Elucidating mechanisms of immunotoxicity by benzotriazole ultraviolet stabilizers in zebra fish (Danio rerio): Implication of the AHR-IL17/IL22 immune pathway

Zhitong Li a, Xuefang Lianga,*, Wang Liua, Yaqian Zhaoa, Huiting Yang a, Wenjing Lia, Ondrej Adamovskyc, Christopher J. Martyniukb

a Inner Mongolia Key Laboratory of Environmental Pollution Control & Waste Resource Reuse, School of Ecology and Environment, Inner Mongolia University, Hohhot, 010021, China
b Department of Physiological Sciences and Center for Environmental and Human Toxicology, University of Florida Genetics Institute, Interdisciplinary Program in Biomedical Sciences Neuroscience, College of Veterinary Medicine, University of Florida, Gainesville, FL, 32611, USA
c Research Centre for Toxic Compounds in the Environment (RECETOX), Faculty of Science, Masaryk University, Brno, Czech Republic

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A B S T R A C T
Benzotriazole ultraviolet stabilizers (BUVSs) are widely used additives in industrial materials and personal care products that protect products from ultraviolet damage. Due to their high production volume and potential to bioaccumulate, BUVSs are an environmental pollutant of concern. In this study, juvenile zebrafish (Danio rerio) were exposed to 4 BUVSs (UV-234, UV-326, UV-329, and UV-P) at 10 and 100 μg/L for 28 d. BUVSs induced hepatic vacuolization and nuclei pyknosis in the liver following 100 μg/L UV-234 and UV-329 exposure. Transcriptomic analysis in the liver uncovered pathways related to inflammation that were affected by BUVSs. Based upon these data, we measured the expression levels of 9 genes involved in AHR-IL17/IL22 pathway in zebrafish larvae exposed to each BUVSs at one dose of either 10 or 100 μg/L for 6 days in a second set experiment. Transcript levels of interleukins il17a and il22 were decreased, while il6 mRNA was increased with exposure to UV-234, UV-329, and UV-P. No change to targeted transcripts was observed with UV-326 treatments. Moreover, cyp1a1 and ahr2 levels were increased in larvae treated with 100 μg/L UV-329 or UV-P. Consistent with expression data, protein abundance of IL22 was decreased by 29% with exposure to 100 μg/L UV-P. Taken together, these results demonstrate that exposure to different benzotriazole congeners may be associated with immunotoxicity in zebrafish through the AHR-IL17/IL22 pathway, and this may be associated with hepatic damage with prolonged exposures. This study provides new insight into unique pathways perturbed by specific BUVSs congeners.

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1. Introduction
Benzotriazole ultraviolet stabilizers (BUVSs) are some of the most commonly used additives in industrial and commercial products, such as plastics, rubber, cosmetics and sunscreens to prevent yellowing and degradation of materials. Due to their high production volumes, manufacturing versatility, persistence in environments, and potential for toxicity in organisms, BUVSs have become an emerging contaminant of concern (Apel et al., 2018; Rani et al., 2015; Wick et al., 2016). BUVSs can enter the aquatic ecosystem through wastewater treatment plant discharges (Lu et al., 2017b; Ruan et al., 2012), landfills (Langford et al., 2015), runoffs (Parajulee et al., 2018), and plastic debris (Rani et al., 2017). BUVSs have been reported to be present in surface waters with concentrations ranging from 0.5 to 544.9 ng/L (Garcia-Guerra et al., 2016; Kameda et al., 2011; Liu et al., 2014; Vimalkumar et al., 2018). The log n-octanol-water partition coefficients (logKow) for most BUVSs are higher than 4.3, suggesting that they can readily accumulate in suspended matter, sediments and biota (Lu et al., 2017a; Nakata et al., 2012; Wick et al., 2016). Reports indicate that concentrations of BUVSs can reach up to 73 μg/g dry weight in
sEDiments (Cantwell et al., 2015) and 316 ng/g lipid weight in fish muscle tissues (Kim et al., 2011). Due to the significant bio-accumulation of BUVSs, sub-chronic exposure to these chemicals over time are predicted to pose health risks to aquatic organisms.

