Research Article

Simple and Rapid *In Vitro* Assay for Detecting Human Thyroid Peroxidase Disruption

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Summary

A simple and rapid luminometric assay for the detection of chemical inhibitors of human thyroid peroxidase (hTPO) activity was developed and validated with 10 model compounds. hTPO was derived from the human thyroid follicular cell line Nthy-ori 3-1 and its activity was quantified by measuring the oxidation of luminol in the presence of hydrogen peroxide (H₂O₂), which results in emission of light at 428 nm. In this assay, hTPO activity was shown to be inhibited by 5 known TPO inhibitors and not inhibited by 5 non-inhibitors. Similar results were obtained with porcine TPO (pTPO). The inhibition of hTPO by the model compounds was also tested with guaiacol and Ampliflu Red as alternative indicator substrates. While all substrates allowed the detection of pTPO activity and its inhibition, only the Ampliflu Red and luminol-based methods were sensitive enough to allow the quantification of hTPO activity from Nthy-ori 3-1 cell lysates. Moreover, luminol gave results with a narrower 95% confidence interval and therefore more reliable data. Whole extracts of fast-growing Nthy-ori 3-1 cells circumvent the need for animal-derived thyroid organs, thereby reducing costs, eliminating potential contamination and providing the possibility to study human instead of porcine TPO. Overall, the application of luminol and Nthy-ori 3-1 cell lysate for the detection of the disruption of hTPO activity was found to represent a valuable *in vitro* alternative and a possible candidate for inclusion within a high throughput integrated testing strategy for the detection of compounds that potentially interfere with normal thyroid function *in vivo*.

Keywords: human thyroid peroxidase, thyroid disruption, alternatives to animal testing, Nthy-ori 3-1, *in vitro* assay

1 Introduction

Thyroid hormones are essential for normal development and the regulation of basal metabolism. A deficiency in these hormones due to a lack of iodine during the first two trimesters of pregnancy is prevalent in landlocked areas and leads to cretinism (Cao et al., 1994). The tell-tale signs of cretinism include short stature and mental retardation. Later on in life, thyroid hormone deficiency or excess lead to goitre, which is a swelling of the neck due to the enlargement of the thyroid gland. In the case of deficiency, the symptoms include weight gain, constipation and lethargy while in the case of excess, the symptoms include

adrenergic stimulation as well as hypermetabolism (Dogra et al., 2006). The turn of the 21st century witnessed a worldwide drop in iodine deficiency due to decades of table salt fortification with iodine and a rise in concern over the ability of manmade chemicals to disrupt the thyroid hormone system (Delange et al., 2001; Jomaa, 2014). Addressing endocrine disruption, including the disruption of the thyroid hormone system, has been mandatory in the US since 1996 and the EU since 2006 (USEPA, 1996a,b; European Commission, 2006). Testing for thyroid hormone disruptors has been challenging due to the long duration, costs and unethical nature of the animal experiments involved. This has prompted the Organisation for Economic Co-operation

Abbreviations

AMI, amiodarone hydrochloride; BBP, benzyl butyl phthalate; EC₅₀, effective concentration 50%; HPT axis, hypothalamus-pituitary-thyroid axis; hTPO, human thyroid peroxidase; HTS, high throughput screening; IC₅₀, inhibitory concentration 50%; MMI, methimazole; NAR, naringenin; OUB, ouabain; pTPO, porcine thyroid peroxidase; PTU, propylthiouracil; QE, quercetin; RES, resorcinol; T3, triiodothyronine; T4, tetraiodothyronine; TH, thyroid hormone; TPO, thyroid peroxidase

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and Development (OECD) to search for high-throughput *in vitro* alternatives (Jacobs et al., 2013; OECD, 2014).

