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A rapid assay of human thyroid peroxidase activity

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A B S T R A C T

Impaired synthesis or action of thyroid hormones (THs) during critically sensitive periods of development can have long term adverse effects on health. Development of rapid assays to identify chemicals that impair THs physiology is an important goal for reducing risks from chemical use. Thyroid peroxidase (TPO) is a key enzyme regulating THs synthesis in thyroid gland and a vulnerable target for chemicals that disrupt THs synthesis. To develop a human-relevant, rapid assay for TPO inhibition, we have engineered two cell lines (CHO and LentiX- 293) to express active human TPO (hTPO) enzyme and applied them in a recently-described assay using a stable fluorescent product (Amplex UltraRed). Assay performance was assessed by comparing activity of 19 reference chemicals with known strong, weak or no TPO inhibitory activity. The assay using hTPO from either cell line consistently identified the relative potency of strong to moderate inhibitors and chemicals known to be inactive. Results were less consistent for chemicals reported to be weak inhibitors of rodent TPO, possibly suggesting some species specificity. Our studies support the use of hTPO from stably transfected cell lines to substitute for animal-derived thyroid microsomes for rapid high throughput screening assays to identify and characterize TPO inhibitors.

1. Introduction

Thyroid hormones (THs) are important regulators of metabolism, cardiac function, tissue development and many physiological processes (Zoeller et al., 2007). In particular, TH is an essential mediator of normal brain development and even subtle disruption of TH synthesis or action during critically sensitive stages of brain development can lead to lasting effects on intelligence and behaviour (Berbel et al., 2009; Li et al., 2010; Modesto et al., 2015).

The regulation of TH physiology is complex and there are multiple potential targets, controlling TH synthesis, transport, metabolism or action, where toxicant chemicals can act to impair TH physiology (Zoeller et al., 2007). The synthesis of THs in the thyroid gland involves the uptake of iodide into thyroid follicular cells *via* the sodium iodide symporter (NIS), and the incorporation of iodide into thyroglobulin (Tg) through the action of thyroperoxidase (TPO). Thyroid hormones, released by proteolysis in thyroid epithelial cell lysosomes, are then secreted into the blood stream. The majority of THs in circulation are bound to albumin or TH carrier proteins (transthyretin (TTR) and thyroxine-binding globulin (TBG)) (McLean et al., 2017). TH uptake into target cells is controlled by membrane bound transporters, such as monocarboxylate transporter (MCT) 8, MCT 10 and organic anion transport protein (OATP) 1c1. Conversion of the major circulating TH, thyroxine (T4) into the more active hormone triiodothyronine (T3) is regulated by multiple deiodinases (DIOs) expressed in various tissues. DIO2 (expressed in most TH target tissues) converts T4 to T3 while DIO3 (mainly in brain and fetal tissues) converts T4 into inactive reverse T3 (rT3) (Bianco and Kim, 2006). The activity of DIO1, mainly expressed in liver and kidney, can convert T4 to T3 or rT3. Within the cell, TH plays various roles by regulating target gene expression *via* binding to and activating TH receptors. Environmental chemicals can affect TH action by targeting any one of these processes. Significant efforts have been made to identify chemicals that disrupt or inhibit NIS (Waltz et al., 2010; Hallinger et al., 2017; Dong et al., 2019), TPO (Paul Friedman et al., 2016; Paul et al., 2014), DIOs (Renko et al., 2015; Hornung et al., 2018; Olker et al., 2019), TH binding to serum transporter proteins ((Brouwer and van den Berg, 1986; Meerts et al., 2000; Chauhan et al., 2000) and others), transmembrane transporters (Dong and Wade, 2017) and TH receptors (Freitas et al., 2011; Sun et al., 2012).

TPO has received considerable attention as a target vulnerable to chemical disruptors of TH physiology. TPO is located on the adluminal extracellular membrane of thyroid epithelial cells and catalyzes two critical steps in TH synthesis. TPO mediates the oxidation and coupling of intraluminal iodide to the phenyl ring of tyrosine residues of thyroglobulin. In addition, TPO catalyzes the transfer of iodinated phenyl rings to adjacent tyrosine residues to form the iodotyronine structure (Taurog et al., 1996). Inhibition of TPO is the primary pharmacological treatment for pathological hyperthyroidism (Schmutzler et al., 2007). TPO-inhibition has been recognized as one of the key mechanisms for thyroid disruption (Jacobs et al., 2013; Murk et al., 2013).

Most reports of TPO activity assays monitor the absorbance of an

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unstable amber peroxidase metabolite of guaiacol which has limited utility for the development of rapid high throughput screening (HTS) assays (Paul et al., 2013). More recently, an assay based on the TPO mediated conversion of fluorescent horseradish-peroxidase substrate (Amplex UltraRed, AUR) to a fluorescent product has been described (Paul et al., 2014). This method has been employed to assay > 1000 chemicals using rat thyroid gland microsomes as a source for TPO activity (Paul Friedman et al., 2016; Paul et al., 2014; Jomaa et al., 2015). This exercise demonstrated the utility of this assay to rapidly identify potential thyroid disrupting properties in chemicals with diverse structure. However, the use of rat tissues for this assay presents some issues including the assumption that results from rat TPO are universally applicable to humans and the need to collect tissues from laboratory animals. A previous study has demonstrated that TPO activity derived from a human thyroid follicular cell line Nthy-ori 3-1 can provide the enzyme needed for TPO screening assays (Jomaa et al., 2015). In the current study, we demonstrate that hTPO derived from engineered cell lines can be effectively used in the AUR assay. Reference chemicals tested using such hTPO preparations were compared to data previously reported from studies using the AUR assay with rat microsomal TPO to evaluate the feasibility of using human enzymes from transfected cells to screen for thyroid disrupting chemicals.

