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## Extrapolating *In Vitro* Screening Assay Data for Thyroperoxidase Inhibition to Predict Serum Thyroid Hormones in the Rat

Iman Hassan<sup>1,‡</sup>,

Hisham El-Masri<sup>2,‡</sup>,

Jermaine Ford<sup>3,‡</sup>,

Amanda Brennan<sup>3,‡</sup>,

Sakshi Handa<sup>2,5,‡</sup>,

Katie Paul Friedman<sup>4</sup>,

Mary E. Gilbert<sup>1,‡,\*</sup>

<sup>1</sup>Toxicity Assessment Division, US Environmental Protection Agency, Research Triangle Park, NC, USA

<sup>2</sup>Integrated Systems Toxicology Division, US Environmental Protection Agency, Research Triangle Park, NC, USA

<sup>3</sup>Analytical Chemistry Research Core/Research Cores Unit, US Environmental Protection Agency, Research Triangle Park, NC, USA

<sup>4</sup>National Center for Computational Toxicology, US Environmental Protection Agency, Research Triangle Park, NC, USA

<sup>5</sup>Oak Ridge Institute for Science Education, Oak Ridge, TN, United States

### Abstract

Thyroperoxidase (TPO) is an enzyme essential for thyroid hormone (TH) synthesis and a target site for a number of xenobiotics that disrupt TH homeostasis. An *in vitro* high-throughput screening (HTS) assay for TPO inhibition, the Amplex UltraRed-TPO (AUR-TPO), has been used to screen the ToxCast chemical libraries for this action. Output from this assay would be most useful if it could be readily translated to an *in vivo* response, namely a reduction of TH in serum. To this end the relationship between TPO inhibition *in vitro* and serum TH decreases were examined in rats exposed to two classic TPO inhibitors, propylthiouracil (PTU) and methimazole (MMI) for 4, 7, or 14 days. Serum and gland PTU, MMI, and TH levels were quantified using tandem liquid chromatography mass spectrometry. TPO activity was determined in thyroid gland

\*Address Correspondence to: M.E. Gilbert, PhD, Endocrine Toxicology Branch, Toxicity Assessment Division (MD-B105-05), National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, Phone: (919) 541-4394, Fax: (919) 541-4849, gilbert.mary@epa.gov.

‡National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC, USA

#### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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microsomes treated with PTU or MMI in vitro and ex vivo from thyroid gland microsomes prepared from exposed animals. A quantitative model was constructed by contrasting in vitro and ex vivo AUR-TPO results and the in vivo time-course and dose-response analysis. In vitro:ex vivo correlations of AUR-TPO outputs indicated that less than 30% inhibition of TPO in vitro was sufficient to reduce serum T4 by 20%, a degree of regulatory significance. Although further testing of model estimates using other TPO inhibitors is essential for verification of these initial findings, the results of this study provide a means to translate in vitro screening assay results into predictions of in vivo serum T4 changes to inform risk assessment.

### Keywords

thyroperoxidase; thyroid hormone; mathematical model

## INTRODUCTION

Thyroid hormones (THs) critically modulate many physiological processes, including neurodevelopment, metabolism, and cardiac function (Cioffi *et al.*, 2018; Duncan Bassett and Williams, 2018; Gilbert *et al.*, 2012; Oetting and Yen, 2007; Williams, 2008; Yen, 2001; Zoeller, 2007). The endocrine and neurodevelopmental functions of THs have resulted in increased interest in screening for chemicals that may disrupt TH homeostasis via one or more of many possible mechanisms (EFSA *et al.*, 2018; Paul Friedman *et al.*, 2016; USEPA, 2017). One potential target for disruption is thyroperoxidase (TPO). TPO is a membrane bound enzyme that iodates tyrosine residues of thyroglobulin to form mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) and also couples these molecules to generate the THs tri-iodothyronine (T3) and tetra-iodothyronine (T4 or thyroxine) (Engler *et al.*, 1982a; Taurog, 1976; Taurog *et al.*, 1996). These THs are then released to the serum from the thyroid gland (Engler *et al.*, 1982a; Engler *et al.*, 1982b; Taurog *et al.*, 1996). The role of TPO in the production of thyroid hormone in the gland and its modulation by chemicals that can disrupt TH homeostasis in humans and in rodent models are well-established in the literature (Crofton *et al.*, 2017; Doerge *et al.*, 2002; Pearce, 2006; Zoeller and Crofton, 2005).

Two pharmaceuticals, propylthiouracil (PTU) and methimazole (MMI), are used to treat hyperthyroidism and exert their action by inhibiting TPO and blocking synthesis of thyroid hormones. In a research context, these drugs are often used to investigate developmental and neurological consequences of hypothyroidism (Axelstad *et al.*, 2008; Bernal, 2015; Cooper *et al.*, 1984; Cooper *et al.*, 1983; Gilbert *et al.*, 2014; Gilbert *et al.*, 2016; Hassan *et al.*, 2017b; Hood *et al.*, 1999; Oppenheimer and Schwartz, 1997; Paul *et al.*, 2014; Vickers *et al.*, 2012). Because of their known mechanism(s) and widespread use in *in vivo* toxicology, PTU and MMI have been used extensively in assay development for TPO inhibition (Cooper *et al.*, 1987; Nagasaka and Hidaka, 1976; Paul *et al.*, 2014). However, other environmentally-relevant chemicals have also been reported to inhibit TPO activity, including the ethylene bisdithiocarbamate pesticides (mancozeb, ziram, and zineb); malachite green; isoflavones; the parasiticide malachite green; and resorcinol (Chang and Doerge, 2000; Divi *et al.*, 1997; Divi and Doerge, 1994; Doerge *et al.*, 1998; Kackar *et al.*, 1997; Marinovich, 1997 #677; Marinovich *et al.*, 1997).

Given the physiological importance of TH homeostasis, and the known importance of the TPO enzyme, high-throughput screening (HTS) assays to identify chemicals that may inhibit TPO and possibly impact TH concentrations *in vivo* have been developed. For example, the Amplex Ultra Red (AUR)-TPO inhibition assay was developed as part of the ToxCast program to rapidly identify chemicals that may inhibit TPO (Kavlock *et al.*, 2012; Thomas *et al.*, 2019). To date, over 1800 unique chemicals have been screened with the AUR-TPO assay, (Paul Friedman *et al.*, 2016) and the multi-concentration response data are publicly available as part of the ToxCast data release and CompTox Chemicals Dashboard (EPA's National Center for Computational Toxicology, 2019; Williams *et al.*, 2017). The AUR-TPO assay uses a commercially available fluorogenic substrate, AUR, to detect peroxidase activity in the presence of excess hydrogen peroxide using rat thyroid microsomes as a TPO source (Paul *et al.*, 2014).

The AUR-TPO inhibition assay has identified over 400 xenobiotics as potential TPO inhibitors (Paul Friedman *et al.*, 2016). However, there is an uncertainty in using these data to predict chemicals that may inhibit TPO *in vitro* and thereby decrease TH synthesis. Moreover, the quantitative relationship between TPO inhibition in the AUR-TPO assay and the magnitude of TH change that would be expected as a result of this inhibition is yet to be elucidated. Therefore, the current study aims to fill this gap by developing a quantitative relationship between *in vitro* and decrements of serum T4 *in vivo*. This key quantitative relationship would enable the prioritization of chemicals with sufficient potency and efficacy for further screening in models of greater biological complexity. Translating *in vitro* findings from the AUR-TPO assay to chemical safety evaluation requires a greater understanding of the magnitude of TPO inhibition required in order to observe TH changes *in vivo*. Previous studies suggest that a 20% decrease in maternal serum T4 may result in irreversible and adverse neurodevelopmental outcomes for offspring (Haddow *et al.*, 2002; Henrichs *et al.*, 2010; Korevaar *et al.*, 2016; Pop *et al.*, 2003; Pop *et al.*, 1999; Willoughby *et al.*, 2014). However, it is currently unknown what percent change in AUR-TPO screening would be indicative of a possible 20% change in serum T4 *in vivo*.

The goal of this study was to investigate the applicability of the *in vitro* AUR-TPO inhibition assay to predict *in vivo* thyroid hormone levels. First, an *in vivo* data-set was developed to provide information to construct such a model. Adult male Long-Evans rats were exposed *in vivo* to three concentrations of PTU or MMI in drinking water. Glandular and serum TH, PTU or MMI concentrations were quantified. AUR-TPO assays were conducted *in vitro* using thyroid microsomes treated with MMI or PTU as well as *ex vivo* with thyroid microsomes obtained from the rats orally dosed with PTU or MMI. *In vitro* and *ex vivo* potency for TPO inhibition, measured levels of chemical in thyroid glands, and TH levels were then used quantitatively to predict *in vivo* serum hormone concentrations in the rat. The resultant findings support a relationship between *in vitro* TPO inhibition and predicted TH changes in rats.