The *in vivo* tests to be replaced include the OECD test guideline 407 (TG 407) for a repeated dose 28-day oral toxicity study in rodents that defines serum thyroid hormone levels, thyroid histopathology, as well as pituitary and thyroid organ weights as endpoints (OECD, 2007, p. 407). While it is currently not possible to replicate thyroid histopathology *in vitro*, in a previous study we have searched for potential correlation between the effects of chemicals on pituitary and thyroid organ weights *in vivo* and the effects of the same set of chemicals on cellular proliferation *in vitro* using pituitary and thyroid model cell lines. To this end, 11 thyroid-active compounds were tested and it was concluded that *in vitro* cellular proliferation alone correlates poorly with pituitary and thyroid organ weight change *in vivo* (Jomaa et al., 2013). This is likely due to the complex multi-organ paradigm that the thyroid system represents (Jomaa, 2014). A remaining *in vivo* endpoint that may prove suitable for the development of *in vitro* alternatives is the serum level of thyroid hormones, which is under the control of a feedback mechanism that accounts for hormonogenesis, metabolism and excretion.

Maternal thyroid deficiency is associated with subtle deficits in the neuropsychological development of the fetus (Haddow et al., 1999). Thyroid dysmorphogenesis can be due to either genetic mutations or chemicals present in our food and environment. There is a high prevalence of thyroid peroxidase (TPO) mutations in patients with low levels of thyroid hormones at birth, a condition known as congenital hypothyroidism (Avbelj et al., 2007). Left untreated, this disease negatively affects growth and leads to mental retardation (Rose and Brown, 2006). The environment, through chemical inhibitors, can also affect TPO function. These chemical TPO inhibitors include the anti-thyroid drugs propylthiouracil (PTU) and methimazole (MMI), the industrial chemicals benzophenone 2 (BP2) and bisphenol A (BPA), and also foods rich in glucosinolates and flavonoids (Alexander and Zenker, 1986; Divi and Doerge, 1996; Schmutzler et al., 2007). Eating large quantities of cabbage, a source of glucosinolates, can lead to hypothyroidism and in extreme cases even coma (Chu and Seltzer, 2010; Dolan et al., 2010). Moreover, infants fed soy formulae, which are rich in flavonoids, can develop soybean goitre (Shepard et al., 1960).

TPO is a heme-dependent enzyme that, in the presence of H_2O_2 , catalyses the iodination and coupling of tyrosyl residues on thyroglobulin (TG), which are important steps in the biosynthesis of the thyroid hormones triiodothyronine (T3) and tetraiodothyronine (T4).

Even though the reduction in thyroid hormone levels due to the inhibition by chemicals of human TPO (hTPO) activity is a cause of concern, there is still no effective assay suited for hTPO-based high throughput screening (HTS). In a new scoping document on *in vitro* and *ex vivo* assays for the identification of modulators of thyroid hormone signaling, the OECD concluded that it was necessary to develop a screening assay for the inhibition of TPO that does not use animal tissue and is suited for HTS (Jacobs et al., 2013; OECD, 2014). Moreover, the OECD mentioned a study by Takamaya et al. that found rat TPO to be

in some cases more than 455 times more sensitive to xenobiotic inhibitors than monkey TPO, emphasizing the potential for substantial interspecies differences (Takayama et al., 1986). The aim of the present study is to address these issues by developing an assay that a) uses hTPO instead of TPO from animal tissue, and b) is suited for HTS.

2 Materials and methods

Chemicals

Methimazole (MMI), propylthiouracil (PTU), quercetin (QE), naringenin (NAR), resorcinol (RES), triiodothyronine (T3), tetraiodothyronine (T4), ouabain (OUB), benzyl butyl phthalate (BBP) and amiodarone hydrochloride (AMI) were of high purity and were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Unless otherwise specified, all reagents were also obtained from Sigma-Aldrich Chemie.

Cell culture

Nthy-ori 3-1, a normal thyroid follicular epithelial cell line, was obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, UK) and grown as a monolayer in RPMI-1640 medium (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS). The cells were cultured at 37°C in a humid atmosphere containing 5% (v/v) CO_2 and passaged enzymatically twice a week.