2. Materials and methods

Chemicals: All test chemicals, their sources and identifiers are listed in Table 1. All chemicals were solubilized in DMSO to yield concentrated stock solutions of 620 mM from which working dilutions in DMSO were prepared and transferred to compound plates. Working stocks for each chemical in stock compound plates were 62, 21, 7, 2.3, 0.8, 0.26, 0.1, 0.03, 0.01, 0.003, 0.001, 0.0003, 0.0001, 0.00003 and 0 mM. From these stocks, assay working dilutions were prepared on the day of assay by 10 times dilution with potassium phosphate buffer (0.1 M, PH7.4).

Establishing Lenti-X 293 T cells overexpressing hTPO: Full length cDNA of hTPO (BC095448) cloned in the lentivirus vector pXL304 (pXL304-hTPO) was obtained from TransOMIC Technologies Inc. (Huntsville, AL). Packaging plasmid pCMV-dR8.2 d*vpr* (Addgene, Plasmid #8455) and envelop plasmid pCMV-VSV-G (Addgene, Plasmid #8454) were gifts from Dr. Robert Weinberg (Stewart et al., 2003). Lenti-X™ 293 T cell line was purchased from Takara Bio USA, Inc. (Cat# 632180, Mountain View, CA). Selecting antibiotic Blasticidin S HCl (10 mg/ml) was obtained from Thermo Fisher Scientific (Cat # A1113903, Waltham, MA). Polybrene was obtained from Sigma-Aldrich Canada Co. (Cat # H9268-5G, Oakville, ON).

For generating lentiviral particles: Lenti-X™ 293 T cells were seeded at 4×10^6 /10 ml in 10 cm culture dishes with culture medium (DMEM supplemented with 10% newborn calf serum; 100 units/ml penicillin G sodium and 100 µg/ml streptomycin sulfate). On Day 2, 2 ml of fresh medium without antibiotics was added for 2 h. Transfection was conducted with FugeneHD (Roche) as per manufacturer instructions. A ratio of 3:1 of Fugene reagent to DNA was applied to each plate. DNA component comprised of the plasmids pCMV-dR8.2, pCMV-VSV-G and pXL304-hTPO presented as a 2:1:2. On Day 4, supernatant was collected and centrifuged for 10 min at 800*g*. The lentiviral particles were stored at −80C until being used.

For lentiviral transduction: Fresh Lenti-X™ 293 T cells were seeded in 10 cm culture dishes at 2×10^6/10 ml medium and cultured for 24 h. Medium was replaced with 2 ml lentiviral particles supplemented with 8 µg/ml polybrene for 24 h; then was replaced with 10 ml regular culture medium for 6 h prior to adding the selection antibiotic blasticidine (8 µg/ml). Lenti-X™ 293 T cells overexpressing hTPO (LentiX-TPO) were maintained for another 10 days with selection medium containing blasticidine to ensure overexpression of hTPO.

CHO-TPO cell culture: Stable transfection of Chinese Hamster Ovary (CHO) cells to express full length human TPO cDNA (CHO-TPO) is described elsewhere (Gora et al., 2004). CHO-TPO and CHO-Control (stably transfected with control cDNA) cells were cultured in Ham's F12 medium supplemented with 2 mM L-glutamine, 10% FBS, antibiotic-antimycotic solution (Cat. No A5955, Sigma-Aldrich) and 300 µg/ml Hygromycin B until 80% confluence.

TPO Preparation from engineered cell lines: Cells cultured to confluence in 10 cm dishes were collected (trypsin/EDTA) and pelleted by centrifuge (5 min at 150 *g* at 4C). Cells were washed twice with 4 ml PBS. Cell pellet from 8 dishes was lysed with 13 ml of 0.1% sodium deoxycholate and incubated on ice for 20 min. The lysed cells were centrifuged for 5 min at 9500*g* to separate the soluble protein fraction from debris (Jomaa et al., 2015). Protein concentration of the supernatant was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific) following the manufacturer's protocol. Cell lysates were aliquoted and kept at −80C until use.

TPO preparation from rat thyroid microsomes: All animal handling procedures adhered to the Canadian Council on Animal Care guidelines and were approved by the Health Canada Animal Care Committee prior to the initiation of the study. Ten thyroid glands from adult F344 female rats were homogenized in 50 ml extraction buffer (potassium phosphate

Table 1
Source and expected activity of reference chemicals tested.

Chemical name	CAS #	Abbr.	Source	Activity[a]
Methimazole	60-56-0	MMI	Sigma	Reference
Amino-1,2,4-triazole	61-82-5	AMT	Sigma	Pos
2,2′,4,4′-Tetrahydroxybenzophenone	131-55-5	BP2	Sigma	Pos
Genistein	446-72-0	GEN	Sigma	Pos
2-Mercaptobenzothiazole	149-30-4	MBT	Aldrich	Pos
6-Propyl-2-thiouracil	51-52-5	PTU	Sigma	Pos
Resorcinol	108-46-3	RSC	Sigma	Pos
4-Nonylphenol	104-40-5	4NP	Sigma	Weak Pos
Daidzein	486-66-8	DDZ	Sigma	Weak Pos
Ethylene thiourea	96-45-7	ETU	Sigma	Weak Pos
Iopanoic acid	96-83-3	IOA	Sigma	Weak Pos
Sulfamethazine	57-68-1	SMZ	Sigma	Weak Pos
Triclosan	3380-34-5	TCS	Sigma	Weak Pos
Dibutyl phthalate	84-74-2	DBP	Aldrich	Neg
Bis(2-ethylhexyl) phthalate	117-81-7	DEHP	Sigma	Neg
Diethyl phthalate	84-66-2	DEP	Aldrich	Neg
3,5-Dimethylpyrazole-1-methanol	85264-33-1	DPM	Aldrich	Neg
Methyl 2-methylbenzoate	89-71-4	MMB	Aldrich	Neg
Sodium perchlorate	7601-89-0	NaPER	Sigma	Neg

[a] Based on activity reported by Paul et al. (2014) and Paul Friedman et al. (2016).

