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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

black plates and the fluorescence was measured with the microplate reader (Infinite M200, Tecan, Salzburg, Austria). The transport rates were determined by subtracting the measured fluorescence of transfected cells with the fluorescence of non-transfected (control) cells and by normalization of the obtained calibration curves for each substrate and protein content. Calibration curves for fluorescent dyes were generated in the 0.1% SDS and in the cell matrix dissolved in the 0.1% SDS. Total protein concentration was measured using Bradford assay (Bradford, 1976). Using the calibration curves and total protein content, uptake of the fluorescent substrates was expressed as nM of substrates per mg of protein per minute.

After determination of transport kinetics for fluorescent dyes, Lucifer yellow (LY) and 6-carboxyfluorescein (6-CF) were used in subsequent inhibition assays. Inhibition measurements were based on the co-exposure of transfected cells and mock control with the determined model substrate and potential interactor. The cells were preincubated for 10 min in transport medium, then for 40 s with the test compounds, followed by 15 min of incubation with LY and 6-CF. Concentrations of the model substrates used were in the linear part of the previously determined concentration-response curves. The initial interaction screens were performed with one concentration of the tested compounds (100 μM). For the two of the most potent interactors (< 50% model substrate uptake), detailed dose response experiments were performed and the respective IC50 values determined.

2.5. Western blotting and cell localization

Cells were collected from 2 wells of a 6-well microplate 24 h after transfection and lysed in NP-40 (Nonidet buffer) with protease inhibitors cocktail AEBSF (Sigma-Aldrich, Taufkirchen, Germany) for 30 min on ice. After the lysis, cells were briefly sonicated (5 s at 5 μm) and centrifuged at 4000*g* for 20 min at 4 °C. Protein concentration in total cell lysate (TCL) was measured using Bradford assay (Bradford, 1976). Western blot analysis was performed using Mini-PROTEAN 3 Cell electrophoresis chamber for polyacrylamide gel electrophoresis, together with Mini Trans-Blot Cell transfer system (Bio-Rad Laboratories, CA, USA) for wet transfer. Proteins (20 μg per lane) were separated by electrophoresis in gradient (3–12%) polyacrylamide gel with 0.1% sodium dodecyl sulfate added and then transferred to the polyvinylidene difluoride membrane (Millipore, MA, US) via wet transfer (1 h at 80 V, with 0.025% SDS). Blocking was performed in blocking solution containing 5% low fat milk, 50 mM Tris, 150 mM NaCl and 0.05% Tween 20 for 1 h. Membranes were washed and incubated for 1 h with anti-Xpress antibody (Invitrogen, ThermoFisher Scientific, MA, USA) diluted 2500 ×. Goat anti-mouse IgG-HRP (diluted 5000 ×) was used as secondary antibody with one hour incubation period (Santa Cruz Biotechnology, CA, USA). The proteins were visualized by chemiluminescence (Abcam, Cambridge, UK). Protein size was estimated by use of the protein marker (ThermoFisher Scientific, MA, USA). Additionally, the PVDF membrane was stained with Coomassie R-250 (Fig. S2). The blot was rinsed in DI water, saturated in methanol, the PVDF membrane stained with 0.1% Coomassie R-250 in 40% MeOH for 20 s, destained with 50% methanol, and rinsed extensively with DI water.

For live cell imaging, HEK293T cells were grown on glass coverslips in 24-well culture plates. Cells were transiently transfected using pDest-YFP vector (kindly provided by Dr. Ivan Ahel, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK). Twenty four hours after transfection coverslips were transferred on microscope slides on a drop of HEPES buffered solution (130 mM NaCl, 0.65 mM KCL, 1.2 mM KH_2PO_4, 3.2 mM $MgSO_4$, 5 mM Taurine, 5 mM HEPES, 5.5 mM Glucose, 15 mM Saccharose, 5 mM BDM and 0.35 mM KOH) and YFP fluorescence was observed under fluorescent microscope (FITC filter). Cell localization of Oat transporters was also confirmed by immunocytochemistry (Fig. S1).

2.6. Data analysis

All assays were performed in 2–4 independent experiments run in triplicates. Data shown represent mean ± standard errors (SE) or standard deviations (SD). All calculations were performed using GraphPad Prism 6 for Windows as described below. The kinetic parameters, K_m and V_m values were calculated using the Michaelis-Menten equation (Eq. (1)),

$$V = \frac{V_m \times [S]}{S + K_m} \quad (1)$$

where V is velocity (nanomoles of substrate per milligram of proteins per minute), V_m is maximal velocity, [S] is substrate concentration and K_m is the Michaelis Menten constant.

3. Results

3.1. Zebrafish oat2a–e conserved synteny analysis with other fish species

Zebrafish *oat2* genes are localized on two chromosomes, 11 and 17. Four *oat2* co-orthologs, *oat2b* (*slc22a7* (1 of many)), *oat2c* (*slc22a7b.2*), *oat2d* (*slc22b.3*), *oat2e* (*slc22b.1*), are localized within the cluster with reversed orientation on chromosome 17 at 21.99 Mbp (Fig. 1). Neighboring genes near the *oat2* cluster on chromosome 17 show syntenic relationship with two *oat2* orthologs in stickleback, cave fish and tetraodon. *ryr2b*, *actn2b* and *slc16a9a* are localized upstream of *oat2b* ortholog in stickleback and determine syntenic relationship with zebrafish ortholog. Two downstream neighboring genes, *ttbk1b* and *slc8a1b*, and an upstream neighboring gene *crip3*, showed syntenic relationship with the second *oat2* ortholog in stickleback, *oat2e*. *oat2a* (*slc22a7a*) on chromosome 11 and its neighboring genes, show syntenic relationship with *oat2a* orthologs in cave fish and tetraodon. *oat2* genes are also present in medaka and cod. However, their *oat2* ortholog does not show syntenic relationship with zebrafish orthologs. One *oat2* ortholog in medaka and cod, respectively, show synteny with *oat2* ortholog in stickleback, and with the zebrafish *oat2* cluster on chromosome 17 (Fig. 1).

3.2. Protein identification and cell localization

To identify zebrafish Oat2a–e proteins overexpressed in transiently transfected cells, Western blot analysis was performed. The analysis was based on the expression of specific tags fused together with the Oat2 proteins in the heterologous expression system. Western blot analysis revealed protein bands of four zebrafish Oat2 proteins (Oat2a, b, d, and e) that correspond to the size of approximately 60 kDa, as expected. However, protein band of Oat2c was not obtained. Estimated size of zebrafish Oat2 transporters are: Oat2a – 62.27 kDa, Oat2b – 61.21 kDa, Oat2c – 62.18 kDa, Oat2d – 62.92 kDa, Oat2e – 60.63 kDa.