FRTL-5, a rat thyroid cell line (obtained from CLS, Germany), was cultured as a monolayer in tissue-culture flasks (obtained from Corning, Badhoevedorp, The Netherlands) at 37°C in a humid atmosphere containing 5% (v/v) CO_2 and passaged once a week with an interim refresh of the medium, which consisted of Coon's Modified F-12 medium (Biochrom, Berlin, Germany) supplemented with 5% FCS and a mixture of six hormones/growth factors (this medium is referred to as 6H5, reflecting the presence of 6 hormones/growth factors and 5% FCS), i.e., bovine thyroid stimulating hormone (1 mIU/ml), insulin (10 μ g/ml), hydrocortisone (10 nM), apo-transferrin (5 μ g/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml) and somatostatin (10 ng/ml). All these hormones/growth factors were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). The 6H5 cell culture medium was supplemented with 1 mM non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (Difco, Amsterdam, The Netherlands). Enzymatic passaging was performed using a cell detachment and disaggregation solution containing 20 units/ml collagenase, 0.075% trypsin and 2% chicken serum (CTC) in PBS. Collagenase and chicken serum were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands) while trypsin was obtained from Difco (Amsterdam, The Netherlands). The FRTL-5 cell line was used as a reference cell line that is positive for TPO (Suzuki et al., 1998).

The human hepatocellular carcinoma (HepG2) cell line was used as a reference cell line that is negative for TPO activity. HepG2 were cultured in DMEM/F12 medium containing 10% FCS, passaged twice a week and kept at 37°C in a humid atmosphere containing 5% (v/v) CO_2 .

TPO preparation

TPO from Nthy-ori 3-1 cells was prepared as a whole cell extract. Briefly, cells were washed with phosphate buffered saline (PBS), scraped, resuspended in a small volume of PBS and spun down for 5 min at 150 x g. The supernatant was discarded and the cell pellet lysed by resuspension in 0.1% sodium deoxycholate (DC, in PBS) and incubation on ice for 20 min. The lysed cells were centrifuged for 5 min at 11,350 x g to separate the soluble protein fraction from unlysed cells and debris. Protein concentration of the supernatant was then measured using the Pierce BCA protein assay (Pierce, Etten-Leur, The Netherlands) following the manufacturer's protocol. Cell lysates from one 75 cm² cell culture flask that was ≥ 80% confluent were resuspended in 2 ml of 0.1% DC and had a protein content of 0.1-0.2 mg/ml. With 100 μl used per 96-well plate quadruplicate, one 75 cm² cell culture flask was therefore sufficient for testing ~ 20 different concentrations of a test compound or controls. For long-term storage, cell lysates were kept at -80°C. Relative TPO gene expression in the Nthy-ori 3-1 cell line has been previously quantified by others (Tuncel et al., 2007). In our experiments, TPO activity in cell extracts, which is proportional to relative fluorescence (for Ampliflu Red) or relative luminescence (for luminol) obtained from cell lysates containing 0.1-0.2 mg/ml total protein, was consistently above the limit of quantitation (LOQ) assessed as the mean of the blank + 10 times the standard deviation.

TPO from porcine thyroid glands was prepared essentially as described previously (Hosoya et al., 1985), with some modifications. Briefly, porcine thyroid tissue was obtained at the slaughterhouse (Visser, Lunteren, The Netherlands) and homogenized in sterile buffer (0.25 M sucrose; 20 mM Tris/HCl, pH 7.4; 100 mM KCl; 40 mM NaCl; 10 mM MgCl₂) with 10 strokes at 24,000 rpm using an Ultra-Turrax. The sample was homogenized further by 10 strokes in a Potter-Elvehjem tissue grinder (VWR, Amsterdam, The Netherlands). The ground tissue was centrifuged for 10 min at 375 x g. The supernatant was then diluted to 50 mg/ml of tissue in sterile buffer.

