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Effects of inducers of cytochrome P450s on enrofloxacin *N*-deethylation in crucian carp *Carassius auratus gibelio*

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ABSTRACT

In this study with crucian carp (*Carassius auratus gibelio*), the effect on enrofloxacin (EF) and its metabolite ciprofloxacin (CF) and on the activity of cytochrome P450 1A (CYP1A) and cytochrome P450 3A (CYP3A) was estimated following the oral administration of rifampicin (RIF) (12 mg/kg) and β-naphthoflavone (BNF) (12 mg/kg), respectively. First, reversed-phase high-performance liquid chromatography (RP-HPLC) was used to detect the pharmacokinetics of EF with continual blood sampling. In RIF-treated, BNF-treated and control groups, the value of the C_{maxCF}/C_{maxEF} ratio was 4.41, 0.81 and 0.95, and the corresponding value of the $AUC_{0\text{-}t\text{-}CF}/AUC_{0\text{-}t\text{-}EF}$ ratio was 3.69, 1.84 and 1.76, respectively. In the RIF-treated, BNF-treated and control groups, the MRT values of EF were 26.57, 27.45 and 30.88 h, and the corresponding values for CF were 5.79, 35.18 and 38.11 h, respectively. Based on these results for crucian carp, the accumulation and elimination of EF and CF in the RIF-treated group were more rapid than in BNF-treated and control groups. Second, liver microsomes were pretreated with the inducer of CYP1A for BNF and that of CYP3A for RIF, and then the enzymatic activities of CYP1A and CYP3A were measured, respectively. The activities of ethoxyresorufin-*O*-deethylation (EROD) and erythromycin-*N*-demethylation (ERND) increased significantly ($P < 0.05$) for CYP1A and CYP3A, respectively. However, in further experiments on the formation of CF, the level of EF *N*-deethylation was significantly induced by RIF and inhibited by ketoconazole (KTZ) for CYP3A but had no influence for CYP1A, BNF and berberine chloride (BER). We concluded that CYP3A might be responsible for the *N*-deethylation of EF and because of this activity, could also serve as a toxicity biomarker in crucian carp.

1. Introduction

Cytochrome P450s (CYP450) play a crucial role in drug metabolism and, as a major class of detoxification enzymes in terrestrial and aquatic organisms, aid in the metabolism of various endogenous and exogenous substrates (Snyder, 2000). For example, the CYP3A enzymes participate in the metabolism of more than 50% of drugs (Shirasaka et al., 2013), and the CYP1A subfamily also participates in the metabolism of numerous drugs and is also used as a biomarker for the effects of pollutants in aquatic organisms (Havelková et al., 2007). Additionally, the activity of CYP450s is particularly prone to inhibition or induction by endogenous and exogenous compounds, which affects drug metabolism (Beijer et al., 2013). Several xenobiotic compounds that are inducers or inhibitors of the primary CYP450s associated with drug metabolism can change the activity of enzymes involved in drug metabolism (Marcella et al., 2006; Riley and Grime, 2004). For example, rifampicin (RIF) and β-naphthoflavone (BNF) are typical inducers (Kim et al., 2008; Li et al., 2008), whereas ketoconazole (KTZ) and berberine chloride (BER) are typical inhibitors of CYP3A and CYP1A, respectively (Hasselberg et al., 2008; Zhou et al., 2011). The inducers and inhibitors of CYP450s influence the metabolism of substrate drugs, which leads easily to drug toxicity in aquatic animals (Hassan et al., 2015). Although the role of CYP450s in the disposition of drugs in aquatic animals remains poorly understood, the CYP1A and CYP3A subfamily is used as a biomarker for effects of pollution and toxicity in aquatic organisms and the environment (Hassan et al., 2015; Havelková et al., 2007).

Enrofloxacin (EF), a fluoroquinolone antibiotic with broad-spectrum antibacterial activity, is widely used to control bacterial infection in veterinary medicine (Schwarz and Chaslus-Dancla,

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2001). The metabolism of EF has been investigated in various aquatic and reptile species following administration, and the absorption and bioavailability of EF is high (Rocca et al., 2004). Ciprofloxacin (CF) is a major metabolite of EF in different species and is formed by the de-ethylation of EF. Moreover, CF is also a potent antimicrobial agent and contributes to the activity of EF (Cadenas et al., 2013). Therefore, EF and CF are widely used in aquaculture for the treatment of bacterial diseases of fish. The metabolism and distribution of EF and CF in aquatic animals has been investigated previously (Cadenas et al., 2013; Liang et al., 2014), and the excessive residuals of EF and CF negatively affect the environment and cause toxicity to aquatic animals. The influence of CYP450 on the *N*-deethylation of EF residues remains poorly understood, as well as EF residues from *N*-deethylation influenced by CYP450 are also used as a biomarker for evaluating toxicity in aquatic animals were limited. Research studies concerning the EF residue elimination metabolized by CYP450 and EF *N*-deethylation as a biomarker for evaluating the pollution in fresh water environment are urgent.

Crucian carp *Carassius auratus gibelio* is one of the most economically important freshwater fish in China. However, crucian carp farming has suffered serious economic losses as a result of infectious disease (Wang et al., 2001), and various antibiotic drugs are widely used to control aquatic animal diseases (Noga, 2010). However, the irresponsible use of antibiotics has led to disposition of residues in the edible parts of treated aquatic animals (Benbrook, 2002). In this study, the *N*-deethylation of EF catalysed by CYP450 was investigated to determine drug residues in crucian carp, with the goals to reduce the risks of EF and CF accumulation and to improve resistance to bacteria. Furthermore, the use of the EF *N*-deethylation in crucian carp as a biomarker for evaluating the toxicity of environmental pollutants in fresh water is also investigated in this study.

