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Phenotypically anchored transcriptome profiling of developmental exposure to the antimicrobial agent, triclosan, reveals hepatotoxicity in embryonic zebrafish

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ABSTRACT

Triclosan (TCS) is an antimicrobial agent commonly found in a variety of personal care products and cosmetics. TCS readily enters the environment through wastewater and is detected in human plasma, urine, and breast milk due to its widespread use. Studies have implicated TCS as a disruptor of thyroid and estrogen signaling; therefore, research examining the developmental effects of TCS is warranted. In this study, we used embryonic zebrafish to investigate the developmental toxicity and potential mechanism of action of TCS. Embryos were exposed to graded concentrations of TCS from 6 to 120 hours post-fertilization (hpf) and the concentration where 80% of the animals had mortality or morbidity at 120 hpf (EC_{80}) was calculated. Transcriptomic profiling was conducted on embryos exposed to the EC_{80} (7.37 μM). We identified a total of 922 significant differentially expressed transcripts (FDR adjusted P-value ≤ 0.05; fold change ≥ 2). Pathway and gene ontology enrichment analyses identified biological networks and transcriptional hubs involving normal liver functioning, suggesting TCS may be hepatotoxic in zebrafish. Tissue-specific gene enrichment analysis further supported the role of the liver as a target organ for TCS toxicity. We also examined the *in vitro* bioactivity profile of TCS reported by the ToxCast screening program. TCS had a diverse bioactivity profile and was a hit in 217 of the 385 assay endpoints we identified. We observed similarities in gene expression and hepatic steatosis assays; however, hit data for TCS were more concordant with the hypothesized CAR/PXR activity of TCS from rodent and human *in vitro* studies.

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1. Introduction

Triclosan (TCS) is a commonly used antimicrobial agent found in many personal care products including hand soaps, toothpastes, various cosmetics, textiles, and some plastics. TCS enters the environment through the general use and disposal of TCS-containing products, and is commonly found in wastewater effluent. Incomplete removal of TCS from wastewater treatment plants, and use of biosolids as soil amendments, results in the release of TCS into aquatic and terrestrial environments (Andrade et al., 2015; Barber et al., 2015; Davis et al., 2015; Dhillon et al., 2015; Kerrigan et al., 2015). Due to its prevalence in many consumer products, a large proportion of humans are directly exposed to TCS. In urine samples collected by the National Health and Nutrition Examination Survey in 2003–2004, TCS was detected in 74.6% of samples from adults and children 6 years or older with a range of 2.3–3790 μg/l, which was attributed to differing degrees of elective use of TCS-containing products (Calafat et al., 2008). Additionally, TCS has been detected in both adult and infant blood and breast milk, increasing concern for potential risk to human development (Allmyr et al., 2006; Calafat et al., 2008; Pycke et al., 2014; Arbuckle et al., 2015; Azzouz et al., 2016; Han et al., 2016).

Efforts among regulatory agencies seek to gain a better understanding of potential hazards from chemicals that are prevalent in the environment or that lack safety information. Leading this effort is the US Environmental Protection Agency National Center for Computational Toxicology's Toxicity Forecaster screening program (ToxCast) (Dix et al., 2007). ToxCast provides comprehensive high-throughput bioactivity data across a wide spectrum of toxicological and biological pathways and was developed to screen and prioritize thousands of chemicals; identifying those that pose the most hazard to human health and the environment for more testing. As part of the ToxCast program, a population-based *in vitro*-to-*in vivo* extrapolation model was developed to estimate oral equivalent doses (OED) for 35 environmental chemicals,

Abbreviations: TCS, Triclosan; EC_{80}, 80% effective concentration; EC_{50}, 50% effective concentration; EC_{20}, 20% effective concentration; Hpf, hours post-fertilization.

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including TCS (Rotroff et al., 2010). Only TCS and one other chemical from this screen had equivalent doses (average OED of three ToxCast assays: 0.0096 mg/kg/day) that overlapped with estimated chronic aggregate human exposure levels (0.13 mg/kg/day). Due to the concerns about human exposure, bioactivity, and potential unknown developmental effects, the use of TCS in consumer products has come into question. The FDA is currently evaluating the safety and efficacy of TCS (Bergstrom, 2014; Kuehn, 2014), the state of Minnesota recently banned the sale of TCS-containing products beginning in 2017, and several companies (including Johnson & Johnson, Colgate-Palmolive, and Avon) have or are in the process of removing TCS from their products.

