands were typically localized to a trachea portion separated from the trunk of the carcass. Euthanasia by decapitation was approved for this and other thyroid hormone studies in this laboratory because the acute stress induced by carbon dioxide asphyxiation would potentially result in altered thyroid hormones and neurochemistry.[46,47] No anesthetics or analgesics were used because these drugs can alter the expression of hepatic metabolic enzymes involved in the regulation of thyroid hormones.[48−50] All animal procedures were approved in advance by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory of the U.S. EPA.

Thyroid Microsomes. Rat thyroid microsomes pools were made from 60 pooled rat thyroids totaling 1−1.5 g of tissue; only a fraction of the microsome pools generated were used for this work. Thyroid microsomes were prepared as described previously.[33,43] Briefly, frozen

Table 1. 21-Chemical Training Set Description and Source

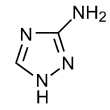
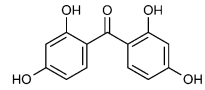
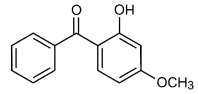
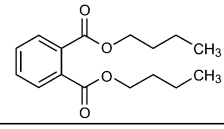
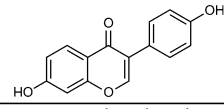
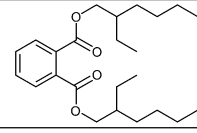
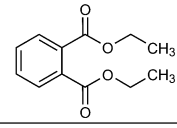
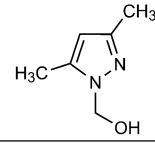
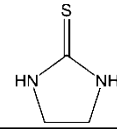
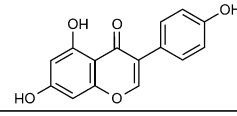
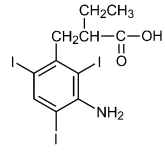
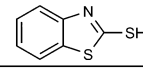
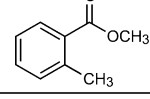
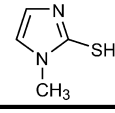
#	Chemical Name	Abbr.	CAS #	Source	Structure
1	3-Amino-1,2,4-Triazole (Amitrole)	AMT	61-82-5	Sigma-Aldrich	
2	2,2',4,4'-Tetrahydroxy-benzophenone	BP2	131-55-5	Aldrich	
3	2-hydroxy-4-methoxybenzophenone	BP3	131-57-7	Aldrich	
4	Dibutylphthalate	DBP	84-74-2	Aldrich	
5	Daidzein	DDZ	486-66-8	Sigma-Aldrich	
6	Diethylhexylphthalate	DEHP	117-81-7	Fluka	
7	Diethylphthalate	DEP	84-66-2	Sigma-Aldrich	
8	3,5-Dimethylpyrazole-1-methanol	DPM	85264-33-1	Aldrich	
9	Ethylene thiourea	ETU	96-45-7	TCI	
10	Genistein	GEN	446-72-0	Sigma-Aldrich	
11	Iopanoic Acid	IOA	96-83-3	TCI	
12	2-mercaptobenzothiazole	MBT	149-30-4	Fluka	
13	Methyl 2-methyl-benzoate	MMB	89-71-4	Aldrich	
14	Methimazole	MMI	60-56-0	Sigma	

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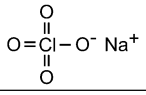
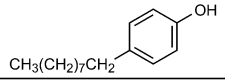
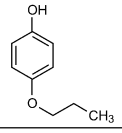
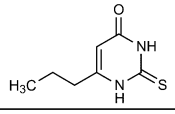
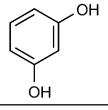
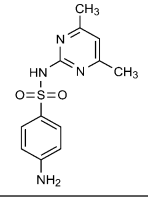
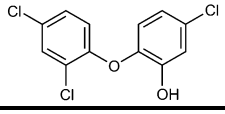
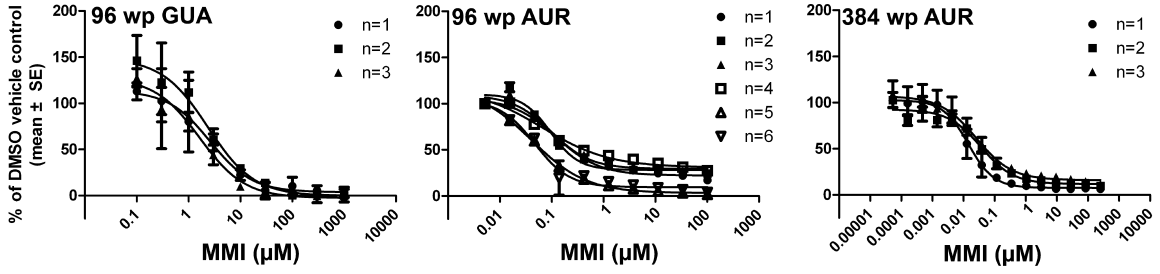
Table 1. continued

#	Chemical Name	Abbr.	CAS #	Source	Structure
15	Sodium Perchlorate	NaPER	7601-89-0	Sigma	
16	4-nonylphenol	4NP	104-40-5	Fluka	
17	4-propoxyphenol	4POP	18979-50-5	Aldrich	
18	6-propylthiouracil	PTU	51-52-5	Sigma	
19	Resorcinol	RSC	108-46-3	Sigma-Aldrich	
20	Sulfamethazine	SMZ	57-68-1	Sigma-Aldrich	
21	Triclosan	TCS	3380-34-5	Aldrich	



Assay Format	96-GUA	96-AUR	384-AUR
Dynamic range (mean ± SE)	127 ± 80	18.2 ± 10	11.5 ± 2.4
Z' (mean ± SE)	0.62 ± 0.16	0.86 ± 0.025	0.93 ± 0.021
Average CV (%)	92.0	6.24	16.1
IC_{50}	2.20	0.093	0.025
N	3	6	3
μg protein/reaction	50	12.5	5