Although the effects of BUVSs on antioxidant response (Giraudo et al., 2017) and endocrine systems in aquatic organisms have been investigated (Fent et al., 2014; Liang et al., 2017a; Zhuang et al., 2017), other toxicity pathways have yet to be characterized. In a recent study, UV-234 and UV-320 perturbed mitochondrial bioenergetics, embryonic development, and locomotor activity of early-stage zebrafish, suggesting metabolic processes and mitochon-dria may be targets for BUVSs toxicity (Liang et al., 2019). Another study measured the distribution of BUVSs in white sucker (Catostomus commersonii) tissues and showed that the liver is a major site for accumulation of BUVSs in fish (Lu et al., 2017a). In our previous study, benzo(a)pyrene - the parent compound of BUVSs – was reported to dysregulate hepatic proteomic responses and induce liver damage in male Chinese rare minnow (Gobiocypris rarus) (Liang et al., 2017b). In mammals, it was reported that UV-320 caused hepatocyte hypertrophy and hepatic necrosis in rats (Hirata-Koizumi et al., 2007). While studies indicate that the liver is a likely target of BUVSs, their toxic action on the hepatic system of fish, and the underlying mechanisms, are not completely elucidated.

The immune system is a frequent target of toxic insult following subchronic or acute exposure to environmental chemicals. Numerous xenobiotic compounds (e.g. pesticides, pharmaceuticals, plasticizers) are known to modulate immune parameters of fish, which can alter immune function and increase sensitivity to infections, leading to overall immunomodulation (Germolec et al., 2017; Segner et al., 2012; Xu et al., 2018; Zhang et al., 2019). Studies in mammals have shown that exposure to some BUVSs can result in dermatitis and skin irritation (Yamano et al., 2001), and decrease numbers of red blood cells, hemoglobin, and hematocrit (Hirata-Koizumi et al., 2007). In addition, our previous transcriptomic investigation in zebrafish brain revealed that BUVSs affected pro cesses such as B-cell activation, leukocyte differentiation, T-cell recruitment, immunomodulation, and immuno-stimulation at the molecular level, implying their inflammatory or immunotoxic effects in fish (Li et al., 2019).

The vertebrate immune system is, in part, under aryl hydrocarbon receptor (AHR)-mediated transcriptional regulation (Stockinger et al., 2014). Toll-like receptors (TLRs) and proinflammatory cytokines (e.g. TNFα, IL-1, IL-6) involved in the innate response are regulated by AHR-DRE (dioxin-response elements) sequences (Kerkvliet, 2009). Xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) act as AHR ligands, which can then bind as a ligand-receptor complex to promoters of immune-relevant genes downstream to initiate transcription (Sun et al., 2004). Evidence is accumulating that activation of the AHR pathway in vertebrates results in modulation of immune outcomes. For example, Xu et al. (2018) reported that bisphenol A (BPA) and tetrachlorodibenzo-p-dioxin (TCDD) exposure can up-regulate ah2 expression and modulation of il8, tnfa, and nbkbz2 mRNA levels in zebrafish larvae, which imply an effect on the immune system. In previous studies, BUVSs have shown anti-androgenic activity in vitro (Nagayoshi et al., 2015; Zhuang et al., 2017) and were demonstrated to regulate the expression of AHR pathway-related genes in zebrafish embryos (Fent et al., 2014). Thus, effects of BUVSs on aryl hydrocarbon receptor (AHR) and immune-related pathways are hypothesized to be a mechanism associated with their toxicity.

To better understand the underlying molecular mechanism of BUVSs-induced inflammatory response and investigate their possible adverse outcome in liver, two sets of experiment were carried out in this study. Firstly, juvenile zebrafish (Danio rerio) were exposed to 4 different BUVSs that included UV-234, UV-326, UV-329, and UV-P for 28 d. Histopathological damage in liver was firstly assessed followed by global transcriptome sequencing (RNA-Seq) to explore molecular response on a broad scale. Differentially expressed genes and pathways related to inflammation and immune signaling were observed based on the transcriptomic data. To further elucidate the impact of BUVSs on the innate immune system, immune-related genes and proteins in zebrafish embryos were then examined. The aim of this study was to (1) determine the hepatotoxicity and immunotoxicity of BUVSs on both a transcriptional and physiological level and to (2) reveal the underlying mechanisms that may be associated with adverse outcomes linked to BUVSs exposure.