Guaiacol and Ampliflu Red assays for TPO activity

Reactions were conducted in 96-well plates with a volume of 200 μl per well containing a thyroid tissue protein content of 0.6-1 mg/ml or a cell lysate protein content of 0.1-0.2 mg/ml, 0.1 M potassium phosphate (pH 7.4), and the desired concentration of the test compound added from a 100 times concentrated stock solution in DMSO (1% DMSO final concentration). 1% DMSO does not interfere with either the guaiacol or the Ampliflu Red assays for peroxidase activity (Vogt et al., 2002; Schmutzler et al., 2007; Dabir et al., 2013). The mixture was incubated for 30 min with gentle shaking at 37°C after which the reaction was initiated by addition of 20 μl of either 1 M guaiacol or 1 mM Ampliflu Red and 5 μl of 80 mM H₂O₂. Guaiacol absorbance was measured at 470 nm ($\epsilon = 2.66 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Chance and Maehly, 1955)) and Ampliflu Red fluorescence was measured at 544/590 nm excitation/emission wavelengths, with readings every 30 sec for 10 min on a SpectraMax M2 microplate reader (Molecular Devices, Menlo Park, CA, USA). The

reaction rate was calculated as the slope of a linear fit of the 21 absorbance values using the SoftMax Pro software provided with the microplate reader.

Luminol assay for TPO activity

Reactions were conducted in 96-well plates with a volume of 200 μl per well containing a thyroid tissue protein content of 0.6-1 mg/ml or a cell lysate protein content of 0.1-0.2 mg/ml, 1 M glycine-NaOH (pH 9.0), 1 mM EDTA, and the desired concentration of the test compound added from a 100 times concentrated stock solution in DMSO (1% DMSO final concentration). 1% DMSO does not affect luminol luminescence (Shertzer et al., 2004). The mixture was incubated for 30 min with gentle shaking at 37°C after which the reaction was initiated by the addition of 20 μl of luminol mix containing 1 M glycine-NaOH (pH 9.0), 1 mM EDTA and 400 μM luminol. Following a 4 sec delay, 5 μl of 80 mM H₂O₂ was automatically added. Luminescence was measured as relative luminescence units (RLUs) integrated over 10 sec using a Luminoskan Ascent luminometer from Labsystems (Helsinki, Finland).

Data analysis

In the guaiacol, Ampliflu Red and luminol assays for TPO activity, data points are representative of three independent experiments (N = 3) and four replicate wells per data point in each experiment. Raw data from quadruplicate wells were averaged, converted to percent of solvent control and represented graphically as the means of independent experiments with bars representing the standard error of that mean (SEM). Nonlinear curve-fitting described in the results was done using the Hill equation with the help of GraphPad Prism software version 5.04 (GraphPad Software, San Diego, CA, USA). *In vitro*, relative IC₅₀ values were calculated from the fitted models as the concentration of the tested compound that gave 50% of the maximal response. A response was considered positive when the squared Pearson's coefficient of correlation (r^2), as an indication of goodness-of-fit of a logistic dose-response model, had a value of 0.8 or higher. The dynamic range, Z' factor (Zhang et al., 1999) and average assay coefficient of variation (CV) were calculated in order to describe the performance of the luminol assay for TPO activity. The Z' factor is defined in terms of the standard deviations (SD) and the means of the positive and negative (DMSO) controls with the following formula:

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

3 Results

3.1 Compound selection

Five model compounds known to inhibit TPO *in vitro* were selected, i.e., MMI, PTU, QE, NAR and RES. Five thyroid-active compounds that interfere with the thyroid hormone system through a non-TPO mode of action were also selected, i.e., T3, T4, BBP, OUB and AMI. Table 1 details the CAS numbers, common use or origin, chemical structure and a representative literature reference pertaining to existing guaiacol-based

Tab. 1: Selected model compounds

Test compounds used in this study with a brief description of their use or nature and their chemical structure. A dash represents a lack of literature on the compound's effect on TPO.