## MATERIALS AND METHODS

### Chemicals

Methimazole (3-methyl-1H-imidazole-2-thione; MMI; CAS No. 60-56-0; purity >98%) and 6-propyl-2-thiouracil (6-propyl-2-sulfanylidene-1H-pyrimidin-4-one; PTU; CAS No. 51-52-5; purity 99.9%) were used in the *in vivo* dose response studies and analytical grade MMI and PTU (purity 99%) was used for LC/MS/MS and *in vitro* experiments were obtained from Sigma Chemical Company (St Louis, Missouri). The following stable isotope internal standards, 3,3'-diiodothyronine-<sup>13</sup>[C6] (T2-<sup>13</sup>[C6]); 3,3',5-triiodothyronine-<sup>13</sup>[C6] HCl (T3-<sup>13</sup>[C6]); L-thyroxine-<sup>13</sup>[C6] HCl (T4-<sup>13</sup>[C6]); 3,3',5'-triiodothyronine-<sup>13</sup>[C6] HCl (reverseT3(rT3-<sup>13</sup>[C6]) were purchased from IsoSciences (King of Prussia, PA) and (MIT-<sup>13</sup>[C6]) was purchased from Toronto Research Chemicals (North York, ON, Canada). Thyroid hormone standards 3,3', 5,5'-tetraiodo-L-thyronine (T4), 3,3',5-triiodo-L-thyronine (T3) as the sodium salt, 3,3',5'-triiodo-L-thyronine (rT3), 3,5-diiodo-L-thyronine (3,5-T2), 3,3'-diiodo-L-thyronine (3,3'-T2), 3,5-diiodo-L-tyrosine (DIT), and 3-iodo-L-tyrosine (MIT) were purchased as neat materials from Toronto Research Chemicals (North York, ON, Canada) to be used as calibration and spiking standards for the target analytes. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, Fisher Chemical) and Amplex UltraRed reagent (Invitrogen, ThermoFisher) were used for the TPO inhibition *in vitro* assay.

### Animal dosing

Adult male (post-natal day 55–60) Long Evans rats (n=64) were obtained from Charles River (Raleigh, NC) and shipped to the animal housing facility at the U.S. EPA. Animal experiments were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and conducted under Institutional Animal Care and Use Committee approved protocol maintained in AALAC accredited facility. On arrival to the facility, animals were individually housed in standard hanging plastic cages and housed under controlled conditions (20–24°C; 40–50% humidity) with 12:12 h light/dark cycle and allowed to acclimate one week before the study began. Rats were fed standard Purina Rat chow 5001 and provided food and water *ad libitum*. The iodine content in standard Purina rat chow 5001 far exceeds that required for optimal hormone synthesis (1000 vs 275 ng/gm, see Gilbert *et al.* (2013) and Fisher *et al.* (2013)) indicating iodine supply is not likely contributing to decrements in hormone synthesis under the conditions of this study. Animals were weight-ranked and randomly assigned to treatment groups and exposed to either PTU (0, 1, 3, and 10 parts per million (ppm) or MMI (0, 3, 30, 200 ppm) in drinking water for 4, 7, or 14 days (n=8 per dose/time point). Animals were weighed three times per week. At the end of each exposure duration, animals were euthanized by decapitation between 0800 and 1200 h and the time of sacrifice was counterbalanced by dose group. Trunk blood was collected, and thyroid glands were removed and weighed. One lobe of each gland was stored in RNALater reagent for gene expression analysis and the other was snap frozen in liquid nitrogen and used for microsomes, hormone, and chemical analysis. Blood was left to clot on ice, centrifuged at 3000 × g at 4 °C for 30 min, and serum was removed and stored at –80 °C.

### Quantification of PTU and MMI in the serum and thyroid gland

Serum and thyroid gland samples were prepared and the levels of PTU or MMI were quantified using TSQ Quantum liquid chromatography/tandem mass spectrometry (LC/MS/MS; ThermoFisher) as described in (Hassan *et al.*, 2017a; Wo niak *et al.*, 2014) with minor modification in the quantification method. Briefly, a Shimadzu LC-20AD system and SCIEX 4000 QTrap Mass Spectrometer were used to quantify levels of MMI or PTU in the serum and thyroid gland. Chromatographic separation was performed on a Phenomenex Kinetex HILIC column (2.6  $\mu\text{m}$ , 100 mm  $\times$  3 mm) maintained at 30°C. A linear gradient using 15 mM ammonium formate in water (pH 3.2) as mobile phase A and acetonitrile as mobile phase B were used at a flow rate of 0.4 mL min<sup>-1</sup>. The starting mobile phase composition was 10% A and 90% B. After 2.5 minutes, mobile phase A increased to 90% for 3 minutes. The column was re-equilibrated to starting conditions for 5 minutes. Analytes were detected by positive ion electrospray ionization.

#### Quality control for chemical measurements:

Matrix-matched calibration standards were used for the quantitation of PTU or MMI levels in thyroid glands. Calibration standards were prepared and analyzed in the same manner as project samples. The calibration curve used a minimum of 5 points over a range of 0.25 to 62.5 ng/mL. The coefficient of determination for the calibration curve was 0.99. Each sample batch consisted of a matrix-matched method blank and MMI-spiked laboratory control sample and duplicate with a continuing calibration verification sample run after approximately every 20 samples in the batch. All quality control samples were within 80–120% of the nominal spike amount.

### Quantification of THs in the serum and thyroid gland

Sample preparation and analysis of serum T3 and T4 and thyroid gland levels of iodotyrosines (MIT, DIT), T3, T4, and reverse T3 (rT3) were based on methods previously described by (Hassan *et al.*, 2017b), with some modifications. Briefly, following solid phase extraction, thyroid hormone extracts were reconstituted with 100  $\mu\text{L}$  of 5% acetonitrile: 95% H<sub>2</sub>O (v/v) with 0.1% formic acid. The extracts were analyzed using an AB Sciex (Framingham, MA) Exion AC UHPLC-Qtrap 6500+ Linear Ion Trap LC/MS/MS system. Chromatographic separation of the pre-hormones and hormones was performed using a Restek (Bellefonte, PA) Raptor Biphenyl column (2.6  $\mu\text{m}$ , 100 mm  $\times$  2.1 mm).

#### Quality control for hormone measurements:

Single component stock standards were prepared in acetonitrile from neat materials. A mixed intermediate standard with each component present at a concentration of 1  $\mu\text{g}/\text{mL}$  was used to prepare a set of 9 intermediate standards by serial dilution over a concentration of 100 to 100,000 pg/mL in methanol. A 100  $\mu\text{L}$  aliquot of each intermediate standard was combined in an autosampler vial with 100  $\mu\text{L}$  of a 100 ng/mL solution of the IS in methanol. Each standard/IS mixture was evaporated under nitrogen on a TurboVap (Caliper Life Sciences, Waltham, MA) set to 35 °C. The dried standard mixture was resuspended in 1 mL of 0.1% formic acid in 95:5 water: methanol to be used for calibration.

Two ion transitions were monitored for each target analyte. The analytes were qualitatively identified based on retention time relative to the internal standard and calibration standard, and the ratio of the peak areas of the monitored ion transitions. The ion ratios of identified target analytes were within 30% of the average ion ratio for standards. Solvent-based calibration standards were used for quantitation of the thyroid hormones in cell media over a range of 10 – 10,000 pg/mL. Each curve used a minimum of five sequential points. The correlation coefficients of the curves were 0.99. The calibration was verified with a second source standard. The lower limit of quantitation (LLOQ) for each analyte was set to the concentration of the lowest calibration standard that gave an acceptable ion ratio, and acceptable recovery of  $\pm 30\%$  of the spike amount. Each sample batch consisted of a method blank, a laboratory control sample (blank spike), and a continuing calibration verification sample prepared in solvent. The calculated concentrations for thyroid hormones in the laboratory control sample and continuing calibration verification sample were within 70–130% of actual spike amount.

### **Analysis of serum thyroid-stimulating hormone (TSH)**

Determination of serum TSH levels was carried out by radioimmunoassay (RIA) based on a method previously reported in (Louis *et al.*, 2017; Stoker *et al.*, 2010; Thibodeaux *et al.*, 2003). The limit of detection for TSH assay was 0.3 ng/ml and intra-assay coefficient of variation was 1.95%.

### **Thyroid peroxidase inhibition *in vitro* and *ex-vivo* with AUR assay**

Thyroid gland microsomes (N=8/group pooled) were prepared from rats exposed to PTU or MMI for 4-days and from untreated rats using a method previously described (Paul *et al.*, 2013; Taurog *et al.*, 1996). The mean protein content of the microsomes was determined using Pierce BCA Protein Assay Kit (ThermoFisher) following the manufacture's protocol. Protein concentration ranged from 0.85 – 1.64 mg/ml and average thyroid gland weight ranged from 6.5–7 mg for PTU dosed animals while average thyroid gland weight for MMI treated rats ranged from 16–19 mg.

