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Early temporal effects of three thyroid hormone synthesis inhibitors in *Xenopus laevis*

Joseph E. Tietge[a,*], Brian C. Butterworth[a], Jonathan T. Haselman[a], Gary W. Holcombe[a], Michael W. Hornung[a], Joseph J. Korte[a], Patricia A. Kosian[a], Marilyn Wolfe[b], Sigmund J. Degitz[a]

[a] *Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. EPA, 6201 Congdon Blvd., Duluth, MN 55804, United States*
[b] *Experimental Pathology Laboratories, Inc., Sterling, VA, United States*

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ABSTRACT

Thyroid axis disruption is an important consideration when evaluating risks associated with chemicals. Bioassay methods that include thyroid-related endpoints have been developed in a variety of species, including amphibians, whose metamorphic development is thyroid hormone (TH)-dependent. Inhibition of TH synthesis in these species leads to developmental delay, and assays designed to capture these effects take several weeks to complete. In an effort to develop a shorter term approach, the early responses of various endpoints were evaluated in *Xenopus laevis* throughout 8 d of exposure to three TH synthesis inhibitors: methimazole (100 mg/L), 6-propylthiouracil (6-PTU) (20 mg/L), and perchlorate (4 mg/L). Endpoints included thyroid gland histology and cell numbers, circulating TH concentrations, and thyroidal TH and associated iodo-compounds. Thyroidal 3,5-diodo-L-tyrosine (DIT) and thyroxine (T4) were significantly reduced from day 2 onward by all three chemicals, while 3-monoiodo-L-tyrosine (MIT) was significantly reduced by methimazole and perchlorate, but not by 6-PTU. These reductions were the earliest indicators of TH synthesis inhibition. Histological effects were apparent on day 4 and became more exaggerated through day 8. However, reductions in circulating T4 and increases in thyroid gland cell numbers were not apparent until day 6. Reductions of thyroidal MIT, DIT, and T4 and circulating T4 are indicative of inhibitory effects of the chemicals on TH synthesis. Changes in thyroid histology and cell number represent compensatory effects modulated by circulating TSH. These observations establish a basis for the development of short term amphibian-based methods to evaluate thyroid axis effects using a suite of diagnostic endpoints.

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1. Introduction

Assessing the impact of chemicals on thyroid function is an important component of a comprehensive screening program for endocrine disruption (DeVito et al., 1999). Several chemicals have been shown to alter normal thyroid function (Brucker-Davis, 1998) and an assortment of tests has been developed in a variety of vertebrate species to assess thyroid disruption using both *in vivo* and *in vitro* approaches (Zoeller and Tan, 2007). The relatively conservative nature of many components of the hypothalamic–pituitary–thyroid (HPT) axis among vertebrates suggests that extrapolation of chemical effects among species may be feasible (Zoeller and Tan, 2007). The amphibian HPT axis is a well studied system (Brown and Cai, 2007; Dodd and Dodd, 1976; Fort et al., 2007) with unique characteristics that make it amenable to bioassay procedures. For example, metamorphic development of many anurans is a thyroid hormone (TH)-dependent process that can be inhibited by exposure to chemicals which inhibit TH synthesis. Based on this knowledge, metamorphic development of *Xenopus laevis* has been proposed as a potential model system upon which a bioassay for thyroid hormone disruption could be developed (Degitz et al., 2005; Opitz et al., 2005; U.S. Environmental Protection Agency, 1998). In response to this proposal, a testing protocol has been developed which exposes premetamorphic larvae at the hind limb bud stage to a test chemical for 21 d and relies on thyroid gland histology and morphological indicators of development as its primary endpoints (OECD, 2009).

Thyroid gland histology is the most sensitive and diagnostic endpoint considered in the 21-d protocol. Thyroid glands of *X. laevis* exposed to TH synthesis inhibitors exhibit follicular cell hyperplasia and hypertrophy, alterations in colloid, and glandular growth; changes generally associated with the compensatory mechanisms which are thought to be modulated by increased circulating thyroid stimulating hormone (TSH) concentrations in response to declin-

* Corresponding author. Tel.: +1 218 529 5176; fax: +1 218 529 5003.
E-mail address: tietge.joe@epa.gov (J.E. Tietge).

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ing TH concentrations in the blood. These changes occur at TH synthesis inhibitor concentrations below those that result in the retardation of metamorphic development and their expression is relatively rapid, being clearly observable following 8 d of exposure to some chemicals (Degitz et al., 2005; Tietge et al., 2005). The changes observed in *X. laevis* thyroid glands are consistent with those observed in other species exposed to thyroid synthesis inhibitors (Hooth et al., 2001; Capen, 1994) suggesting further that this model system may be representative of other vertebrate thyroid systems.

The value of this assay, like any bioassay approach, is largely determined by the duration and cost of the test as compared to the diagnostic information gained. To improve the value of this model system, a shorter bioassay with more diagnostic endpoints is desirable. In order to achieve that goal, the early temporal responses of several endpoints associated with thyroid axis disruption need to be evaluated and compared to the results obtained through longer term studies. Consistent with this approach, early temporal histological and selected transcriptional responses of *X. laevis* larvae exposed to perchlorate and ethylenethiourea (ETU) were recently evaluated (Opitz et al., 2009). The histological responses observed in that study following exposure to perchlorate and ETU were similar to those noted above, including: follicular cell hyperplasia and hypertrophy, diffuse glandular hypertrophy, and alterations in follicular size. The temporal responses of several thyroidal genes were also evaluated and three genes associated with TH regulation and synthesis, thyroid stimulating hormone receptor (TSHr), sodium iodide symporter (NIS), and thyroperoxidase (TPO), were significantly upregulated following 3–5 d of exposure to each chemical. These results support the concept that a shorter term assay is feasible.