2. Materials and methods

2.1. Experimental animals and chemicals

Zebrafish (ZF, AB strain) were raised in a flow-through system in the School of Ecology and Environment at Inner Mongolia University. Experimental procedures were approved by Institutional Animal Care and Use Committee of Inner Mongolia University. Fish were kept in the following conditions: temperature of 28 ± 1 °C, 14:10 h light: dark photoperiod, mean water pH of 7.2 ± 1.0, and conductivity of 500 μS/cm. Fish were fed newly hatched brine shrimp (Artemia nauplii) twice a day. Zebrafish mating and embryo exposure procedures have been described previously by Liang et al. (2019).

UV-stabilizers UV-234 (CAS number 70321-86-7, >99% purity), UV-326 (CAS number 3896-11-5, >99% purity), UV-329 (CAS number 3147-75-9, >98% purity), and UV-P (CAS number 2440-22-4, >99% purity) were purchased from J&K Chemical Ltd. (USA). Stock solution of BUVSs were prepared by dilution in DMSO. Then, 10 and 100 mg/L stock solutions were mixed with distilled-deionized water (for juvenile exposure) or embryo rearing medium (ERM, for embryos exposure) (Liang et al., 2019) to yield the final concentrations of either BUVSs at 10 or 100 μg/L. A vehicle treatment of DMSO served as control (0.1%, vol/vol).

2.2. Experiment 1- hepatotoxicity assessment in juvenile ZF

2.2.1. Exposure experiment

The first experimental regime was conducted according to our previous report (Li et al., 2019). Briefly, juvenile zebrafish (~2 months) were randomly divided into 36 beakers (4L) with 0.1% DMSO (solvent control), 10, or 100 μg/L either BUVSs (n = 4). Each beaker contained 10 fish and a 28-day semi-static waterborne exposure was carried out. At the end of the exposure, fish were anesthetized on ice and livers were dissected. Within a beaker, two fish livers were sampled for histopathological analysis and the other 8 livers were processed for transcriptome analysis. Livers were harvested from four zebrafish from each replicate beaker and pooled for RNA-Seq. The remaining livers from the remaining four fish in a beaker were pooled for qPCR. Thus, there were 4 biological replicates for qPCR derived from each of the 4 beakers. Samples were flash-frozen using liquid nitrogen and stored at −80 °C until subsequent RNA extraction.

2.2.2. Histological examination

The histopathological analysis of BUVSs-exposed liver was performed as previously described (Liang et al., 2017b). Briefly, liver samples were fixed in 4% paraformaldehyde and dehydrated in ethanol. After being embedded in paraffin wax, the sections were cut at 4 μm and stained with hematoxylin and eosin (H and E stain).
Tissue abnormalities were examined under an optical microscope BX53 (Olympus, Tokyo, Japan). In each image, 5 fields of visions were randomly selected for inspection and the number of different cells (e.g. nucleated cells and akaryotes) were counted (n = 8). A student's t-test was performed to determine whether the liver damage occurred and p < 0.05 was considered to be statistical significance.

2.2.3. Transcriptomic profiling and pathways analysis
The high dose (100 μg/L) BUVSS-treated juvenile zebrafish were used for RNA-Seq as per Li et al. (2019). After extraction, RNA samples with a RIN value > 8.0 were used for library construction. Following this, the libraries were subjected to sequencing using the Illumina HiSeq 2500 platform. Following quality check and filtering, there were 18.4, 17.6, 16.5, and 17.5 million RNA-Seq clean reads obtained from the control, UV-234, UV-326, UV-329, and UV-P groups, respectively. Differentially expressed genes (DEGs, |log2FC (fold change)| > 1, p < 0.05) imported into Pathway Studio 10.0 for pathways analysis. Further details were provided in Supplemental Materials.