Figure 1. Assay performance with MMI in 96- and 384-well formats. All assay data were normalized to the DMSO vehicle-control value for each plate run. The average DMSO vehicle values by plate were 1.95, 2.83, and 2.95 absorbance units for the 96-well plate GUA assay; 10 476, 10 508, 24 410, 34 217, 41 088, and 46 646 relative fluorescence units for the 96-well plate AUR assay; and 40 340, 33 958, and 44 074 relative fluorescence units for the 384-well plate AUR assay.

thyroid glands 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 0.01 g of thyroid tissue. This homogenate was processed briefly with a Teflon mortar and pestle. This homogenate was then centrifuged at 29.4*g* for 10 min at 4 °C to remove larger debris and then ultracentrifuged at 151 515*g* for 60 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in homogenization buffer (without catalase) with 0.25 mL of buffer per 0.01 g of thyroid tissue using a Teflon-coated mortar and pestle. Glycerol (5%) was added to the final microsomal preparation, and aliquots were stored at −80 °C until they were thawed one time for use. A Bradford protein assay was performed to determine the total protein content of each of the microsome lots used in this study; the mean protein content of the

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five sample pools used in this study was 0.87 mg/mL, with a range of 0.65−1.12 mg/mL.

Chemicals. The chemical names, abbreviations, CAS nos., and sources for all chemicals are presented in Table 1. MMI was used for initial assay development as the positive-control compound for validation. A 21-chemical training set, including MMI, was employed to demonstrate the performance of the assay. Fourteen chemicals in the training set had been previously reported as TPO inhibitors (MMI, AMT, BP2, DDZ, DPM, ETU, GEN, MBT, 4NP, 4POP, PTU, RSC, NaPER, and SMZ), and seven of the chemicals in the training set were selected because of previously reported negative activity (BP3, DBP, DEHP, DEP, IOA, MMB, and TCS). The phthalates (DEP, DBP, and DEHP), IOA, and TCS have previously demonstrated effects on thyroid-related end-points but via mechanisms other than TPO inhibition; NaPER acts predominantly via NIS inhibition but has demonstrated TPO inhibition at millimolar concentrations in guaiacol oxidation assays.[43] All chemicals were solubilized in DMSO at 62 or 124 mM.

Guaiacol Oxidation Assay. This assay has been described previously for rat microsomes.[33,43,44] The guaiacol oxidation assay is used to measure peroxidase activity; H_2O_2 is the hydrogen donor for TPO-mediated oxidation of guaiacol to yellowish-brown diguaiacol, detected spectrophotometrically over a 60 s kinetic period. All reactions were conducted in 96-well plates with a final reaction volume of 200 μL. Thyroid microsomes (50 μg total or 0.25 μg/L) were incubated with 35 mM guaiacol with test-chemical exposure at 37 °C for 30 s, 300 μM H_2O_2 in 100 mM potassium phosphate buffer (pH 7.4) was added to initiate the reaction, and the oxidation of guaiacol was monitored spectrophotometrically at 450 nm for 120 s. The kinetic reaction was monitored during the linear phase (i.e., 60 s), and the change in absorbance was recorded for analysis. Three biological replicates (n = 3) and three technical replicates per concentration of MMI were employed to determine the optimized assay parameters reported in Figure 1.

Amplex UltraRed TPO (AUR-TPO) Inhibition Assay. Amplex UltraRed (AUR) (Life Technologies, cat. no. A36006) is sold for the sensitive detection of H_2O_2 released from biological samples, including cells, in the presence of excess horseradish peroxidase; AUR is a fluorogenic substrate that is converted from AUR to Amplex UltroxRed by horseradish peroxidase in the presence of H_2O_2. We have repurposed the AUR substrate to detect peroxidase activity (i.e., TPO activity) in the presence of excess H_2O_2 for the AUR-TPO assay. The 96- and 384-well-format AUR-TPO assays contained rat thyroid microsomes, 200 mM potassium phosphate buffer, AUR diluted from a 10 mM DMSO stock in 200 mM phosphate buffer, and H_2O_2. The reaction profile for the 96-well-plate AUR-TPO assay was as follows: 25 μM AUR (75 μL), 12.5 μg of total microsomal protein (10−15 μL), 200 mM phosphate buffer to bring the total assay volume of microsomes plus buffer to 100 μL, and 300 μM H_2O_2 (25 μL), for a total assay volume of 200 μL. The reaction profile for the 384-well-format AUR-TPO assay is scaled to a total assay volume of 80 μL containing 25 μM AUR (50 μL), 5 μg of total microsomal protein (3−8 μL) diluted into 200 mM potassium phosphate buffer to 12 μL volume, and 300 μM H_2O_2 (18 μL). The concentration of thyroid microsomal protein was 0.0625 μg/mL for both the 96- and 384-well formats. First, microsomes and diluent 200 mM potassium phosphate buffer were added to the microplates using an automatic repeating multichannel pipettor (either 100 or 12 μL by plate format). AUR was then added by an automated liquid handler (BioTek MicroFlo; Winooski, VT) with a plastic 5 μL tubing cassette. Test compounds were administered using a BioMek 2000 automated laboratory workstation (Beckman Coulter; Brea, CA) equipped with a high-density replicating tool (HDRT) fitted with either a 96- or 384-pintool with 200 nL slot pins (V&P Scientific; San Diego, CA); both pintools deliver a fixed volume of 327 nL using stainless steel pins that dip into a compound plate and then into the assay plate. The H_2O_2 (300 μM, final concentration) was then added by the BioTeck MicroFlo to initiate the reaction. Microplates were shaken on a fluorescence plate reader (FLUOStar Optima, BMG LabTech; GmbH, Germany) and incubated for 30 min in the dark plate reader until end-point fluorescence was measured using 10 flashes per well at 544 nm/590 nm excitation/emission. The assays were run using opaque, black 96- or 384-well plates (Greiner, BioExpress; Kaysville, UT) and read by the fluorometer from the top position. Six biological replicates and technical duplicates of each MMI concentration used were employed to determine the optimized assay metrics for the 96-well format of the AUR-TPO assay (Figure 1).

