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Response of gene expression in zebrafish exposed to pharmaceutical mixtures: Implications for environmental risk

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ABSTRACT

Complex mixtures of pharmaceutical chemicals in surface waters indicate potential for mixture effects in aquatic organisms. The objective of the present study was to evaluate whether effects on target gene expression and enzymatic activity of individual substances at environmentally relevant concentrations were additive when mixed. Expression of zebrafish cytochrome P4501A (*cyp1a*) and vitellogenin (*vtg*) genes as well as activity of ethoxyresorufin-O-deethylase (EROD) were analyzed after exposure (96 h) to caffeine-Caf, ibuprofen-Ibu, and carbamazepine-Cbz (0.05 and 5 μM), tamoxifen-Tmx (0.003 and 0.3 μM), and after exposure to pharmaceutical mixtures (low mix: 0.05 μM of Caf, Ibu, Cbz and 0.003 μM of Tmx, and high mix: 5 μM of Caf, Ibu, Cbz and 0.3 μM of Tmx). Pharmaceuticals tested individually caused significant down regulation of both *cyp1a* and *vtg*, but EROD activity was not affected. Exposure to low mix did not cause a significant change in gene expression; however, the high mix caused significant up-regulation of *cyp1a* but did not affect *vtg* expression. Up-regulation of *cyp1a* was consistent with induction of EROD activity in larvae exposed to high mix. The complex mixture induced different responses than those observed by the individual substances. Additive toxicity was not supported, and results indicate the need to evaluate complex mixtures rather than models based on individual effects, since in environment drugs are not found in isolation and the effects of their mixtures is poorly understood.

1. Introduction

Pharmaceutical substances are constantly released to the aquatic environment principally through municipal effluents and wastewater treatment plants leading to chronic exposure in aquatic organisms (Brain et al., 2004; Halling-Sørensen et al., 1998; Jones et al., 2005; Nakada et al., 2006). Discharges of these substances are likely to increase in the future because of increases in their production, human population growth, and demographic shifts towards higher proportions of older people that use greater amounts of pharmaceuticals (Daughton and Ternes, 1999). Although present at low concentrations at the ng L^{-1} to μg L^{-1} range (Gómez et al., 2007; Gros et al., 2007; Ternes, 1998; Thomas and Foster, 2004), pharmaceutical substances are found simultaneously as complex mixtures that have unknown and difficult to evaluate effects on aquatic biota (Blasco and DelValls, 2008; Gagné et al., 2006; Lara-Martín et al., 2014; Yoon et al., 2010).

It is possible that individual substances can act in a synergistic or additive manner, which suggests management for environmental protection should take into consideration mixtures of substances rather than models based on individual effects. Pharmaceuticals are an example of substances for which additive effects on toxicity in aquatic organisms have been observed (Cleuvers et al., 2003; Christensen et al., 2007; Henry

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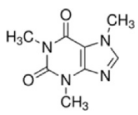
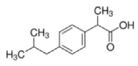
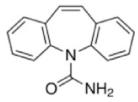
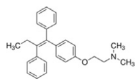
Table 1
Features of the pharmaceuticals selected in this study.

Pharmaceutical (Cas Number)	Structure	Molecular weight (g mol^{-1})	Log K_{ow}	*Group*: Mode of Action
Caffeine (58–08–2)		194.19	−0.07[a]	*Stimulant*: Inhibitory neurotransmitter that suppresses activity in the central nervous system.
Ibuprofen (15687–27–1)		206.28	3.97[a]	*Anti-inflammatory*: Inhibit the enzyme cyclooxygenase.
Carbamazepine (298–46–4)		236.27	2.45[a]	*Anticonvulsant*: Stabilizes the inactivated state of voltage-gated sodium channels.
Tamoxifen (10540–29–1)		371.51	6.3[b]	*Anticarcinogenic*: anti-estrogen (inhibiting agent) in mammary tissue. Estrogen (stimulating agent) in cholesterol metabolism, bone density, and cell proliferation

[a] Yoon et al. (2010).
[b] NTP, 2011.