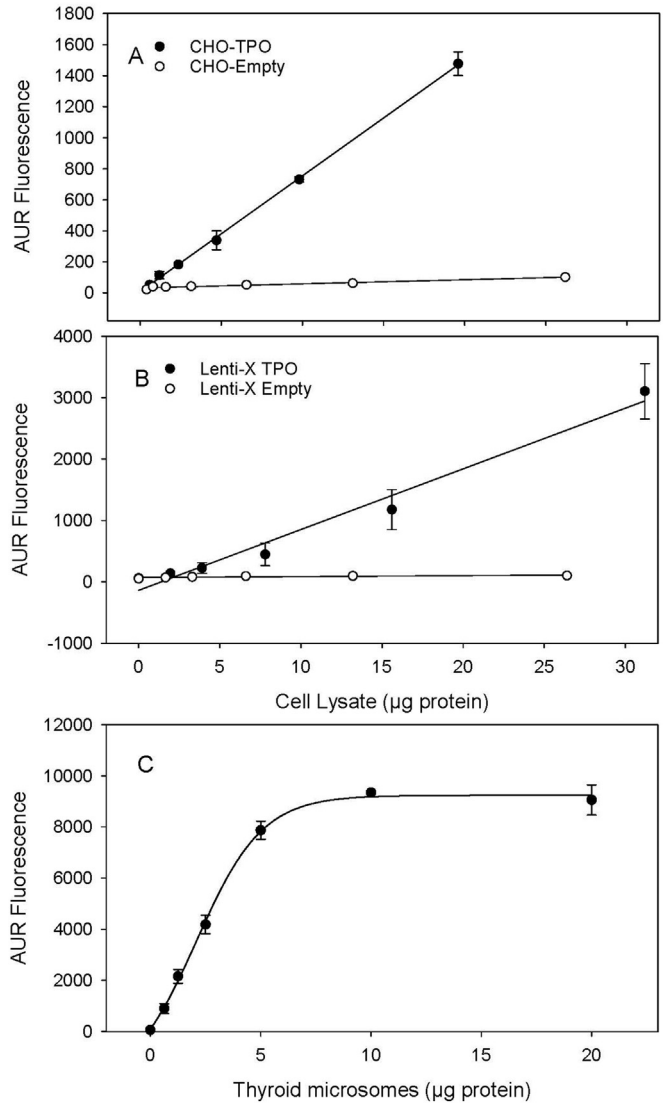

Fig. 1. Concentration response relationship of TPO activity in CHO cells (stably transfected with the empty vector) and CHO-TPO cells (A), LentiX-293 cells (empty vector) and LentiX-TPO cells (B) and rat thyroid microsome extracts (C). A various amount of cell lysates or rat thyroid microsomes were reacted with AUR in the presence of H2O2. Conducted in the black 96-well plates, each condition triplicated, each plate repeat 3 times.

buffer 5 mM, pH 7, supplemented with sucrose 200 mM, EDTA 1 mM and catalase 500 U/ml), centrifuged 10 min at 29.4*g*. Supernatant was collected and ultra-centrifuged at 151515 *g* for 1 h at 4C, the pellet was re-suspend in 7.5 ml extraction buffer without catalase. Protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer's instruction.

Amplex ultraRed (AUR) assay: AUR is a non-fluorescent substrate that is converted by peroxidases (including TPO) in the presence of H_2O_2 to a proprietary resorufin-like molecule with very similar fluorescence characteristics. Working concentration of 10 mM AUR (Cat# A36006, Invitrogen™, Thermo Fisher Scientific) was prepared by adding 340 µl DMSO to a vial of AUR. To assay TPO activity, 20 µg of cell lysate or 5 µg of rat thyroid microsome extract was added to each well of an opaque black 96-well plate containing 25 µM AUR and 8.5 µl test chemicals at different concentrations in a total volume of 200 µl of potassium phosphate buffer. The final concentrations of each chemical tested with the engineered cell lysates were: 253, 84, 28, 9.3, 3.1, 1.04, 0.34, 0.11, 0.038, 0.013, 0.004, 0.001, 0.0004, 0.0001 and 0 µM. The final concentration of vehicle DMSO is 0.4% in all wells. Reaction was initiated by addition of 0.68 µl of 0.3% H_2O_2. Each chemical was tested across 6 orders of magnitude concentration range with each concentration tested in triplicate wells per test and each test repeated a total of 3 times on separate days with separate aliquots of cell lysate. Microplates were shaken for 30 min at room temperature in the dark after which fluorescence was quantified at 544 nm/590 nm excitation/emission using a Fluorometer (Molecular Devices).

Data analysis: Fluorescence intensity was corrected for background (average of enzyme-free wells), then normalized as a percentage of the vehicle control (100%). Mean percentage values were calculated for each separate experiment and concentration dependency was modeled by fitting a four parameter logistic curve (Sigma Plot 12.5, Systat Software Inc. San Jose, CA, USA) from which values of IC_{50} were calculated. Relative inhibitory potency of each chemical was derived by comparing to the known TPO inhibitor methimazole (MMI) (IC_{50} of MMI/ IC_{50} of test chemical).