Screening the 21-Chemical Training Set. For the 21-chemical training set, a single compound plate containing all control and test compound dilutions in DMSO was prepared from chemical stocks and stored at room temperature, away from light, in a desiccator. All training-set experiments with this compound plate were conducted within 2 weeks of the compound-plate preparation. The compound plate was made using 100% DMSO as the diluent; 15 concentrations of each chemical were tested. The compound plate concentrations were 0, 0.00013, 0.00039, 0.00012, 0.00035, 0.0011, 0.0032, 0.0095, 0.028, 0.085, 0.26, 0.77, 2.3, 6.9, 21, and 62 mM, yielding final concentrations in the AUR-TPO assay of 0, 0.000053, 0.00016, 0.00048, 0.0014, 0.0043, 0.013, 0.039, 0.12, 0.35, 1.0, 3.1, 9.4, 28, 84, and 253 μM. The AUR-TPO assay was run using this compound plate on three separate days, with freshly prepared reagents each day, and each with three separate pooled lots of thyroid microsomes for 3 biological replicates or n = 3. Technical duplicates of MMI were performed for every biological replicate (i.e., each MMI concentration appeared twice in each experiment with the training set). There were no technical replicates employed for the test chemicals (i.e., concentrations for a particular chemical were present in a single well per biological replicate).

Data Analysis. For assay development, data were analyzed as a percent of DMSO vehicle control using the raw end-point data for this normalization; the Z' factor,[51] the dynamic range (or signal to background), and average assay coefficient of variation (CV) were calculated using these data in Microsoft Excel (version 7 for Windows). The Z' factor was calculated as follows

$$Z' = 1 - \left(\frac{3\text{SD of positive control signal} + 3\text{SD of DMSO control}}{|\text{mean of positive control signal} - \text{mean of DMSO control}|} \right)$$

and is a measure of assay quality, with a Z' of 0.5−1.0 corresponding to an assay with a suitably high signal-to-background difference and a sufficiently low intersample variability to discriminate clearly between positive and negative test chemicals. A score of less than 0.5 means that the positive and negative signals in the assay are converging, and it may be difficult to discern positive from negative behavior in an HTPS assay with such a low Z' score.[51]

The IC_{50} values for the optimized GUA-TPO and AUR-TPO assays reported in Figure 1 were predicted with a four-parameter Hill model using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA). For assay confirmation with the training set, concentration−response data were analyzed using scripts developed for this purpose, run using R version 2.15.1 (scripts available upon request) (R Core Team 2012). The data were obtained as raw fluorescence units and were first normalized to the mean DMSO vehicle-control value for each replicate, n, obtained from the average of 54 separate wells of vehicle control on each plate and refined by removal of any values that were greater than ±3 times the standard deviation of the mean. The threshold for a positive response in the assay was arbitrarily set at a 20% change from the normalized vehicle-control value. Curves were fit to the data using a four-parameter Hill model, with the Hill slope constrained from −1 to −8, and the top of the inhibition curves constrained to 90−110%. A predicted IC_{50} value was derived from the Hill fit by determining the concentration at 50% of the dynamic range of the fitted response curve. A relative IC_{50} value was derived by determining the 50% inhibitory concentration that corresponds to a strict 50% response. The lowest effect concentration (LEC) was the first measured concentration to surpass the threshold for positive activity (20% activity decrease). The predicted and relative IC_{50} values, maximum response (E_{max}), top limit of the fit (T_{lim}), goodness of fit (r^2), and LEC were calculated and are reported in Table 2. Relative potency to

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Table 2. Hill Model Fit and Potency Data for the 14 Positive TPO Inhibitors in the 21-Chemical Training Set

chemical[a]	predicted IC_{50} (μM)[b]	relative IC_{50} (μM)[c]	E_{max} (% activity)[d]	T_{lim} (% activity)[e]	Hill slope[f]	r^2	lowest effect concentration (LEC, μM)[g]	relative potency to MMI (%)[h]
MMI	0.025	0.031	9.16	98.6	−1.00	0.89	0.0129	100
ETU	0.034	0.038	8.54	107	−1.00	0.97	0.0129	82
PTU	0.12	0.12	5.74	100	−1.00	0.97	0.116	26
BP2	0.16	0.18	9.41	112	−1.00	0.98	0.116	17
MBT	0.45	0.51	3.16	101	−1.00	0.96	0.116	6.1
AMT	1.1	1.3	16.5	111	−1.00	0.96	1.04	2.4
GEN	4.5	6.4	23.9	110	−1.00	0.93	3.13	0.48
4POP	5.3	8.5	16.5	106	−1.01	0.94	3.13	0.36
SMZ	18	25	24	111	−1.03	0.92	28.2	0.12
DDZ	23	33	32.4	111	−1.00	0.92	28.2	0.094
4NP	44	59	42.4	112	−1.00	0.86	28.2	0.053
TCS	142	184	48.9	110	−1.40	0.87	253	0.017
IOA	160	232	58.8	110	−1.00	0.76	253	0.013
RSC	253	317	77.1	111	−1.50	0.48	253	0.0098

[a]Chemical abbreviations: MMI, methimazole; ETU, ethylene thiourea; PTU, 6-propylthiouracil; BP2, benzophenone-2 or 2,2′-4,4′-tetrahydroxybenzophenone; MBT, 2-mercaptobenzothiazole; AMT, amitrole or 3-amino-1,2,4-triazole; GEN, genistein; 4POP, 4-propoxyphenol; SMZ, sulfamethazine; DDZ, daidzein; 4NP, 4-nonylphenol; TCS, triclosan; IOA, iopanoic acid; RSC, resorcinol. [b]Predicted IC_{50} is the 50% inhibitory concentration derived for the data reported herein using a Hill fit to model a top-to-bottom distance and dividing this distance by 2. [c]Relative IC_{50} is the 50% inhibitory concentration derived for the data reported herein that corresponds to the 50% response level on the y axis. [d]E_{max} (% activity) is the maximum inhibition response observed expressed as percent of DMSO vehicle control. [e]The top of the Hill fit (T_{lim}) was constrained within 90−110% of vehicle control. [f]The model Hill slope was constrained from −1 to −8. [g]The lowest effect concentration (LEC, μM) corresponds to the first tested concentration that demonstrated a ≥20% decrease in TPO activity. [h]Relative potency to MMI (%) is calculated as the relative IC_{50} value for MMI divided by the relative IC_{50} for the chemical and multiplied by 100.