Live cell imaging analysis has shown localization of all five zebrafish Oats within the cell membranes (Fig. 3). Green signal could also be seen inside the cells, particularly for Oat2a and Oat2c. These results indicate that all five zebrafish Oat2 co-orthologs are correctly localized (in higher or lower proportion) inside the plasma membrane and may be active in the used expression system.

3.3. Functional characterization of zebrafish Oats using model fluorescent substrates

To identify suitable Oat2a–e model substrates which could be used for functional characterization of zebrafish Oats, we initially tested a range of commercially available anionic dyes from the group of fluoresceins: fluorescein, 5- and 6-carboxyfluorescein (5CF and 6CF), 2′,7′-dichlorofluorescein, 4′,5′-dibromofluorescein; Lucifer yellow (LY), resorufin, resazurin, calcein and eosin Y. Our testing of anionic fluorescent dyes revealed two potential fluorescent substrates of the three

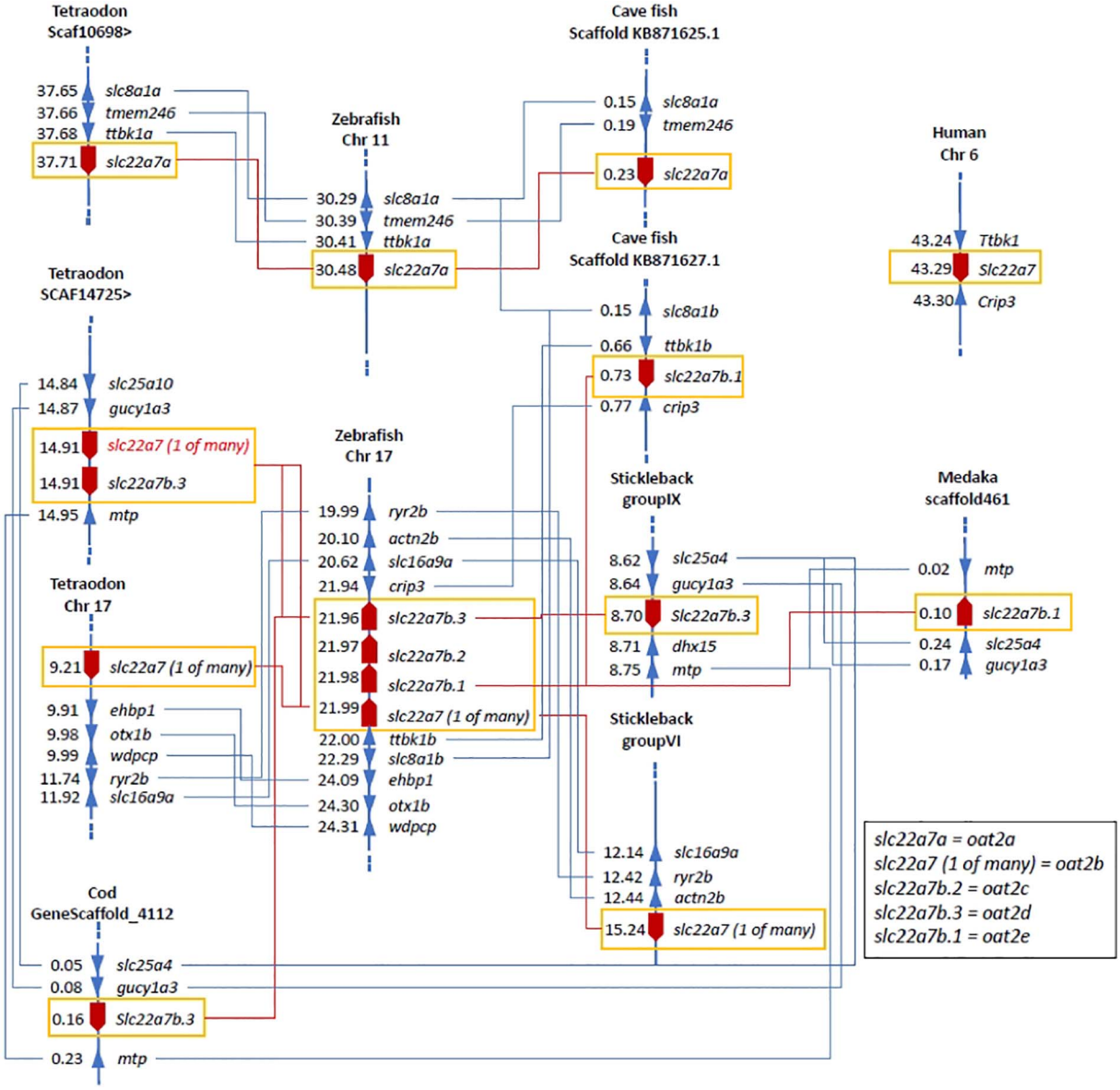

Fig. 1. Conserved synteny analysis of teleost fish slc22a7 genes. Numbers next to the gene names represent megabase pair (Mbp) of particular gene location on the chromosome. Species names: zebrafish, *Danio rerio*; green spotted puffer, *Tetraodon nigroviridis*; cod, *Gadus morhua*; cave fish, *Astyanax mexicanus*; stickleback, *Gasterosteus aculeatus;* medaka, *Oryzias latipes;* human, *Homo sapiens*.

studied Oats: Oat2a, Oat2b and Oat2d. However, Oat2c and Oat2e showed no transport activity with any of the fluorescent dyes tested.

We have identified Lucifer yellow (LY) as fluorescent dye that showed high accumulation in Oat2a transfected HEK293 cells, thus revealing the interaction with Oat2a as a potential substrate. Time (Fig. S1) and dose response assays confirmed that LY is indeed an Oat2a substrate, whose transport followed the classical Michaelis-Menten kinetics. The determined kinetic parameters of the LY transport in transfected cells resulted in Vmax value of 2.21 nmol/mg protein/min and Km of 122 μM (Fig. 4).

Fluorescent dye that showed to be a potent zebrafish Oat2b substrate was 6-carboxyfluorescein, resulting in the determined Vmax value of 32.24 nmol/mg protein/min and Km of 199.7 μM (Fig. 5).

Oat2d showed interaction with two fluorescent dyes – Lucifer yellow (Vmax = 36.31 nmol/mg protein/min, Km = 49.68 μM) and 6-carboxyfluorescein (Vmax = 29.65 nmol/mg protein/min, Km = 266.9 μM) – with better affinity for LY where Michaelis Menten constant of 49.68 μM was actually the lowest of all tested fluorescent dyes with zebrafish Oat2a, b, and d (Fig. 6).