Assay performance: The assay performance was estimated with three parameters: coefficients of variation (CV), signal separation (Z') and signal window (SW). These metrics were estimated using data from multiple dose series experiments using MMI as a model inhibitor. CV for negative control (DMSO) was calculated by the equation (Iversen et al., 2004): $CV = \left(\frac{SD}{\sqrt{n}}\right)/Avg$, where SD indicates standard deviation and AVG refers mean of fluorescence intensity; sample size n is 8 and 12 for CHO-TPO and LentiX-TPO, respectively. Z', indicating the signal separation, was calculated as (Iversen et al., 2004):

$$Z' = \frac{\left(AVG_{max} - \frac{3SD_{max}}{\sqrt{n}}\right) - \left(AVG_{min} + \frac{3SD_{min}}{\sqrt{n}}\right)}{AVG_{max} - AVG_{min}}$$

SW, indicating signal dynamic range, was calculated as (Iversen et al., 2004):

$$SW = \frac{\left(AVG_{max} - \frac{3SD_{max}}{\sqrt{n}}\right) - \left(AVG_{min} + \frac{3SD_{min}}{\sqrt{n}}\right)}{SD_{max}/\sqrt{n)}}$$

AVG_{max} and AVG_{min} are normalized mean of DMSO and MMI at highest concentration, respectively; while SD_{max} and SD_{min} are standard deviation of DMSO and MMI at highest concentration. Sample size n is 8 and 12 for CHO-TPO and LentiX-TPO, respectively.

Acceptable criteria were CV < 20%, Z' > 0.4 and SW > 2, as described in the publication (Iversen et al., 2004) that studied the performance of high throughput assay. Chemicals were considered to be TPO inhibitors based on the criterion of Paul Friedman et al. (2016) if TPO activity was reduced below 80%.

3. Results

3.1. Concentration response relationship of TPO activity with AUR assay

To demonstrate that CHO or LentiX cells can produce sufficient hTPO activity detected using this assay, increasing concentration of lysates from either cell line were incubated with AUR (Fig. 1 A and B). Lysates from either cell line expressing hTPO caused a linear concentration-dependent increase in production of AUR fluorescent product while lysates from cells containing the empty vector had no effect on fluorescence. This strongly indicates that the hTPO transgene is active and generates the only source of peroxidase activity in lysates from these cells. Activity of TPO in rat thyroid microsomes increased in a linear manner with increasing protein concentration but saturated above 5 µg (Fig. 1C). Consequently, 20 µg of protein from lysates of either cell line or 5 µg of rat thyroid microsomes were used for each well to provide TPO activity for routine assay. Aliquoted the lysate and used it only once to avoid re-freezing.

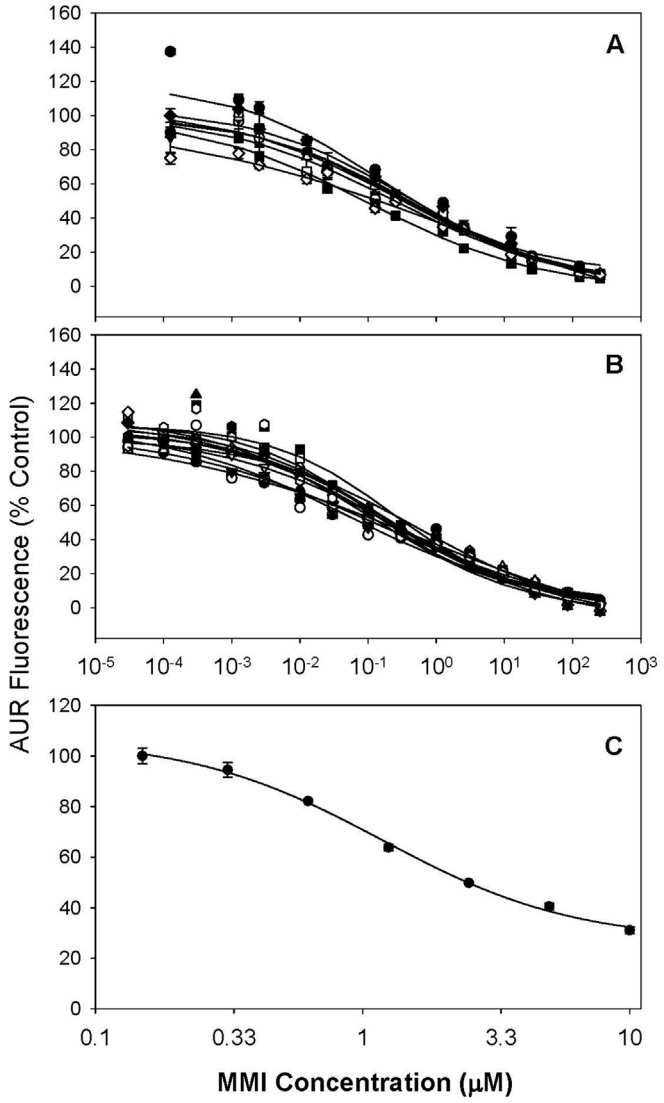

Fig. 2. The dose dependency of MMI inhibition of TPO activity in CHO-TPO cells (A), Lenti-TPO cells (B) and rat thyroid microsome extracts (C). Increasing concentrations of MMI were added to the cell lysate (20 μg protein) or rat thyroid microsome extracts (5 μg) and reacted with AUR in the presence of H2O2. Inhibition of TPO activity with MMI was repeated 8 and 12 times in CHO-TPO cells and LentiX-TPO cells, respectively. Data for rat thyroid microsomes are derived from a single experiment conducted in triplicate. All assay data were normalized to the DMSO vehicle control for each plate.