MMI (%) was determined as the relative IC_{50} value for MMI divided by the relative IC_{50} value for the training-set chemical, multiplied by 100 (Table 2).

RESULTS

MMI was used to demonstrate the comparative assay performance for the 96-well-format GUA-TPO assay and the 96- and 384-well-format versions of the AUR-TPO assay (Figure 1). These concentration−response data were fit using a Hill model, and the resultant IC_{50} values varied by both assay type and plate format, likely owing to distinct affinities for the GUA versus AUR substrates and assay miniaturization. The estimated MMI IC_{50} values were 2.20 μM, 93 nM, and 25 nM for the 96-well GUA-TPO, 96-well AUR-TPO, and 384-well AUR-TPO assays, respectively. All three assay paradigms exhibited favorable Z' factors of greater than 0.5, but the 384-well-format AUR-TPO assay demonstrated the highest Z' factor of 0.93.

Several initial optimization and scaling experiments were conducted to ascertain optimal protein and AUR concentrations along with the associated dynamic range, Z', and IC_{50} for MMI (Table S1). These preliminary experiments demonstrated a maximal dynamic range and a Z' factor for the assay with 12.5 μg/reaction of total thyroid microsomal protein and 25 μM AUR in a 96-well-plate format. The assay was further modified from the GUA oxidation assay by measuring the data at an end point rather than as a kinetic change over a time interval. A 30 min incubation at 37 °C was sufficient for relative fluorescence unit values of the AUR assay to stabilize, as demonstrated by an example reaction profile (Figure S1). The AUR-TPO assay signal was stable for at least 2 h post-initiation of the peroxidase reaction (Figure S1). The assay was tested for solvent sensitivity; DMSO concentrations of 0.16, 0.33, 0.50, and 1.20% did not yield significant effects on assay performance (Figure S2).

Assay performance with a 21-chemical training set is illustrated in Figure 2A,B, with the Hill fit and potency data listed in Table 2. All training-set data are plotted as the percent of vehicle-control (DMSO) activity, with the vehicle-control mean set to 100% activity. Vehicle-control outliers were removed from the DMSO vehicle average (54 wells per n) if they were three times the standard deviation of the mean (3 values out of 162 values were removed). Fourteen chemicals tested positive for TPO inhibition (i.e., these chemicals produced an effect greater than or equal to an arbitrarily set 20% decrease in TPO activity from vehicle control; Figure 2A). A four-parameter Hill model was used to fit the concentration−response data for all fourteen positive chemicals to generate the predicted and relative IC_{50} values, lowest effect concentration (LEC), maximum effect (E_{max}, in percent activity), Hill slope, and r^2 value on the Hill fit, all of which are reflected in Table 2. The LEC represents the lowest measured concentration that yielded a significant response (i.e., greater or equal to a 20% decrease in TPO activity). Eleven of the 14 positive chemicals demonstrated a concentration-responsive effect, with more than one tested concentration satisfying the criteria for a positive effect. Three of the 14 chemicals, IOA, RSC, and TCS, were of extremely low potency, and the LEC was the highest concentration tested (253 μM; Table 2). Seven chemicals tested negative, including BP3, DBP, DEHP, DEP, DPM, MMB, and NaPER (Figure 2B). The assay results demonstrated the following rank-ordered relative potency for the positive chemicals in the set: MMI > ETU > PTU > BP2 > MBT > AMT > GEN > 4POP > SMZ > DDZ > 4NP > TCS > IOA > RSC.

The concordance of the 21-chemical training set data from the AUR-rTPO assay with previously reported literature data is reviewed in Table 3. TCS and IOA were unexpected TPO inhibitors. DPM and NaPER were previously reported to inhibit TPO only at very high concentrations but were negative in this study. In general, the IC_{50} values derived in this study

