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In vitro characterization of zebrafish (*Danio rerio*) organic anion transporters Oat2a-e

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ABSTRACT

OATS/Oats are transmembrane proteins that transport a variety of drugs, environmental toxins and endogenous metabolites into the cell. Zebrafish (*Danio rerio*) has seven OAT orthologs: Oat1, Oat2a–e and Oat3. In this study we specifically address Oat2 (*Slc22a7*) family. Conserved synteny analysis showed localization of zebrafish *oat2* genes on two chromosomes, 11 and 17. All five zebrafish Oats were localized by live cell imaging in membranes of transiently transfected HEK293-T cells, and Oat2a, b, d, and e were confirmed using western blot analysis. Functional studies using the HEK293T cells overexpressing zebrafish Oats revealed two model fluorescent substrates of three Oats: Lucifer yellow for Oat2a and Oat2d (Km 122, and 49.7 μM), and 6-carboxyfluorescein for Oat2b and Oat2d (Km 199.7, and 266.9 μM). The initial screening of a series of diverse endo- and xenobiotics showed interaction with a number of compounds, including cGMP and diclofenac (IC50 27.74, and 19.14 μM) with Oat2a; estrone-3-sulfate and diclofenac (IC50 30.96, and 12.6 μM) with Oat2b; and fumarate and indomethacin (IC50 68.24, and 20.41 μM) with Oat2d. This study provides the first comprehensive data set on Oat2 in zebrafish and offers an important basis for more detailed molecular and (eco)toxicological characterizations of these transporters.

1. Introduction

The organic anion transporters (OATs in humans, Oats in other animal species) are a family of transmembrane proteins able to transport a variety of compounds including drugs, environmental toxins and endogenous metabolites into the cell, playing an essential role in their elimination from the body. They received a lot of attention recently because of their role in transporting of common drugs (antibiotics, antivirals, diuretics, nonsteroidal anti-inflammatory drugs), toxins (mercury, aristolochic acid), and nutrients (vitamins, flavonoids). OATs/Oats belong to the SLC22 (solute carrier 22) subfamily of the major facilitator superfamily (MFS) of transmembrane proteins. Except for OATs/Oats, SLC22 subfamily includes the organic cation transporters (OCTs/Octs) and organic carnitine (zwitterion) transporters (OCTNs/Octns). These groups of transporters share many structural characteristics with other MFS proteins (Nigam et al., 2015). Although their crystal structure is not solved yet, several homology models based on the related bacterial MFS protein glycerol-3-phosphate transporter (GlpT) or lactose permease (LacY) were reported, indicating the structure of 12 α-helices (Perry et al., 2006). Accordingly, OATs/Oats are composed of 540–560 amino acids, comprising 12 transmembrane domains (TMDs) that form the pore and are characterized by two large interconnecting loops (one extracellular and one intracellular), similar to other bacterial and mammalian transporters (Lopez-Nieto et al., 1997). Extracellular LP1, located between TMD1 and TMD2, is important for N-linked glycosylation of the protein and in some cases for its homo-oligomerization (carries conserved cysteine residues for the formation of disulfide cross bridges), while intracellular LP6 is involved in posttranscriptional regulation (protein kinase-mediated phosphorylation) (Brast et al., 2012; Keller et al., 2011; Nigam et al., 2015). Considering their tissue specific expression, although OATs/Oats were initially found in kidney, they are expressed in almost all barrier epithelia of the body (kidney, liver, choroid plexus, intestine, olfactory mucosa, brain, retina, placenta, even in muscle, bone and heart) (Burckhardt, 2012; Mihaljevic et al., 2016).