3.2. Inhibition by methimazole (MMI)

The repeatability of the AUR assay with different sources of hTPO was examined through multiple experiments with the positive control MMI. Extended dose ranges were evaluated 8 times with CHO-TPO (Fig. 2A) and 12 times with LentiX-TPO (Fig. 2B). The curves generated in these experiments followed a similar pattern and the average IC_{50} for MMI from CHO-TPO was 0.49 μM with individual estimates ranging between 0.15 and 2.0 μM (SD = 0.69). For LentiX-TPO, average IC_{50} was 0.23 μM with a range of 0.05 to 0.64 μM (SD = 0.17). These data result in the following estimates of performance: SW was 15 ± 1.0, Z' was 0.91 ± 0.01 and CV was 7.2% for the assay in CHO-TPO cells. The average signal window was 34 ± 3.0, Z' was 0.85 ± 0.19 and CV was 3.8% for the assay in LentiX-TPO cells. The performance of AUR assay using hTPO from both cell lines reached the criteria of HTS (Iversen et al., 2004). MMI inhibition for TPO derived from rat thyroid microsome was examined once and IC_{50} was predicted to be 2.4 μM (Fig. 2C). These data are summarized in Table 2.

3.3. Activity of reference chemicals in hTPO Assay

In addition to the positive reference chemical MMI, 12 other TPO inhibitors with a broad range of potency and 6 chemicals previously reported to have no TPO activity (Paul Friedman et al., 2016; Paul et al., 2014) were tested on both hTPO preparations (Table 1). A subset of 6 inhibitors was also tested using rat thyroid microsomes for comparison (Fig. 3). The values of IC_{50} of PTU and RSC were similar between hTPO and rat TPO (Fig. 3A and C). The potency of MBT inhibiting hTPO in LentiX-TPO cells (IC_{50} = 5.2 μM) was an order of magnitude higher than that observed in rat TPO (0.5 μM, Fig. 3B). The weak inhibitor TCS was not observed to be inhibitory with either hTPO preparation but was detected as a very weak inhibitor (IC_{50} = 109 μM) by the rat microsomal TPO (Fig. 3D). For two weak inhibitors, IOA and SMZ (Fig. 3E and F, respectively), inhibitory activity (< 80% of control activity) was only detected at the highest test concentration, except SMZ for TPO from rat thyroid microsome, which has no inhibitory activity at all concentrations tested.

Curves for the remaining 6 positive substances are compared for both hTPO preparations in Fig. 4. Both hTPO preparations identified strong inhibitors AMT, BP2, and GEN resulting in similar IC_{50} values (Fig. 4 A-C, respectively). Of the chemicals anticipated to be weak inhibitors (Table 1), other than TCS, the criterion for positive (< 80% of control activity) was achieved for the highest concentration tested in at least one of the hTPO preparations (Fig. 4D-F). Weak inhibitory activity of 4NP was found for the highest concentration tested in CHO-TPO cells, while no inhibitory activity was observed in LentiX-TPO cells (Fig. 4E). ETU was found to inhibit the hTPO activity prepared from both cells, but no reliable IC_{50} could be predicted (Fig. 4F).

The values of IC_{50} and relative potency (IC_{50} of MMI / IC_{50} of test chemical) for all inhibitory reference chemicals from either TPO preparations are summarized in Table 3. Of the 6 positive chemicals, all were detected by both hTPO preparations with similar IC_{50} values. Of the 6 weakly positive reference chemicals, DDZ inhibited both hTPO preparations with IC_{50} values calculated to be 19.6 and 3.4 μM for CHO and LentiX preparations, respectively. None of the purported negative reference substances had any impact on activity of either hTPO preparation (data not shown).

4. Discussion

The current study has demonstrated that recombinant hTPO,

Table 2
The performance of AUR-TPO assay.

	CHO-TPO cells	LentiX-TPO cells	Rat thyroid microsome extracts
IC_{50} (μM) average (range)	0.49 (0.15–2.0)	0.23 (0.05–0.64)	2.4
SW (average ± SD)	15 ± 1.0	34 ± 3.0	*
Z' (average ± SD)	0.91 ± 0.01	0.85 ± 0.19	*
CV (%)	7.2	3.8	*
n	8	12	1

* Since the MMI inhibition on the activity of rat TPO was only conducted once, the reliable SW, Z' and CV could not be calculated.