The primary objectives of this study were to investigate the pharmacokinetics of EF and its major metabolite CF after treatment with RIF and BNF in addition to the effect of CYP450 on the elimination of CF and EF. To examine whether CYP450s play a predominant role in the *N*-deethylation of EF, the enzymatic activities of CYP1A and CYP3A were investigated in crucian carp pretreated with BNF or RIF after a single EF injection. Additionally, the effects of CYP450 inducers and inhibitors on EF *N*-deethylation in microsomes from crucian carp were evaluated. Therefore, both the EF N-deethylation influenced by CYP3A and the residues of CF in crucian carp were investigated, and these results will facilitate the future appropriate use of EF for fish safety and disease control in aquaculture.

2. Materials and methods

2.1. Chemicals

Suzhou Hengyi Pharmaceutical (Jiangsu, China) supplied the enrofloxacin (EF > 90%) and ciprofloxacin (CF > 90%). Rifampicin (RIF > 97%), β-naphthoflavone (BNF > 99%), berberine chloride (BER > 99%), ketoconazole (KTZ > 99%) and NADPH were purchased from Sigma-Aldrich (Shanghai, China). Erythromycin was obtained from Sangon (Shanghai, China). Methanol was HPLC grade (Darmstadt, Germany). All other chemicals and solvents were analytical grade (Shanghai, China).

2.2. Fish and drug treatment

Healthy crucian carp (68 fish; average body weight 500 ± 12 g, mean ± SD) were obtained from a farm in Shanghai, China. The fish were transported to a single, large tank containing 500 L of freshwater in the animal house of our institute four weeks before the start of the experiment. Before the initiation of the experiment, eight fish were randomly selected as negative controls and analysed to confirm the absence of EF and CF. The remaining 60 fish were divided into three groups of 20 fish/tank, with each group housed in a separate tank (100 L). Fish were fed twice a day with pellet feed (made in the laboratory). The water was recirculated, the aeration was constant, and the temperature was maintained at 26.0 ± 1.0 °C. In the experiment, the three groups were randomly cultured. The groups (20 individuals/group) were administered by gavage once daily with RIF or BNF at a dose of 12 mg/kg b.w./day (Kim et al., 2008; Li et al., 2008; Yu and Yang, 2010) for seven consecutive days. Solutions of RIF and BNF were mixed thoroughly in corn oil, and the fish were weighed before administering the medicated feed. In the control group, an equal amount of corn oil was administered by gavage. Twenty-four hours after the last feeding interval, ten fish were randomly sampled from each group, anaesthetized using AQUI-S® at a concentration of 10 mg/L (AQUI-S New Zealand Ltd, Lower Hutt, NZ) and processed to obtain liver microsomes. The remaining ten fish in each group, which were not anaesthetized, received a single intraperitoneal injection with EF (10 mg/kg) and were then maintained in separate tanks. Blood (0.3 mL per fish) was collected at 0.083, 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 h post-dosing per fish. Blood samples (0.3 mL) were collected from a tail vein with a 1.0-mL syringe, transferred into 1.5 mL polypropylene centrifuge tubes with 2 mg of heparin sodium and then centrifuged for 10 min at 8000×*g*. The supernatant was collected and frozen at −80 °C. All fish were treated in accordance with the guidelines on the care and use of animals for scientific purposes by the Institutional Animal Care and Use Committee (IACUC) of the East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, China. The IACUC specifically approved this study within the project of Special Funds for Agro-scientific Research in the Public Interest.

2.3. Measurement of enzymatic activity in liver microsomes

Microsomes were prepared as previously described (Novi et al., 1998), with slight modifications. Following a previous study (Zhou et al., 2011), the liver tissues were excised and homogenized with 1.15% KCL and centrifuged at 10,000 ×*g* for 20 min. Subsequently, the supernatant was collected by ultracentrifugation at 105,000×*g* for 60 min. After washing, the liver microsomal pellets obtained were resuspended in 100 mM potassium phosphate (KCL) buffer (supplemented with 1 mM EDTA, pH 7.4) and used to assay enzymatic activity. In *in vitro* experiments, the blank microsomes were incubated with BER or KTZ, the microsomes from the RIF-pretreated group were incubated with KTZ, the microsomes from the BNF-pretreated group were incubated with BER, and the controls were blank microsomes.

To determine the enzymatic activities for CYP1A and CYP3A in crucian carp liver microsomes, the activities of CYP1A-related ethoxyresorufin-*o*-deethylase (EROD) and CYP3A-related erythromycin *N*-demethylase (ERND) were assayed, respectively. First, to determine the EROD activity, 60 μL of 10 mg/mL microsomes, 50 μL of 0.5 mM NADPH, and 7 μL of 0.3 mM 7-ethoxyresorufin were incubated in 0.05 M Tris-HCL (pH = 7.4) with a total volume of 1 mL for 5 min at 27 °C. The reaction was terminated with the addition of 1 mL of methanol. After centrifugation at 5000×*g* for 10 min, the reaction product resorufin in the supernatant was measured fluorometrically (Langea et al., 1999). Second, to assay ERND activity, 100 μL of 10 mg/mL microsomes, 100 μL of 0.5 mM NADPH and 100 μL of 1 mM erythromycin were mixed in 70 mM Tris-HCL (supplemented with 0.07 mM EDTA, pH 7.4) to reach a total volume of 2 mL. After reacting these reagents for 30 min at 27 °C, the ERND