The AUR assay was conducted as described by (Paul *et al.*, 2013; Paul *et al.*, 2014) with minor modifications. In brief, the assay was conducted in a 96-well format using rat thyroid microsomes, 200 mM potassium phosphate buffer, AUR reagent diluted from 10 mM AUR DMSO stock diluted in 200 mM phosphate buffer, and H<sub>2</sub>O<sub>2</sub>. The reaction was conducted by adding 75  $\mu$ L AUR reagent (25  $\mu$ M), 10 – 15  $\mu$ L of microsomal protein (12.5  $\mu$ M), 25  $\mu$ L H<sub>2</sub>O<sub>2</sub> (300  $\mu$ M), and finally 100  $\mu$ L of 200 mM potassium phosphate buffer. A dose response curve for PTU or MMI was included in each plate as positive control for TPO inhibition *in vitro*. The standard curve was constructed using untreated rat thyroid microsomes (obtained from thyroid glands of non-dosed animals) exposed *in vitro* to 0–100  $\mu$ M PTU or MMI, prepared from a 1 mM stock in DMSO. The assay was also conducted with microsomes prepared *ex vivo* from PTU or MMI dosed rats. The plates were incubated for 30 minutes at 37°C in the plate reader (BMG Optima FluoStar with excitation/emission of 544/590). The assay was run three separate times on three different days with fresh reagents prepared for each biological replicate (n=3), with technical duplicates in each plate. *In vitro* concentration

that inhibits enzyme activity by 50% (IC50) were calculated using GraphPad Prism (v5.0, LaJolla, California).

### RNA extraction and Quantitative RT-PCR (qRT-PCR)

Gene expression analysis was performed on thyroid tissues from PTU or MMI treated rats. RNA was extracted and quantitative real-time polymerase chain reactions (qRT-PCR) was performed according to procedure described by (Gilbert *et al.*, 2016).

### Statistical analysis

The dose-response measures of gland and serum PTU, MMI, and thyroid hormone analytes are presented as mean  $\pm$  standard error (SEM) and were plotted using GraphPad Prism (v5.0, LaJolla, California). PTU, MMI, serum, and gland TH concentrations were evaluated using general linear model analysis of variance (ANOVA) conducted using SAS version 9.2 (Cary, NC). Significant main effects were followed by Dunnett post-hoc multiple comparisons testing with  $p < 0.05$  considered statistically significant whereas gene expression significance was set at  $p < 0.01$  which was determined by the alpha level by the square root of the number of genes analyzed. For statistical purposes control values of serum and thyroid gland PTU or MMI which were below the detection limit of the assay were set at the LLOQ as follows: thyroid gland PTU and MMI LLOQ = 7 ng/g and 25 ng/g, respectively; serum PTU and MMI LLOQ = 0.38 ng/ml; All the thyroid gland hormone analytes were above the LLOQ = 0.5 ng/ml; and all serum T4 and T3 were above the LLOQ = 0.1 ng/ml, 0.4 ng/ml, respectively.

### Quantitative *in vitro* to *in vivo* relationship

The quantitative relationships between *in vitro* derived estimates of TPO inhibition and *in vivo* serum thyroid hormone levels were examined computationally. Chemical disposition and dosimetry information, and hormone concentrations in the thyroid gland and serum collected in *in vivo* experiments were used to define two mathematical relationships. First, a relationship between TPO inhibition (as indicated by the AUR-TPO assay) in thyroid gland microsomes from naïve animals and TPO inhibition in microsomes derived *ex vivo* from exposed animals was defined. Best linear fit for the data from both assays was obtained and correlated. Second, a mathematical relationship between gland concentrations and TPO inhibition was developed; i.e., chemical and hormone concentrations in the gland were coupled with *in vitro* IC50 to simulate the degree of TPO inhibition. The simulated degree of TPO inhibition was then used to predict serum T4 levels *in vivo*. This was accomplished by introducing glandular T4, PTU, or MMI concentrations into a one-compartment model to describe TPO inhibition, under steady state condition, as a function of *in vitro* derived IC50 using the following equation:

$$dT4/dt = K_{syn} * \left( 1 - \left( I_{max} * \frac{[PTU \text{ or } MMI]}{[PTU \text{ or } MMI + IC50]} \right) \right) - K_{deg} * T4$$

The values for  $I_{max}$  (the maximal activity of TPO) and IC50 values (1.2  $\mu\text{M}$  for PTU; 0.11  $\mu\text{M}$  for MMI) were obtained from the *in vitro* AUR-TPO assay.  $K_{syn}$  and  $K_{deg}$  are the basal production and degradation rates of T4, respectively. Their values were obtained from (Silva

*et al.*, 1984). Unlike MMI, which remains largely unbound after oral intake, 80 to 90% of PTU remains bound to serum proteins (Jastrzbska, 2015). Therefore, to account for the free portion, the IC<sub>50</sub> value for PTU was divided by 0.24, which is the unbound fraction of PTU, obtained from (Kampmann and Mølholm Hansen, 1983).

To relate glandular T4 levels to serum T4, direct mathematical relationships were constructed. First the percent of TPO T4 synthesis inhibition was determined by dividing gland T4 levels at each dose by T4 in the control animals. The percent TPO inhibition was then multiplied by the serum basal level of T4. The resulting predicted T4 serum levels were then plotted against the experimentally determined ones at each dose for both chemicals.

## RESULTS

### Body and thyroid weights

Body weights were not significantly altered by exposure to PTU or MMI at any time-points (Supplemental Data, Figure 1). Thyroid gland weights were significantly increased 1.3 and 1.6-fold, following 14-days of PTU exposure at doses of 3 and 10 ppm, respectively, [F (3,28) = 11.05; p<0.0001; Figure 1A]. Similarly, 14-day exposure to 30 and 200 ppm MMI significantly increased thyroid gland weights by approximately 1.4 and 1.5-fold compared with controls [F (3,30) = 5.05, p = 0.006; Figure 1B]. Although 7-day exposure to 30 and 200 ppm MMI also appeared to increase thyroid gland weights, the results were not statistically significant (p=0.052) (Figure 1B).

### PTU and MMI concentrations in the thyroid gland and serum of dosed rats

There was a dose-dependent increase in mean PTU concentrations in the thyroid gland and serum at all doses relative to controls with 4, 7, and 14-day exposure to PTU (Table 1, p<0.0001). The levels did not appear to increase with increased exposure duration suggesting that PTU at these concentrations does not accumulate in the gland over time. Similarly, intra-thyroid and serum levels of MMI significantly increased in a dose-dependent manner in animals exposed to 3, 30, and 200 ppm MMI for 4, 7 and 14 days, compared to non-treated controls (p<0.0001; Table 2). Like PTU, MMI did not appear to accumulate in the gland over the duration of exposure. MMI levels at the highest doses were comparable at 4, 7, and 14 days of exposure.

### PTU and MMI decreased thyroid hormone levels in the thyroid gland

As shown in Table 1, treatment with PTU for 4 days resulted in a dose-dependent decrease in glandular THs. Exposure to 3 and 10 ppm PTU for 4 days significantly decreased mean gland concentration of THs (p<0.009). In the 1 ppm and 3 ppm PTU exposed groups, DIT was significantly decreased, whereas exposure to 10 ppm PTU reduced all the analytes (p=0.0001). After 7 days of exposure to PTU, a significant decrease in glandular levels of MIT and DIT at all doses was noted while the two highest doses also decreased rT3, T3, and T4 (p<0.0003). Similarly, 14-day exposure significantly decreased DIT, T3, and T4 at all doses and MIT as well as rT3 were also decreased at the two highest doses (p<0.0001).



A similar dose and time-dependent pattern of hormone analytes was also observed in the glands of animals exposed to MMI (Table 2). The two highest doses of MMI significantly decreased MIT, T3, rT3, and T4, whereas DIT was significantly decreased at all doses in the 4-day treated animals ( $p < 0.0001$ ). Decrease in glandular TH levels observed in the 4, 7 and 14-day dosed rats suggested that MMI is a more potent TPO inhibitor than PTU. Significant dose-dependent declines in DIT, rT3, and T4 at all doses were also observed in the 7-day dosed animals and MIT was significantly decreased at the two highest doses only ( $p < 0.001$ ). Similarly, 14-day exposure to 3, 30, and 200 ppm significantly decreased all the analytes except T3 which was decreased at the two highest doses only ( $p < 0.001$ ).

