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Effects of the pharmaceuticals diclofenac and metoprolol on gene expression levels of enzymes of biotransformation, excretion pathways and estrogenicity in primary hepatocytes of Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

The expression levels of key enzymes of the xenobiotic metabolism and excretion pathways concerning biotransformation phases I (cytochrome P4501A), II (glutathione S-transferase) and III (multidrug resistance protein) and of the estrogenic biomarker vitellogenin (*vtg*) were investigated in primary hepatocytes isolated from male Nile tilapia (*Oreochromis niloticus*) after exposure to diclofenac and metoprolol, two pharmaceuticals prevalent in the aquatic environment worldwide. The lowest test concentration (4×10^{-9} M) was chosen to reflect an environmentally relevant exposure situation. Furthermore concentration dependent effects were investigated. Therefore a series of concentrations higher than the environmentally relevant range were used (10- and 100-fold). Diclofenac significantly induced all chosen biomarkers already at the environmentally relevant concentration indicating that biotransformation and elimination occur via the pathways under investigation. Estrogenic potential of this substance was demonstrated by VTG up-regulation as well. Metoprolol was either less effective than diclofenac or metabolized using different pathways. Key enzymes of the xenobiotic metabolism were less (CYP1A, GST) or not (MDRP) induced and a mild increase in *vtg* mRNA was detected only for 4×10^{-8} M. No concentration-dependency for metoprolol was found.

1. Introduction

Pharmaceutically active compounds (PhACs) are considered as a relatively new environmental concern among a large number of chemical substances contaminating aquatic environments worldwide. They are used in human and veterinary medicine, as well as for agriculture and aquaculture purposes (Dietrich et al., 2002). PhACs are often not or only partly removed during wastewater treatment and are therefore found in the range of nanograms (ng) to micrograms (μg) per liter (L^{-1}) in the aquatic environment (Corcoran et al., 2010).

Non-target organisms, such as fish, are exposed to the PhACs and their metabolites and knowledge is needed to understand interactions with the detoxification mechanisms in these taxa (Wassmur et al., 2013). This is of special interest since different authors found the lowest observed effect concentration in fish to be around 1 μg L^{-1}, hence in the environmentally relevant range (Triebskorn et al., 2004, 2007; Hong et al., 2007).

Diclofenac (DCF) is a non-steroidal anti-inflammatory drug sold in many countries at high quantities annually (Heberer, 2002). DCF has a high persistence in the environment and is found in concentrations up to several μg L^{-1} in waste waters and surface waters (Heberer, 2002; Ashton et al., 2004; Montagner and Jardim, 2011). Recent studies about the effects of environmentally relevant concentrations of DCF are limited to few fish species, mainly rainbow trout (*Oncorhynchus mykiss*) and Japanese medaka (*Oryzias latipes*; Schwaiger et al., 2004; Triebskorn et al., 2004, 2007; Hong et al., 2007; Cuklev et al., 2011).

Metoprolol (MTP) is a ß-blocker used to treat cardiovascular diseases (Regårdh et al., 1974). Since fish, like all vertebrates, possess ß-receptors in the heart, liver and gonads, there is a high probability that they will be affected from exposure to ß-blockers (Santos et al., 2010). Concentrations as high as 2.2 μg L^{-1} were already detected in surface waters (Ternes, 1998). Studies on the impacts of MTP on fish are scarce. Triebskorn et al. (2007) found ultrastructural effects in trout liver caused by MTP concentrations as low as 1 μg L^{-1}.

Here, we investigated in vitro the effects of DCF and MTP on primary hepatocytes of male *Oreochromis niloticus* with regard to xenobiotic metabolism and excretion pathways as well as endocrine disruption potential using the estrogenic biomarker vitellogenin (VTG). *O. niloticus* is an economically important aquaculture species, frequently used as a model for toxicological research (Costa et al., 2012). Primary hepatocytes are considered as the closest available in vitro experimental

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model in terms of normal fish liver physiology (Schmid et al., 2000; Zaja et al., 2008). The isolated hepatocytes possess all benefits of intact cells such as functional organelles and enzyme interactions (Segner, 1998) but in comparison to in vivo assays fewer animals are needed for toxicity testing (Schirmer, 2006).

The biotransformation pathway is mainly located in the liver and typically consists of three major phases. Phase I and II lead to more easily excretable hydrophilic metabolites of the chemicals by activation and conjugation whereas phase III is used for immediate transportation and final excretion. Cytochrome-P450-monooxygenases are the key enzymes of phase I activation. The isoform CYP1A is widely used as a biomarker in ecotoxicological studies due to its inducibility by many different environmental pollutants (Van der Oost et al., 2003). Furthermore, it is used as an indicator of cellular toxicity caused by chemicals (Sarasquete and Segner, 2000). Glutathione-S-transferases (GST) are a major group of detoxification enzymes of phase II (Sheehan et al., 2001). GSTα, as used in this study, is one of the most expressed classes of cytosolic GSTs in mammals (Fu and Xie, 2006; Li et al., 2010). Finally, different efflux pumps such as multidrug resistance proteins (MDRP; ABCC) are involved in the cellular defense by regulating accumulation and excretion (Leslie et al., 2005). Depending on the transported compounds, ABC transporters function also in phase 0 of the biotransformation pathway, especially if they act as a first line of defense preventing unmodified compounds from accumulating in the cell (Bard, 2000; Sturm and Segner, 2005). Knowledge of the presence and function of ABC efflux transporters in fish is relatively modest (Zaja et al., 2008). For Nile tilapia, the MDRP was first described recently by Ziková et al. (2010).