Therefore, due to reliable protein identification, correct cell localization, and finally identification of suitable model fluorescent substrates, we have continued with functional analysis of the three out of five zebrafish Oat2s: Oat2a, Oat2b and Oat2d.

3.4. Interaction of zebrafish Oat2a–e with selected interactors of mammalian OAT2/Oat2

Following optimization of the transport activity assays, we have tested a series of known interactors of mammalian OATs/Oats, including potential physiological interactors (cGMP, Kreb's cycle intermediates: α-ketoglutarate, succinate, fumarate; creatinine and prostaglandin E2), and xenobiotic substrates (mostly pharmaceutical compounds known to be interactors of mammalian OAT2/Oat2, e.g., diclofenac, erythromycin and indomethacin) (Figs. 7, 8 and 9). It is important to note that this type of transport assay does not distinguish between an interactor that is transported by the protein and one that is a non-competitive inhibitor. It reveals, however, whether a compound interferes (or not) with transport of the model fluorescence substrate.

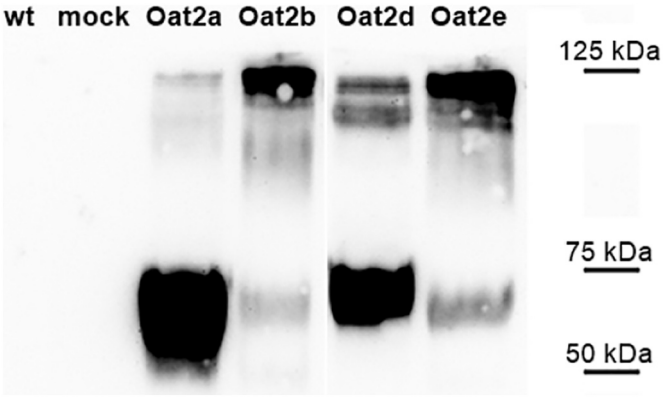

Fig. 2. Western blot analysis of zebrafish Oat2a–e proteins expressed in transfected cells. Total cell lysate (TCL) of HEK293/Oat2a–e overexpressing cells shows protein bands of four Oat2s (Oat2a, b, d, and e) that correspond the size of monomeric protein (approx. 60 kDa). Oat2a is present as a monomer, whereas other Oat2 proteins show monomeric, and possible homo- or heterodimeric forms. Western blots were performed in the presence of reducing agent dithiothreitol (DTT) with anti-Xpress antibody, and visualized with chemiluminescence.

Among the two anionic dyes listed above, we chose Lucifer yellow for further Oat2a and Oat2d functional analysis, due to high affinity of these two Oats toward LY. For Oat2b functional analysis we used 6-carboxyfluorescein, which was the only anionic dye transported by Oat2b.

As is shown in Fig. 7, the most potent endogenous interactors of Oat2a were glutamate (29% LY uptake), prostaglandin E_2 (44.43% LY uptake), cGMP (45.02% LY uptake), testosterone (51.47% LY uptake), estrone-3-sulfate (54.16% LY uptake) and deoxycholic acid (58.19% LY uptake). Apart from endogenous interactors, our screening revealed numerous xenobiotic interactors. The highest inhibition (0% LY uptake) was observed with MK571, an established cysteinyl leukotriene-based inhibitor of multidrug resistance protein (MRP). Other potent xenobiotics were diclofenac, indomethacin and erythromycin, which reduced LY uptake up to 18.23, 23.39 and 48.94%, respectively (Fig. 7).

The most potent endogenous interactors of Oat2b were prostaglandin E_2 (3.38% 6-CF uptake), estrone 3-sulfate (24.95% 6-CF uptake), adenine (36.46% 6-CF uptake), testosterone (41.04% 6-CF uptake) and cGMP (41.55% 6-CF uptake). Xenobiotics that showed the highest inhibition rate were diclofenac, indomethacin, tetracycline and erythromycin, which reduced LY uptake up to 7.52, 17.15, 30.45 and 41.71%, respectively (Fig. 8).

Oat2d has also shown interaction with numerous endo- and xenobiotics. Endogenous compounds that showed the highest inhibition rate were α-ketoglutarate (21.20% LY uptake), deoxycholic acid (29.04% LY uptake) and bilirubin (48.81% LY uptake). Xenobiotic that has shown the highest inhibition of Oat2d transport (0% LY uptake) was MK571, followed by tetracycline (10.90% LY uptake), diclofenac (33.34% LY uptake), p-aminohippurate (35.78% LY uptake) and indomethacin(43.07% LY uptake) (Fig. 9).

After initial screening of the described endogenous and xenobiotic compounds, we have selected two of the most potent interactors, one endogenous compound and one xenobiotic, for concentration dependent inhibition assays for each of the three functional Oats (Oat2a, b, and d).

As can be seen, cGMP and diclofenac both showed concentration dependent inhibition of Oat2a mediated LY transport (IC_{50} = 27.74 μM and 19.14 μM, respectively) (Fig. 10).

Two of the most potent Oat2b interactors, estrone-3-sulfate and diclofenac, clearly showed dose dependent inhibition of Oat2b mediated 6-CF uptake (IC_{50} = 30.96 μM and 12.6 μM, respectively) (Fig. 11).

Finally, both of the most potent tested Oat2d interactors, fumarate and indomethacin, showed inhibition of Oat2d mediated LY uptake (IC_{50} = 68.24 μM and 20.41 μM, respectively) (Fig. 12).

4. Discussion

Membrane transporters are one of the crucial determinants in absorption, distribution, metabolism and excretion (ADME) both of physiological compounds and xenobiotic substances (Klaassen and Lu, 2008). Despite being an integral determinant of the ADME processes, organic anion transporters have not been thoroughly investigated in non-mammalian species. Therefore, using the zebrafish model in this study we present the first characterization of subfamily 2 of fish organic anion transporters. Our major goal was to obtain knowledge that can provide first insights into potential roles of Oats in relation to their physiological and/or defensive functions in zebrafish, which could be further correlated with OAT/Oat functions recognized in higher vertebrates and human.

Zebrafish Slc22 transporters share structural similarity with mammalian SLC22: they possess 12 α-helical TMDs, large extracellular LP1 and intracellular LP6, and intracellular orientation of C and N termini (Burckhardt and Wolff, 2000; Koepsell, 2011), indicating high degree of conservation of their secondary structure in vertebrates. Through extensive genome search and phylogenetic analysis of SLC22 genes, we recently identified seven organic anion transporters in zebrafish: Oat1, Oat2a, Oat2b, Oat2c, Oat2d, Oat2e and Oat3 (Mihaljevic et al., 2016). Oat2 genes have shown one-to-many orthologies, possibly as a consequence of the teleost-specific whole genome duplication (WGD). Oat2a–e have shown expression in various zebrafish tissues, with the highest levels determined in testes, kidney and intestine (Mihaljevic et al., 2016).