### **PTU and MMI decreased serum T3, T4, and increased TSH**

Mean serum levels of T3 and T4 were moderately to markedly decreased with concomitant increases in TSH as a function of dose and duration of PTU (Figure A-C) exposures. Treatment with doses greater than 1 ppm PTU for 4 and 7 days decreased serum T3 (Figure 2A) and T4 (Figure 2B). Serum T3 declined by 58%, and 84% ( $p < 0.0001$ ) whereas T4 was decreased by 51%, and 58% ( $p < 0.0002$ ) with exposure to 3 and 10 ppm for 4 days, respectively (Figure 2A and B). Exposure to 3 ppm PTU for 7 days decreased serum T3 and T4 by 47% and 61% as well as 89% and 71% in the 10 ppm PTU treated group, and 14-day exposure to PTU resulted in significant declines in serum T3 and T4 at all doses ( $p < 0.0001$ ). Specifically, serum T4 declined by 27%, 71%, and 94%, while T3 was decreased 23%, 41%, and 97%, with exposure to 1, 3, and 10 ppm PTU, respectively. Coinciding with declines in serum T4, TSH was increased by 3.5, and 5-fold with exposure to PTU for 7 days and by 5 and 9-fold at the two highest doses with exposure extended to 14 days ( $p < 0.0001$ , Figure 2C).

Exposure to MMI also significantly decreased serum thyroid hormones. As shown in Figure 2D and 2E, T3 and T4 significantly declined with exposure to doses greater than 3 ppm for 4 days (Figure 2D and E). Unlike PTU however, TSH was simultaneously elevated in animals exposed to 30 ppm MMI after 4-days (Figure 2F). Specifically, TSH levels were significantly increased by 3-fold in rats exposed to 30 ppm for 4-days ( $p < 0.0001$ ). Exposure to 30 and 200 ppm MMI resulted in 3.3 and 3.5-increase in TSH levels in 7-day exposed rats and 5.7 and 7.5-fold increase in 14-day exposed rats ( $p < 0.0001$ ). MMI at the doses used here resulted in severe T3 declines at the two highest doses and at all time points. After 4-day exposure to 30 and 200 ppm MMI, T3 decreased by 95.2% and 95.4%, whereas T4 was declined at all doses by 18%, 73% and 74%, respectively ( $p < 0.0001$ ). Exposure to the two highest MMI doses for 7 days also significantly decreased T3 by 75% and 82% while T4 declined by 67% and 78% ( $p < 0.0001$ ). Similarly, a marked decrease in T3 was noted in 14-day group dosed with 30 and 200 ppm MMI whereas T4 significantly declined at all doses ( $p < 0.0001$ ).

To further characterize the impact of MMI and PTU on thyroid hormone synthesis, glandular mRNA levels of TH regulating genes were determined (Supplemental Data Figure 2). Increased expression of *Slc5a5*, a transcript encoding for sodium-iodine symporter (NIS), suggests that the gland is responding to the activation of the feedback loop including

elevation of serum TSH. Increases in *Tpo* and *Dio1* were seen at the highest dose of PTU and were restricted to the 7-day timepoint.

### PTU and MMI inhibit TPO activity *in vitro* and *ex vivo*

PTU was less potent inhibitor of TPO *in vitro* with an IC<sub>50</sub> of 1.2 μM compared with 0.11 μM for MMI (Figure 3A and C). Statistically significant inhibition of TPO activity was also seen in microsomes from *ex vivo* glands from rats exposed to 3 and 10 ppm PTU for 4 days [F (3,20) = 25.83, p<0.0001; Figure 3B], and 3, 30, and 200 ppm of MMI-treated animals [F (3,12) = 14.10, p=0.0003; Figure 3D]. The Z' factor, a score that distinguishes signal from noise, was calculated for AUR-TPO inhibition assay for both PTU and MMI, and values that exceeded 0.94 were obtained (Zhang *et al.*, 1999).

The quantitative relationship between *in vitro* and *ex vivo* measures of TPO activity after 4 days of exposure to PTU or MMI are summarized in Figure 4. For both chemicals, the TPO activity from the *in vitro* assay was plotted against the *ex vivo* assay results. The estimated slope for the best linear fit for PTU was 0.89 with an R<sup>2</sup> = 0.86 (Figure 4A) and for MMI was 0.84 with an R<sup>2</sup> = 0.71 (Figure 4B). For both PTU and MMI, the best fit line approached unity. Based on this *in vitro-ex vivo* relationship, it was possible to estimate the anticipated *in vivo* level of reduced TPO activity, permitting the derivation of the corresponding serum T4 level for a given degree of *in vitro* TPO inhibition. Using the linear correlation equation in Figure 4A or B, TPO activity *in vitro* was converted to TPO inhibition *ex vivo*, expressed as a percent of control, and plotted against observed serum T4 levels for both PTU (Figure 4C) and MMI (Figure 4D). This analysis revealed that 10–30% of *in vitro* TPO inhibition corresponded with a 20% reduction of serum T4.

### Predicting serum T4 with *in vitro* TPO inhibition

TPO inhibition was simulated using gland concentration of chemical and T4 for all three timepoints to provide a detailed quantitative description of inhibition over time (Figures 5 and 6). Simulation of the 4-day exposure duration resulted in better fit of thyroid gland T4 at lower doses than higher doses for both PTU (Figure 5A) and MMI (Figure 6A). The 7-day exposure duration to PTU (Figure 5A) or MMI (Figure 6A) resulted in the best fits at all doses, while longer exposures of 14 days provided better fits at higher doses. The simulated degree of TPO inhibition was then used to predict the PTU- (Figure 5B) and MMI- driven (Figure 6B) declines in serum T4. *In vitro* IC<sub>50</sub> values of 1.2 μM and 0.11 μM for PTU and MMI, respectively, provided a reasonable fit for the observed data across exposure duration. Furthermore, the simulated percent decline in TPO activity was applied to the basal concentration of serum T4 and was compared with measured serum T4 level for each drug. A simple index value was calculated to quantify the discrepancy between the predicted serum T4 levels and the measured values according to a method published by (Krishnan *et al.*, 1995). The index values for 4, 7, and 14 day PTU exposure was 8, 5, and 2%, respectively while the index values for MMI exposure was 7, 7, and 4% for 4, 7, 14-day treatments, suggesting an increased concordance between predicted serum T4 levels, based on the simulated degree of TPO inhibition, and measured serum T4 levels with increased duration.

The predicted levels of decline in serum T4 based on serum levels of PTU and MMI for all time-points were 3-fold lower than the measured serum T4 levels for both drugs at the 4- and 7-day exposure durations (Figure 5 C, D; Figure 6 C, D). These differences largely disappear after 14 days of exposure (Figures 5E and 6E). These observations suggest that in addition to concentration of chemical in the gland, kinetic factors including hormone half-life and storage in the gland also contribute to this relationship. The convergence of the predicted and measured serum levels over time may reflect time required to deplete reserved T4 levels in the face of diminished synthesis in the gland (Choksi *et al.*, 2003). Overall, these results suggest that serum T4, a more readily obtainable measure, reflects the TH status in the gland due to TPO inhibition, and it can be calculated using estimates of the degree of the inhibition from *in vitro* studies when duration of exposure is taken into consideration. These observations may have implications for design of *in vivo* targeted test strategies, where accuracy of risk estimates is improved with exposure durations that are more than 4 days.

## DISCUSSION

Alterations in serum TH concentrations represent a common downstream indicator of xenobiotic-induced thyroid disruption *in vivo*. HTS assays are increasingly being used to identify and prioritize potential endocrine disrupting chemicals for further testing. Several *in vitro* assays have been developed to screen for those that interfere with the thyroid axis (Murk *et al.*, 2013; OECD, 2017; Olker *et al.*, 2019; Wang *et al.*, 2018). However, the value of these *in vitro* experiments would be greatly enhanced if their output could be translated to *in vivo* measures of regulatory importance. Two major factors influence the fidelity of translating *in vitro* potency factors to biologically meaningful parameters, and this study addressed both of these challenges for TPO inhibition data generated using the AUR-TPO assay. First, it was important to determine if TPO inhibition observed in the *in vitro* assay could be recapitulated *in vivo*. The second factor was the applicability of the quantitative findings obtained from *in vitro* measures (TPO inhibition) to those achieved *in vivo* (serum THs). These factors were directly assessed by comparing *in vitro* and *ex vivo* estimates of TPO inhibition in the AUR-TPO assay following exposure to two prototypical TPO inhibitors.

*In vivo* dose-dependent reductions in thyroid gland concentrations of THs provided quantitative estimates of synthesis inhibition by these well-known TPO inhibitors, PTU and MMI. This action resulted in decreased hormone available for release and drove the subsequent declines in T3 and T4 observed in the serum. Consistent with numerous reports in the literature, effects on serum THs by both drugs occurred at lower doses as exposure duration increased (Cooper *et al.*, 1984; Cooper *et al.*, 1983; Hood *et al.*, 1999). In addition, in the current study, concentrations of PTU and MMI in the serum and the thyroid gland reached steady state by the 4-day timepoint and remained constant over all exposure durations. Therefore, the greater declines in hormones in both gland and serum for MMI and PTU likely reflect the diminution of the reserve capacity of the thyroid gland as exposure duration increases (Choksi *et al.*, 2003). In addition, the half life of serum T4, which is 0.5–1 day, may also play a role in the T4 decrements observed in the serum when T4 synthesis is inhibited in the gland.