Molecular Signatures Database (MSigDB) v6.2 (http://software.broadinstitute.org/gsea/index.jsp) was used to map the DEGs onto Gene Sets of “immunologic signatures”. FDR q-value was set to 0.05. A total of 434 immune-related DEGs were further analyzed based on the KEGG database (http://software.broadinstitute.org/gsea/index.jsp). Forty-nine genes involved in “cytokine-cytokine receptor interaction” and 14 involved in “intestinal immune network for IgA production” from KEGG database were further screened. Among these DEGs, 18 were also detected in the brain transcriptome of our previous study (Li et al., 2019). Based on these screening, il17, il22 and other genes associated with these proteins were selected for further verification.

2.3. Experiment 2- toxicity of BUVSS in innate immune system of ZF embryos

A second experiment was designed based on the transcriptomic data of the first experiment. To validate the effects of BUVSS on the innate immune system of ZF, embryos were exposed to BUVSS for 6 days. ZF embryos have been widely used as a model for innate immunity research, due to its independence from the adaptive immune response, which is functionally mature after 4–6 weeks post-fertilization (Novoa and Figueras, 2012; Trede et al., 2004).

2.3.1. Exposure experiment
Exposure experiments were followed using TRIZol® Reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s protocol. After purification and quantitation, the cDNA was synthesized with 500 ng total RNA using Transcriptor First Strand cDNA Synthesis Kit as per the manufacturer’s instructions (TIANGEN Biotech, Beijing). The cDNA was generated using a Veriti Thermal Cycler (Applied Biosystems, USA) with the following steps: 42 °C for 3 min, 42 °C for 15 min, 95 °C for 3 min, and a final cycle of 4 °C for 5 min. Further details are provided in Supplemental Materials.

2.3.2. RNA extraction and cDNA synthesis
Extraction of RNA from livers (Experiment 1) or larvae (Experiment 2) was performed using 1 mL TRIZol® Reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturers protocol. After purification and quantitation, the cDNA was synthesized with 500 ng total RNA using Transcriptor First Strand cDNA Synthesis Kit as per the manufacturer’s instructions (TIANGEN Biotech, Beijing). The cDNA was generated using a Veriti Thermal Cycler (Applied Biosystems, USA) with the following steps: 42 °C for 3 min, 42 °C for 15 min, 95 °C for 3 min, and a final cycle of 4 °C for 5 min. Further details are provided in Supplemental Materials.

2.3.3. Real-time PCR
Transcriptomic data from this study and that from Li et al. (2019) were used to screen the candidate list for genes responsive to BUVSS. Several genes were identified as candidates, and we focused on assessing these in zebrafish larvae. These genes included interleukin 1 beta (il1b), il6, interleukin 6 receptor (il6r), il17a, il22, transforming growth factor, beta 1 (tgf1b), argyl hydrocarbon receptor (ahr), ahr2, and cytochrome P450 family 1 subfamily A member 1 (cyp1a1). Table S1 shows the primers pairs for target genes. In addition, 4 genes that included ano1, ddx19, hsd17b12a, and pknama were verified by qRT-PCR to confirm the results of RNA-Seq.

Real-time PCR was performed using the CFX96™ Real-Time PCR Detection System (BioRad) as per Liang et al. (2019). Sample volume for Real-time PCR was 20 μL, consisting of the TB Green™ Premix Ex Taq™ II (Takara, Japan), 200 nM forward primer and 200 nM reverse primer. Reference genes β-actin and ef1α were most stable combination (M = 0.97, CV = 0.39) and used to normalize all target genes. Normalized gene expression was extracted using CFX Manager™ software with the relative delta-delta Ct method. Further details on real-time PCR assays can be found in Supplemental Materials and Liang et al. (2019).