Compound	Abbr.	CAS No.	Description	Chemical structure	<i>In vitro</i> TPO inhibition literature (guaiacol assay)	<i>In vivo</i> thyroid disruption literature
Methimazole	MMI	60-56-0	Antithyroid drug		Taurog et al., 1996	Hood et al., 1999
Propylthiouracil	PTU	51-52-5	Antithyroid drug		Hosoya, 1963	OECD, 2006
Quercetin	QE	117-39-5	Plant copigment		Divi and Doerge, 1996	Giuliani et al., 2014
Naringenin	NAR	480-41-1	Plant copigment		Divi and Doerge, 1996	Panda and Kar, 2014
Resorcinol	RES	108-46-3	Antiseptic used in cosmetics		Divi and Doerge, 1994	Berthezène et al., 1979
Triiodothyronine	T3	6893-02-3	Endogenous hormone		—	Soukup et al., 2001
Thyroxine	T4	51-48-9	Endogenous hormone		—	OECD, 2006

Compound	Abbr.	CAS No.	Description	Chemical structure	<i>In vitro</i> TPO inhibition literature (guaiacol assay)	<i>In vivo</i> thyroid disruption literature
Benzyl butyl phthalate	BBP	85-86-7	Plasticizer		–	Nagao et al., 2000
Ouabain	OUB	630-60-4	Cardiotonic and antiarrhythmic drug		–	Wardlaw, 1986
Amiodarone HCl	AMI	19774-82-4	Antiarrhythmic drug		–	Safran et al., 1986

in vitro tests of effects of these compounds on TPO activity as well as the *in vivo* effects of these compounds on the thyroid hormone system. All test compounds and reagents were of high purity and were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands).

3.2 Luminol assay for TPO activity

Figure 1 shows that the protein content of the porcine thyroid homogenate and Nthy-ori 3-1 lysates correlate linearly with the luminescence that is produced in the luminol assay. For thyroid homogenate the squared coefficient of correlation (r^2) was 0.96 and for the Nthy-ori 3-1 lysate the r^2 was 0.98. Based on results obtained with MMI (N = 3) as a positive control, the luminol

assay for TPO activity had a dynamic range of 25.4 ± 4.3 (mean $\pm$ SEM), a Z' factor of 0.74 ± 0.021 (mean $\pm$ SEM) and an average CV (%) of 4.94. In the luminol assay, the reaction was started with H_2O_2 that was added by the automatic dispenser in the luminometer followed by a 2 second delay before measurement and integration of the signal over 10 seconds. The luminol signal decays more quickly than Ampliflu Red, however, the integration procedure enables reproducible results (%CV = 4.94 for MMI) irrespective of the decay of the luminescence signal. The fact that the luminol signal can be measured within a shorter period of time allowed for a more rapid assay. In case of the Ampliflu Red assay, the reaction proceeded linearly over the measured reaction period of 10 min.

3.3 TPO inhibition

In Figure 2 dose response curves for the 5 tested TPO inhibitors are presented based on three separate experiments with four replicate wells per concentration. It can be seen that the luminol assay detects the disruption of TPO activity by the 5 selected TPO inhibitors in a dose-dependent manner. Moreover, while the Ampliflu Red (Paul et al., 2014), guaiacol and luminol (Tab. 1) methods are all able to detect pTPO activity that is present in

porcine thyroid tissue samples, the guaiacol assay was negative with hTPO from the Nthy-ori 3-1 human thyroid cell line (data not shown), and only the Ampliflu Red and luminol methods were able to detect hTPO activity in lysates from Nthy-ori 3-1 human thyroid cells cultured *in vitro* (Tab. 1). The selected non-inhibitors of TPO, namely, T3, T4, BBP, OUB, and AMI were also all correctly identified as non-inhibitors by both the Ampliflu Red method and the luminol method (Fig. 3).

Fig. 1: TPO-mediated light emission upon oxidation of luminol

a) Porcine thyroid gland homogenate and b) human Nthy-ori 3-1 thyroid cell lysate dependent enzymatic activity in terms of RLU emitted upon oxidation of luminol in the presence of H_2O_2 .

Fig. 2: Effect of TPO inhibitors in the Ampliflu Red and luminol assay

Effect of MMI, PTU, quercetin, naringenin and resorcinol on a) hTPO activity in the Ampliflu Red assay and on b) pTPO and c) hTPO activity in the luminol assay.

Fig. 3: Effect of TPO non-inhibitors in the Ampliflu Red and luminol assay

Effect of T3, T4, ouabain, BBP and amiodarone on a) hTPO activity in the Ampliflu Red assay and on b) pTPO and c) hTPO activity in the luminol assay.