Additionally, primary hepatocytes can provide initial information on the endocrine activity of test substances (Kloas et al., 1999; Navas and Segner, 2006). VTG genes coding for the synthesis of egg yolk protein precursor are expressed in the liver. The induction of VTG in male fish is an indicator of exposure to estrogen or estrogen-like chemicals (Sumpter and Jobling, 1995). Many pharmaceuticals may disrupt the endocrine systems of non-target organisms (Kloas et al., 2009; Massarsky et al., 2011).

In this study, primary hepatocytes of *O. niloticus* were exposed to increasing concentrations of DCF and MTP, respectively, where the lowest concentrations are in the range of the concentrations found in surface waters worldwide. The main objective of the study was a first evaluation of the effects of DCF and MTP on xenobiotic metabolism and elimination in Nile tilapia primary hepatocytes. Therefore, established biomarkers (CYP1A, GST, MDRP; Ziková et al., 2010) were used. The second objective was the analysis of the endocrine-disruption potential of the test substances concerning estrogenicity, and thus VTG gene expression in primary hepatocytes of male fish was analyzed.

2. Material and methods

2.1. Fish

Male Nile tilapia (*O. niloticus*), weighing between 100 and 200 g, were obtained from the stock of Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany. Prior to cell isolation, fish were kept in an aerated 800 L tank with water flow-through (26 ± 1 °C, natural photoperiod) for several weeks and fed daily ad libitum with a commercial fish food (Aller Aqua Primo, Golßen, Germany).

2.2. Chemicals

All chemicals were obtained from Sigma Aldrich (Steinheim, Germany) or as indicated otherwise. Diclofenac sodium salt (CAS: 15307-79-6) and metoprolol tartrate (CAS: 56392-17-7) with a purity of >98% were dissolved in Milli-Q-grade water. 17 α-Ethinylestradiol (CAS: 57-63-6) with a purity of >98% was dissolved in ethanol (p.a.) and substances were used for exposure stock preparation to reveal final concentrations as indicated in Table 1.

2.3. Primary hepatocyte isolation

Hepatocytes were isolated according to the method described by Schmid et al. (2000) with slight modification. Briefly, fish were anesthetized by immersion in Tricaine methanesulfonate (MS222; 8 g L^{-1}) and injected with heparin (3000 U dissolved in 0.6 mL water; Roth, Karlsruhe, Germany). Perfusion of liver, carried out at room temperature, was performed retrogradly by inserting a catheter (1 mm diameter) via the *bulbus arteriosus* into the heart. First, calcium–magnesium free (CMF) medium (100 mL; Schmid et al., 2000) was used to wash out blood. Next, tissue was digested for approximately 20 min with calcium–magnesium containing (CMC) medium (50 mL; Schmid et al., 2000), supplemented with collagenase D and H (0.25 mg mL^{-1} each; Roche, Mannheim, Germany). The liver was then perfused with 50 mL CMF medium and removed from the body cavity. Finally, the liver was transferred into ice-cold CMF medium and minced with a scalpel. The cell suspension was filtered through meshes of 250 and 50 μm, centrifuged (4 °C, 70 g for 5 min, 50 g for 5 min, 30 g for 5 min) and the resulting cell pellet was resuspended in minimum essential medium (MEM; Schmid et al., 2000). Cell density was counted in a Neubauer chamber. Hepatocytes were seeded at a density of 1.5×10^6 cells mL^{-1} MEM in sterile culture discs (35 mm diameter) and incubated overnight at 20 °C under normal air conditions in an incubator (Heraeus, Hanau, Germany).

2.4. Hepatocyte treatment

Following overnight culture without exposure to chemicals, hepatocytes were exposed to test substances for 24 h (Table 1) for target gene transcription analysis. MEM was changed every 12 h and 2/3 of the culture medium including test substance concentrations were replaced twice. 17 α-Ethinylestradiol (EE_2) was included as a positive control for VTG induction (for concentrations, see Table 1); MEM-only was used as control. After 24 h of exposure, culture medium was removed and 700 μL Trizol (Invitrogen, Darmstadt, Germany) was added. Cells were harvested and immediately frozen in liquid nitrogen and stored at −80 °C.

2.5. RNA extraction and reverse transcription into cDNA

Total RNA was extracted using TRIZOL reagent (Invitrogen, Darmstadt, Germany) according to manufacturer's protocol. The amount and purity of extracted RNA was quantified by UV absorbance measurements (260/280 and 230/280 ratio) using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany). Complementary DNA (cDNA) was reversely transcribed using Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT): 17 μL of RNA (55 ng μL^{-1}) was heated together with 3 μL Poly(dt)-primer (1:40 diluted; Biometra, Göttingen, Germany) having a final concentration of 4 μM at 70 °C for 3 min, and

Table 1
Experimental design of *Oreochromis niloticus* primary hepatocyte exposure.

	Control	Diclofenac			Metoprolol			EE_2
[M]	0	4×10^{-9}	4×10^{-8}	4×10^{-7}	4×10^{-9}	4×10^{-8}	4×10^{-7}	1×10^{-6}
[μg L^{-1}]	0	1.27	12.72	127.24	1.07	10.69	106.94	0.3

EE_2: 17 α-Ethinylestradiol.