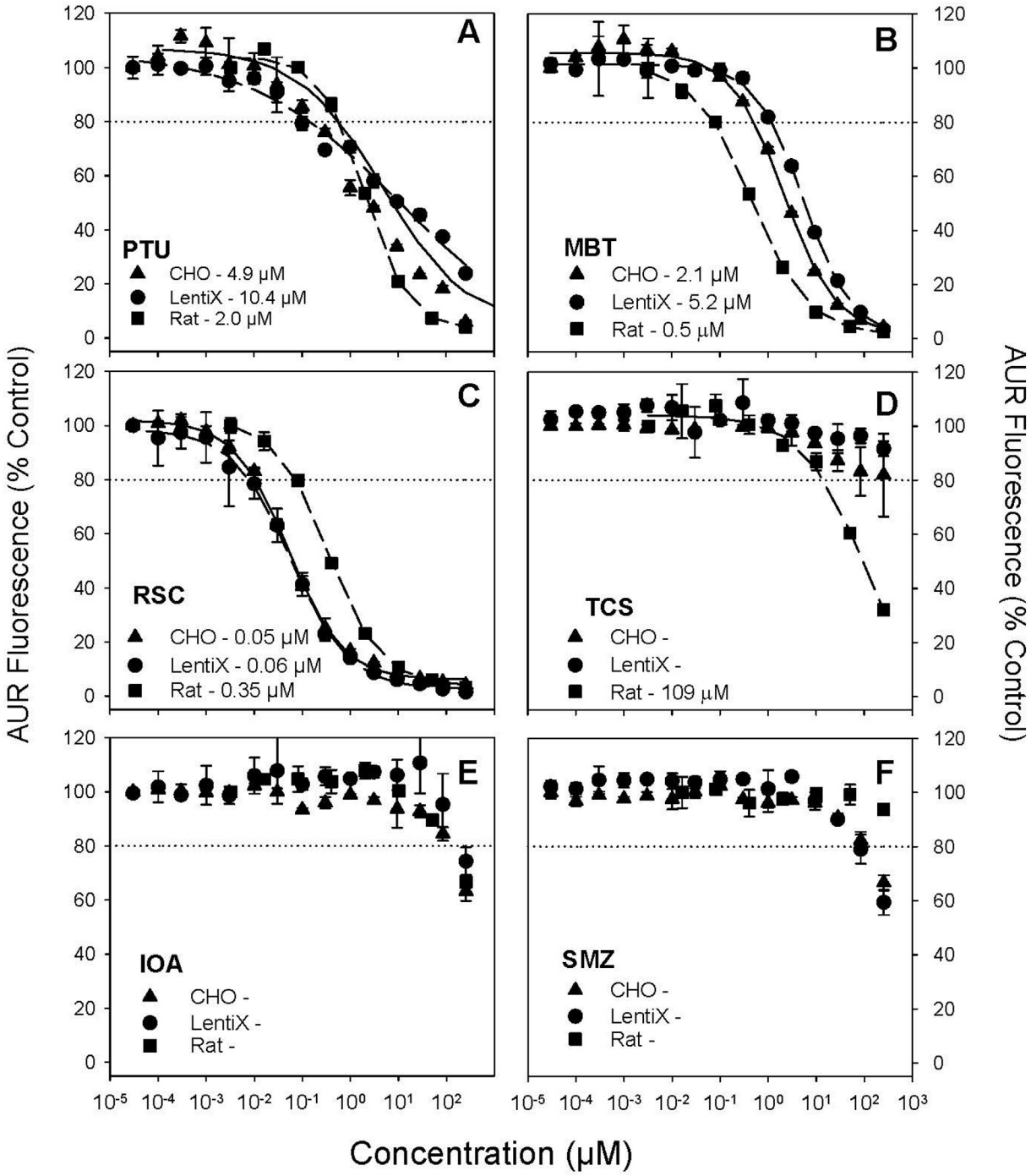

Fig. 3. Comparison of dose response curves for reference chemicals between human and rat TPO. Increasing concentrations of 6 reference chemicals were added to wells containing lysates (20 µg protein) of CHO-TPO cells, LentiX-TPO cells or thyroid microsome extracts (5 µg). Values represent the mean of three replicate experiments, each with fresh reagents and treatments in triplicates.

produced from stably transfected cells lines, can provide sufficient enzyme activity to be assayed consistently using the peroxidase AUR substrate. Consequently, the AUR TPO assay can be developed using the human target enzyme, thereby lessening the use of laboratory animals for toxicological screening and enhancing the human-relevance of resulting data. The use of AUR to assay TPO activity was first described with rat thyroid microsomes as a source of enzyme activity (Paul et al., 2014). Its use to assay hTPO derived from the human thyroid follicular cell line Nthy-ori 3-1 was subsequently reported by Jomaa and collaborators (Jomaa et al., 2015). We describe two transgenic cell lines expressing hTPO that can also provide enzyme activity to screen for TPO inhibition using a human-relevant assay.

The advantage of AUR over the more widely reported assay format using guaiacol (Doerge and Takazawa, 1990; Freyberger and Ahr, 2006; Nakashima and Taurog, 1978) is that the peroxidase product of AUR, resorufin, is stable at ambient temperature allowing the assay to be quantified by a single read of fluorescence after a defined reaction period rather than a kinetic assay. This property provides increased flexibility making the AUR-based TPO assay amenable for use in very high throughput screening, as it has been used to test TPO inhibitory activity of chemicals from the large chemical libraries of ToxCast (Paul Friedman et al., 2016).