Concentration-dependent reductions in *in vitro* TPO inhibition assays were also in agreement with previous reports (Paul *et al.*, 2013; Paul *et al.*, 2014). Importantly, consistent with *in vitro* findings, TPO was also inhibited in microsomes prepared *ex vivo* following *in vivo* dosing. The *in vitro* and *ex vivo* estimates of TPO inhibition for both PTU and MMI were highly correlated with *in vivo* measurements of hormone concentrations in the gland and the serum, providing a means to incorporate, in a quantitative manner, chemical action on gland hormone synthesis to a downstream serum hormone response familiar to regulators.

Based on these data, a simple one compartment computational model was constructed to estimate the degree of TPO inhibition in the gland as a function of *in vitro* IC50 and the internal dose of PTU or MMI, and glandular T4 concentrations. Simulations using glandular T4 to predict serum T4 at the shorter duration exposures to PTU or MMI resulted in drastic declines in the predicted serum T4 compared with measured concentrations. However, after 14 days of treatment, the predicted serum T4 was closely aligned with measured serum T4 in both PTU and MMI models. Incorporating parameters of gland capacity beyond the one compartment model may improve model fit and predictions for shorter exposure durations.

Although the primary action of both chemicals is to inhibit TPO, MMI and PTU differ from one another in two ways that could potentially influence their downstream effects on serum THs. A higher glandular PTU concentration relative to MMI concentration (80  $\mu\text{M}$  vs 25  $\mu\text{M}$ ) was required to produce a 60% decrement in gland T4, and is consistent with previous observations that MMI is more potent suppressor of TH production (Supplementary Data Figure 3). This same pattern was also reflected in serum hormone profiles for MMI and PTU. Distinct from MMI, PTU also impacts TH synthesis and regulation through inhibition of deiodinase activity (Dio1). This action within the thyroid gland may negatively influence iodine recycling to reduce TH synthesis with resultant decrements in serum T4 concentrations. In contrast, inhibition of Dio1 in the serum and liver would be expected to augment serum T4 by blocking its conversion to T3 (Schneider *et al.*, 2006). However, due to the high iodine content of the Purina rat chow and relatively low doses of PTU administered in the current study, it is likely the primary action of PTU on serum thyroid hormone is through its action on TPO.

Collectively, these findings indicate that when combined with estimates of gland concentration derived from dosimetry models, the output of the *in vitro* AUR-TPO assay may be used to predict chemical effects on serum TH. Incorporation of the *in vitro* IC50 to the *in silico* model over-predicted the decline in serum T4 in the short-term, but it was well aligned with declines in serum T4 observed with 14-days of exposure. Although limited to a single *ex vivo* assessment, a TPO activity decrease *in vitro* AUR-TPO assay corresponded to a similar percent activity declines observed *ex vivo*. The relationship between *in vitro* AUR-TPO inhibition and *ex vivo* AUR-TPO inhibition approached unity. Establishing this relationship permitted the derivation of a minimal *in vitro* estimate of TPO inhibition that would be required to reduce serum T4 *in vivo*. Based on the associations between decrements in serum T4 in pregnant women and adverse neurodevelopmental outcomes in their children (Haddow *et al.*, 1999; Korevaar *et al.*, 2016; Morreale de Escobar *et al.*, 2000) the US EPA considers 20% decline in circulating T4 levels in animal studies as a regulatory level of concern (US EPA 2011). Our analysis suggests that 10–30% TPO inhibition *in*

*vitro* may be sufficient to reduce serum T4 by 20% after 7–14 days of exposure. Currently, the threshold for a positive response in the AUR-TPO assay as analyzed for the ToxCast program is 20%; now, for at least two chemicals, we have established that this threshold for a positive seems reasonable in terms of the anticipated *in vivo* change in serum THs that might occur as a result.

A limitation in interpreting the current work is that the mathematical models developed here to relate TPO inhibition *in vitro* to rat serum TH *in vivo* may not apply to all chemicals screened in the AUR-TPO assay. Additional chemicals would have to be examined using a similar paradigm to confirm the relationship between *in vitro* TPO inhibition and *in vivo* serum TH changes in a rat model. Indeed, findings in the current study contrast with those reported by Chang and Doerge (2000). Using a guaiacol-based TPO assay, Chang *et al.* reported comparable levels of TPO inhibition *in vitro* and *ex vivo* in response to exposure to the isoflavone genistein, but despite nearly 80% inhibition in the TPO assay, no effects were observed on serum hormones in exposed animals. Genistein was also positive in the TPO-AUR assay results of Paul *et al.* (2014), but less potent than MMI or PTU, with an IC50 value of 4.5  $\mu\text{M}$ . It is not clear if the results reported for genistein by Chang *et al.* differ due to mechanistic differences in how genistein inhibits TPO activity, due to differences in the TPO assay technology, or due to some other factor. Thus, other chemicals identified in the AUR-TPO assay would require confirmation of any potential to alter serum hormones, either in biologically complex *in vitro* models or *in vivo* models. Additional future work could address the variability in the mathematical relationship between TPO inhibition using the AUR-TPO assay and *in vivo* serum TH changes in rat models.