Conserved synteny analysis performed in this study confirmed multiple duplications of zebrafish *oat2* genes (Fig. 1). It showed double conserved synteny of five zebrafish *oat2* genes on chromosomes 11 (oat2a) and 17 (*oat2b-e*) with human *OAT2* on chromosome 6 (Fig. 1). Zebrafish has five *oat2* co-orthologs, while other fish species have less (Tetraodon – 4, cave fish – 2, stickleback – 2, cod – 1 and medaka – 1). Zebrafish *oat2a* has direct orthology with Tetraodon and cave fish *slc22a7a* genes. Therefore, gene environment of the fish *oat2* ortholog cluster is relatively conserved.

The molecular weight of zebrafish Oat2a–e is approximately 60 kDa, as shown by the Western blot analysis (Fig. 2), which corresponds to the molecular weight predicted from the amino acid sequences of each protein. Zebrafish Oat2a–e are similar in size to Oat2 proteins found in other animal species: mouse and rat Oat2s are 52–66 kDa in size (Simonson et al., 1994; Ljubojevic et al., 2007), whereas the molecular weight of human OAT2 is 59.8 kDa. Furthermore, it appears that Oat2b, d, and e, may be present in monomeric and dimeric forms (Fig. 2). The large extracellular loop of all Slc22 proteins is glycosylated, contains six conserved cysteins that may form disulfide bridges (Pelis et al., 2012), and plays a pivotal role for the oligomerization, as has been demonstrated for rat Oct1 (Keller et al., 2011) and human OCT2 (Brast et al., 2012). Among OATs/Oats, oligomerization has only been demonstrated in human OAT1, where it may depend on interactions of the TMDs 6 of two monomers (Duan et al., 2011). As oligomerization may be important for membrane trafficking (Keller et al., 2011; Brast et al., 2012), this topic remains to be addressed in more detail in follow up studies.

As HEK293 cells are not polarized and lack well defined apical and basolateral membranes, while mammalian OATs/Oats are in the expressing tissues mostly localized on basolateral membranes (Nies et al., 2011), that fact could present a problem for a correct localization in the cell membrane. Nevertheless, all five zebrafish Oat2 co-orthologs showed localization within plasma membranes of the transiently transfected HEK293 cells (Fig. 3), indicating that all transfected proteins could be functionally active in transporting compounds across plasma membranes. This result confirmed the used heterologous expression system as a suitable in vitro tool for initial characterization of zebrafish Oat2 transporters. Nevertheless, the performed live cell imaging has shown that Oat2a, Oat2c and Oat2e remain inside the cell at a

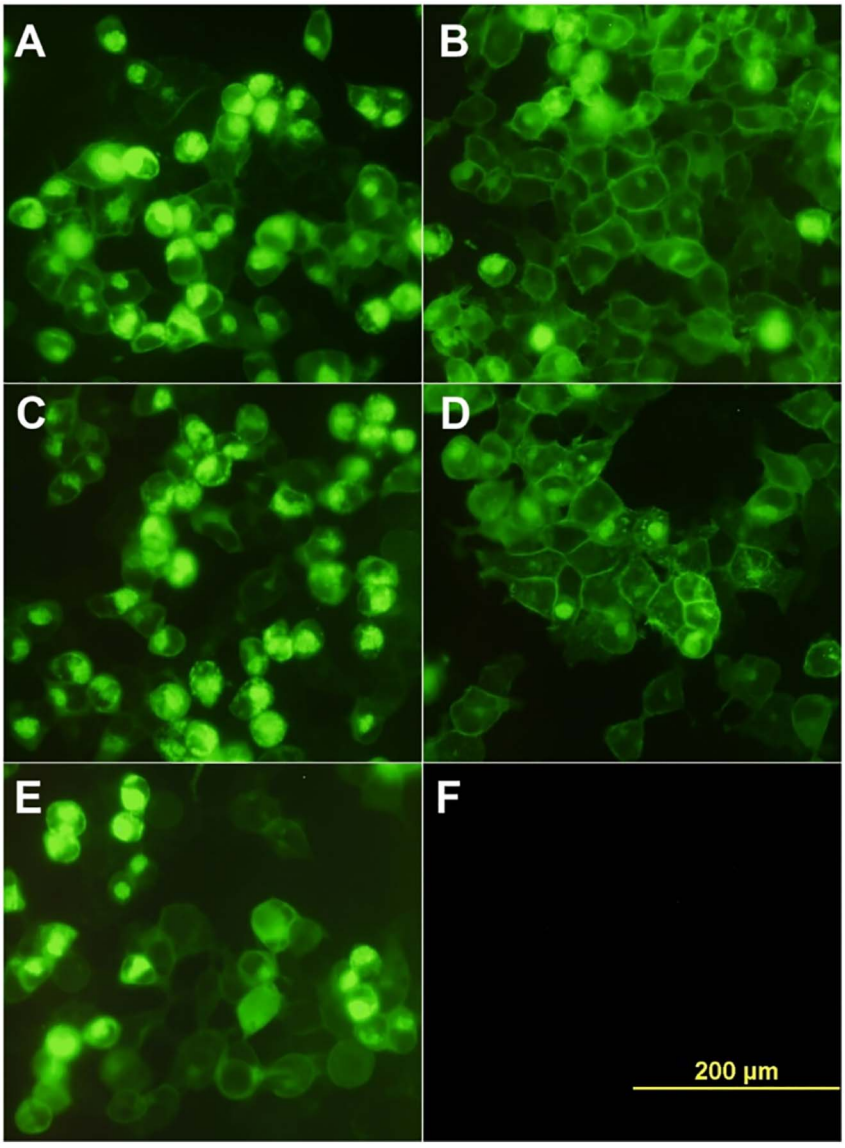

Fig. 3. Live cell imaging on HEK293T cells transiently transfected with pDest-YFP vector containing specific zebrafish *oat2* gene: A) Oat2a, B) Oat2b, C) Oat2c, D) Oat2d, E) Oat2e, and F) mock transfected cells. YFP-labeled Oat2a–e fluorescence was observed with fluorescent microscope using the FITC filter.