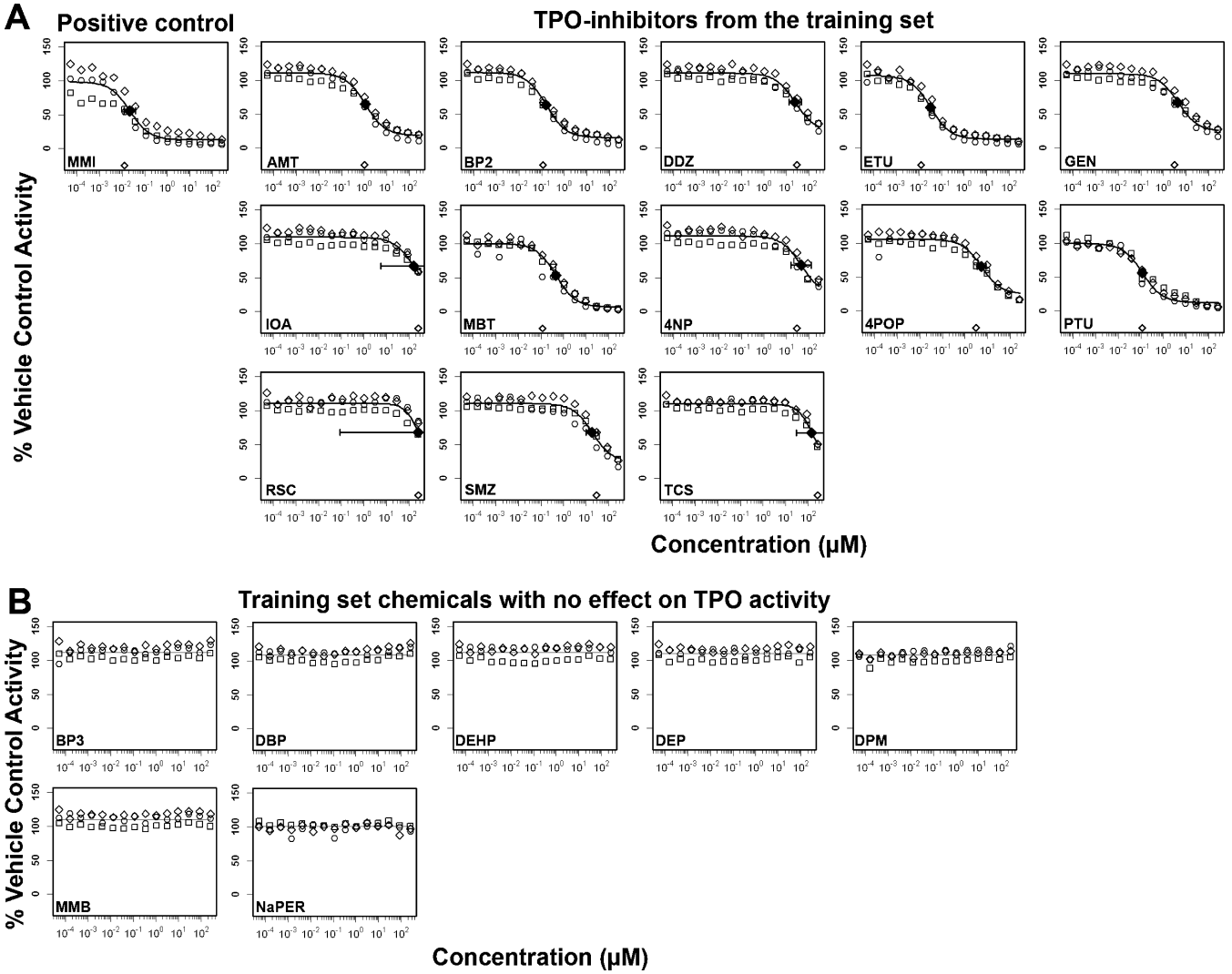

Figure 2. Results from a 21-chemical training set in 384-well format. (A) Positive TPO inhibitors (n = 3) are shown in each panel with a corresponding abbreviation for the chemical concentration−response data illustrated. The positive control was MMI. All chemicals were tested at the same 15 concentrations ranging from 0.05 nM to 253 μM. Each of the 3 biological replicates is represented by a different symbol type (clear diamonds, circles, and squares). The predicted IC_{50} and associated SE values are represented by a black diamond with bounded error bars. The clear diamond located at the x axis represents the LEC, the first concentration tested that meets or exceeds the threshold for a positive effect (≥20% decrease in TPO activity). (B) Negative TPO inhibitors (n = 3), seven chemicals in total, are shown in each panel with a corresponding abbreviation for the chemical concentration−response data illustrated.

were lower than those derived from other methods, suggesting that this method may be more sensitive.

DISCUSSION

The present study describes a novel use of the AUR substrate in a screening assay paradigm to detect potential TPO-inhibiting chemicals and thus addresses the critical need for a high-throughput assay to detect TDCs that may act via inhibition of TPO-catalyzed TH synthesis. The methodological approach employed confirms a previous report of successful measurement of TPO activity with AUR using rat and human thyroid microsomes,[52] but a unique assay chemistry was used herein. This study expands the use of AUR as a TPO substrate to a 384-well-plate high-throughput screening assay to identify TPO-inhibiting chemicals. Assay optimization in 96-well and 384-well formats was followed by characterization of the assay responses to a 21-chemical training set composed of chemicals previously reported either to inhibit or have no effect on thyroperoxidase activity. As a whole, the current work demonstrates that AUR can be used as an alternative H^+ donor in place of guaiacol to yield a HTPS assay that can be measured at a single time-point and with one-tenth of the animal tissue previously required in the rat thyroid microsome-based GUA-TPO assay.

The objective of developing the AUR-TPO assay was to establish an assay method for high-throughput preliminary screening of a large number of chemicals for potential TPO-inhibiting activity, thereby prioritizing a more limited number of chemicals for confirmatory testing using orthogonal assay models, including guaiacol oxidation and/or analytical chemistry methods as well as animal models including the thyroid explant assay, the 7-day amphibian metamorphosis assay, the 4-day rat assay, and/or developmental rat assays. Thus, miniaturizing the AUR-TPO assay to a 384-well format increases the screening utility of the AUR-TPO assay and enables further testing to focus on suspected TPO inhibitors.

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Table 3. Available in Vitro Data on the TPO-Inhibiting Activities of the 21-Chemical Training Set