In conclusion, herein we explored a quantitative model to support translation of AUR-TPO inhibition assay results for prioritization of putative TH disruptors. In this case study, the dose-dependent declines in gland and serum THs and internal doses of PTU and MMI in the serum and thyroid gland were used to develop a computational model to link *in vitro* TPO inhibition to serum TH reductions *in vivo*. This work highlights methodology important for extrapolating *in vitro* assay data to *in vivo* effects. Further, this work demonstrates quantitative linkages from the molecular initiating event, to target tissue responses, to measurable events at the organism level within an adverse outcome pathway context (Ankley *et al.*, 2010; Edwards *et al.*, 2016; Villeneuve *et al.*, 2014). Although further testing with other TPO inhibitors is necessary to improve confidence in the generalizability of this model to other chemicals, the approach provides a simple scheme to use *in vitro* assay results to predict an *in vivo* endpoint important for regulatory toxicology. On a practical basis, this work provides evidence to support a selection of a conservative threshold for a positive response in the AUR-TPO HTS assay for use in prioritization and screening-level assessment.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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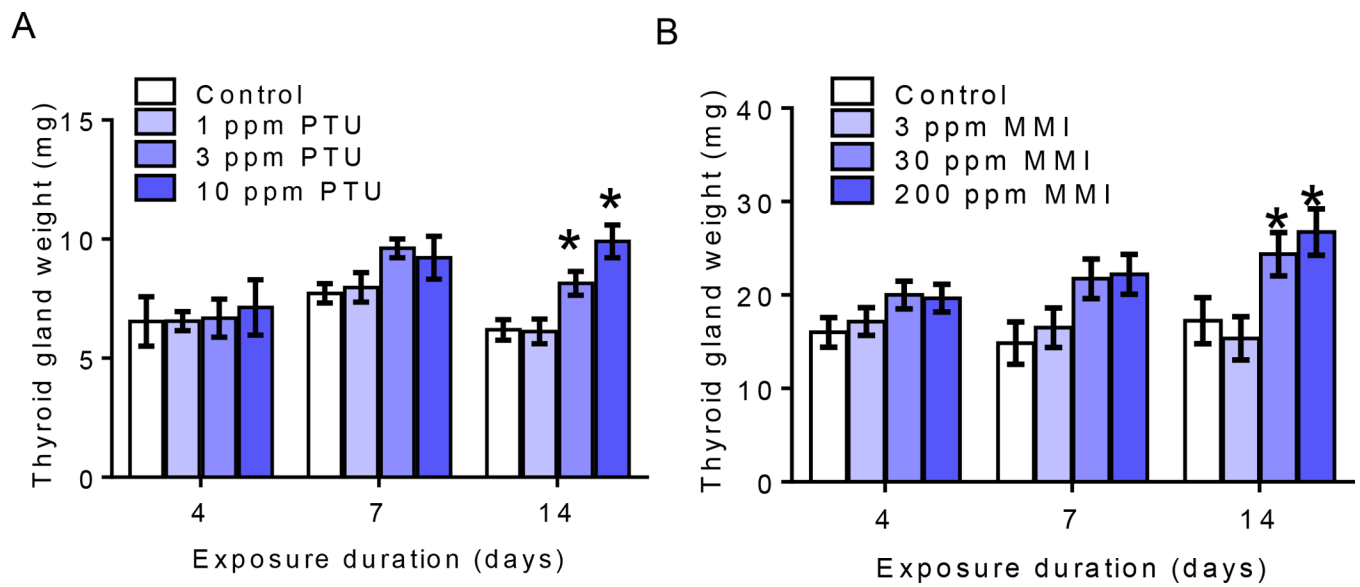
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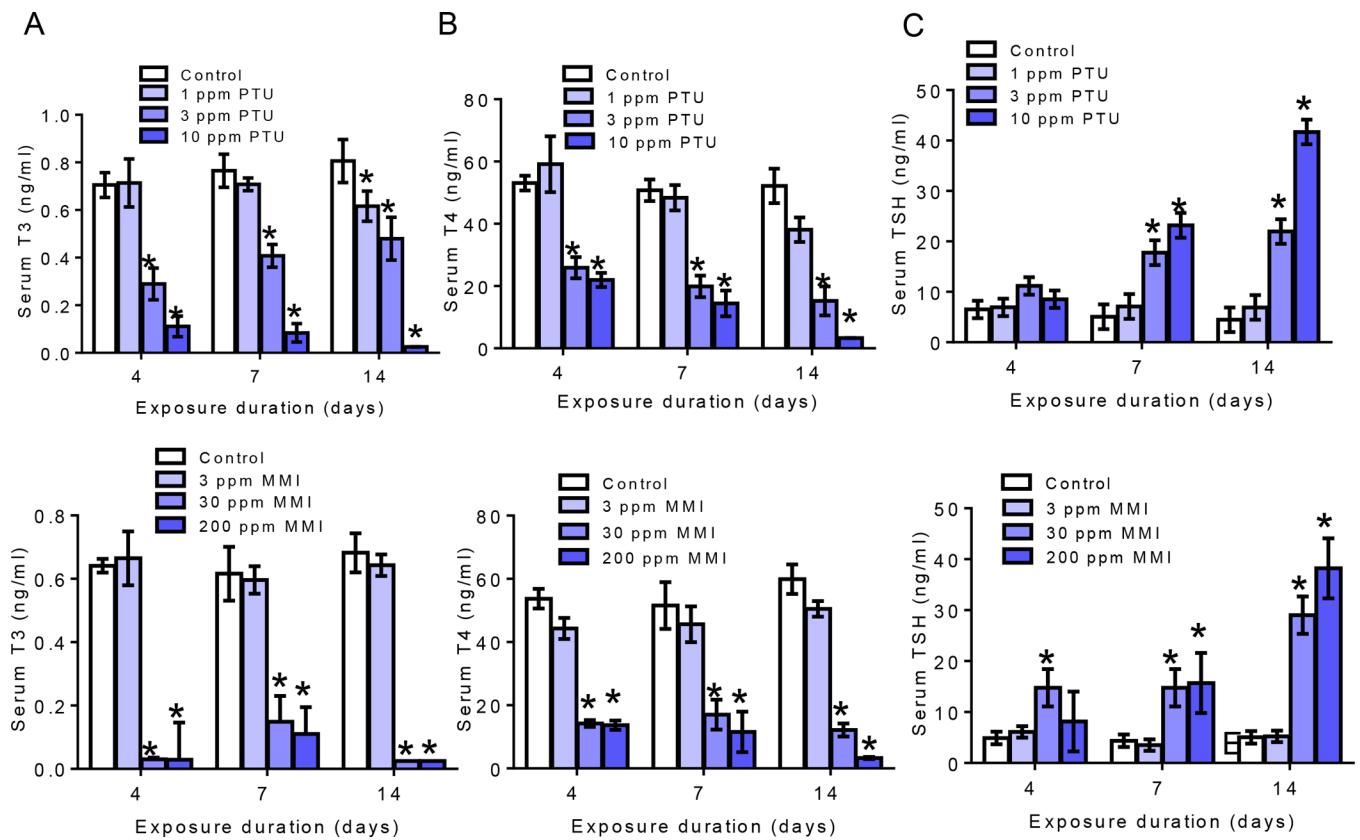
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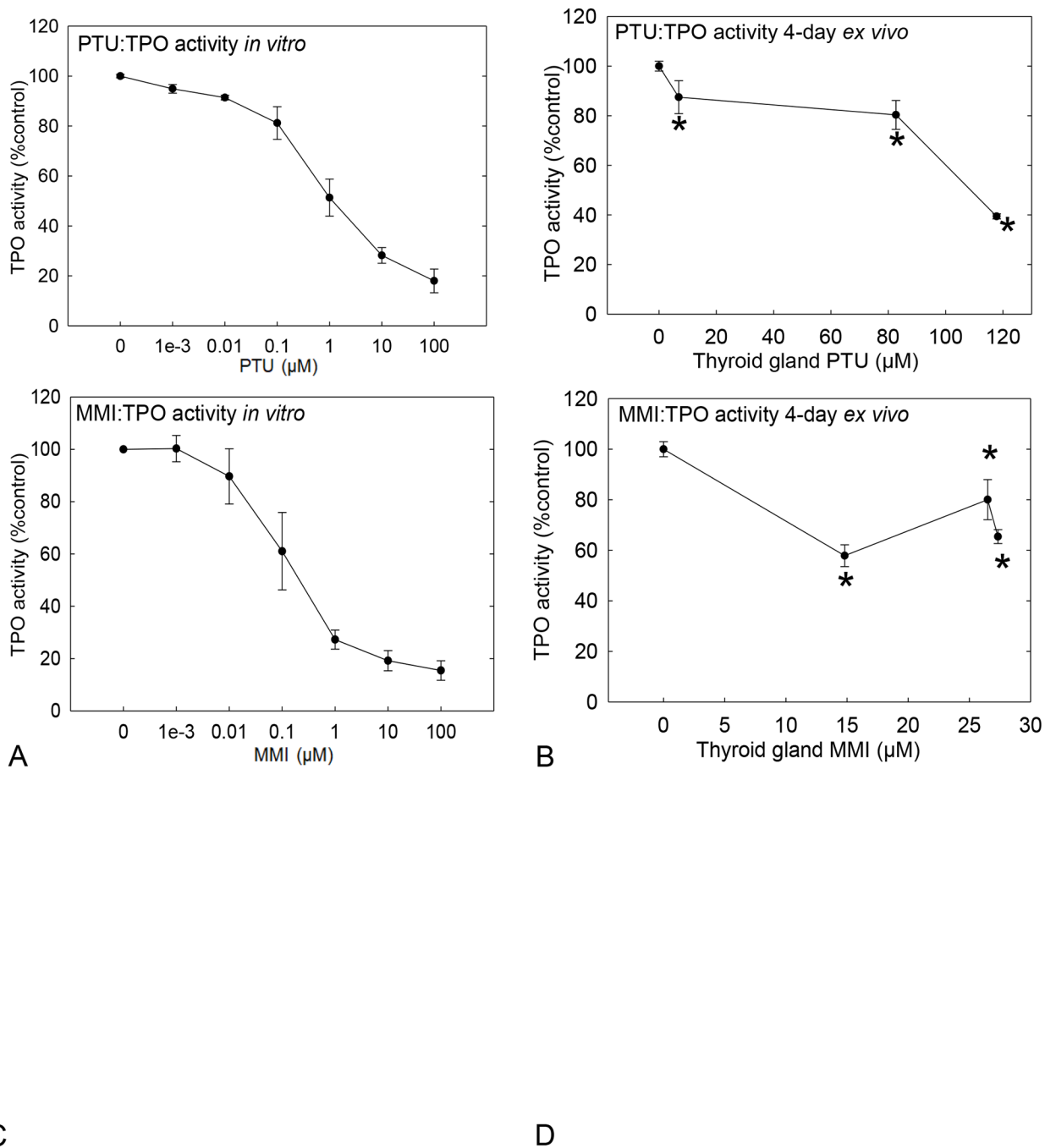
**Figure 1. Thyroid gland weights.**

A) Thyroid gland weight was significantly increased with exposure to 3 and 10 ppm PTU for 14 days B) Thyroid gland weight was also increased with exposure to 30 and 200 ppm MMI for 14 days. An N = 8 was analyzed per treatment per timepoint; \*represent  $p < 0.01$  and bar graph shows mean  $\pm$  SEM.