In terms of the transport mechanism, prototypical OATs/Oats such as Oat1 are secondary active transporters. OATs/Oats mediated influx typically involves the exchange, or countertransport with another solute, in most cases intracellular organic anions (e.g., dicarboxylate or α-ketoglutarate for OAT1, OAT3, OAT4, Oat6, Oat8; or succinate for OAT2) against negative membrane potential inside the cell, and this transport requires the input of energy. The required intracellular to extracellular dicarboxilate gradient is maintained by the Na+/dicarboxylate co-transport (approximately 60%) and by the intracellular

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production via metabolism (approximately 40%) (Dantzler, 2002). OATs/Oats are thought to be part of a so-called "tertiary" transport system involving the organic anion transporter, the Na^+-K^+-ATPase, and the sodium-dicarboxylate cotransporter (Nigam et al., 2015). They primarily transport organic anions, but are also capable of transporting a variety of organic cationic drugs (e.g., cimetidine), as well as metabolites like creatinine, and possibly polyamines and carnitine (Ahn et al., 2009, 2011; Kusuhara et al., 1999; Vallon et al., 2012). Their substrates are characterized by a small size (< 500 Da), a hydrophobic region (optimally 8–10 Å in length), and a negative charge (Ullrich and Rumrich, 1988; Fritzsch et al., 1989). Complete transport through epithelial cells involves transporters at the basolateral and apical surfaces, and is often a combination of the SLC ("uptake/influx") transporters and ABC ("efflux") transporters. For example, in kidneys it appears that basolateral Oat1 and Oat3 uptake of organic anions is coupled to apically located efflux transporters including ABCC2 and ABCC4 (Nigam et al., 2015).

Yet, despite their physiological importance and role in cellular detoxification, the knowledge about uptake transporters in non-mammalian species is scarce, and the same is true for zebrafish (*Danio rerio*) as an increasingly important vertebrate model species. There is a growing interest in zebrafish due to numerous advantages of this model organism: small size, simple breeding, high reproduction rate, fast development, external fertilization, transparent embryo and developmental stages, and finally a fully sequenced genome. In addition, the teleost specific whole genome duplication (WGD) provided the evolutionary driving force in generating enormous number of newly functional genes, whose research can provide new understanding of human gene changes linked to numerous diseases. Nevertheless, the WGD is the reason why zebrafish often has two paralogs that correspond to the single gene in other vertebrate species, including humans (Ravi and Venkatesh, 2008). Despite the additional round of the genome duplication in fish, however, large portions of vertebrate genes and cellular pathways are evolutionary conserved in vertebrates, and findings on zebrafish can generally be translated to other vertebrate species (Busby et al., 2010).

In this study we specifically address members of the Organic anion transporter 2 family in zebrafish (OAT2/Oat2, gene symbol SLC22A7/Slc22a7). OAT2/Oat2 was previously cloned from rat, human and mouse, and its isoforms range from 535 to 546 amino acids in size. Rat Oat2 shares 88 and 79% amino acid sequence identity with mouse Oat2 and human OAT2, respectively (Simonson et al., 1994; Sun et al., 2001; Kobayashi et al., 2002). Tissue expression profile showed the highest expression of human OAT2 mRNA in liver and kidneys, followed by pancreas, small intestine, lung, brain, spinal cord and heart. In human and male rats, OAT2/Oat2 is mainly expressed in liver, where it mediates hepatic excretion of endogenous substrates such as glutamate, glutarate, urate, L-ascorbate, cyclic nucleotides, prostaglandin E2 and F2, estrone-3-sulfate, dehydroepiandrosterone and α-ketoglutarate, along with transport of xenobiotics such as salicylate, erythromycin, tetracycline, ranitidine, 5-fluorouracil, methotrexate, taxol, aflatoxin B1 and other drugs and toxins (Nigam et al., 2015). Oat2 was also shown to interact with various drugs including diuretics, antibiotics, antiviral, antineoplastic and nonsteroidal anti-inflammatory drugs. Its mRNA expression has shown age, sex and species dependence (Burckhardt, 2012). Human OAT2 was immunolocalized in basolateral membrane of proximal tubules (Enomoto et al., 2002a, 2002b), whereas in rats and mice Oat2 was found at the apical membrane in late S3 segments of proximal tubules (Ljubojevic et al., 2007), as well as in cortical thick ascending limbs of Henle's loop and collecting ducts (Kojima et al., 2002). OAT2/Oat2 in liver is presumed to be localized in the sinusoidal membrane of hepatocytes (basolateral membrane) (Burckhardt, 2012). Human OAT2 functions as an antiporter, exchanging intracellular succinate or fumarate against extracellular organic anions. This transport could be fueled by sodium dependent dicarboxylate transporter (NaDC3 in human, NaDC1 in rodents) that takes up succinate into the cells (Burckhardt, 2012). In liver, human OAT2 mediates the efflux of glutamate into the sinusoids and the uptake of various endogenous compounds, drugs and toxins into hepatocytes, whereas in kidney it participates in the tubular secretion of urate and various other organic anions including drugs and toxins. OAT2 may also be involved in the adjustment of intracellular cGMP concentration in hepatocytes and renal proximal tubule cells (Koepsell, 2013).

