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A multi-omic approach to elucidate low-dose effects of xenobiotics in zebrafish (*Danio rerio*) larvae

Susie S.Y. Huang[a,b,*], Jonathan P. Benskin[c], Nik Veldhoen[b], Bharat Chandramouli[a], Heather Butler[a], Caren C. Helbing[b], John R. Cosgrove[a]

[a] SGS AXYS, Sidney, BC, Canada
[b] Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada
[c] Department of Environmental Science and Analytical Chemistry (ACES), Stockholm University, Stockholm, Sweden

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ABSTRACT

Regulatory-approved toxicity assays such as the OECD Fish Embryo Toxicity Assay (TG236) allow correlation of chemical exposure to adverse morphological phenotypes. However, these assays are ineffective in assessing sub-lethal (*i.e.* low-dose) effects, or differentiating between similar phenotypes induced by different chemicals. Inclusion of multi-omic analyses in studies investigating xenobiotic action provides improved characterization of biological response, thereby enhancing prediction of toxicological outcomes in whole animals in the absence of morphological effects. In the current study, we assessed perturbations in both the metabolome and transcriptome of zebrafish (*Danio rerio*; ZF) larvae exposed from 96 to 120 h post fertilization to environmental concentrations of acetaminophen (APAP), diphenhydramine (DH), carbamazepine (CBZ), and fluoxetine (FLX); common pharmaceuticals with known mechanisms of action. Multi-omic responses were evaluated independently and integrated to identify molecular interactions and biological relevance of the responses. Results indicated chemical- and dose-specific changes suggesting differences in the time scale of transcript abundance and metabolite production. Increased impact on the metabolome relative to the transcriptome in FLX-treated animals suggests a stronger post-translational effect of the treatment. In contrast, the transcriptome showed higher sensitivity to perturbation in DH-exposed animals. Integration of 'omic' responses using multivariate approaches provided additional insights not obtained by independent 'omic' analyses and demonstrated that the most distinct overall response profiles were induced following low-dose exposure for all 4 pharmaceuticals. Importantly, changes in transcript abundance corroborated with predictions from metabolomic enrichment analyses and the identified perturbed biological pathways aligned with known xenobiotic mechanisms of action. This work demonstrates that a multi-omic toxicological approach, coupled with a sensitive animal model such as ZF larvae, can help characterize the toxicological relevance of acute low-dose chemical exposures.

1. Introduction

Increases in global use of pharmaceuticals and personal care products (PPCPs) have led to the widespread distribution of these substances into the aquatic environment. Although concentrations of PPCPs are low (*i.e.* <μg/L) compared with most environmental contaminants, PPCPs are designed to be bioactive at low concentrations (Berninger et al., 2011). Thus, exposure risks of active pharmaceutical ingredients (APIs) towards non-target aquatic organisms are likely to be related to the intended therapeutic mode-of-action (MOA) (Ankley et al., 2007; Berninger and Brooks, 2010). Several laboratory-based studies on fish have reported effects of short-term exposure to APIs in accordance with known mammalian MOAs; however, the concentrations used in these studies were typically much higher than those found in the environment (Corcoran et al., 2010). Chronic effects of exposure to environmental concentrations of APIs have been observed in limited teleost studies. Exposure to fluoxetine (FLX) as low as 1 μg/L affects mating behavior and predator avoidance in adult fathead minnow (*Pimephales promelas*), while chronic, low-level exposure to carbamazepine (CBZ) can induce atretic oocytes and altered ovarian histology in female zebrafish (ZF; *Danio rerio*) (Galus et al.,

* Corresponding author at: SGS AXYS, 2045 Mills Road, Sidney, British Columbia, Canada V8L 5X2. Tel.: +1 250 655 5800;fax: +1 250 655 5811.
E-mail address: shuang@axys.com (S.S.Y. Huang).

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2013b; Weinberger and Klaper, 2014). Short-term (96 h) exposure to CBZ (0.5 μg/L) and acetaminophen (APAP; <5 μg/L) increases developmental abnormalities and mortality in ZF embryos, respectively (David and Pancharatna, 2009; Galus et al., 2013a). Results from studies such as these validate the feasibility and highlight the importance of correlating biological function to sub-lethal exposure in assessing the potential deleterious environmental impacts of PPCPs. However, more sensitive, reliable, and rapid screening methods for the assessment of environmentally relevant concentrations of PPCPs are needed to accommodate the demands of large scale chemical screening and environmental monitoring efforts. Furthermore, a reduction in exposure time by using sensitive life stages would reduce monitoring costs and expedite testing (Barron and Adelman, 1984).

The high level of conservation in genomic and metabolic pathways between fish and mammals makes teleost an effective vertebrate model in toxicological testing (Williams et al., 2014). Furthermore, early life stages in fish have a reduced capacity to metabolize xenobiotics compared with that of equivalent life stages in mammals (Andersson and Förlin, 1992). Thus, the embryonic or larval stages represent a more sensitive toxicological model than adults, particularly in addressing adverse effects of low-level chemical exposure. In addition, the use of early life stages of teleosts is not regulated by the current legislation on animal experimentation (ex. EU Directive 2010/63/EU and Canadian Council of Animal Care) and can be used as an alternative approach in animal testing.

