Morphological and molecular analysis of the toxicity of pharmaceutical-derived aquatic contaminants (PPCPs) in zebrafish

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**Background**

Contents of emerging concern (DECs) are ubiquitous aquatic contaminants, include pharmaceutical- and personal-care products (PPCPs). Due to the exponential increase in pharmaceutical consumption in recent years, pharmaceutical compounds have been detected at elevated concentrations globally in surface water, finding their way into waterways through sewage discharge containing unmetabolized drugs and improper drug disposal. As these medications are specifically designed to promote biological activity at low doses, there is increasing concern that pharmaceutical-derived aquatic contaminants could pose as potential environmental stressors to non-target organisms.

Due to their relative reactivity, the toxicological implications of pharmaceutical-derived aquatic contaminants, specifically the APIs (1), qualitatively characterizes toxicological responses to both single-chemical and mixtures of PPCPs in zebrafish embryos by morphological and 2D conduct molecular analysis of PPCP toxicity in order to better understand potential mechanisms of toxicity.

Thus, the research question is: What are the toxicological implications of PPCP's both singly and in complex mixtures and can molecular analysis be applied to this specific class of contaminants for enhanced understanding of toxicity? It was hypothesized that contaminant compounds in a mixture would result in an additive toxic effect which could potentially induce measurable changes on the molecular level.

**Purpose**

The overarching objective of this study was to elucidate the toxicological implications of pharmaceutical-derived aquatic compounds. Specifically, the study was to: 1) qualitatively characterize toxicological responses to both single-chemical and mixtures of PPCPs in zebrafish embryos through morphological analysis and 2) conduct molecular analysis of PPCP toxicity in order to better understand potential mechanisms of toxicity.

**Experimental Compounds**

Triamterene and gemfibrozil are pharmaceutical-derived contaminants that have been detected at elevated concentrations not just in the Puget Sound but globally. However, research regarding the aquatic toxicity of triamterene is very limited. Gemfibrozil has been identified as a cause of liver toxicity in humans and is a specific example of this type of toxicity. Triamterene is a diuretic primarily used to treat hypertension and edema. Gemfibrozil is a lipid-regulating fibrate used to treat hypertriglyceridemia.

**Morphometric Analysis**

Morphometric measurements were extracted from images of zebrafish embryos (48 hpf) for various morphological endpoints (eye area, whole-body length, yolk sac size, cardiac abnormalities). Values were assessed for the following cardiac morphology/functioning endpoints: enlarged atrium, enlarged ventricle, heart rate, regurgitation, and spontaneous cardiac arrest. A binary (1, 0) system was used to indicate the presence or absence of a specific condition and the resultant sum was used as a composite measure of cardiac morphology/functioning (output CA score).

The response addition (RA) model was used to arrive at the predicted mixture response based on single-chemical trials:

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\text{Mixture response} = (\text{probability A} + \text{probability B}) - (\text{probability A} \times \text{probability B})
\]

Where A and B are endpoints from single-chemical exposures.

**Lipid Analysis Using Thin Layer Chromatography Coupled with Flame Ionization Detection (TLC-FID)**

**Method Development**

Sample Preparation:
- Extracts will be concentrated with methanol.
- Sodium sulfate and magnesium perchlorate will be used to remove water.
- Lipid levels are significantly elevated following single-chemical gemfibrozil exposure.
- No significant differences are observed between lipid percent values in control embryos and those in triamterene and mixture treated embryos.
- Chromatography of triamterene and gemfibrozil end-products using TLC-FID reveals specific endpoints.
- Triamterene-exposed embryos do not have significantly different cholesterol levels or triacylglycerol content as compared to water control values.
- Mixture-exposed embryos show lower triglyceride levels and higher cholesterol levels than control embryos. Lipid composition in mixture-exposed embryos is therefore distinct from control embryos.

**Future Research Directions**

- Identifying and characterizing which specific metabolic pathway is induced by triamterene and gemfibrozil mixtures.
- Conducting gene expression analysis of exposed embryos using qPCR to determine potential genomic changes.
- Identifying triamterene mechanism of toxicity in fish by specifically analyzing sodium channel function in exposed embryos.
- Exploring exposure biomarkers in early life stage fish to next generation tests for ecological assessment. However, they have yet to be applied to this specific class of contaminants. Therefore, identifying novel molecular biomarkers of toxicity is a viable and promising next step.

**Implications**

- While experimental concentrations were not consistent with environmentally detected levels, results indicate that PPCPs pose as environmental stressors for non-target organisms.
- Morphometric analysis revealed sub-lethal toxic effects in early life stage fish which cannot be detected by currently available tests.
- Highlights the need for toxicity assays to take into account the effect of complex PPCP mixtures in order to more accurately predict environmental effects.
- Mixture prediction results using RA model indicate that PPCPs with different mechanisms of action could induce additive toxic effects and, for certain endpoints, potentially metabolically interact to produce synergistic toxic effects.
- TLC-FID method allowed for novel examination of potential molecular mechanisms underlying PPCP toxicity.
- Lipid analysis suggests that PPCPs could be inducing similar metabolic pathways in fish as they do in humans.
- Shows the potential for evidence-based decision-making advocating for increased policy regulation regarding the disposal of pharmaceutical waste.

**Conclusion/Discussion**

Results indicate that gemfibrozil and triamterene induce sub-lethal toxic effects in zebrafish embryos. Increased observed heart physiological abnormalities suggest that triamterene is toxic to the developing fish because with pericardial blood pooling being a sensitive measure of cardiotoxicity.

Gemfibrozil induced a similar dose-dependent increase in cardiac defects. The gemfibrozil-induced injury phenotypes, however, also included extracardiac abnormalities, with a small eye-phosphoglucomutase (glycoglycerol), enlarged yolk sac, and a reduced whole-body length.

The eye and whole-body length growth are highly linked to embryonic development processes, result suggest that gemfibrozil induces a developmental delay during a postembryonic stage.

Observed mixture toxicity was consistent with additive toxicity as determined by RA model for all toxicological endpoints. However, with yolk sac area, observed toxicity is consistently above predicted values for all mixture concentrations—while data values are not significantly different from predicted ones, this trend in the data suggest that the drugs may be interacting to produce greater than additive toxicity in terms of yolk sac enlargement.

The lipid analysis is not aligned following triamterene-gemfibrozil mixture exposure, which indicates that the triamterene does not interact with lipid metabolism.

As total percent body values did not significantly follow following gemfibrozil exposure, results indicate that gemfibrozil blocks lipid metabolism. Future investigation suggests that gemfibrozil may be acting through the inhibition of lipid uptake or by altering metabolic pathways. Inhibition of this enzyme can be a common effect: instead of increasing lipase activity, it may be inhibiting lipase, thereby blocking lipase activity.

Due to distinctive lipid makeup in mixtures-exposed embryos, results suggest that triamterene and gemfibrozil may be interacting to produce toxicity through a different metabolic pathway than single-chemical exposures.