Based on these data, to perform the initial toxicological characterization of zebrafish Oat2 subfamily in this study we aimed at cloning, heterologous expression and development of transport activity assays that can be used for a detailed analyses of Oat2 transporters and their interactions with both physiological and xenobiotic substances.

2. Materials and methods

2.1. Chemicals

All tested compounds, model fluorescent substrates and interactors alike were purchased from Sigma-Aldrich (Taufkirchen, Germany), except ethidium bromide which was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany).

2.2. Conserved synteny analysis

Conserved synteny analysis between zebrafish and other teleost genes of interest were made using Genomicus (http://www.genomicus.biologie.ens.fr/genomicus), a conserved synteny browser synchronized with genomes from the Ensembl database (Louis et al., 2013).

2.3. Cloning and heterologous expression

A full-length zebrafish *oat2(a–e)* sequence was obtained from zebrafish cDNA by polymerase chain reaction using high fidelity Phusion DNA polymerase (Thermo Scientific, MA, USA) and specifically designed primers with *Not*I and *Hin*dIII restriction sites on the forward, and *Kpn*I and *Xba*I restriction sites on the *oat2(a–e)* reverse primers. An amplified DNA fragment was cloned into a linearized pJET 2.0 vector (Invitrogen, Carlsbad, CA). Zebrafish *oat2a–e* sequences were verified by DNA sequencing using automated capillary electrophoresis (ABI PRISM® 3100-Avant Genetic Analyzer) at the Ruđer Bošković Institute DNA Service (Zagreb, Croatia). Sequenced genes of each clone were compared to the reported gene sequences from the NCBI and ENSEMBL databases. The verified *oat2a–e* sequences were subcloned into the pcDNA3.1(+) and pcDNA3.1/His vectors (Invitrogen, Carlsbad, CA). Transient transfection of human embryonic kidney cells (HEK293T) was based on the previously described procedure by Popovic et al. (2013) using polyethyleneimine (PEI) as the transfection reagent.

In order to evaluate transfection efficiency, separate cells were transfected with pcDNA3.1/His/LacZ plasmid (Invitrogen, Carlsbad, CA) and transfection efficiency was evaluated 24 h after transfection with the LacZ staining protocol (Sambrook et al., 1989).

2.4. Transport assays

Transfected cells that showed over 70% transfection efficiency were used in transport assays 24 h post transfection. DMEM-FBS was removed from the cells grown in 48-well plates and cells were preincubated in 200 µL of the transport medium (145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 5 mM glucose, 5 mM HEPES and 0.5 mM $MgCl_2$) for 10 min at 37 °C. To assess transport and dose responses of fluorescent substrates, 50 µL of five times concentrated fluorescent substrates were added to the preincubation medium and incubated 15 min at 37 °C. Incubation time was chosen based on the time response assay (Fig. S3). After the incubation, the cells were washed two times with 250 µL of pre-chilled transport medium and lysed with 0.1% of sodium dodecyl sulfate (SDS) for 30 min. Lysed cells were transferred to the 96-well