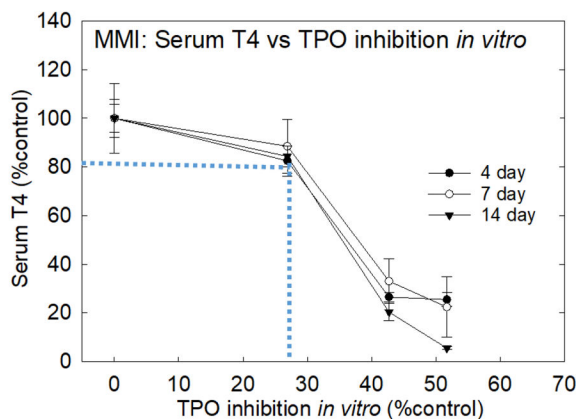
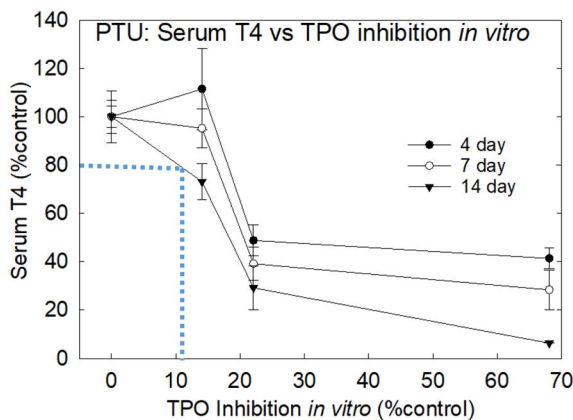
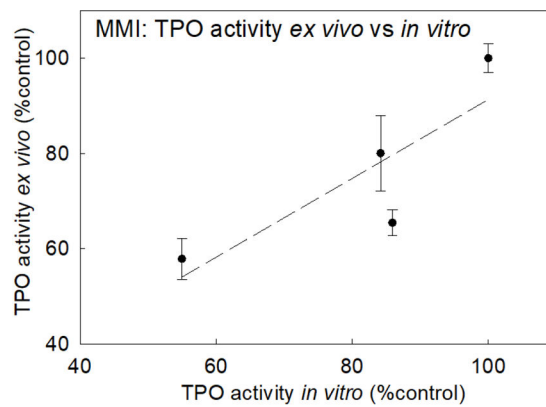
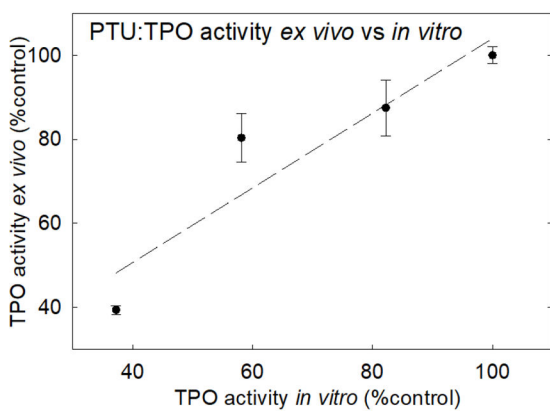
**Figure 2. Serum thyroid hormones.**

A-C) Serum T3, T4, and TSH levels for PTU treated animals. Both T3 and T4 levels were significantly decreased with exposure to 3 and 10 ppm PTU at all exposure durations with corollary increase in TSH at 3 and 10 ppm at 7 and 14-day exposures only. D-F) T3, T4, and TSH levels in the serum of MMI treated animals. Exposure to 30 and 200 ppm MMI significantly reduced serum T3 and T4 at all exposure durations with significant increase in TSH with exposure to 30 and 200 ppm for 7 and 14 days and 30 ppm only for 4 days. The bar graphs are presented as mean  $\pm$ SEM and statistically significant is designated as \* $p < 0.05$  N=8 per dose/time-point.



**Figure 3. *In vitro* and *ex vivo* TPO inhibition determined by AUR-TPO inhibition.**

Assay with MMI and PTU in a 96-well format. A) *In vitro* assay was performed with PTU on rat thyroid microsomes of non-treated rats exposed *in vitro* to 0–100  $\mu\text{M}$  PTU N=6.  $\text{IC}_{50} = 1.21\mu\text{M}$ . B) TPO activity was inhibited at all doses in the *ex vivo* microsomes obtained from thyroid gland of 4-day PTU exposed animals. C) TPO inhibition measured from MMI exposed rat thyroid microsome *in vitro*  $\text{IC}_{50} = 0.11\mu\text{M}$ . D) TPO inhibition was also demonstrated at all doses in *ex vivo* microsomes from thyroid gland of 4-day MMI treated animals N=4  $Z' > 0.94$ . \* represent  $p < 0.05$ , and error bars  $\pm$  SEM



A

B

$$R^2=0.86$$

$$Y = 15.17 + 0.89X$$

$$R^2=0.71$$

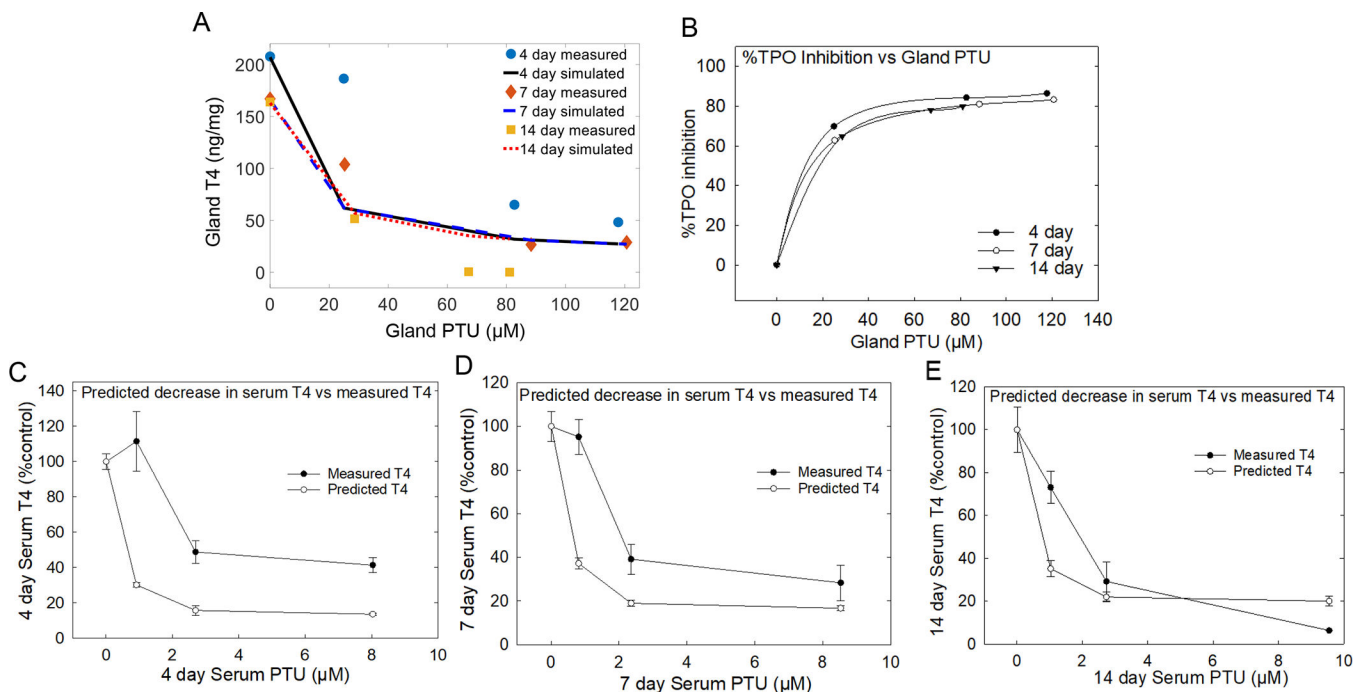
$$Y = 8.51 + 0.84X$$

C

D

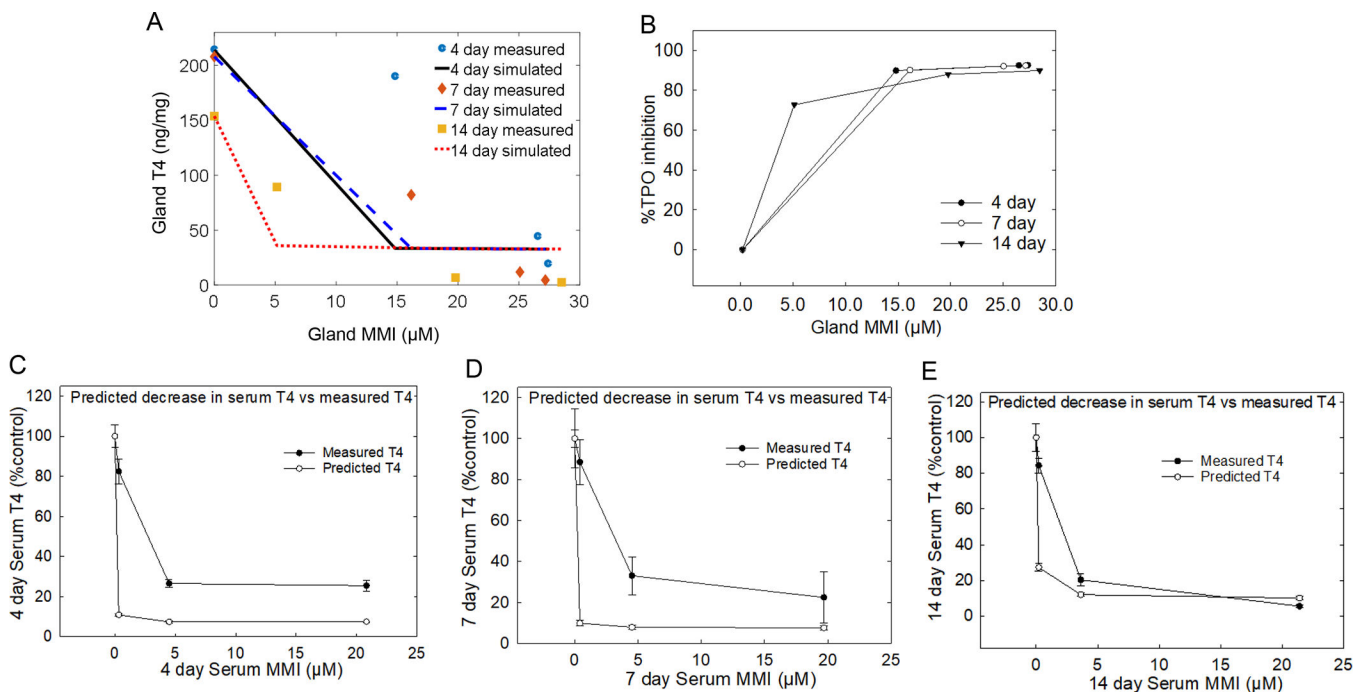
**Figure 4: Quantitative relationships *ex vivo* and *in vitro* extrapolation.**