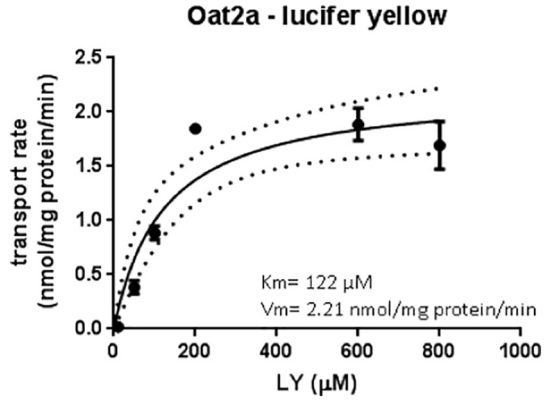

Fig. 4. Dose-response of zebrafish Oat2a transport of the fluorescent model substrate Lucifer yellow (LY) (Km = 122 µM) expressed as transport rate (nmol/mg protein/min) over LY concentration (µM) after 15 min incubation with LY. Each data point represents the mean ± SE from triplicate determinations. Dotted lines represent confidence intervals.

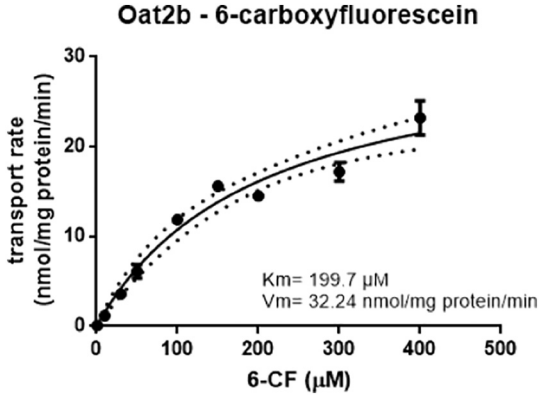

Fig. 5. Dose-response curve of the Oat2b mediated transport of fluorescent substrate 6-carboxyfluorescein (Km = 199.7 µM) expressed as transport rate (nmol/mg protein/min) over 6-CF concentration (µM) after 15 min incubation with 6-CF. Each data point represents the mean ± SE from triplicate determinations. Dotted lines represent confidence intervals.

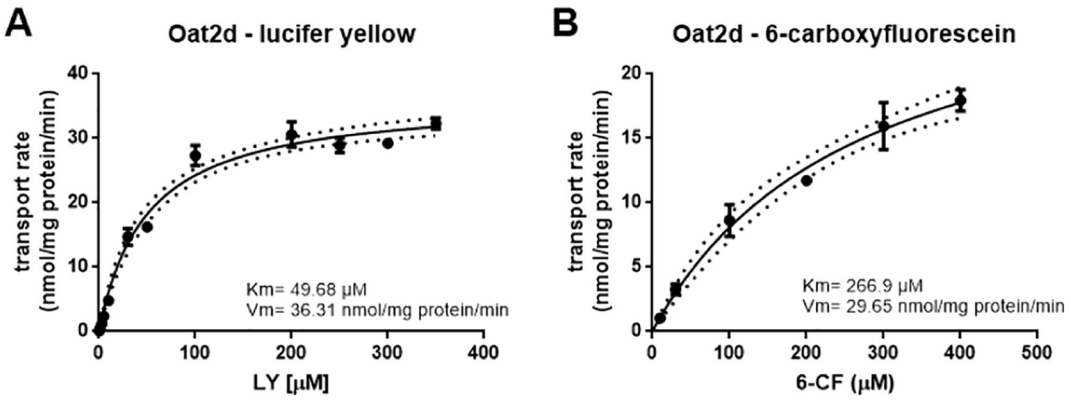

Fig. 6. Dose-response curve of Oat2d mediated transport of model fluorescent substrates: A) Lucifer yellow (Km = 49.68 µM) expressed as transport rate (nmol/mg protein/min) over LY concentration (µM) after 15 min incubation with LY; and B) 6-carboxyfluorescein (Km = 266.9 µM) expressed as transport rate (nmol/mg protein/min) over 6-CF concentration (µM) after 15 min incubation with 6-CF. Each data point represents the mean ± SE from triplicate determinations. Dotted lines represent confidence intervals.