and Black, 2007). Pharmaceuticals may induce cytochrome P450 enzyme activity (CYP1A) by binding to the aryl hydrocarbon receptor (AhR). The induction of CYP1A1 gene transcription by the aryl hydrocarbon begins by their binding and activating the AhR, a cytosolic protein that, on ligand binding, translocates to the nucleus and with its partner, the aryl hydrocarbon nuclear translocator, interacts with the promoter of the CYP1A1 gene (Rowlands and Gustafsson, 1997). This results in an up-regulation of transcription and a subsequent increase in CYP1A1 mRNA and enzyme levels. Consequently, this mechanism is reflected in the rated of deethylation of the substrate 7-ethoxyresorufin by cytochrome P450 (CYP) to give the product resorufin. The 7-ethoxyresorufin O-deethylase (EROD) activity is a biomarker used to determine AhR agonist exposure to certain polyhalogenated aromatic hydrocarbons (PHAHs) and polycyclic aromatic hydrocarbons (PAHs) (Bucheli and Fent, 1995; Gokøyr and Förlin, 1992) Responses of the cytochrome P450 system are used as biomarkers of oxidative stress in fish (Bucheli et al., 1995). The Induction of CYP1A by pharmaceuticals may generate reactive oxygen species (ROS) (Van der Berg et al., 1998). When a substrate is metabolized by cytochrome P450 consumes one molecule of molecular oxygen leading to an oxidized substrate plus a molecule of water as a by-product. However, for most CYPs, depending on the nature of the substrate, the reaction is "uncoupled", consuming more O_2 than the metabolized substrate and producing activated oxygen or O_2^- (Gonzalez and Tukey, 2006).

It has been demonstrated that mixtures of substances that induce *cyp1a* a gene that encodes for CYP1A, the induction is consistent with the addition of the individual induction activities of each substance (Hook et al., 2008; Filby et al., 2007). Induction of *cyp1a* and the activity of CYP1A are important because increased generation of ROS can lead to oxidative stress and damage to biomolecules, or abnormally enhance the CYP1A metabolism of endogenous substrates (Rifkind, 2006). It is also considered of greatest concern for risk assessment pharmaceuticals that bind the estrogen and induce estrogenic effects in fish such as oral contraceptives, hormone replacement therapies, motor deficits associated with menopause, hypoestrogenism, and the management of some pre- and postmenopausal symptoms (Laurenson et al., 2014). In particular, mixtures of estrogenic substances can cause effects [e.g., induction of vitellogenin genes (*vtg*) or vitellogenin lipoprotein (Vg) production] in fish that are reported to be equivalent to the addition of their individual activities (Filby et al., 2007; Thorpe et al., 2006, 2001). In addition, even when concentrations of individual substances in a mixture are below levels that cause estrogenic effects as single substances, their combined effect within a mixture can be sufficient to induce estrogenicity in fish (Brian et al., 2005; Ketan and Collins, 2007). Consequently, analysis of the potential for substances to influence fish vitellogenin (*vtg* or VG) is frequently used as a biomarker of estrogenic activity (Filby et al., 2007). Although numerous studies have examined toxicological impacts of pharmaceuticals, the biological activity of these substances in non-target organisms remain uncertain, and interactions among pharmaceuticals when present in mixtures can be considerably more complicated than that of additive toxicity based on a single mechanism of action. The toxicity of pharmaceutical mixtures in fish has been investigated at various levels of biological organization and results have not yet provided a clear direction on how management of this environmental issue should be approached. At issue is whether management of complex mixtures can be addressed by understanding effects of individual substances and predicting fish responses to complex mixtures, or whether each possible mixture combination leads to a unique organism response that must be assessed independently.

The objective of the present research is to investigate whether effects of individual substances on expression of target genes (*vtg* and *cyp1a*) in zebrafish larvae can be used to predict the change in expression when fish are exposed to substance mixtures. To support observations on changes in *cyp1a* expression, evaluation of CYP1A enzymatic activity was assessed in parallel by evaluation of ethoxyresorufin O-deethylase (EROD) activity. Pharmaceuticals from different therapeutic groups (Table 1) were selected for this study based on their frequency of use, presence as mixtures in surface waters, and concentrations in municipal effluents (Table 2). Caffeine (Caf) is a potent stimulant of the central nervous system (Nikolau et al., 2007) and has been used as a marker for residual wastewater in the environment due to its persistance. The anti-inflammatory non-prescription drug ibuprofen (Ibu) is used as an analgesic and antipyretic and, in addition to naproxen, is one of the most abundant anti-inflammatory drugs found in municipal effluents (Miège et al., 2009; Stuer-Lauridsen et al., 2000). Carbamazepine (Cbz) is a psychiatric drug prescribed as an anticonvulsant and mood-stabilizer applied in the treatment of epilepsy,