Integration of multi-omics technologies provides unprecedented potential to identify and link molecular initiating events; hence, to better predict toxicological outcomes and gain further insight into the mechanistic aspects of chemical effects (Cavill et al., 2015; Santos et al., 2009). This systems biology approach has been frequently applied in medical and pharmaceutical research for diagnostic purposes and target identification (Edwards and Preston, 2008) but such integrative assessments remain relatively unexplored in the area of ecotoxicology. An early integrative approach incorporating transcriptomic and metabolomic information was conducted on stickleback (*Gasterosteus aculeatus*; Williams et al., 2009) and only a handful of multi-omics studies have been conducted on aquatic organisms in subsequent years (Benskin et al., 2014; Chen et al., 2016; Ji et al., 2013; Katsiadaki et al., 2010; Santos et al., 2009; Soanes et al., 2011; Wu et al., 2013a, 2013b).

We previously reported that the 96 to 120 h post fertilization (hpf) time point is an appropriate developmental stage in ZF for obtaining fingerprint-like metabolomic profiles associated with 13 different xenobiotics exposed at environmentally relevant concentrations (Huang et al., 2016). Since the objectives of that work were to assess the sensitivity, specificity, and reproducibility of the technical approach, no attempts were made at biological interpretation. In the present work, we selected metabolomic datasets associated with 4 substances tested in Huang et al. (2016) and supplemented these data with additional transcriptomic data in the same ZF larvae model. The objectives were two-fold: firstly, to compare perturbations in the transcript and metabolite profiles induced by environmental concentrations of common PPCPs with known MOAs in terms of sensitive and specificity of response; and secondly, to integrate the 'omic datasets to identify molecular interactions and biological relevance of the responses.

2. Materials and methods

2.1. Chemicals and exposure

Acetaminophen (APAP; CAS# 103-90-2, purity ≥99.0%), diphenhydramine hydrochloride (DH; CAS # 147-24-0) and carbamazepine (CBZ; CAS # 298-46-4) were purchased from Sigma-Aldrich (St. Louis, MO, USA) whereas fluoxetine hydrochloride (FLX; CAS# 59333-67-4) was purchased from US Pharmacopeia (Rockville, MO, USA). Test solutions were prepared in HE3 embryo media and 100% EtOH was used as carrier resulting in a final EtOH concentration of 0.1% in all test solutions. Details of chemical selection and concentration range can be found in the Supporting Information (SI) and Table S1. A total of 3 different exposure concentrations were prepared for each chemical and are referred to throughout the manuscript according to suffixes L, M, and H, corresponding to low, medium, and high dose, respectively. For example, the lowest APAP dose is referred to as APAP-L.

Details on animal handling, experimental design, and morphological effects can be found in SI and in Huang et al. (2016) In brief, the 24 h blind exposures consisted of 5 biological replicates per treatment and a total of 88 ZF larvae (96 hpf) per group. HE3 (untreated control; UT) and 0.1% EtOH (vehicle control; VEH) groups were included with every chemical exposure. At the end of the chemical exposure trials, 80 animals were collected, washed with ice-cold HE3 media, flash-frozen on dry ice, and stored at −80 °C for metabolomic analysis. The remainder 8 larvae from each group were collected, euthanized, and immersed in *RNAlater* (1 mL) tissue preservation solution (Life Technologies Inc., Burlington, ON, Canada) at 4 °C overnight prior to transfer to −80 °C for storage pending transcriptomic analysis.

2.2. Chemical analyses

Details of metabolite extraction, instrumental analysis, and full lists of analytes, internal standards, and abbreviations are as described by Huang et al. (2016). In brief, metabolites were measured using LC- or FI-MS/MS and concentrations were determined by isotope dilution or internal standard approaches (Benskin et al., 2014).

2.3. qPCR primer design and reactions

DNA primers were designed *de novo* or derived from published information as indicated (Table S2). The gene targets were chosen based on 1) their importance as ZF developmental markers (Table S3), 2) their potential to be induced by the board range of xenobiotics tested in our previous study (Huang et al., 2016) as informed by literature and/or 3) their relative invariance during early development stages and to the chemicals under investigation (*i.e.* housekeeping genes). The former two criteria provide information relating to potential xenobiotic perturbations while the last criterion allows for appropriate input normalization during the assay procedure. The extent of qPCR validation as well as the experimental conditions employed was considered when selecting primer sequences from the literature.

All DNA primers were purchased from Integrated DNA Technologies (Coralville, IA, USA) and resuspended in PCR-grade water. Each 15 μL qPCR reaction contained 0.01% Tween 20, 0.8% glycerol, 10 mM Tris-HCl (pH 8.3 at 20 °C), 50 mM KCl, 3 mM $MgCl_2$, 40,000-fold dilution of SYBR Green I (Life Technologies), 200 μM dNTPs (Bioline USA Inc., Taunton, MA, USA), 69.4 nM ROX reference dye (Life Technologies), one unit of Immolase DNA polymerase (Bioline), 2 μL or 20-fold diluted cDNA, and 5 pM of each of forward and reverse DNA primers. Samples were measured in quadruplicate on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

The thermocycle program consisted of an initial enzyme activation step at 95 °C (9 min), followed by 40 cycles of 95 °C denaturation (15 s), 60 °C or 64 °C annealing (30 s) depending on target, and 72 °C elongation (30 s). A negative control for each target using a template reaction without cDNA and a positive inter-run