2.3.4. Enzyme-linked immunosorbent assay

The protein abundance of IL17A and IL22 were measured using enzyme-linked immunosorbent assay (ELISA) test kits (Meimian, Jiangsu, China) as per the manufacturer’s instructions. Briefly, ~200 larvae for each treatment (control, 10 and 100 μg/L UV-234, UV-329 or UV-P) were homogenized in 200 μL 1× PBS. There were 4 biological replicates in each treatment (n = 4). The samples were then disrupted on ice by intermittent sonic oscillation. After centrifugation at 5000×g for 15 min at 4 °C, the supernatants were collected and 10 μL from each biological replicate was used for antibody detection according to the protocol. The absorbance was measured at 450 nm. The detection limit of IL17A and IL22 were 0.25–9 ng/L and 1–40 ng/L respectively. The average coefficient of variation between replicates and within plates were less than 10% and 12% respectively.

2.4. Statistical analysis

Kolmogorov-Smirnov test was used to assess the normality of data. Logarithmic transformation was performed to ensure data conformed to the assumption of normality. For RNA-Seq, the threshold of DEGs was set to be |log2FC|>1 and p < 0.05. For qPCR assay, one-way ANOVA followed by a Dunnett’s post-hoc test was employed to compare each BUVSS treatment with the control. Protein data (ELISA) were tested for differences between the control and treatment groups using a One-Way ANOVA, followed by Holm-Sidak’s multiple comparisons test. Quantitative data are expressed as mean ± SEM and p-value <0.05 indicated a significant difference between groups. All statistical analyses were performed in GraphPad Prism 6.
3. Results

3.1. Chronic exposure to BUVSs may induce liver damage in juvenile zebrafish

During 28-d exposure, there were no significant changes in mortalities and deformities of juvenile ZF. However, histological changes in liver were observed following high doses of BUVSs exposure (Fig. 1). In normal liver histology, the hepatocytes were polygonal with a central spherical nucleus and interconnected (Fig. 1A). In contrast, nuclei pyknosis and increases in cellular vacuolization were observed in hepatic tissues of juvenile ZF following exposure to 100 μg/L UV-234 (Fig. 1B). Additionally, the livers of juveniles treated with 100 μg/L UV-329 exhibited noticeable disorganization of the hepatic parenchyma and nuclear pyknosis (Fig. 1D). However, no notable changes were detected in the fish treated with UV-326 or UV-P (Fig. 1C and E).

3.2. Transcriptomic analysis of juvenile zebrafish liver in response to BUVSs

There were 659, 1068, 1047, and 875 DEGs detected in juvenile zebrafish liver following 100 μg/L UV-234, UV-326, UV-329 and UV-P exposure, respectively (Table S2). Among these DEGs, 219, 222, 127, 180 were up-regulated while 440, 846, 920, 695 were down-regulated in UV-234, UV-326, UV-329 and UV-P group, respectively (Table S2). A total of 434 immune-related DEGs were identified following BUVSs exposure, and 18 involved in “cytokine –cytokine receptor interaction” were both detected in brain and liver when compared (Fig. 2). Among these genes, interleukins that included il17a, il22, il6, il1b were significantly increased in livers treated with UV-326, UV-329 and UV-P (log2FC = 1.26–3.38) (Table S3).

Gene Set Enrichment Analysis (GSEA) revealed that the expressions of 296 entities were differentially altered by BUVSs. Among these pathways, 23, 15, 117 and 16 pathways were uniquely affected in the liver following UV-234, UV-326, UV-329, and UV-P exposure, respectively (Fig. S1, Table S4). There were 6 pathways including “IL13R - > STAT signaling” commonly identified in all 4 BUVSs treatments (Fig. S1). Additionally, several biological processes that were shared among BUVSs were related to inflammatory response, immune system activation, cell proliferation, cell differentiation, and myogenesis (Fig. 3). Among these clustered adverse processes, specific subpopulations of T cells were involved, including neutrophil recruitment, IL-17 signaling in diseases, Th1 and Th17 cell activation in disease states and differentiation of the T cells (Table S4).

3.3. BUVSs altered genes expressions related to AHR-IL17A/IL22 pathway

To confirm RNA-seq data, we selected 4 DEGs affected by 3 or 4 BUVSs that included ano1, ddx19, hsd17b12, and pfkma. For each 100 μg/L BUVSs groups, the expression of these genes measured by RT-PCR corresponded to that obtained from RNA-Seq (Fig. S2). The transcript levels of all 4 genes showed a decreasing trend following BUVSs exposure (Fig. S2). For 10 μg/L BUVSs treatments, mRNA levels of target genes were generally consistent with that observed in zebrafish treated with 100 μg/L BUVSs (Fig. S2).