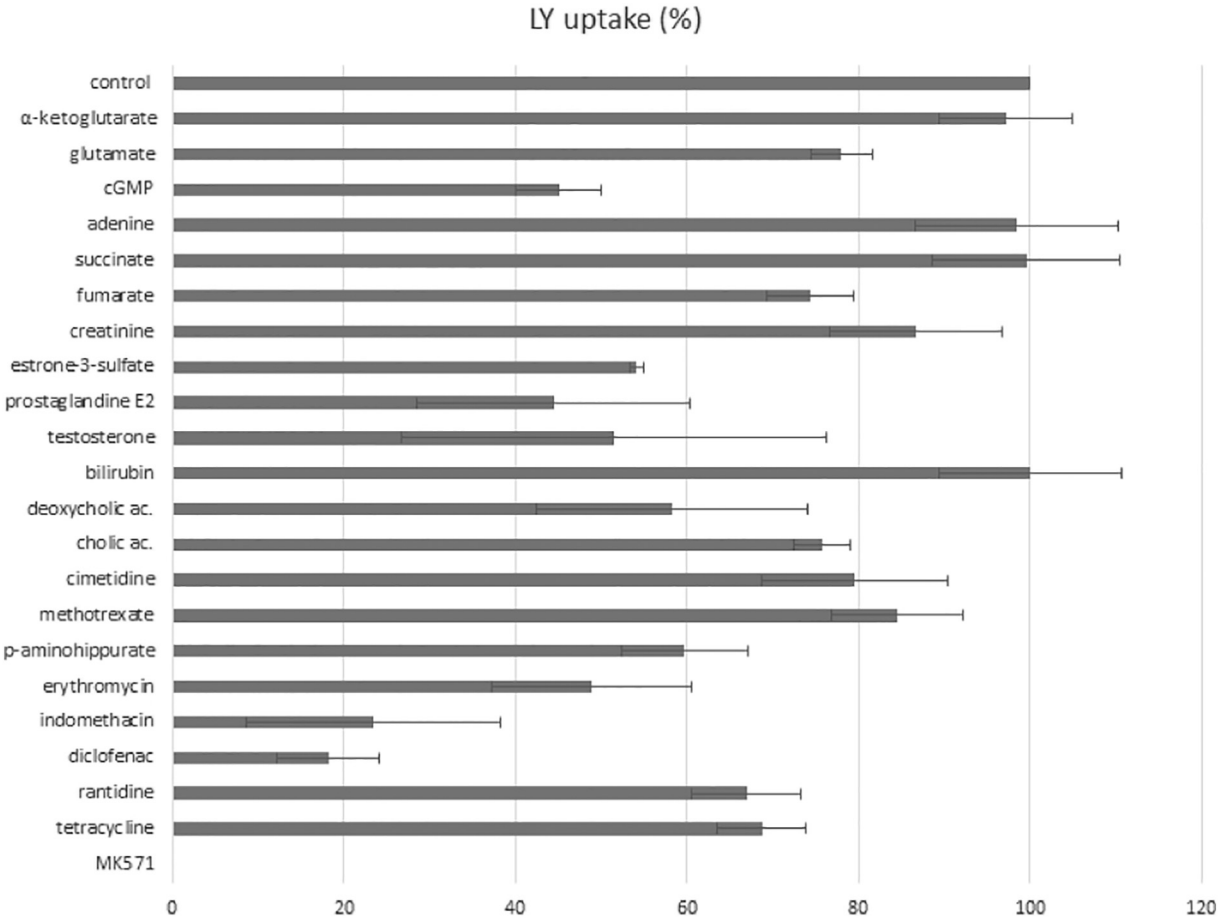

Fig. 7. Interaction of zebrafish Oat2a with known substrates and inhibitors of mammalian OAT2/Oat2 subfamily members. Data are expressed as mean percentage (%) ± SD from triplicate determinations of LY uptake after co-incubation with each interactor (100 µM) relative to LY uptake in the absence of interactor which is set to 100%.

higher proportion when compared to Oat2b and Oat2d, probably as a result of either protein processing in the cytosol or in the ER/Golgi system. Alternatively, the proteins could be partly retained in intracellular membranous compartments where they could transporting their substrates (Hotchkiss et al., 2015).

In the next step of our study we aimed at identifying model fluorescent substrate(s) of zebrafish Oats that would enable development of a high-throughput screening protocol, enabling identification of interactors and a detailed in vitro characterization of substrate preferences of these transporters. We tested a range of commercially available anionic dyes: fluorescein, 5- and 6-carboxyfluorescein (5CF and 6CF), 2′,7′-dichlorofluorescein, 4′,5′-dibromofluorescein, Lucifer yellow (LY), resorufin, resazurin, calcein and eosin Y. Two of the tested fluorescent dyes were shown to be substrates for three organic anion transporters in zebrafish: LY uptake was mediated by Oat2a and Oat2d, whereas 6CF was transported by Oat2b and Oat2d. The highest

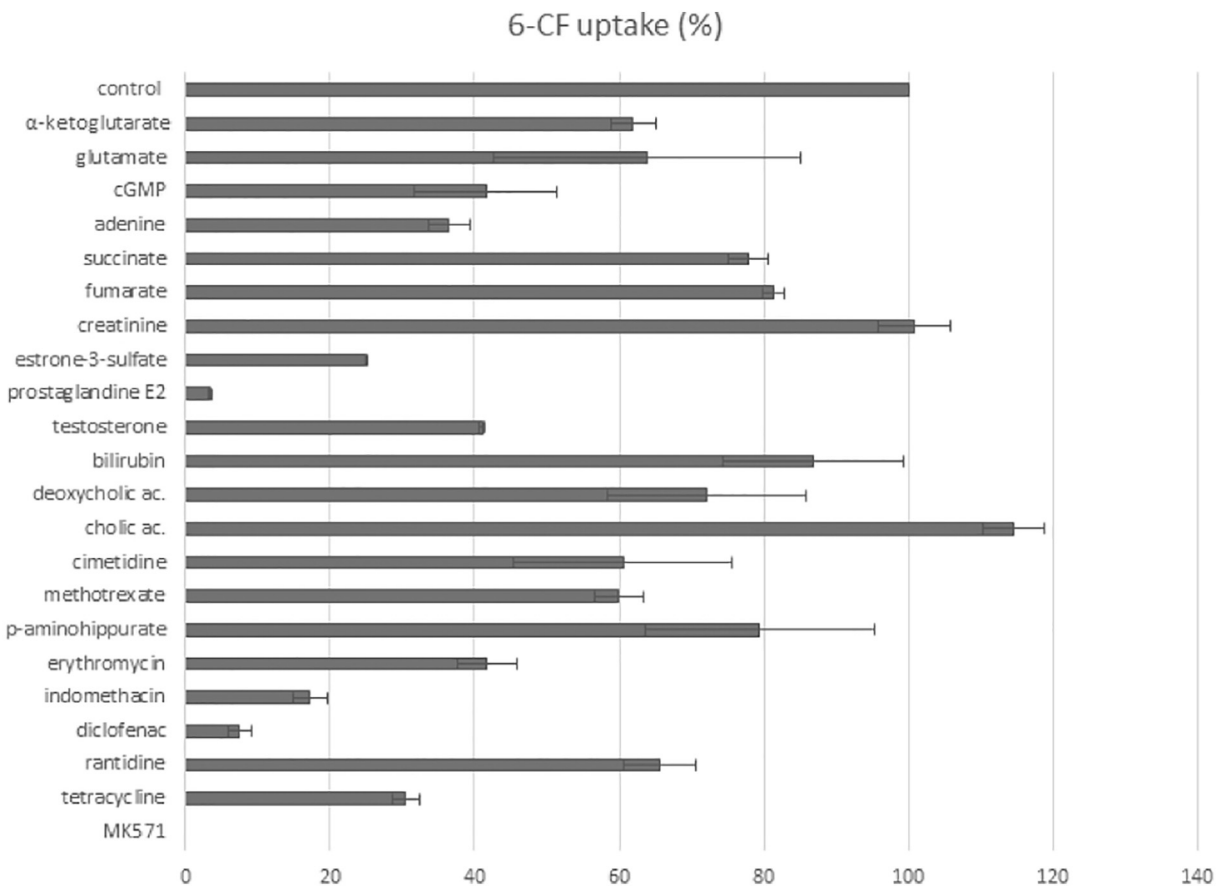

Fig. 8. Interaction of zebrafish Oat2b with known substrates and inhibitors of mammalian OAT2/Oat2 subfamily members. Data are expressed as mean percentage (%) ± SD from triplicate determinations of 6-CF uptake after co-incubation with each interactor (100 μM) relative to 6-CF uptake in the absence of interactor which is set to 100%.

substrate affinity was detected between Oat2d and LY (Km = 49.68 μM, Fig. 6.A). After the determination of transport kinetics for fluorescent dyes, these model fluorescent substrates were used in the inhibition assays based on co-exposure of transfected cells and non-transfected control with determined model substrate and potential interactor.