Tab. 2: TPO inhibition by model compounds

IC₅₀ values of the compounds as TPO inhibitors in the guaiacol assay, Ampliflu Red assay and luminol assay. CI stands for confidence interval (n=3). r² is indicated for the goodness-of-fit of a logistic dose-response model.

Compound	IC ₅₀ μ M (95% CI or reference) guaiacol assay		IC ₅₀ μ M (95% CI) Ampliflu Red assay		IC ₅₀ μ M (95% CI) luminol assay			
	pTPO	r ²	hTPO	r ²	pTPO	r ²	hTPO	r ²
MMI	4.0 (Taurog, 1970)	–	2.7 (0.4 - 19.9)	0.95	5.7 (3.0 - 10.7)	0.95	4.0 (1.6 - 10.1)	0.91
RES	6.5 (Taurog, 1970)	–	18.7 (6.8 - 51.9)	0.84	5.8 (4.0 - 8.3)	0.99	5.0 (3.6 - 6.9)	0.98
QE	8.4 (1.2 - 60.1)	0.78	7.7 (3.9 - 15.4)	0.96	10.0 (6.7 - 14.9)	0.99	4.1 (3.2 - 5.3)	0.99
PTU	10.7 (5.1 - 22.2)	0.92	35.2 (10.4 - 119.1)	0.86	41.6 (24.9 - 69.5)	0.94	16.4 (10.0 - 27.0)	0.91
NAR	10 - 150 (Divi and Doerge, 1996)	–	59.0 (30.4 - 114.6)	0.94	67.5 (29.24 - 155.6)	0.95	55.5 (43.2 - 71.3)	0.98

A comparison of the concentrations resulting in 50% inhibition (IC₅₀s) presented in Table 2 reveals that the luminol method detects the effects of the tested compounds with a narrower 95% confidence interval (CI). The overall potency ranking for pTPO inhibition was MMI > RES > QE > PTU > NAR. The potency ranking for hTPO inhibition was MMI > QE > RES > PTU > NAR.

3.4 Confounders of peroxidase activity measurements in samples from *in vivo* or *in vitro* origin

In order to find out whether contamination of thyroid tissue with blood or muscle tissue could be a confounder when analysing thyroid peroxidase activity in samples from animal origin, hemoglobin-rich muscle tissue homogenates were tested for peroxidase activity and compared to results obtained for thyroid homogenates. Figure 4a shows that hemoglobin-rich muscle tissue has peroxidase activity as well.

Confounding of measurement of TPO activity when using samples from *in vitro* cell lines could come from non-TPO

based activity in cells from non-thyroid origin. The cell lines used in this experiment were grown under controlled conditions to ensure uniformity and kept at below passage 30 in order to retain as much of the characteristics of their organ of origin as possible. Figure 4b shows that in the luminol assay using cell lysates from follicular rat thyroid cells grown using 5% serum (FRTL-5), from human Nthy-ori 3-1 cells or from human hepatocellular carcinoma (HepG2) cells, only cell lysates from cells of thyroidal origin have peroxidase activity.

4 Discussion

The aim of the present study was to address the pitfalls of animal tissue-based assays using guaiacol as a substrate by developing an assay that a) uses human TPO (hTPO) instead of TPO from animal tissue, and b) uses a substrate that is suited for HTS.

The results obtained reveal that lysates of the Nthy-ori 3-1 human thyroid cell line retain peroxidase activity that can be detected in the Ampliflu Red assay and luminol assay but not

Fig. 4: *In vivo* and *in vitro* TPO activity

a) Peroxidase activity can be detected from both thyroid homogenates and muscle tissue. b) *In vitro* peroxidase activity is present in thyroid cell lines only.