All values plotted as percent control. A) *Ex vivo* measure of TPO inhibition versus TPO activity for PTU exposure was determined using AUR assay. B) *Ex vivo* TPO activity correlate well to glandular PTU levels as a linear relationship. C) Examining the relationship between TPO inhibition *in vitro*, the dotted blue line demonstrates that 10% inhibition in TPO activity *in vitro* by PTU corresponds to 20% decrement in serum T4 level at 14-day exposure duration. D) For MMI, 20% decline in serum T4 corresponded to 28% TPO inhibition *in vitro* in the 14-day dosed rats.



**Figure 5. PTU computational model and extrapolation of *in vitro* responses to *in vivo* serum T4 levels.**

A) *in vitro* PTU IC50 value of 1.2 µM used to estimate the degree of TPO inhibition for all exposure to points. TPO estimate was better at all doses with 7 and 14-day exposures than 4-day time point and the estimation for 4-day were better at higher PTU doses. B) Degree of TPO inhibition is translated to percent TPO inhibition at all timepoints versus gland PTU levels. C, D, and E) Results demonstrate that the percent TPO decrease, obtained from the simulated degree of TPO inhibition, when applied to serum T4 provides levels that closely aligns with measured serum T4 at 14 days of exposure compared with 4 and 7-day exposures. Error bars represent ± SEM.



**Figure 6. MMI computational model and extrapolation of *in vitro* responses to *in vivo* serum T4 levels.**

A) Glandular T4 and MMI introduced into an *in-silico* model to estimate the degree of TPO inhibition resulted in stronger estimation at the lower doses compared with higher doses at all time-points. However, the 7 and 14-day simulations of TPO inhibition provided a better fit of the measured data compared with 4-day estimation of TPO inhibition. B) Translation of the simulated degree of TPO inhibition to %TPO inhibition C, D, and E) finding show when percent TPO inhibition is applied to serum T4 it provides a good prediction of declines in serum T4 compared in the 14-day dosed group compared with the 4 and 7-day dosed groups. Error bars represent  $\pm$  SEM.



**Table 1:**

Serum and Thyroid Gland PTU levels and Gland Hormone Concentrations

PTU exposure duration/doses (ppm) 4 day	Serum PTU (ng/ml)	Gland PTU (ng/g)	Hormone Concentrations in the Thyroid Gland (ng/mg)				
			MIT	DIT	T3	rT3	T4
0	<0.38 <sup>1</sup>	<7 <sup>1</sup>	35 (9)	344 (96)	18 (4)	10 (2)	207 (39)
1	156 (20)	4,246 (1170)	30 (11)	186 (66) <sup>*</sup>	18 (6)	9 (3)	186 (30)
3	459 (56)	14,075 (2284)	32 (7)	142 (44) <sup>*</sup>	13 (2)	8 (2)	65 (39)
10	1,367 (139)	20,055 (2725)	12 (7) <sup>*</sup>	94 (34) <sup>*</sup>	5 (2) <sup>*</sup>	3 (1) <sup>*</sup>	48 (19) <sup>*</sup>
7 day							
0	<0.38 <sup>1</sup>	<7 <sup>1</sup>	37 (12)	640 (113)	21 (4)	6 (1)	168 (27)
1	138 (15)	4,297 (1170)	9 (1) <sup>*</sup>	204 (29) <sup>*</sup>	19 (3)	5 (0.7)	104 (20)
3	412 (41)	15,037 (2284)	7 (1) <sup>*</sup>	60 (23) <sup>*</sup>	6 (1) <sup>*</sup>	2 (0.5)	27 (9) <sup>*</sup>
10	1,589 (131)	20,542 (2725)	3 (1) <sup>*</sup>	62 (26) <sup>*</sup>	3 (1) <sup>*</sup>	0.7 (0.3) <sup>*</sup>	29 (9) <sup>*</sup>
14 day							
0	<0.38 <sup>1</sup>	<7 <sup>1</sup>	14 (2)	630 (60)	24 (2)	6 (1)	164 (14)
1	175 (49)	4,867 (1170)	13 (1)	148 (36) <sup>*</sup>	17 (2) <sup>*</sup>	5 (0.8)	51 (11) <sup>*</sup>
3	453 (50)	11,438 (2889)	3 (1) <sup>*</sup>	34 (16) <sup>*</sup>	2 (0.5) <sup>*</sup>	0.7 (0.3) <sup>*</sup>	0.6 (0.4) <sup>*</sup>
10	1,626 (113)	13,800 (2725)	4 (0.11) <sup>*</sup>	6 (1) <sup>*</sup>	0.1 (0.02) <sup>*</sup>	0.1 (0.02) <sup>*</sup>	0.2 (0.04) <sup>*</sup>

Serum PTU LLOQ = 0.38 ng/ml; Gland PTU LLOQ = 7 ng/g Gland TH LLOQ = 0.5 ng/ml

<sup>\*</sup> indicates significantly different from control group where p<0.05

<sup>1</sup> ppm = parts per million

<sup>2</sup> PTU = 6-propylthiouracil; (n=8/dose/group)

MIT, 3-monoiodotyrosine, DIT, 3,5-diiodotyrosine, rT3, 3,3',5'-triiodothyronine, T3, 3,3',5'-triiodothyronine, T4, thyroxine.

**Table 2:**

Serum and Thyroid Gland MMI levels and Gland Hormone Concentrations

MMI exposure duration/doses (ppm) 4 day	Serum MMI (ng/ml)	Gland MMI (ng/g)	Hormone Concentrations in the Thyroid Gland (ng/mg)				
			MIT	DIT	T3	rT3	T4
0	<0.38 <sup>1</sup>	<25 <sup>1</sup>	28 (6)	617 (73)	36 (4)	5 (0.6)	215 (23)
3	37 (7)	1,698 (350)	25 (6)	485 (92)	31 (8)	5 (0.9)	190 (50)
30	512 (59)	3,135 (381)	5 (1) *	76 (19) *	3 (0.6) *	0.8 (0.2) *	20 (5) *
200	2,376 (222)	3,038 (504)	9 (3) *	163 (46) *	7 (2) *	1 (0.3) *	45 (13) *
7 day							
0	<0.38 <sup>1</sup>	<25 <sup>1</sup>	23 (7)	653 (147)	29 (8)	9 (2)	208 (40)
3	47 (8)	1,850 (350)	14 (0.3)	266 (54) *	18 (3)	3 (0.4) *	82 (16) *
30	517 (64)	2,871 (430)	4 (1) *	41 (18) *	2 (0.8) *	0.5 (0.1) *	12 (4) *
200	2,249 (264)	3,110 (227)	1 (0.2) *	12 (3) *	0.6 (0.1) *	0.2 (0.1) *	5 (1) *
14 day							
0	<0.38 <sup>1</sup>	<25 <sup>1</sup>	17 (4)	415 (79)	32 (7)	6 (1)	154 (29)
3	24 (6)	589 (144)	8 (2) *	146 (40) *	18 (2)	4 (0.4) *	89 (10) *
30	415 (47)	2,266 (150)	2 (0.3) *	13 (2) *	1 (0.3) *	0.4 (0.1) *	7 (1) *
200	2,448 (192)	3,263 (345)	0.7 (0.1) *	6 (2) *	0.4 (0.1) *	0.1 (0.03) *	2 (0.6) *

Serum MMI LLOQ = 0.38 ng/ml; Gland MMI LLOQ = 25 ng/g and thyroid gland LLOQ = 0.1 ng/ml

\*\* indicates significantly different from control group where p<0.05

<sup>1</sup> ppm = parts per million

<sup>2</sup> MMI = methimazole; (n=8/dose/group)

<sup>4</sup> MIT, 3-monoiodotyrosine, DIT, 3,5-diodotyrosine, rT3, 3,3',5'-triiodothyronine, T3, 3,3',5-triiodothyronine, T4, thyroxine.