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Effect of human pharmaceuticals common to aquatic environments on hepatic CYP1A and CYP3A-like activities in rainbow trout (*Oncorhynchus mykiss*): An *in vitro* study

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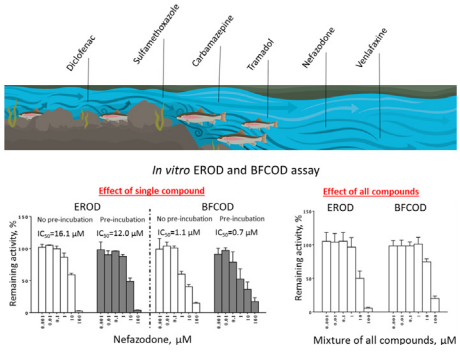
HIGHLIGHTS

- The inhibitory potency of six medicines on rainbow trout liver microsomes was studied.
- Two CYP mediated reaction were measured: EROD and BFCOD.
- Nefazodone inhibited EROD and BFCOD in a non-competitive manner.
- Neither single nor mixture of six pharmaceuticals inhibit enzyme activity at environmentally relevant concentrations.
- Mixture of all compounds exhibit inhibition pattern at 10 and 100 μM.

GRAPHICAL ABSTRACT



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ABSTRACT

This study examined the ability of several human pharmaceuticals to modulate hepatic piscine CYP-mediated monooxygenase activities. Effects of six pharmaceuticals: diclofenac, sulfamethoxazole, tramadol, carbamazepine, venlafaxine and nefazodone, were investigated *in vitro* in rainbow trout hepatic microsomes. The reactions of 7-ethoxyresorufin-O-deethylase (EROD) and benzyloxy-4-trifluoromethylcoumarin-O-debenzyloxylase (BFCOD), were used as markers for hepatic CYP1A and CYP3A-like activities, respectively. Our results showed that EROD and BFCOD activities were both affected by nefazodone. Nefazodone inhibited EROD in a dose dependent manner and was found to be a potent non-competitive inhibitor of EROD with a K_i value of 6.6 μM. BFCOD activity was inhibited non-competitively in the presence of nefazodone with K_i value of 30.7 μM. BFCOD activity was slightly reduced only by the highest concentration of carbamazepine. Diclofenac, sulfamethoxazole, tramadol, and venlafaxine did not affect the activity of either EROD or BFCOD. We further exposed microsomal fraction to mixtures of six pharmaceuticals to investigate potential inhibition. The results showed that EROD and BFCOD activity was inhibited on 94% and 80%, respectively at higher tested concentration. To

Abbreviations: K_i, inhibition constant or equilibrium dissociation constant for the enzyme–inhibitor complex; K_m, Michaelis–Menten constant; ER, 7-ethoxyresorufin; EROD, 7-ethoxyresorufin-O-deethylase; BFC, 7-benzyloxy-4-trifluoromethylcoumarin; BFCOD, benzyloxy-4-trifluoromethylcoumarin O-debenzylase; CYP, cytochrome P450; PRTH, primary cultures of rainbow trout hepatocytes.

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our knowledge, this is the first report to demonstrate an inhibitory effect of nefazodone on hepatic CYP1A and CYP3A-like proteins in rainbow trout.

1. Introduction

Various studies regularly report the presence of human pharmaceuticals in different aquatic compartments, and this issue is gaining more attention around the world (Andersson et al., 2011). Many factors affect contamination of aquatic environments, including a trend in increasing prescription drug sales (EvaluatePharma®, 2017), irrational use of medicines (Mao et al., 2015) demographics of a given population in certain area, seasonal variability (Chen et al., 2016) and improper water treatment processes at sewage treatment plant facilities (Björklund et al., 2016). This resulted in persistency of some pharmaceuticals, such as analgesics, antibiotics, antidepressants, and anticonvulsants classes in waste water treatment plants effluents (Fick et al., 2011; Grabicova et al., 2017; Koba et al., 2018). It is still challenging to evaluate the combined effect of all pharmaceuticals at the concentrations found in aquatic environment on living organisms. Even trace levels of discharged pharmaceuticals might have a potency to affect non-target organisms (Grzesiuk et al., 2018).

As in other vertebrates, fish metabolises xenobiotics through the phase I and II biotransformation enzymes (Uno et al., 2012). The most important group of phase I enzymes is cytochrome P450 (CYP). These enzymes play a major role in the clearance of human pharmaceuticals and other environmental pollutants. Compared to other enzymes CYPs are unspecific and metabolize pharmaceuticals with diverse structure. Additionally, differences between mammalian and piscine receptor regulation may translate to differences in CYP substrate specificities and catalytic activities (Burkina et al., 2016).