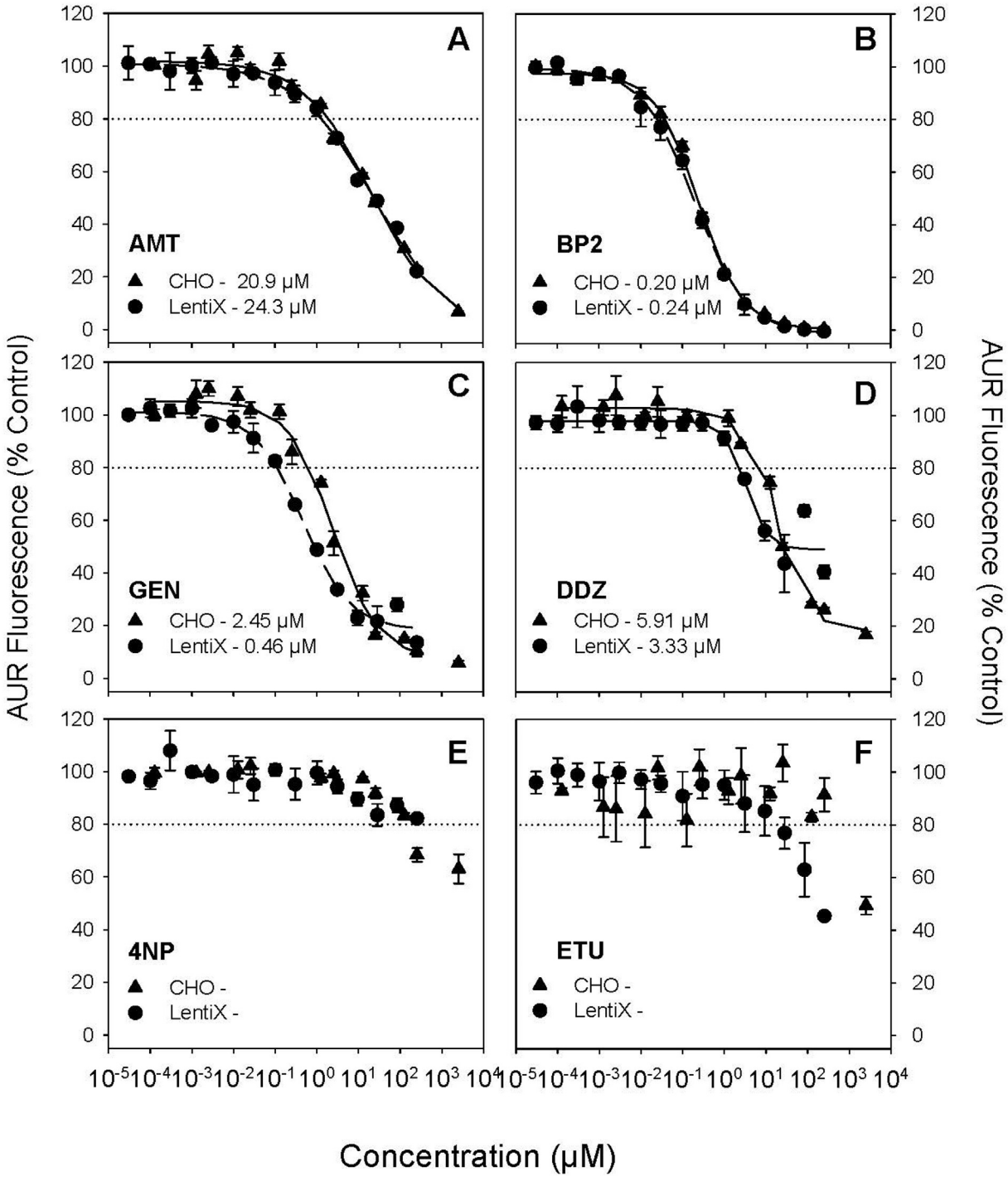

Fig. 4. Comparison of dose response curves for reference chemicals between lysates of human TPO expressing cells. Increasing concentrations of 6 reference chemicals were added to wells containing lysates (20 µg protein) of CHO-TPO cells or LentiX-TPO cells. Values represent the mean of at least three replicate experiments, each with fresh reagents and treatments in triplicates.

Our results with rat microsome extracts were consistent with previous work with this TPO source. Rat TPO was strongly inhibited by MMI, PTU and MBT. However, previous reports of RSC activity, the most potent inhibitor in our models, are mixed as Paul et al. (Paul Friedman et al., 2016) observed very potent inhibition of rat microsomal TPO activity; an early study from the same research group reported only weak activity (Paul et al., 2014). An unrelated study reported that RSC is 27 times more potent than PTU (Lindsay et al., 1992). Despite *in vitro* observations of RSC potency in TPO inhibition, there is little *in vivo* evidence that RSC exposure leads to impaired TH synthesis to the same extent of other potent TPO inhibitors MMI or PTU. Exposure of breeding rats to RSC did not cause reductions of blood THs levels nor induced developmental toxicity or neurotoxicity (Welsch et al., 2008; IPCS, 2006). Yet both MMI and PTU exposure in similarly designed studies lead to marked reductions in TH synthesis, impaired TH signalling and neurodevelopmental delays and impairments (reviewed in (Motonaga et al., 2016)). The discordance of *in vitro vs in vivo* potency of RSC is likely explained by rapid absorption, metabolism and excretion of administrated RSC that resulted in minimal residence of the active agent in the thyroid gland (Merker et al., 1982; Kim and

Table 3
IC_{50} and relative potency of test chemicals.

	CHO-TPO assay		LentiX-TPO assay		Rat thyroid microsome TPO assay		Previously Reported	
Chemical	IC50	Relative potency	IC50	Relative potency	IC50	Relative potency	IC50[a]	Relative potency
MMI	0.49	1.00	0.23	1.00	2.4	1.00	0.06	1
RSC	0.05	9.8	0.06	3.83	0.345	6.96	0.025	2.4
BP2	0.20	0.41	0.24	0.96	–	–	0.17	0.35
MBT	2.10	0.23	5.2	0.044	0.5	4.80	0.36	0.17
GEN	2.45	0.20	0.45	0.511	–	–	3.5	0.017
PTU	4.9	0.10	10.4	0.022	2	1.20	0.23	0.26
DDZ	5.91	0.08	3.33	0.069	–	–	10	0.006
AMT	20.9	0.023	24.3	0.009	–	–	1.1	0.024
ETU	> 253	N/A[b]	198	0.0011	–	–	7.8	0.0077
SMZ	> 253	N/A	> 253	N/A	–	–	18	0.0012
TCS	> 253	N/A	> 253	N/A	109	0.022	142	0.00017
IOA	> 253	N/A	> 253	N/A	–	–	160	0.00013
4NP	> 253	N/A	> 253	N/A	–	–	44	0.00053

-, Not tested.
[a] Values from Paul et al. (2014) and Paul Friedman et al. (2016).
[b] N/A, No activity.