To investigate the immunotoxic responses to BUVSs of zebrafish larvae, the mRNA levels of 9 related genes that included il1b, il6, il6r, il17a, il22, tgfβ1, ahr, ahr2 and cyp1a1 were measured. After 6 d exposure, il1b, il6r, tgfβ1 and ahr in zebrafish larvae were not altered by BUVSs (Fig. 4A, C, F and G). However, the mRNA level of il6 was elevated by 2.1-fold in the 100 μg/L UV-329 group (mean rank diff. = −0.16, p = 0.01) (Fig. 4B). Furthermore, levels of il17a were significantly down-regulated by 2.1 (mean rank diff. = 0.67, p = 0.03) and 1.7-fold (mean rank diff. = 0.52, p = 0.02) in fish from
the 100 µg/L UV-234 and 10 µg/L UV-P treatments, respectively (Fig. 4D). Additionally, the expression level of il17a in both the 10 and 100 µg/L UV-329 treated fish showed a significant decrease by 2 and 2.1-fold, respectively (mean rank diff. = 0.62, p = 0.01; mean
rank diff. = 0.67, p = 0.008) (Fig. 4D). A reduction of \( \text{il22} \) was also observed in fish from both 10 \( \mu \)g/L (2.9 fold, mean rank diff. = 0.59, \( p = 0.0003 \)) and 100 \( \mu \)g/L UV-234 groups (4.2 fold, mean rank diff. = 0.69, \( p < 0.0001 \)), while a decrease of \( \text{il22} \) was detected in fish from the 10 \( \mu \)g/L UV-P treatment (3.5-fold, mean rank diff. = 0.64, \( p = 0.03 \)) (Fig. 4E). Following exposure to 100 \( \mu \)g/L UV-P, transcripts of \( \text{ahr2} \) and \( \text{cyp1a1} \) were significantly up-regulated in fish by 1.9-fold (mean rank diff. = 0.63, \( p = 0.01 \)) and 3.5-fold (mean rank diff. = 1.06, \( p < 0.0001 \)) respectively (Fig. 4H and I). Moreover, an increase of \( \text{ahr2} \) was observed in fish from the 100 \( \mu \)g/L UV-329 group (1.7-fold, mean rank diff. = 0.49, \( p = 0.02 \)) (Fig. 4H). However, no significant alteration in any transcript was detected for UV-326 treatments (Fig. 4A–I).

### 3.4. Immune response at protein level

\( \text{Il17a} \) and \( \text{Il22} \) levels were measured in zebrafish larvae after 6 d of exposure (n = 4). \( \text{Il22} \) was significantly reduced by 29% (\( F_{(2, 9)} = 4.64, p = 0.03 \)) in the zebrafish larvae exposed to 100 \( \mu \)g/L UV-P treatments compared to control group (Fig. 5B). However, no changes of \( \text{Il17a} \) and \( \text{Il22} \) concentrations were examined in any other exposure groups (Fig. 5A and B).

### 4. Discussion

Benzotriazole ultraviolet stabilizers (BUVSs) are subsets of benzotriazole moieties attached together with a phenolic group, which are used as UV light absorbers in plastic products and cosmetics (Cantwell et al., 2015; Parajulee et al., 2018). In contrast to benzotriazole (logKow = 1.23), BUVSs are highly hydrophobic (logKow > 4.3) and readily distribute in sediment and biota (Lu et al., 2016, 2017a; Nakata et al., 2012; Wick et al., 2016). Lu et al. (2017a) reported that the liver is a major tissue that accumulates BUVSs in fish, implying that these compounds can exert toxicity in hepatic tissue. Despite adverse effects that include lower heart rates (Liang et al., 2017a), hatching delays, altered mitochondrial bioenergetics, and effects on locomotor activity in zebrafish embryos/larvae (Liang et al., 2019), hepatotoxicity of BUVSs in fish has not been fully investigated to date.

