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Interaction of environmental contaminants with zebrafish organic anion transporting polypeptide, Oatp1d1 (*Slco1d1*)

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ABSTRACT

Polyspecific transporters from the organic anion transporting polypeptide (OATP/Oatp) superfamily mediate the uptake of a wide range of compounds. In zebrafish, Oatp1d1 transports conjugated steroid hormones and cortisol. It is predominantly expressed in the liver, brain and testes. In this study we have characterized the transport of xenobiotics by the zebrafish Oatp1d1 transporter. We developed a novel assay for assessing Oatp1d1 interactors using the fluorescent probe Lucifer yellow and transient transfection in HEK293 cells. Our data showed that numerous environmental contaminants interact with zebrafish Oatp1d1. Oatp1d1 mediated the transport of diclofenac with very high affinity, followed by high affinity towards perfluorooctanesulfonic acid (PFOS), nonylphenol, gemfibrozil and 17α-ethinylestradiol; moderate affinity towards carbaryl, diazinon and caffeine; and low affinity towards metolachlor. Importantly, many environmental chemicals acted as strong inhibitors of Oatp1d1. A strong inhibition of Oatp1d1 transport activity was found by perfluorooctanoic acid (PFOA), chlorpyrifos-methyl, estrone (E1) and 17β-estradiol (E2), followed by moderate to low inhibition by diethyl phthalate, bisphenol A, 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4 tetrahydronapthalene and clofibrate. In this study we identified Oatp1d1 as a first Solute Carrier (SLC) transporter involved in the transport of a wide range of xenobiotics in fish. Considering that Oatps in zebrafish have not been characterized before, our work on zebrafish Oatp1d1 offers important new insights on the understanding of uptake processes of environmental contaminants, and contributes to the better characterization of zebrafish as a model species.

Introduction

Membrane transporters are crucial determinants of toxicokinetics and are recognized as important factors in toxicological response to various xenobiotics (Klaassen and Lu, 2008). In the past decades, so-called uptake transporters have been characterized and recognized as key players in mediating the entrance of metabolites and foreign compounds into the cell by means of facilitated diffusion. Together with the efflux transporters from the ATP binding cassette (ABC) and multidrug and toxin extrusion (MATE) families, uptake transporters from the organic anion transporting polypeptides (OATP/Oatp) superfamily (gene name *SLCO*, old gene name *SLC21*) are important determinants of cellular concentrations and effects of xenobiotics (Klaassen and Lu, 2008).

OATP/Oatp superfamily (OATPs in humans, Oatps in all other species; gene symbol *SLCO*/*Slco*) includes polyspecific transporters that mediate uptake of compounds across cell membranes of eukaryotes. OATPs transport a wide range of endogenous (steroid hormones, bile salts, prostaglandins etc.) and exogenous compounds (pharmaceuticals, natural toxins). The classification in mammals includes six families named OATP1-6/Oatp1-6. The OATP1 family in humans encompasses four genes: OATP1A2, OATP1B1, OATP1B3 and OATP1C1 (Hagenbuch and Meier, 2004). Ubiquitously expressed OATP1A2 mediates transport of physiologically important compounds such as steroid and thyroid hormones, bilirubin, bile salts and eicosanoids. Along with the role in the balance of hormones and bile salts, OATP1A2 has been shown to transport various pharmaceuticals. Given its ubiquitous distribution it is probably important for the tissue-specific disposition, pharmacokinetics and toxicity of xenobiotics (Badagnani et al., 2006). Unlike OATP1A2, OATP1B1 and OATP1B3 are liver specific transporters with a largely overlapping substrate range that includes steroid conjugates, bile salts and bilirubin. However, their affinities for the same substrates can significantly differ (Hagenbuch and Gui, 2008). OATP1B1 and OATP1B3 have been extensively studied due to the fact that they are largely responsible for the disposition of drugs (e.g., lipid lowering agents and chemotherapeutics) and their subsequent elimination through bile. OATP1C1, unlike most OATPs, has a narrow substrate range that principally includes thyroid hormones: thyroxine (T4), triiodothyronine (T3), reverse triiodothyronine (rT3) and their sulfated

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conjugates. OATP1C1 is predominantly expressed at the blood brain barrier (BBB) and is not implicated in the transport of xenobiotics (Roth et al., 2012).