Concerns have been raised about the potential for TCS to perturb endocrine functioning given the structural similarities of TCS to thyroid hormones and some estrogens. In terms of thyroid bioactivity, studies in amphibians have shown that exposure to TCS accelerates thyroid hormone-dependent metamorphosis and alters expression of several amphibian thyroid hormone-dependent biomarker genes, including increases in proliferating cell nuclear antigen and decreases in thyroid receptor beta (Veldhoen et al., 2006; Marlatt et al., 2013). In contrast, exposures to TCS caused a dose-dependent decrease in total serum thyroxine (T4) levels in rats at various life stages (Crofton et al., 2007; Zorrilla et al., 2009; Paul et al., 2010a; Paul et al., 2010b; Stoker et al., 2010; Paul et al., 2012; Axelstad et al., 2013). It has been hypothesized that TCS decreases serum T4 through increased hepatic catabolism mediated by activation of the constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR) xenobiotic metabolism pathways (Crofton et al., 2007). Activation of these receptor pathways leads to upregulation in phase I and II enzymes including several cytochrome P450s, sulfotransferases, and UDP-glucuronosyltransferases (UGTs) which enhances the elimination of circulating T4 by the liver. Indeed, TCS activated the human PXR and differentially affected human CAR isoforms (Jacobs et al., 2005; Paul et al., 2013). Furthermore, *in vivo* studies in rats exploring this hypothesis have shown transcriptional activation of *Cyp2b* and *Cyp3a*, and increased PROD and UGT enzymatic activity (Zorrilla et al., 2009; Paul et al., 2012). Studies that have targeted TCS estrogenic bioactivity have shown that it interacts with the ER. Although there are conflicting *in vitro* studies showing that TCS has estrogenic and anti-estrogenic activity, the majority of *in vivo* studies have demonstrated that TCS is a potential ER agonist (Ishibashi et al., 2004; Ahn et al., 2008; Gee et al., 2008; Stoker et al., 2010; Huang et al., 2014; Yueh et al., 2015; Yueh and Tukey, 2016). TCS increased hepatic vitellogenin gene expression in adult male medaka (Ishibashi et al., 2004), as well as led to precocious puberty in female rats and enhanced the effects of ethinyl estradiol in a rat pubertal study and uterotrophic assay, respectively (Stoker et al., 2010; Louis et al., 2013). TCS also increased estrogen response element luciferase activity in transfected rat pituitary GH3 cells (Jung et al., 2012). In the same study, three-day exposures to TCS significantly increased uterine weight, C3 expression, and uterine *CaBP-9k* expression in immature rats (Jung et al., 2012). Collectively, these data suggest that TCS disrupts endocrine activity, and more research is needed to fully elucidate the mechanisms of the observed endocrine effects.

The zebrafish is an ideal *in vivo* model for studying the developmental and reproductive toxicity of chemicals. It's rapid, external development and transparency make it amenable to high-throughput chemical screens examining a wide range of biological endpoints (Padilla et al., 2012; Truong et al., 2014; Noyes et al., 2015). Zebrafish share high genetic homogeneity to humans, with 71% of human genes having an ortholog (Howe et al., 2013), and many developmental and biological signaling pathways conserved between zebrafish and humans. The effects of TCS have previously been studied in both the developing and adult zebrafish (Tatarazako et al., 2004; Oliveira et al., 2009; Padilla et al., 2012; Truong et al., 2014; Chen et al., 2015). Dietary exposure of adult zebrafish to TCS for 21 days resulted in hyperplasia of thyroid tissues, with increased thyroid follicle size and number, and increased mRNA expression of the thyroid sodium-iodide symporter (NIS) and thyroid-stimulating hormone (TSH) (Pinto et al., 2013). Developmental exposure to TCS caused changes in lipid droplet accumulation and decreased the expression of several transcripts involved in β-oxidation of fatty acids in larval zebrafish (Ho et al., 2016). However, to our knowledge, no studies have taken a global, transcriptomic approach to examine the possible mechanism of the developmental toxicity of TCS in zebrafish. Whole-genome transcriptomics in embryonic zebrafish represents an untargeted, unbiased method to investigate putative mechanisms of action for various compounds, as the full repertoire of the genome is expressed over the course of development. Transcriptomic studies of 48 hpf embryos have identified differential mechanisms and upstream transcriptional regulation after exposure to phenotypically anchored concentrations of polycyclic aromatic compounds and their oxygenated derivatives (Goodale et al., 2013; Goodale et al., 2015). Similarly, transcriptomic analysis of select estrogenic and anti-estrogenic compounds detected disruption of several distinct endocrine-related biological pathways, supporting the use of 48 hpf embryos in a mechanistic context to identify potential endocrine disrupting compounds (Schiller et al., 2013). For these reasons, we hypothesized that transcriptomics at 48 hpf would provide meaningful insight into the mechanism of TCS developmental toxicity in zebrafish and provide information regarding the endocrine disrupting potential of TCS.

Herein, we used a phenotypically anchored toxicogenomics approach to investigate the mechanism of toxicity after developmental exposure of embryonic zebrafish to TCS. Zebrafish were exposed from 6 to 120 hpf to TCS to identify the concentration at which 80% of exposed embryos have an adverse effect (EC_{80}) in our model system, and whole-genome transcriptomics was conducted using high-density microarrays to examine the effects of TCS on the 48 hpf zebrafish transcriptome. TCS exposure induced robust transcriptional changes with a majority of transcripts being significantly decreased. Downstream functional analysis of the transcriptional responses indicated disruption of many processes related to normal liver functioning, but no responses that would indicate disruption of thyroid or estrogen signaling at the early developmental life-stage tested.

2. Methods

2.1. Chemicals

Triclosan (CAS: 3380-34-5; 99.7%) was obtained from Sigma Aldrich (catalog number PHR1338), and stock solutions were prepared in dimethyl sulfoxide (DMSO; Avantor Performance Materials, Center Valley, PA) at a concentration of 10 mM. For all experiments, the maximum DMSO percentage for all exposure concentrations, including control exposures, was 0.64%.

2.2. Zebrafish husbandry

Wild-type 5D zebrafish were used for all experiments. Zebrafish were maintained at the Sinnhuber Aquatic Research Laboratory on a 28 °C recirculating water system with a 14:10 hour light/dark photoperiod. Embryos were collected in the morning from group spawns of adult zebrafish. In brief, large group tanks of adult zebrafish with a 1:1 male female ratio were set up one day prior to spawning. In the morning, embryos were collected from the tanks using an internal collection apparatus. Embryos were cleaned, age staged in spans of no more than 1 h, and kept in petri dishes under the same conditions as adults prior to chemical exposure (Kimmel et al., 1995). Animal handling and use were conducted according to Institutional Animal Care and Use Committee procedures at Oregon State University.

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