In the present study, histopathological abnormalities were detected in the liver of juvenile zebrafish following high-dose (100 \( \mu \)g/L) UV-234 and UV-329 exposure. Their parent compound benzotriazole has been shown to induce hepatotoxicity in fish based on molecular and histopathological changes (Duan et al., 2017; Liang et al., 2017a, b). UV-320 has been shown to cause
hepatocyte hypertrophy and hepatic necrosis in rats (Hirata-Koizumi et al., 2007). The chemical also induces hepatotoxicity by influencing the peroxisome proliferator-activated receptor α (PPARα) pathway (Hirata-Koizumi et al., 2016), which is related to cell proliferation, immune response, and inflammation (Chinetti et al., 2003). It has been reported that inflammatory responses caused by exogenous chemicals can induce hepatotoxicity (Dyk et al., 2012; Toughan et al., 2018). For example, Liu et al. (2016a, b) demonstrated that exposure to tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), which is used as a flame retardant and plasticizer, can upregulate the expression of several inflammation biomarkers (e.g. IL13, IL26, IL6) at the mRNA or protein level, leading to hepatic damage in zebrafish. These studies indicate that the observed histopathological changes in this study may be the result of inflammatory response to BUVSs exposure.

Our transcriptomic data revealed that after sub-chronic exposure to BUVSs, several cytokines (e.g. il17, il22, il6, il1b, tgfβ1, etc.) as well as inflammatory pathways, were significantly altered in expression. The upregulation of specific cytokines related to Th17 signaling (IL-17 and IL-22) indicated that BUVSs have a potency to disrupt immune-related processes. The key function of Th17 cells is to stimulate the recruitment of neutrophils via production of chemokines, and thus induce inflammation (Zheng et al., 2007). Th17 cells also generate IL-17 that stimulates liver nonparenchymal cells and monocytes/myeloid dendritic cells to produce proinflammatory cytokines and chemokines, inducing liver inflammation (Ouyang and Kolls, 2008). The involvement of T cell–mediated immune response is supported by upregulation of IL-6 and IL-1b, both enhancers of an adaptive immunity (Dienz and Rincon, 2009). In response to injury, the inflammatory cells (i.e. macrophages and neutrophils) secrete various cytokines that included IL-1, IL-6, IL-8 as well as TNF-α into the bloodstream, stimulating the production of acute phase proteins in hepatocytes to protect tissue from damage (Roy et al., 2016). These results further support the linkage between liver injury and immune disturbance following BUVSs exposure. Furthermore, hepatotoxicity in response to BUVSs may be attributed, in part, to the perturbation of cytokines.

To better understand the underlying mechanisms of BUVSs-induced immunotoxicity, several biomarkers of inflammation were evaluated in early-staged fish. Cytokines and genes related to the innate immune system can be major targets for environmental immunostimulants in fish (Liu et al., 2016a, b; Wester et al., 1994). As a result, we employed ZF embryos to validate the effects of BUVSs on the innate immunity, for the inactivation of adaptive immune system during this period (Novoa and Figueras, 2012; Trede et al., 2004). In the present study, an increase of il6 and a decrease of il17a and il22 were detected in larval zebrafish in response to either one dose of either UV-234, UV-329, or UV-P, while ahr2 was increased with exposure to UV-329 and UV-P. Upon activation, AHR can stimulate expression of downstream IL-22 and IL-17 (Nikoopour et al., 2015; Zhou, 2016). Expression of IL17 requires TGF-β and IL-6, IL-21 or IL-23 as a secondary inflammatory stimulus, while IL22 can be induced only by IL-6 (Liang et al., 2006; Zheng et al., 2007). It is reported that both IL17 and IL22 regulate tissue inflammation and each plays a protective role against gut and liver injury (Buckley et al., 2017; Park et al., 2005; Radaeva et al., 2004). In terms of infection, IL17 and IL22 upregulated cytokines and chemokines to recruit neutrophils to the sites of inflammation (Werner et al., 2011; Zindl et al., 2013). In a previous study, IL-22 knockdown in zebrafish embryos enhanced the expression of pro-inflammatory cytokine in bacteria-stimulated fish and induced mortality (Werner et al., 2011). The downregulation of il17a and il22 suggests that inflammation may be induced following BUVSs exposure. Noteworthy, the cytokine profile in juvenile fish exposed for 28 days followed a different pattern compared with 6-day old larvae; in the liver of juveniles, as il17 and il22 were increased. This may be due to the kinetics of cytokines production during the development of adaptive immunity (Novoa and Figueras, 2012). Nevertheless, liver damage in juvenile zebrafish was induced by UV-234 and UV-329 after 28d exposure, which was accompanied by altered expression of il17a and il22. We posit that BUVSs overwhelm biotransformation enzymes in the liver over a 28-day exposure, leading to hepatotoxicity, followed by an inflammatory response and invasion of immune cells to clear cellular debris, which can explain the tissue pathology observed.