symbol	TPO inhibitor in the literature (±)	system/method(s)	literature IC_{50} (μM) value(s)	reaction inhibited, if known	ref	IC_{50} (μM) in current Study
AMT	+	LPO, spectrophotometric production of MIT;[39] guaiacol oxidation assay[2]	12.6[30]	irreversible inhibition of the enzyme (suicide substrate)	30, 39	1.1
BP2	+	recombinant hTPO from stably transfected FTC-238 cell line in the guaiacol oxidation assay[32]	0.5	potential competition with iodide	32	0.16
BP3	−	recombinant hTPO from stably transfected FTC-238 cell line, guaiacol oxidation assay[53]	NA	NA	53	NA
DDZ	+	porcine TPO, analytical measurement of tyrosine iodination;[37] rat thyroid microsomes, guaiacol oxidation assay[33]	0.143[33]	irreversible inhibition of the enzyme (suicide substrate); prevented by excess iodide	33, 37	23
DBP	−	porcine and rat thyroid microsomes, guaiacol oxidation assay	NA	NA	43	NA
DEHP	−	porcine and rat thyroid microsomes, guaiacol oxidation assay	NA	NA	43	NA
DEP	−	porcine and rat thyroid microsomes, guaiacol oxidation assay	NA	NA	43	NA
DPM	−	porcine and rat thyroid microsomes, guaiacol oxidation assay	171−3100	NA	43	NA
ETU	+	porcine TPO; porcine thyroid microsomes, guaiacol oxidation assay	>1000[65]	potential competition with iodide; metabolism of ETU by TPO; inhibition of TPO-catalyzed iodination[56]	56, 65	0.034
GEN	+	hTPO, pTPO analytical measurement of tyrosine iodination,[37] rat thyroid microsomes, guaiacol oxidation assay,[33] recombinant hTPO from stably transfected FTC-238 cell line in the guaiacol oxidation assay[68]	0.050[37] and 50[68]	irreversible inhibition of the enzyme (suicide substrate); prevented by excess iodide[33]	33, 37, 68	4.5
IOA	−	porcine and rat thyroid microsomes, guaiacol oxidation assay	NA	NA	43	160
MBT	+	porcine thyroid microsomes with guaiacol oxidation assay	11.5	unknown	44	0.45
MMI	+	many models	0.5−10	ireversible inhibition	44, 52, 53	0.025
MMB	ND	NA	NA	NA	NA	NA
4NP	+	recombinant hTPO from transfected FTC-133 cells	57	unknown	68	44
4POP	+	porcine and rat thyroid microsomes, guaiacol oxidation assay	1760−6000	unknown	43	5.3
PTU	+	porcine TPO, porcine thyroid microsomes	4[65] and 100[30]	irreversible inhibition of the enzyme (suicide substrate)	30, 65	0.12
RSC	+	TPO-, LPO-catalyzed tyrosine iodination and guaiacol oxidation	3.48[38] and 0.27[75]	irreversible inhibition of the enzyme due to production of a reactive species via oxidation of resorcinol that covalently binds to the heme-containing active site to block enzymatic activity permanently	38, 70, 75	253
NaPER	+	porcine TPO (Hosoya) guaiacol oxidation assay; porcine and rat thyroid microsomes, guaiacol oxidation assay	9650−21 149[43]	low potency inhibition of MIT and DIT formation	30, 43, 76	NA
SMZ	+	TPO- and LPO catalyzed iodination of tyrosine	300−500	reversible inhibition	54	18
TCS	−	rat thyroid microsomes, guaiacol oxidation assay	NA	not previously reported to be an inhibitor	43	142

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Assay miniaturization to a 384-well format maintained data interpretability, dynamic range, and Z' for the assay. In fact, the Z' factor was improved with the semiautomated 384-well-plate reaction profile such that the resolution of even low-potency TPO inhibitors appears feasible. This also suggests the possibility that this microsomal assay could be conducted in a further miniaturized platform (e.g., 1536-well plates); however, microsomal preparation and the heterogeneity of the preparation may act as limitations on the utility of the AUR-TPO screening assay in a higher-density format. The intra-laboratory reproducibility of this assay is high, as indicated by Z' factors of 0.86 and 0.93 for the 96- and 384-well formats, respectively. Minor differences in quantitative prediction of IC_{50} for positive controls and other chemicals may result from biological variability inherent to microsomal pools made from 60 individual rat samples, as illustrated by the 96-well-plate assay data for MMI in Figure 1. For the 96- and 384-well-plate assay formats, the amount of thyroid microsomal protein used per well was 12.5 and 5 μg, respectively, representing reductions of 75 and 90% in the amount of protein used per well when compared to previous work with the rat-based 96-well-plate guaiacol oxidation assay.[43] A recently published study of porcine thyroid microsomal TPO activity employed 150−200 μg of total protein per well in a 96-well-plate format.[44] The current work demonstrates that the use of AUR and a reaction profile such as the one reported here may drastically increase the amount of testing and screening that can be performed with limited animal tissues.

The AUR-TPO 21-chemical screen generally confirmed previous literature reports of TPO inhibition by xenobiotics.[33,38,39,43,53−56] Some quantitative differences were apparent between our model system and previously used models, and a few unexpected qualitative results for chemicals with little data reported previously emerged. Twelve of the 14 positive chemicals (MMI, ETU, PTU, BP2, MBT, AMT, GEN, 4POP, SMZ, DDZ, 4NP, and RSC) were previously demonstrated to inhibit TPO in various model systems (Table 3); unique positive observations were made for two chemicals, TCS and IOA. In a previous study, TCS had no effect on rat TPO activity.[43] The LEC for TCS in this work was 253 μM, with a predicted IC_{50} value of 142 μM; both of these concentrations fall within the previously tested range.[43] TCS is a known thyroid-disrupting chemical,[57−59] so with only one previous contradictory report using a different testing method, TCS may be a true positive. Depending on the cell model employed, TCS would most likely be overwhelmingly cytotoxic at 253 μM,[60] making potential TPO inhibition likely secondary to other effects that would occur at lower TCS tissue concentrations. The same previous report also demonstrated no effect of IOA on rat or porcine TPO activity at tested concentrations from 33 to 333 μM.[43] In the current work, the LEC for IOA was 253 μM, with a predicted IC_{50} value of 160 μM. IOA is a known TDC that inhibits peripheral deiodination of T4 to T3.[61,62] Because both TCS and IOA appear to act as TPO inhibitors in this screening assay at only the highest concentrations tested, these would be considered chemicals with very low potency for TPO inhibition. The positive responses for TCS and IOA herein suggest the AUR-TPO 384-well-format assay sensitivity, in comparison to previously published methods, may allow for the detection of low-potency TPO inhibitors.

The AUR-TPO method failed to detect NaPER and DPM as TPO inhibitors despite previous reports of their effects on TPO activity. NaPER has been previously reported to inhibit TPO at concentrations that exceeded 5 mM,[43] so we would not have expected to see this effect because these concentrations far exceeded the tested concentration range in this work. NaPER is a known inhibitor of the sodium-iodide symporter and was more potent than MMI in vivo in the *Xenopus laevis* tadpole assay[63] and in vitro in an amphibian thyroid explant culture,[64] signifying that inhibition of iodide uptake would be the predominant mechanism for reduction of T4 synthesis by NaPER. One previous report of inhibition of TPO by DPM suggested that its potency varied somewhat across species, with IC_{50} value ranges of 1930−4400 and 104−266 μM for porcine and rat TPO, respectively.[43] These previous data suggest that DPM may inhibit TPO but with very low potency and at concentrations approaching or overlapping with cytotoxicity in an amphibian thyroid explant culture (Michael Hornung, personal communication).