Our initial screening assay revealed interaction of Oat2a, b, and d with numerous endo- and xenobiotics, confirming polyspecific properties of related transporters (Figs. 7, 8 and 9). Among potential physiological substrates, Oat2a and Oat2b showed strong interaction with prostaglandin E_2 (44.43% and 3.38% uptake of the model substrate, respectively). Prostaglandin E_2 was identified as a substrate for human OAT2 with Km value of 0.71 μM (Enomoto et al., 2002a, 2002b; Kimura et al., 2002). Furthermore, HEK-293 T cells possess endogenous multidrug resistance proteins MRP2 and MRP4 (Cheung et al., 2014) which may extrude the prostaglandins accumulated by Oats. Therefore, MK571, an established cysteinyl leukotriene-based inhibitor of MRPs, was used as a model MRP inhibitor by Henjakovic et al. (2015), but it turned out to be a potent inhibitor of human OAT2 as well. Similarly, in our study MK571 has shown the highest inhibition of all three zebrafish Oats among all tested xenobiotics, resulting in complete inhibition of the LY transport (i.e. 0% uptake).

Previous reports have identified cGMP as a possible physiological substrate for human OAT1, OAT2, and OAT3 (Cheng et al., 2012; Cropp et al., 2008). Importantly, interaction with cGMP was confirmed for all three zebrafish Oats (Oat2a = 45.02%, Oat2b = 41.55% and Oat2d = 89.27% uptake of the model substrate). The determined uptake of cGMP via Oats could prevent loss of this intracellular second messenger and prolong its action. Additionally, Oats may also clear cGMP from blood by uptake into cells and subsequent release into the tubular lumen via MRP2 and MRP4 (Henjakovic et al., 2015). It has also been discussed that increased renal cGMP levels may have an antifibrotic benefit (Schinner et al., 2015).

A 19-carbon androgen, testosterone, has shown interaction with zebrafish Oats with percentage of the model substrate uptake of 41.04, 51.47 and 85.93 for Oat2b, Oat2a and Oat2d, respectively. Oat2a, b, and d are all expressed in zebrafish testes, and this result may suggest their possible role in transporting this steroid hormone in male zebrafish. Additionally, it was observed that testosterone treatment can cause a remarkable decrease of Oat2 protein expression in castrated male rats (Ljubojevic et al., 2007) and mice (Buist and Klaassen, 2004; Cheng et al., 2008).

Among xenobiotic compounds, besides MK571 which showed a complete inhibition of zebrafish Oats transport, the strongest interaction was observed with nonsteroidal anti-inflammatory drugs diclofenac and indomethacin, and antibiotics tetracycline and erythromycin. Human OAT2 was shown to transport a number of pharmacologically active agents, and has been increasingly recognized in terms of its role in drug disposition (Shen et al., 2016). Out of these four pharmaceuticals, three have been well characterized as substrates of human OAT2: erythromycin and tetracycline (Babu et al., 2002, Kobayashi et al., 2005, Yee et al., 2013), and diclofenac (Zhang et al., 2016). Indomethacin has been identified as a potent inhibitor of human OAT2 (IC_{50} values 2.1 to 6.5 μM) (Shen et al., 2015, Zhang et al., 2015), OAT1 (IC_{50} values 3 to 10 μM), and OAT3 (IC_{50} values 0.61 to 5.95 μM) (Khamdang et al., 2002). H-2 receptor antagonist cimetidine (CMD) has

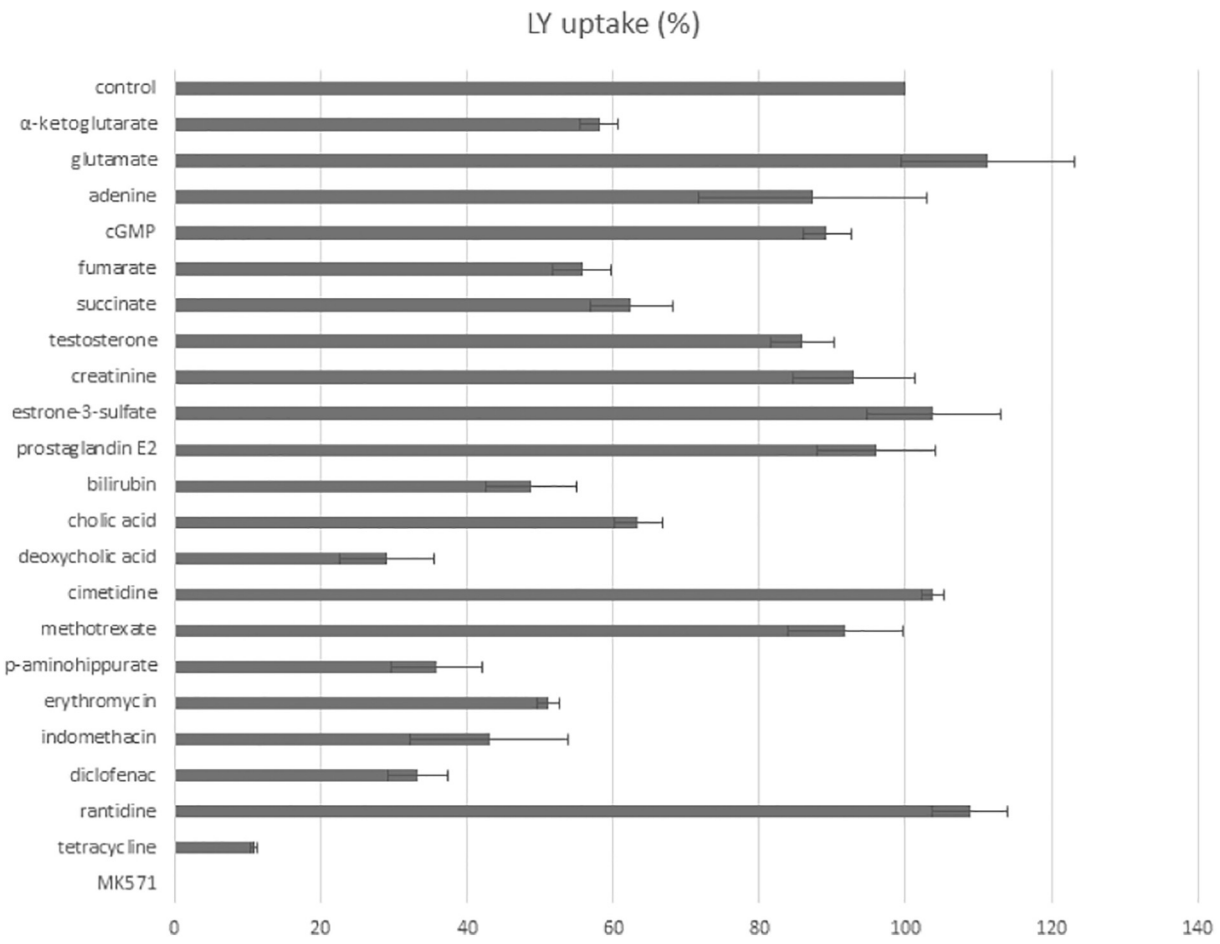

Fig. 9. Interaction of zebrafish Oat2d with known substrates and inhibitors of mammalian OAT2/Oat2 subfamily members. Data are expressed as mean percentage (%) ± SD from triplicate determinations of LY uptake after co-incubation with each interactor (100 µM) relative to LY uptake in the absence of interactor which is set to 100%.