Xenobiotics (e.g., BaP and PAHs) have been proposed to interact with AHR in immune cells, which may disrupt the differentiation of Th17 cells and the production of IL-22 in innate lymphoid cells, leading to inflammation-mediated toxicity (Adamovsky et al., 2018). In this study, an increase of ahr2 and cyp1a1 mRNA and an inhibition of IL22 protein was observed following 100 μg/L UV-P treatment, suggesting that UV-P may regulate immune response via an AHR-CYP feedback loop. AHR plays a pivotal immunological role in pro- and anti-inflammatory responses as well as regulating cytokines IL-22 and IL-17 (Benson and Shepherd, 2011; Trifari et al., 2009). Upon activation by exogenous or endogenous ligand, the cytosolic AHRs translocate into the nucleus and act as a heterodimeric complex on xenobiotic response elements (XREs) (Denison et al., 2011; Larigot et al., 2018). As markers of AHR activation, cytochrome P450 1 (CYP1) family enzymes are regulated by XREs and could attenuate AHR in a negative feedback pathway (Chiaro et al., 2007; Ma, 2001; Schiering et al., 2018). Thus, many AHR ligands are considered nontoxic due to their rapid metabolization by phase I
metabolizing enzymes such as CYP1A1, which limit their ability to remain active as AHR ligands. It has been shown that CYP1 inhibition induces IL-22 by AHR activation in human immune cells, implying that the CYP pathway can regulate immune responses (Effner et al., 2017; Schiering et al., 2017). In a previous in vivo study, UV stabilizers UV-P, UV-9, and UV-090 showed their significant ligand activity toward human AHR, and such ligand stability was not affected following the addition of CYP1A1 (Nagayoshi et al., 2015). In addition, Fent et al. (2014) determined that UV-P increased cyp1a1 and ahr1 in zebrafish embryos at doses higher than 16 μg/L and at 690 μg/L, respectively. The authors demonstrated that UV-P can activate AHR pathways and modulate transcripts of phase I and phase II enzymes in zebrafish. In this study, we also observed the regulation of cyp1a1 and ahr2 in response to UV-P. However, in juvenile fish, liver damage in response to UV-P was not observed under the conditions tested. We propose that this may be attributed to the regulation of expression of CYP1 and Il22, which may lead to a compensatory response to UV-P toxicity.

In conclusion, this study evaluated the histopathological and molecular (transcriptomics, mRNA and ELISA) responses in juvenile/larval zebrafish for 4 BUVSSs (UV-234, UV-326, UV-329, UV-P). The data support the hypothesis that BUVSs can lead to hepatoxicity and affect the inflammatory response, potentially through the AHR-IL17/IL22 pathways. However, different BUVS appear to exert differing degrees of immunotoxicity via unique mechanisms; UV-P may inhibit IL22 by increasing ahr2 and cyp1a1 expression, while UV-234 may target cytokines il17a and il22 to induce hepatic abnormality. Furthermore, UV-329 activated ahr2 and altered downstream cytokines il6 and il17a (Fig. 6). Further studies on adverse effects of BUVSS after long-term exposure and their metabolites should be conducted to confirm precise pathways of immunotoxicity.

CRediT authorship contribution statement


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Appendix A. Supplementary data

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