Currently, 582 pharmaceutical compounds are listed for environmental risk assessment (Fick et al., 2010; Roos et al., 2012). The metabolism pathways and pharmacokinetic profiles of those compounds are known for humans however, little is known about metabolic clearance in fish. As occurs in mammals, the expression of piscine CYP is controlled by nuclear receptors, which can be induced or inhibited by environmental pollutants, including pharmaceutically active compounds (Burkina et al., 2015). The pharmaceuticals diclofenac, sulfamethoxazole, tramadol, carbamazepine, venlafaxine and nefazadone are common in the aquatic environment. These compounds are ranked 15–342 of 582 compounds slated for environmental risk assessment (Roos et al., 2012), and their concentrations in water are typically vary from 10 to 2000 ng/L.

The metabolism of these compounds by mammalian liver microsomes is well documented, including the enzymes involved. Hepatic enzymes CYP2D6, CYP3A4, CYP2C9 and CYP2C8 are involved in the metabolism of diclofenac (Bort et al., 1999; VandenBrink and Isoherranen, 2010), sulfamethoxazole (Wen et al., 2002), tramadol (Coller et al., 2012), carbamazepine (Nakamura et al., 2003), venlafaxine (Fogelman et al., 1999; Nichols et al., 2009; Oganesian et al., 2009) and nefazodone (Davis et al., 1997; von Moltke et al., 1999; Kalgutkar et al., 2005). Mammalian CYP2B, 2C and 2D do not have piscine orthologues (Burkina et al., 2016), and the enzymes that are involved in the metabolism of these compounds in fish are unknown. The most studied enzymes in fish are 7-ethoxyresorufin-O-deethylase (EROD) and benzyloxy-4-trifluoromethylcoumarin-O-debenzyloxylase (BFCOD), which are frequently used for monitoring CYP1A and CYP3A-like enzymatic activities, respectively, due to their involvement in xenobiotic metabolism, particularly their induction by environmental pollutants.

The aim of the present study was to examine *in vitro* effects of six pharmaceuticals, which are repeatedly found in wastewater effluent, on EROD (CYP1A) and BFCOD (CYP3A) activities. The effects of each compound alone and in combination were examined.

2. Material and methods

2.1. Chemicals

The test substances sulfamethoxazole (CAS Number: 723-46-6, purity > 99%), diclofenac (CAS Number: 15307-79-6, purity 92%), tramadol (CAS Number: 36282-47-0, purity 89%), venlafaxine (CAS Number: 99300-78-4, purity 98%), carbamazepine (CAS Number: 298-46-4, purity >99%) and nefazodone (CAS Number: 82752-99-6, purity 92%) were purchased from Sigma Aldrich (Europe). Stock solutions (25 mM) of sulfamethoxazole, diclofenac and tramadol were prepared in methanol, whereas venlafaxine, carbamazepine and nefazodone were prepared in DMSO. Stock solutions were further diluted from 25 mM to 0.00025 mM to obtain several concentrations ranging from 100 to 0.001 μM. 7-ethoxyresorufin (ER), resorufin, 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxy-4-trifluoromethylcoumarin (HFC) and NADPH were purchased from Sigma Aldrich (Sweden). Acetonitrile and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany).

2.2. Fish

Rainbow trout (n = 16), length 30 ± 2.0 cm (mean ± standard deviation) and weight 382 ± 66 g (mean ± standard deviation), were purchased from a local commercial hatchery (Vodňany, Czech Republic). Fish were handled according to national and institutional guidelines for the protection of human and animal welfare. The fish were acclimated for 14 days before being sacrificed. During the acclimation period, the fish were fed commercial fish food (Bio Mar, Denmark) at 1% of body weight per day. Fish were not fed 24 h prior to sampling to avoid post-prandial effects during the assay.

Fish were sacrificed according to the ethical rules of the EU-harmonized Animal Welfare Act of the Czech Republic. The unit is licensed (No. 53100/2013-MZE-17214) according to the Czech National Directive (the Law against Animal Cruelty, No. 246/1992). Before sampling, fish were anesthetized in an ice bath and their spinal cords were immediately cut. Liver samples were collected from fish and stored at −80 °C until use. Hepatic microsomes were prepared from each fish by differential centrifugation as described previously (Burkina et al., 2013). Microsomes were suspended in 20% glycerol and stored in −80 °C until use for EROD and BFCOD assays. The protein content of the microsomes was determined by the method of Smith et al. (1985) using bovine serum albumin as a standard. The microsomes were diluted to a protein content of 10 mg/ml prior to use.