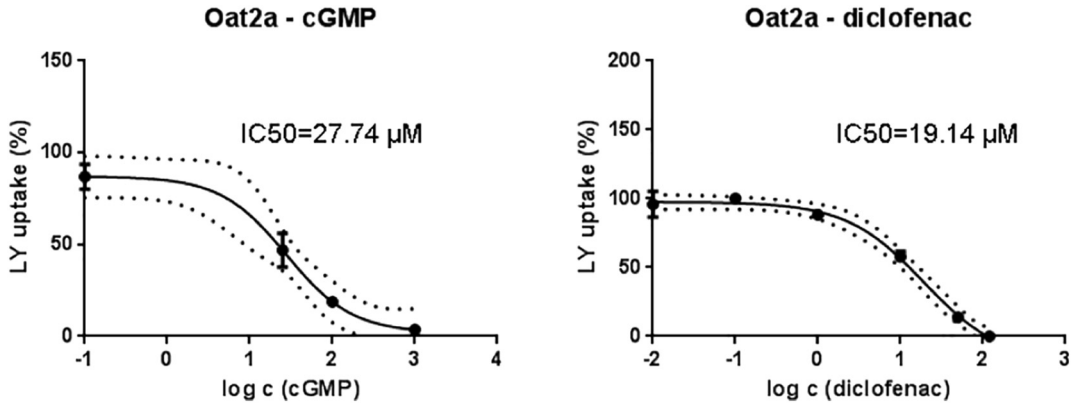

Fig. 10. Concentration dependent inhibition of Oat2a mediated LY uptake by cGMP and diclofenac (µM). Values on X-axis were transformed to logarithmic scale (log X). Each data point represents the mean ± SD from triplicate determinations. Dotted lines represent confidence intervals.

been described as a human OAT2 substrate by Tahara et al. (2005). In this study, CMD only inhibited Oat2b transport (60.55% of 6-CF uptake), while it had no effect on Oat2a and Oat2b transport (Figs. 7, 8 and 9).

In conclusion, the literature describing OAT2/Oat2 is rapidly evolving, with numerous contradicting publications regarding the transport mechanism, tissue distribution, and substrate preference. These differences can be partly attributed to various experimental systems and protocols used as well as to more than one transcript variants used in studies with this transporter (e.g., there are 3 transcript variants for human OAT2). This study provides the first comprehensive data set on Oat2 transporters in zebrafish as an important vertebrate model species. It also reveals similarities and differences in comparison to human and mammalian orthologs and confirms their broad ligand selectivity. Finally, we have identified new model substrates for three zebrafish Oat2 proteins, and standardized high throughput in vitro assays for identification of their interactors. Based on these initial insights and the assays developed, the putative physiological and/or defensive role of zebrafish Oats can be more specifically addressed in follow-up studies through detailed functional characterization of single transporter(s) in suitable expression systems, and by zebrafish functional genomics studies using targeted gene knockdowns.

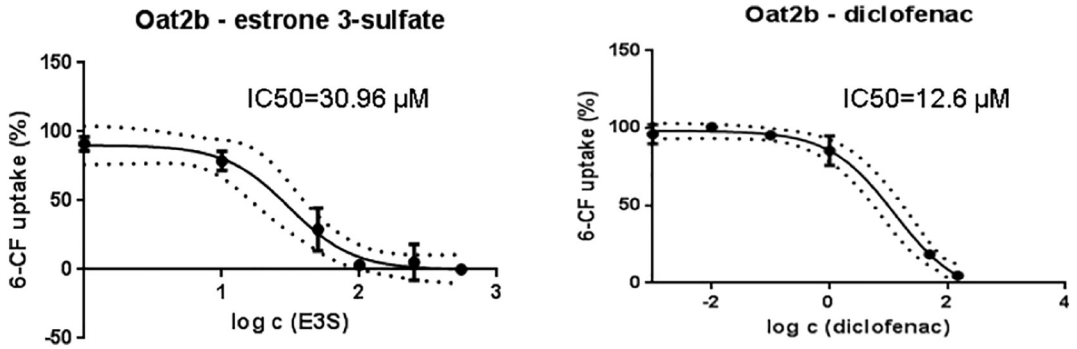

Fig. 11. Concentration dependent inhibition of Oat2b mediated 6-CF uptake by estrone-3-sulfate and diclofenac (µM). Values on X-axis were transformed to logarithmic scale (log X). Each data point represents the mean ± SD from triplicate determinations. Dotted lines represent confidence intervals.

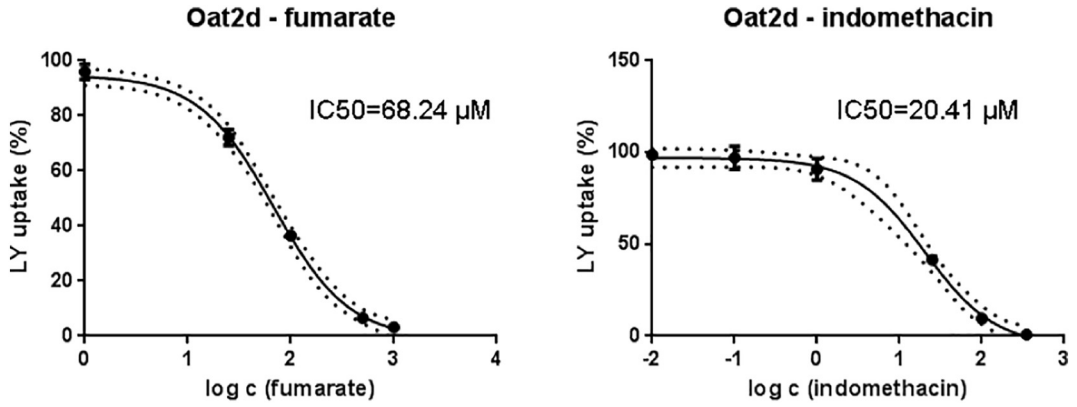

Fig. 12. Concentration dependent inhibition of Oat2d mediated LY uptake by fumarate and indomethacin (µM). Values on X-axis were transformed to logarithmic scale (log X). Each data point represents the mean ± SD from triplicate determinations. Dotted lines represent confidence intervals.

Transparency document

The Transparency document associated with this document can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2017.09.026.

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