Our previous work offered the first comprehensive analysis of OATP/Oatp superfamily in zebrafish (Popovic et al., 2010), which was followed by the detailed molecular characterization of a novel transporter, Oatp1d1 (Popovic et al., 2013). Oatp1d1 orthologs are present within the entire teleost group, thus making the Oatp1d subfamily relevant in all fish, not just cyprinids (Popovic et al., 2013). Oatp1d1 is a high affinity transporter of conjugated steroid hormones: estrone-3-sulfate (E3S), estradiol-17β-glucuronide (E17ß-glucuronide) and dehydroepiandrosterone sulfate (DHEAS); whereas non-conjugated steroids like estradiol, progesterone, androstenedione, dihydrotestosterone and testosterone are strong inhibitors of Oatp1d1. Oatp1d1 is also involved in corticosteroid metabolism. Similar to OATP1A2, Oatp1d1 transports cortisol (Popovic et al., 2013), a crucial hormone in fish that acts as both glucocorticoid and mineralocorticoid, given that fish do not possess corticosterone and aldosterone (James, 2011; Pippal et al., 2011). Considering high expression of Oatp1d1 in the liver, we proposed that Oatp1d1 is crucial for the uptake and subsequent elimination of excess steroid hormone metabolites through bile, similar to the role of OATP1A2, OATP1B1 and OATP1B3 (Hagenbuch and Gui, 2008). Furthermore, high expression of Oatp1d1 in testes may imply possible involvement in the uptake of DHEAS as a precursor for androgen synthesis, similar to the role of the OATP6/Oatp6 family in mammalian testes (Klaassen and Lu, 2008; Suzuki et al., 2003).

Uptake of DHEAS by Oatp1d1 may be of critical importance in the brain, considering its action as a neurosteroid and high expression of Oatp1d1 in the brain (Popovic et al., 2013). Inhibition of Oatp1d1 by non-conjugated steroids could reduce the uptake of conjugated steroid hormones in target tissues, depending on the fine hormonal balance in the plasma. In that sense, transporters, and more specifically Oatp1d1, would be involved in the negative feedback loop regulation of steroid hormone synthesis (James, 2011).

We have previously shown that Oatp1d1 has one and the same substrate binding site for estrone-3-sulfate (E3S) and anionic dye Lucifer yellow (LY), thus enabling Michealis–Menten analysis to distinguish between substrates and inhibitors among compounds of interest (Popovic et al., 2013). When comparing the number of substrate binding sites, Oatp1d1 has one binding site which is similar to OATP1A2, whereas OATP1B1, OATP1B3 and probably OATP1C1 have two binding sites for E3S (Hirano et al., 2006; Noe et al., 2007; Roth et al., 2011; Tamai et al., 2000).

To date, ABC transporters have been extensively studied in the field of aquatic toxicology (e.g., Caminada et al., 2008; Della Torre et al., 2012; Fischer et al., 2013; Lončar et al., 2010; Zaja et al., 2013). In contrast, studies on uptake transporters from the OATP/Oatp superfamily are scarce. Cai et al. (2002) and Meier-Abt et al. (2005) investigated Oatp transporter in the fish, little skate (*Raja erinacea*), Nakao et al. (2006) analyzed Oatps in birds, and recently Muzzio et al. (2014) investigated thyroid hormone transport in teleosts. Given the fact that OATPs/Oatps are recognized as a rate-limiting step in the disposition of drugs in mammals (Shitara et al., 2013), more research is needed on non-mammalian vertebrates including fish.

The human functional orthologs of zebrafish Oatp1d1, OATP1B1 and OATP1B3 (Popovic et al., 2013), mediate the uptake of drugs into hepatocytes, enabling their elimination through bile. Another functional orthologs of Oatp1d1, OATP1A2 and rodent Oatp1a4 (Popovic et al., 2013), are ubiquitously expressed in zebrafish tissues. They have important physiological function in the brain and intestine, and are found to be crucial for the pharmacokinetics and toxicity of certain drugs (Abe et al., 1999; Badagnani et al., 2006; König et al., 2000; Lee et al., 2005; Ose et al., 2010). Considering functional similarity of zebrafish Oatp1d1 with OATP1B1, OATP1B3 and OATP1A2, which are recognized as important determinants of drug transport in humans, and the fact that Oatp1d1 shows interaction with numerous pharmaceuticals (Popovic et al., 2013), we hypothesize that Oatp1d1 may be crucial for the transport of environmental chemicals.