For several positive chemicals in the 21-chemical training set, the IC_{50} values were lower than those from previous reports using the guaiacol oxidation assay and other methods. Previously derived IC_{50} values for MMI-induced TPO inhibition have ranged from 1 to 10 μM,[27,32] including one previous report of an AUR-based TPO-inhibition assay.[52] Here, we report a more potent IC_{50} value for MMI of 0.025 μM, suggesting a potential increased sensitivity of this screening assay when compared to published methods. Using AUR as an H^+ donor in place of guaiacol or iodide and the further miniaturization of the assay from a 96-well format to a 384-well format may have resulted in potency changes for MMI and other chemicals in the training set when compared to previously reported values from different TPO-inhibition assays. Relative potency to MMI may be a more relevant dose−response evaluation metric for this assay in terms of prioritizing positive chemicals for further dose−response analysis.

The relative potency values for MBT-induced TPO inhibition in the AUR-TPO assay and a previously described porcine thyroid microsome system are very similar;[43] the IC_{50} value with porcine thyroid microsomes was 11.5 μM with a relative potency to MMI of 5.8%,[44] and in this study, the IC_{50} value for MBT was 0.451 μM with a relative potency to MMI of 6.1%. The PTU IC_{50} values for TPO inhibition have been previously reported in the 1−15 μM range.[43,65] PTU was twice as potent as MMI in rat thyroid microsomal TPO-inhibition studies using the guaiacol oxidation assay, but was about one-fifth as potent as MMI for TPO from porcine thyroid microsomes.[43] A similar thiourea, ETU, a degradant of ethylenebisdithiocarbamate pesticides, demonstrated limited efficacy and potency in a guaiacol oxidation assay with porcine TPO compared to PTU.[65] In the current study, both thiourea compounds, PTU and ETU, demonstrated high relative potency to MMI, but ETU appeared to be more potent than PTU.

The UV-blocking agent BP2 moderately decreased T4 and increased TSH in rats at high doses of 333 and 1000 mg/kg/day after 5 days of exposure, but intriguingly, ex vivo TPO activity in the thyroids of these rats was unchanged despite in vitro TPO inhibition;[32] the authors suggest that akin to the effects of isoflavones on TPO activity[33] the presence of iodide can prevent TPO inhibition. The study that originally identified BP2 as an in vitro TPO inhibitor suggested that BP2 was 20-fold more potent than MMI in a guaiacol oxidation assay with a human recombinant TPO protein fraction, with IC_{50} values of

0.5 and 10 μM for BP2 and MMI, respectively.[32] BP2 inhibited TPO with a relative potency to MMI of 17% and was not more potent than MMI in the present study.

AMT, an herbicide known to cause thyroid tumors in rodents,[66] consistent with TPO inhibition,[67] was previously identified as a suicide substrate of LPO[39] and an inhibitor of porcine TPO with an IC_{50} value of 12.6 μM.[30] AMT was confirmed as a TPO inhibitor in the present work, with an IC_{50} value of 1.11 μM and a relative potency to MMI of 2.4%.

The isoflavones GEN and DDZ have been previously described as TPO inhibitors.[28,33,37,55] High dietary isoflavone exposure may result in human goiters and rodent thyroid tumors, and an in vivo rat study demonstrated IC_{50} values for TPO inhibition correlated to serum genistein and daidzein concentrations in the low micromolar range (0.2−0.6 μM).[28] The IC_{50} values reported herein for GEN and DDZ were 4.45 and 23.3 μM, respectively. The IC_{50} values for rat TPO inhibition in vitro by GEN and DDZ have been previously reported as IC_{50} 0.05 and 0.143 μM, respectively,[28] suggesting that GEN is approximately 4 times more potent than DDZ for rat TPO inhibition, consistent with our observation that GEN was approximately 5 times more potent than DDZ.

Phenolic estrogens 4POP and 4NP both tested positive for TPO inhibition, with IC_{50} values of 5.34 and 43.5 μM, respectively, and with weak relative potency to MMI. Our previous work with rat and porcine thyroid microsomes in the guaiacol oxidation assay demonstrated negative results for 4NP, contrary to reports of 4NP-induced TPO inhibition,[68] and positive results for 4POP, but with very low potency.[43]

RSC, another phenolic compound in many personal-care products and in deposits of coal and shale, has demonstrated low-potency and low-efficacy antithyroid effects in mammals[69] and had also previously been identified as a TPO inhibitor.[38,70] RSC was a low-potency TPO inhibitor in the AUR-TPO assay, with an IC_{50} value and LEC at the highest concentration tested, 253 μM.

Conservation of TH regulation and TPO function across species[2,3,28] implies that disruption of TH synthesis in an in vitro rat thyroid microsome-based system could be predictive of human TPO inhibition. An amino acid sequence alignment of the putative catalytic domain of rat and human TPO demonstrated a maximum identity of 80%, illustrating a high degree of amino acid homology while still underscoring that species-specific differences may exist between rat and human TPO.[43] The sequence homology between the putative catalytic domains of the rat and porcine thyroperoxidase enzymes demonstrated 75% maximum identity; however, porcine and rat thyroid microsomal TPO activity in responses to a 12-chemical training set were qualitatively concordant and yielded similar relative potency values,[43] suggesting general species conservation of TPO responsivity to chemical exposures.

Beyond the obvious limitations in extrapolating to in vivo human TPO functional response from in vitro rat-based TPO activity is the somewhat nebulous question of whether the AUR-TPO screening assay may be used to detect diverse inhibitors of TPO function. Because TPO acts as an enzymatic catalyst of the organification of iodine, in the addition of iodide to Tg to form MIT and DIT residues, and in the coupling of iodinated residues to form T3 and T4, more than one enzymatic process may be disturbed. Furthermore, some compounds may act as suicide inhibitors that form reactive intermediates that covalently bind TPO and permanently alter TPO structure and function, whereas others may be reversible or competitive inhibitors of TPO-catalyzed reactions. Thus, it is plausible that one screening assay that does not recapitulate the exact sequence of reactions may fail to detect all of the potential TPO-inhibiting chemicals or chemical classes. In the present work, however, both reversible inhibitors and irreversible inhibitors were detected; for instance, SMZ and ETU are thought to inhibit TPO-catalyzed iodination without production of a reactive intermediate,[54,65] which occurs for AMT-, DDZ-, GEN-, and RSC-induced TPO inhibition.[37−39,70] On the basis of these observations, the AUR-TPO assay may detect TPO inhibitors that act via different mechanisms of action to prevent TPO-catalyzed synthesis of THs.