Matthews, 1987). Such observations remind us that interpreting *in vitro* HTS assay for TPO, like that for other mechanisms of toxicity, must consider test chemical ADME (absorption, distribution, metabolism and excretion).

Current results for some weaker reference chemicals diverged from expectation. Of the 6 weak chemicals tested, only one chemical (DDZ) inhibited hTPO to a sufficient extent to allow potency estimates (IC_{50}) to be calculated. Among the others, there was no indication of hTPO inhibitory activity (TCS) or inhibitory activity (< 80% control TPO activity) was only observed in at least one of two cell lines at the highest dose tested. TCS was found weakly inhibit rat TPO, consistent with the previous reports (Paul et al., 2014). In the current study, a reliable IC_{50} could not be estimated for ETU using hTPO from either cell lines; while IC_{50} of ETU using rat thyroid microsome TPO was estimated to be 0.03 μM or 7.8 μM but > 1000 μM using porcine thyroid microsome extracts (Paul Friedman et al., 2016; Paul et al., 2014; Doerge and Takazawa, 1990; Freyberger and Ahr, 2006). The basis for this discrepancy is not known but might reflect species difference in chemical sensitivity or an issue with the specific lot of this chemical as we do not have the capacity to confirm the structure or purity of any chemical beyond the documentation received from the supplier. Inhibitory activity of SMZ is not consistent based on the available literatures. Inhibition of porcine TPO by SMZ was very weak with an IC_{50} > 300 μM (Doerge and Decker, 1994), yet SMZ inhibited rat microsomal TPO with IC_{50} of 18 μM (Paul et al., 2014). SMZ was not identified to inhibit rat TPO in the current study, but weakly inhibit hTPO activity derived from both cell lines. Reports of IOA potency were mixed with no effect on TPO activity in both rat and porcine thyroid microsome extracts reported by (Paul et al., 2013), but IC_{50} 160 μM in rat thyroid was reported in another study by the same research group (Paul et al., 2014). We observed weakly inhibitory activity of IOA in the current study. Similarly, the observed potency of 4NP varies: inhibitory activity was observed in studies with a cell line overexpressing hTPO (Schmutzler et al., 2007; Schmutzler et al., 2004) and rat thyroid microsome extracts (Paul Friedman et al., 2016; Paul et al., 2014), while no activity observed in another study with pig or rat thyroid microsome extracts (Paul et al., 2013). We observed the weak inhibition in hTPO derived from only one of cell lines overexpressing hTPO in the current study. This difference may suggest that cellular background factors presenting in the hTPO extracts prepared from the different host cells from different species background (Human HEK293 *vs* Chinese Hamster Ovary) may underlie some difference in response. Alternatively, these may reflect species difference in enzyme sensitivity or inconsistencies in the quality/purity of chemical preparations across studies.

In contrast with weakly acting substances, the current study consistently identified hTPO inhibitor activity of reference chemicals expected to be potent to moderate inhibitors and consistently identified the chemicals expected to be inactive of TPO inhibitory. In Table 1, all 7 chemicals anticipated being positive were observed to inhibit hTPO from either cell line with IC_{50} of < 25 μM; and all 6 negative chemicals were observed to be inactive in both cell lysates (data not shown). In the current study, the potency of MMI appeared to be higher for hTPO than for rat microsomal TPO with IC_{50} values of 0.49 and 0.23 μM for the two hTPO preparations compared to 2.4 μM rat TPO (Table 3). As we have followed the convention of Paul et al. (Paul et al., 2014) in using the results derived from MMI as the reference to estimate relative potency of test chemicals, this observed difference potency between hTPO preparations and rat TPO has a considerable effect on estimates of relative potency (Table 3). These results do indicate that hTPO preparations can be used effectively to identify chemicals strongly or moderately inhibiting TPO activity.

Although there are advantages using hTPO from engineered cell lines to screen chemicals as mentioned above, there are some uncertainty and limitations. For example, as the cell lines used to generate the enzyme in the current study are not the physiologically appropriate source, it is possible that the enzyme conformation, post-translational modifications and cell-specific context may differ from that of enzyme produced in the native, physiologically-relevant source (*i.e.* the intrafollicular facet of the human thyroid epithelial cell). Each of these differences could theoretically result in subtle differences in substrate, co-factor binding or some other aspect that influences susceptibility to inhibition. One of the hTPO sources (CHO-TPO) used herein is well-recognized by the broad panel of TPO-specific conformation-sensitive human (IgG and Fab fragments), rabbit and murine antibodies (Godlewska et al., 2014). Moreover, it contains high quantity of N-glycans and forms dimers and could be effectively delivered to the cell membrane and exhibits enzymatic activity at the cell surface (Godlewska et al., 2014). Similar characterization of hTPO from LentiX cells is not available but nor is there definitive evidence that transgenic enzyme derived from non-thyroid cells performs sufficiently distinctly to alter the predictive capacity of the assay. Such evidence could be generated by a direct comparison of assay responses to diverse reference chemicals between hTPO from transgenic cells *vs* TPO from microsomes of human thyroid tissue. However, it seems unlikely that such differences should alter enzyme activity to the extent that the assay based on transgenic enzymes should fail to detect inhibition by true potent chemicals.

In conclusion, we describe two cell lines that efficiently produce human TPO activity that can be used in AUR-based HTS assay to effectively characterize TPO inhibitors. Screening assays based on these

cells or that described by Jomaa and collaborators (Jomaa et al., 2015) produce data relevant to human molecular targets of thyroid disrupting chemicals as well as reduce the use of animals in toxicity screening.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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