The objective of this study is to further evaluate the early temporal responses of additional endpoints related to TH synthesis inhibition besides thyroid gland histology and expression of selected genes. These endpoints include circulating T4 and T3 as indicators of overall thyroid function, thyroid gland T4, MIT, and DIT as indicators of TH synthesis, thyroid histology as an indicator of TSH-mediated compensation, and thyroid cell numbers as an alternate indicator of hyperplasia associated with compensation. These endpoints were evaluated throughout 8 d of exposure to three different chemicals known to inhibit TH synthesis: perchlorate, 6-propylthiouracil (6-PTU), and methimazole. These studies were initiated with prometamorphic organisms, a stage which coincides with the onset of thyroid gland function and normally increasing TH concentrations in the blood (Dodd and Dodd, 1976; LeLoup and Buscaglia, 1977). Perchlorate, 6-PTU, and methimazole were each tested at single concentrations of 4, 20, and 100 mg/L, respectively. These concentrations were selected based on previous studies which demonstrated that these were maximally effective concentrations in terms of metamorphic inhibition.

2. Materials and methods

2.1. Biological procedures

X. laevis tadpoles used for the exposures were obtained from an in-house culture. Reproduction was induced with human chorionic gonadotropin injections and the resultant embryos were held in clean water at 21 °C. Tadpoles were fed a mixture of TetraFin® (Tetra Sales, Blacksburg, VA, USA), Spirulina algae discs (The Wardley Corporation, Secaucus, NJ, USA), Silver Cup Trout Starter (Nelson & Sons Inc., Murray, UT, USA) and <24 h old live brine shrimp (Bio-Marine® Brand, Bio-Marine Inc., Hawthorne, CA, USA). The TetraFin®, algae discs, and trout starter were all blended in Lake Superior water prior to feeding. Tadpoles in all tanks were fed twice/day Monday through Friday and a double amount once/day on the weekend. After 21 d, tadpoles were netted from the tanks, anesthetized using 100 mg/L of MS-222 buffered with 200 mg/L of sodium bicarbonate, and the developmental stage of each tadpole was determined according to Nieuwkoop and Faber (NF) (1994). Tadpoles were placed in clean water to recover from anesthesia. After recovery, the tadpoles at stage NF54 were randomly distributed to the exposure tanks.

2.2. Exposure

Three different studies were conducted using the same exposure protocol to collect the data presented herein. Thyroid gland histology was analyzed from a study conducted in October 2004. Circulating TH concentrations and thyroid gland MIT, DIT, and T4 concentrations were measured from a study conducted in February 2007. Thyroid cell number data were measured from a study conducted in July 2007. No mortality or other overt signs of toxicity were observed in any of the studies. In each study, there were four replicate tanks of one exposure concentration for each chemical and controls. The water flow rate to each 4.0 L tank was 25 mL/min which resulted in 9 tank volume additions per day. Exposures were conducted at 21 °C in Lake Superior water which underwent ultraviolet light sterilization. The photoperiod was 12 h light:12 h dark. Exposure tanks were immersed in a water bath system to maintain temperature uniformity.

All test chemicals (methimazole, 6-PTU, and sodium perchlorate) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions for methimazole and sodium perchlorate were made in a 19 L glass carboy by dissolving each chemical in water using a stir plate and a magnetic stir bar. Stock solutions for 6-PTU were made in a 19 L glass carboy using a high speed top stirrer to dissolve it in water at 40 °C. Nominal aqueous chemical concentrations of methimazole, 6-PTU, and perchlorate were 100 mg/L, 20 mg/L, and 4 mg/L, respectively. Exposure concentrations of each chemical in each test were analytically verified by either HPLC-DAD (methimazole and 6-PTU) or anion chromatography (perchlorate). Actual concentrations were within 5% of the nominal values for all chemicals in all tests and concentrations did not change throughout each study (data not shown).

2.3. Tissue sampling

Prior to any tissue sampling, larvae were anesthetized with 150 mg/L MS-222. For serum collection, larvae were placed in a dry dissection tray and the pericardial membrane was carefully pulled aside to expose the aorta. Excess pericardial fluid was then removed by blotting with filter paper. The aorta was severed for blood collection using micro-surgical scissors and whole blood was collected using either 10 or 25 μL capillary tubes. Blood was allowed to clot on ice for 4 h and was subsequently centrifuged at 13,700 × *g* for 2 min in a serological centrifuge. Serum was transferred to vials and stored at −80 °C until analysis by HPLC/ICP-MS. Serum samples from four individuals were pooled from each replicate tank at 1, 2, 4, and 6 d, yielding a serum sample size of 4 for each chemical at every time point.

Thyroid glands used for MIT, DIT, and T4 analysis were dissected with fine tip forceps from larvae held in cold L-15 media. The free glands were transferred to vials and stored at −80 °C until processing for analysis by HPLC/ICP-MS. Paired glands from four individuals were pooled from each replicate tank at 1, 2, 4, and 6 d, yielding a sample size of 4 for each chemical at every time point.

Anesthetized larvae were decapitated and head sections were fixed in Davidson's fixative for 48 h. The head samples were rinsed in water and stored in 10% neutral buffered formalin. The samples were dehydrated in a graded ethanol series and embedded in paraffin using standard histological procedures. Head samples were

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