A key finding in this study is the reduction in the number of animals required to assess TPO inhibition. In the present work, 21 chemicals were evaluated at 15 concentrations with three biological replicates, each with a single technical replicate; this design required the thyroid tissue from a sum of approximately 14 rats. To evaluate TPO inhibition using the GUA-TPO assay and the same experimental design would have required a minimum of 140 rats. Evaluation of TPO inhibition for 21 chemicals in a single in vivo rat study using four doses and eight animals in each dose group would have required at least 672 rats and likely more. The AUR-TPO assay with rat thyroid microsomes demonstrates a major reduction in animal use for thyroid-disruptor screening and enables future comparison to in vivo rat studies for TPO inhibitors of interest.

Obstacles to employing the AUR-TPO assay for screening chemical libraries to detect TPO-inhibition activity include common constraints, such as difficulty in resolving the effects of proteases, detergents, or generic protein inhibitors from chemicals with specific antithyroid effects.[71] As with any loss-of-signal screening assay, a compound that decreases activity nonspecifically, such as a protease, would likely appear as a false positive in this assay. Because the current version of the AUR-TPO is a biochemical assay, cytotoxicity and protein synthesis are not limitations on the assay, but high salt concentrations or chemicals that can precipitate ions may affect protein activity. Assessment of the chemical library for fluorescent chemicals that might interfere with the fluorescent signal used in the AUR-TPO assay would provide useful data for confirming true positive and negative chemicals.[72] Despite these limitations, the AUR-TPO screening assay described in the current work represents the first implementation of a rapid, 384-well-format screen for TPO inhibitors. Parallel assays to evaluate nonspecific protein inhibition (e.g., inhibition of a constitutively active luciferase enzyme in the presence of all necessary cofactors and cytoxicity in a cell line) may be appropriate for distinguishing positive and equivocal screening results from chemicals that may be broad-spectrum protein inhibitors or cytotoxicants. Subsequent to a HTPS effort, confirmed positives would need to be further evaluated in models of increasing complexity to characterize further the potential biological hazard of these compounds.[73,74] This follow-up testing would include using the GUA-TPO and perhaps other low-throughput TPO inhibition assays to discern potentially the mechanism of TPO inhibition and to confirm unique findings. Biologically relevant targeted testing could be performed, for example, with the cultured amphibian thyroid explant system, amphibian short term, or rat 4 day exposures, which have been previously used to demonstrate the effects of many chemicals including MMI, PTU, and NaPER on TH production.[64] This system requires intact thyroids and contains all of the additional components required for TH synthesis,

including the sodium-iodide symporter (NIS), iodide, catalytic generators of hydrogen peroxide, and so forth, and would provide a confirmation of TPO inhibition and a potential to assess if TH synthesis is disrupted. However, this is a less specific test system that combines more than one molecular-initiating event for disruption of TH synthesis (i.e., a chemical could inhibit TPO, NIS, or other enzymatic processes key to synthesis and liberation of THs from the gland). Further whole-animal targeted testing could include a 7-day amphibian prometamorphic larvae screen and/or a 4-day rat thyroid hormone screen.[18,19,44]

This is the first high-throughput screening assay development effort for TPO-inhibiting chemicals using microsomes from animal tissues. The use of this AUR-TPO assay in a tiered screening approach represents a novel first step toward increasing capacity for TDC identification. Future work will strengthen the understanding of how TPO inhibition, as a molecular-initiating event, is associated with downstream changes within an adverse outcome pathway(s) for amphibian and human development. Understanding the correlation between in vivo effects manifested in the 7-day amphibian and 4-day rat thyroid hormone screening assays will aid interpretation of any findings from in vitro TPO inhibition assays, further reduce animal usage for identification of TDCs, and support subsequent risk assessment.

■ ASSOCIATED CONTENT

Supporting Information

Summary of selected optimization experiments for the design of the AUR-TPO assays, including a description of trials with varied concentrations of protein and the AUR substrate and the resultant IC_{50}, Z', and dynamic range obtained; AUR-TPO reaction profile over time, including stabilization of the reaction over 30 min, MMI inhibition curves after 30 min, and stability information for the assay signal over a 120 min period; and AUR-TPO assay response in the presence of 0.16, 0.33, 0.50, and 1.2% DMSO. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

AMT, amitrole; AUR, Amplex UltraRed substrate; AUR-TPO, Amplex UltraRed Thyroperoxidase activity assay; BP2, 2,2′,4,4′-tetrahydroxy-benzophenone; BP3, 2-hydroxy-4-methoxybenzophenone; CV, coefficient of variation; DBP, dibutylphthalate; DDZ, daidzein; DEHP, diethylhexylphthalate; DEP, diethylphthalate; DMSO, dimethyl sulfoxide; DPM, 3,5-dimethylpyrazole-1-methanol; E_{max}, maximum response; ETU, ethylene thiourea; GEN, genistein; GUA, guaiacol; HTPS, high-throughput screening; IC_{50}, 50% inhibitory concentration; IOA, iopanoic acid; LEC, lowest effect concentration; MBT, 2-mercaptobenzothiazole; MMB, methyl 2-methyl-benzoate; MMI, methimazole; NaPER, sodium perchlorate; 4NP, 4-nonylphenol; 4POP, 4-propoxyphenol; PTU, 6-propylthiouracil; r^2, goodness of fit; RSC, resorcinol; SMZ, sulfamethazine; T4, thyroxine; TCS, triclosan; TDC, thyroid-disrupting chemical; TH, thyroid hormone; T_{lim}, top limit of the curve fit; TPO, thyroperoxidase; Z', Z prime

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