In order to test our hypothesis, we developed a novel assay for assessing Oatp1d1 interactors using the fluorescent probe Lucifer yellow (LY) and transient transfection in HEK293 cells, and determined the potency and type of interaction with zebrafish Oatp1d1 for a series of diverse environmental contaminants frequently found in the aquatic environment. The methodological protocol developed in this study can serve as a useful tool for better understanding of toxicokinetics of environmental contaminants and/or drugs in zebrafish as a model species.

Material and methods

Chemicals. Lucifer yellow (LY) and all other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Carl Roth GMBH (Karlsruhe, Germany) or Alfa Aesar (Ward Hill, MA, USA).

Cloning and heterologous expression in HEK293 cells. Full-length zebrafish Oatp1d1 (corresponding to the GeneBank accession No. NP_001082802) was amplified from the zebrafish liver using the primer pair 5′- TTAGCGGCCGCATGAGTACGGAGAAGAAGAAG -3′ (forward) and 5′- TTAGGTACCTCTAGACTTCAGATGGTGGTCTCCTG -3′ (reverse) with high fidelity Phusion DNA polymerase (Finnzymes, Vantaa, Finland). The amplicon was cloned into pJET2.0 vector (Invitrogen, Carlsbad, CA) and the sequence verified by sequencing (VBC-Biotech Services GmbH, Vienna, Austria). Slco1d1 cDNA located between NotI and KpnI multiple cloning sites was subcloned into expression vector pcDNA3.1 and pcDNA3.1/His (Invitrogen, Carlsbad, CA). HEK293 cells were transiently transfected using the polyethyleneimine (PEI) reagent in 48-well plates according to Tom et al. (2008). Cells were in parallel transfected with pcDNA3.1/His/LacZ plasmid and transfection efficiency evaluated 24 h after transfection with the LacZ staining protocol (Sambrook and Russell, 2001). Transport experiments were conducted 24 h post transfection, when transfection efficiency was above 70%. HEK293 cells were grown in the complete growth medium that consisted of DMEM high glucose (Dulbecco's modified Eagle medium) (Life technologies, CA, US) and 10% fetal bovine serum (FBS; Invitrogen, CA, USA) in the 5% CO_2 incubator at 37 °C.

Transport measurements. For the purpose of the transport assay, HEK293 cells overexpressing Oatp1d1 were preincubated in the transport medium (145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose and 5 mM HEPES, pH 7.4) for 10 min at 37 °C. To assess transport, the medium was removed and the same medium containing the substrate was added. After incubation, the cells were rapidly washed three times with ice-cold phosphate-buffered saline (PBS), lysed in 500 µl of 0.1% sodium dodecyl sulfate (SDS) for 30 min, transferred to 96-well black microplates (Sigma-Aldrich, Taufkirchen, Germany) and fluorescence was measured using a microplate reader (Infinite M200, Tecan, Salzburg, Austria). Fluorescence was measured at the following wavelengths: LY, 425/540 nm; fluorescein, 492/522 nm; dihydrofluorescein, 490/522 nm; 5-carboxyfluorescein and 6-carboxyfluorescein at 492/524 nm; rhodamine B, 535/590 nm; resazurin, 530/590 nm; and Eosin Y, 510/556 nm. Calibration curve for LY was generated in the 0.1% SDS and in the cell matrix dissolved in the 0.1% SDS. Linear calibration curves were the same in the SDS and in the dissolved cell matrix. Using the calibration curves, uptake of LY was calculated as nmol of substrate per mg of protein. Total cell protein content was determined according to the manufacturer's instructions using the DC protein assay kit (Bio-Rad Laboratories, CA, USA). Inhibition assay using LY as a fluorescent substrate was performed in the same transport medium with incubation time of 30 min and LY concentration of 5 µM. When interacting compounds showed inhibition of LY uptake above 20% at the interactor concentration of 100 µM, K_i values were determined. Compounds with K_i values in nanomolar range (<1 µM) were considered to be very strong interactors, compounds