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Cross-species comparison of fluoxetine metabolism with fish liver microsomes

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ABSTRACT

Pharmaceuticals discharged in municipal wastewater are of emerging concern because of their potential for inducing biological effects in aquatic organisms. Selective serotonin reuptake inhibitors (SSRIs), pharmaceuticals prescribed to treat chronic depression, have been detected in receiving and wastewaters. Fluoxetine is a highly prescribed model SSRI used to assess impacts of antidepressants on aquatic organisms. In this study, *in vitro* hepatic fluoxetine metabolism was determined in several model fish species: rainbow trout, goldfish, zebrafish and killifish. Incubation of fluoxetine with hepatic microsomes from trout pre-treated with carbamazepine showed a time-dependant loss of fluoxetine, concomitant with an increase in norfluoxetine, the major mammalian demethylated metabolite. However, fluoxetine was not well metabolized in reactions with hepatic microsomes from untreated fish. Fluoxetine loss was greater than norfluoxetine production, indicating that norfluoxetine is not the predominant fluoxetine biotransformation product in fish. Furthermore, norfluoxetine was often undetected, possibly indicating that fluoxetine demethylation is a minor metabolic pathway in fish. Inter-species differences in fluoxetine metabolism were not evident because of high intra-species variability, although killifish appeared to have the highest hepatic metabolic capacity for fluoxetine. Fluoxetine metabolism in mammals is catalyzed by cytochrome P450 (CYP) enzymes. Trout were exposed to known CYP inducers, carbamazepine and 3-methylcholanthrene, to assess potential induction of hepatic fluoxetine metabolism. Microsomes from 3-methylcholanthrene treated fish did not induce detectable changes in fluoxetine concentrations *in vitro*, indicating that fish CYP1s are not involved in fluoxetine metabolism; the CYPs involved are still unclear. Identification of metabolites other than norfluoxetine warrants further investigation.

1. Introduction

Pharmaceuticals are discharged from wastewater treatment plants into surface waters at annual rates of tonnes per year in both Europe and North America (Daughton and Ternes, 1999; Lindberg et al., 2005). Selective serotonin reuptake inhibitors (SSRIs) and other pharmaceuticals used to treat chronic depression have been detected in wastewater and in surface waters in Europe and North America (Lamas et al., 2004; Vasskog et al., 2006; Lajeunesse et al., 2008; Schultz and Furlong, 2008; Metcalfe et al., 2009) and in the tissues of fish collected near municipal wastewater discharges (Brooks et al., 2005; Chu and Metcalfe, 2007). It has recently been established that SSRIs bioconcentrate in the tissues of fish (Paterson and Metcalfe, 2008) and more specifically, in the liver (Nakamura et al., 2008).

SSRIs were developed as antidepressants with a specific mode of action; SSRIs target the reuptake of the brain neurotransmitter, serotonin, at the synaptic cleft. Fish produce serotonin (Khetan and Collins, 2007), and possess both serotonin receptors (Yamaguchi and Brenner, 1997) and a serotonin transporter. Since the teleost serotonin neuroendocrine system is analogous to that found in mammals (Gould et al., 2007), fish may show the same biological responses to SSRIs and may be susceptible to the same adverse effects that are associated with SSRI exposure in mammals.

Fluoxetine, the active ingredient in Prozac™, is a highly prescribed SSRI that has been detected in the aquatic environment and is often used as a model compound for assessing SSRI impacts on aquatic organisms. Lister et al. (2009) reported that exposure of zebrafish (*Danio rerio*) to fluoxetine for 7 d at environmentally relevant concentrations (0.32–32 μg L^{-1}) can significantly decrease egg production. Japanese medaka (*Oryzias latipes*) exposed to fluoxetine at concentrations as low as 0.1 μg L^{-1} for 4 weeks showed significantly elevated plasma estradiol and developmental deformities among offspring (Foran et al., 2004). In goldfish (*Carassius auratus*), fluoxetine (five injections of 5 μg g^{-1} over 14 d) decreased transcript levels in the brain of isotocin, the fish homolog of the mammalian neuropeptide oxytocin, indicating a mechanistic link

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between fluoxetine exposure and reproductive dysfunction (Mennigen et al., 2008). Gulf toadfish (*Opsanus beta*) exhibited decreased plasma osmolality and increased urea excretion with 24 h fluoxetine treatment at concentrations as low as 25 $\mu g\ g^{-1}$ (Morando et al., 2009). Changes in feeding behavior were noted in fathead minnows (*Pimephales promelas*) exposed to fluoxetine for 7 d (LOEC = 51 $\mu g\ L^{-1}$; Stanley et al., 2007) and altered levels of the neuropeptides that regulate feeding behavior were observed in goldfish injected with 5 $\mu g\ g^{-1}$ every 3 d for 13 d (Mennigen et al., 2009). These responses observed in fish exposed to fluoxetine are analogous to the clinical side effects of fluoxetine observed in some humans, including appetite loss and sexual dysfunction (Brambilla et al., 2005).

While the biological responses to SSRI exposure have been investigated in fish species, studies on the biotransformation of SSRIs in fish are lacking. Recently, it has been documented that Japanese medaka are capable of *in vivo* fluoxetine metabolism and the production of norfluoxetine, the major fluoxetine metabolite in mammals (Nakamura et al., 2008; Paterson and Metcalfe, 2008). In medaka, moderate bioaccumulation factors (i.e. <200) for fluoxetine have been observed and the half-life for fluoxetine was found to be relatively long at 9.4 d (Nakamura et al., 2008; Paterson and Metcalfe, 2008). In humans, the half-life of fluoxetine is 1–4 d (Hiemke and Hartter, 2000), suggesting that metabolic conversion of fluoxetine is significantly slower in fish.