in the guaiacol assay. The greater sensitivity of luminol in comparison with guaiacol supports previous findings where luminol was found to be 3-fold more sensitive than guaiacol in detecting TPO activity (Kaczur et al., 1997). While already used for the detection of TPO inhibition by anti-TPO antibodies and in the detection of the inhibition of HRP by chemical inhibitors (Kaczur et al., 1997; Navas Díaz et al., 1998), the present study reports the application of luminol as a highly sensitive and reliable substrate that can be applied to detect the effect of chemical inhibitors on TPO activity. Figure 2 shows that all compounds that tested positive for inhibition in the guaiacol assay also tested positive for inhibition in the luminol assay. These results are in accordance with the known *in vitro* effect of these compounds on TPO activity (Tab. 1). Moreover, while the Ampliflu Red assay was also able to detect compounds known to inhibit TPO activity, the 95% CI of the IC₅₀ of the compounds tested was wider and therefore the Ampliflu Red-based assay is regarded to be less reliable than the luminol-based assay (Tab. 2).

Even though the guaiacol oxidation assay is widely used to detect peroxidase activity, the apparent lack of sensitivity is one of several drawbacks since guaiacol is also readily oxidized upon exposure to air and light, which means that necessary precautions have to be put in place to avoid premature color change. Moreover, the measurement time needed is 2-10 minutes, compared to 10 seconds for the luminol assay. Compound purity could be yet another drawback as it has been previously reported that guaiacol from Aldrich Chemical Co. contained contaminants that lead to low values for peroxidase activity (Taurog et al., 1992).

The successful use of TPO derived from the human follicular thyroid cell line Nthy-ori 3-1 avoids the use of animal tissue and eliminates interspecies differences when evaluating risks for the human population. Such interspecies differences exist and have been previously reported (Takayama et al., 1986; Paul et al., 2013) and are also illustrated in the present study by the difference in potency ranking observed between pTPO and hTPO in the luminol assay. Moreover, animal sources of TPO are often contaminated with blood hemoglobin that also can act as a peroxidase, a fact that can be attributed to the catalytic heme moiety present in both hemoglobin and TPO (Harauchi and Yoshizaki, 1982). In this study, it was demonstrated that hTPO from Nthy-ori 3-1 cells can be easily used in combination with a luminol assay method to detect thyroid-active compounds whose mechanism of action involves TPO inhibition. While other studies have reported increased *in vitro* TPO protein levels and activity in cells transfected with a TPO expression construct (Marinovich et al., 1995), the present study shows that the luminol assay is sensitive enough to detect the activity of endogenously expressed TPO from cells of the commercially-available human Nthy-ori 3-1 cell line. This overcomes the inability to measure TPO activity in samples derived from this cell line when using guaiacol as a substrate. It can therefore be concluded that there is no apparent need for using porcine TPO instead of human TPO. The relative high speed, stability and sensitivity of the luminol-based assay relative to the guaiacol-based assay fulfilled our objective to have an assay suited for HTS.

The results obtained with hTPO also mirror the effect of the TPO inhibitors tested in humans (Divi and Doerge, 1994; Nakamura et al., 2007). hTPO inhibition profiles derived from both the luminol and Ampliflu Red assay showed that MMI followed by quercetin and resorcinol were the most potent of the inhibitors tested. PTU was less potent than MMI, a finding that is supported in clinical studies (Nakamura et al., 2007), whereas naringenin was the least potent TPO inhibitor tested, showing no reported effects in humans. The association between the topical application of resorcinol in ointments and thyroid dysfunction in humans has a long history but even though rodent studies support causality, the higher sensitivity of rodents to thyroid disruption means that causality in humans is still considered inconclusive (Lynch et al., 2002). The resorcinol derivative quercetin, which is present in plant components of the human diet, is thought to be offsetting the benefit of iodized salt in parts of India that are affected by endemic goitre and while further human studies are necessary to confirm causality, recent rodent studies support the notion that quercetin inhibits thyroid function (Divi and Doerge, 1994; Giuliani et al., 2014). Overall, these antioxidants inhibit TPO *in vitro* and are associated with thyroid dysfunction *in vivo*, and this is accurately reflected in the luminol assay.

In conclusion, the combination of luminol as a chemiluminescent indicator of TPO activity and the Nthy-ori 3-1 cell line as a source of hTPO results in an alternative assay for the detection of TPO inhibitors that holds promise for inclusion within a high throughput integrated testing strategy.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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