Biotransformation of xenobiotics in vertebrates is a multi-phase process, the first of which usually involves the cytochrome P450 (CYP) superfamily of enzymes (Andersson and Forlin, 1992; Xu et al., 2005). In humans, CYP 3A4 is responsible for 50% of all human drug metabolism, followed by 2D6, 2C9 and 1A2 (Hemeryck and Belpaire, 2002). Enzymes from the CYP2 and CYP3 families are responsible for SSRI metabolism in mammals. In humans, fluoxetine is primarily metabolized into norfluoxetine through demethylation by CYP2D6 and to a lesser extent, by CYPs 2C9, 3A4 and 2C19 (Fig. 1). However, fish lack CYP2C and 2D homologs. It has been reported that fish are capable of metabolizing some typical mammalian CYP2 and CYP3 substrates (Buhler and Wang-Buhler, 1998). However, studies of drug metabolism in fish are extremely limited and the metabolic fate and CYPs responsible for the metabolism of fluoxetine and other drugs in fish are largely unknown.

The present study was designed to investigate the *in vitro* hepatic metabolism of fluoxetine by fish using a comparative approach. Liver microsomes from several model fish species (rainbow trout, goldfish, killifish, zebrafish) were used to assess basal metabolism of fluoxetine in fish. To investigate the potential to alter *in vitro* hepatic fluoxetine metabolism, trout were exposed to the CYP inducers carbamazepine (CBZ) and 3-methylcholanthrene (3-MC). The mammalian CYP enzymes responsible for fluoxetine metabolism are inducible and assay optimization with trout indicated that

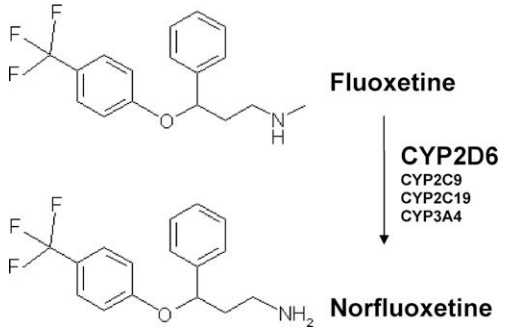

Fig. 1. The chemical structure of fluoxetine and its active demethylated metabolite, norfluoxetine. The cytochrome P450 (CYP) enzyme isoforms associated with the demethylation of fluoxetine to norfluoxetine in humans are listed. CYP2D6 is the dominant human CYP responsible for this metabolism.

overall metabolism was not high in at least this fish species. Although data are not yet clear on the induction pathway of CBZ in fish, CBZ induces the CYPs responsible for fluoxetine metabolism in mammals. Secondly, 3-MC is known to significantly induce a number of CYPs in fish. These compounds were therefore chosen for pre-treatment of fish to assess induction of fluoxetine metabolism and provide evidence as to which CYPs may be involved in fluoxetine metabolism in fish liver. The rates of metabolism in untreated and exposed fish microsomes were compared to that found in rat and rabbit liver microsomes. Resolving the capacity of aquatic vertebrate species to metabolize fluoxetine and other pharmaceuticals detected in the environment will contribute to our understanding of the ecological risks associated with the release of pharmaceuticals into the aquatic environment.

2. Materials and methods

2.1. Animals

Goldfish (2–7 g body weight) were purchased from a pet store, held at room temperature in recirculating dechlorinated tap water and fed staple flake food daily. Mummichog killifish (*Fundulus heteroclitus*; 1.5–5 g body weight) were wild caught and purchased from Aquatic Research Organisms (New Hampshire, USA). The killifish were held at 19–20 °C in 10% recirculating seawater and fed staple flake food 5 d per week supplemented with frozen brine shrimp (*Artemia* sp). Rainbow trout (*Oncorhynchus mykiss*) were purchased from Humber Springs trout hatchery (100–130 g body weight; Mono Mills, Ontario) or Lynnwood Acres Trout Farm (180–230 g body weight; Campbellcroft, Ontario). The trout were kept at 10–15 °C in flow-through water and fed floating trout pellets 3 d per week. Wild caught zebrafish (200–500 mg body weight) were purchased from DAP International (Etobicoke, Ontario). Zebrafish were kept in a semi-recirculating system at 28–29 °C and fed three times per day, alternating between tropical flake food and fresh brine shrimp. All fish were held for a minimum of 2 weeks before use. Rainbow trout livers were large enough to use single fish replicates; other species were pooled due to their small liver size. Goldfish were juveniles and consisted of 21–25 fish per pool. Sexually mature zebrafish and killifish were separated into male and female pools. Zebrafish and killifish pools consisted of 35–42 and 8–13 fish, respectively.

Livers from four male Sprague–Dawley rats were pooled and used as a mammalian control in metabolism assays. Commercially available rabbit liver microsomes (Sigma–Aldrich, St. Louis, MO) that express high CYP2B activity were used for optimization of the *in vitro* assay and as a mammalian comparison.

2.2. In vivo P450 induction with 3-MC or CBZ treatment

Female rainbow trout (180–230 g) were held in filtered Otonabee River water. Food was withdrawn from the fish at 2 d prior to injection. Five fish per treatment were injected i.p. with CBZ or 3-MC at a dose of 20 mg kg^{-1} in corn oil. At 5 d post-injection, the fish were sacrificed by an overdose of MS-222. Preliminary experiments with trout sacrificed between 1 and 7 d post-injection revealed maximum induction after 5 d (data not shown). This is similar to that observed in previous studies of the time-course for induction of CYP proteins in fish (Celander et al., 1993; Pretti et al., 2001).

2.3. Fluoxetine metabolism assay

Livers were collected and either placed directly on ice or flash-frozen in liquid nitrogen and stored at −80 °C until microsomal