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Interaction of environmental contaminants with zebrafish (*Danio rerio*) multidrug and toxin extrusion protein 7 (Mate7/Slc47a7)

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Abstract

Zebrafish Mate7 belongs to solute carrier protein superfamily and specifically to subfamily of multidrug and toxin extruders. It is co-orthologous to mammalian Mates, and is ubiquitously expressed in zebrafish tissues with the highest expression in kidney. It has been shown to interact with both endogenous (steroid hormones) and xenobiotic compounds (pharmaceuticals), implying its role in efflux of toxic compounds. The objective of our study was to analyse interaction of environmental contaminants with zebrafish Mate7 using a newly developed high throughput screening (HTS) Mate7 assay. A full-length zebrafish *mate7* sequence was obtained from zebrafish cDNA originating from male kidney, and a stable expression of Mate7 in genetically engineered HEK293 Flp-In cells was achieved. Stable Mate7 transfectants were then used for development and optimization of a new HTS cellular uptake protocol, with DAPI and ASP+ as model fluorescent substrates. The developed assay was used for identifying zebrafish Mate 7 interactors and discerning the type of interaction. A series of 89 diverse environmental contaminants, including industrial chemicals, pesticides, and pharmaceuticals, was tested and highly effective Mate7 interactors were identified in all of the

aforementioned groups. Some of the inhibitors identified could be of environmental concern because they may potentially impair Mate7 efflux function, lowering the fish defence capacity against environmental contaminants, or interfering with transport of yet unidentified physiological substrates. In addition, we found significant differences between zebrafish Mate7 and mammalian Mates' substrate preferences, a finding that should be taken into consideration when using zebrafish as a model organism in toxicokinetics studies.

Key Words: Mate7, environmental contaminants, high throughput screening assay, HEK293 Flp-In cells

Introduction

Trafficking of compounds through cells and tissues is significantly determined by passive diffusion and active uptake and efflux. After a xenobiotic compound has entered the cell it is metabolized by phase I and II detoxification enzymes and eventually eliminated from the cell via efflux transporters. MATE (multidrug and toxin extrusion) proteins, together with ABC transporters have been shown to constitute an important part of the mammalian cellular detoxification system. They function as efflux transporters that mediate predominantly the elimination of cationic compounds (e.g., metformin, cimetidine, MPP+, TEA) but can also transport anionic (E3S) and zwitterionic (e.g., cephalexin, cefradine) compounds (Damme et al., 2011).

MATE/Mate proteins (MATEs in humans and mice, Mates in all other species; gene name *SLC47* in humans, *Slc47* in mice, and *slc47* in all other species) belong to the superfamily of solute carriers (SLCs). They function as bidirectional transporters, with the efflux of substrates linked to the proton-coupled electroneutral exchange (Tsuda et al., 2007). MATEs/Mates are 400–600 amino acids long and consist of 12–13 transmembrane domains (TMDs) with an intracellular N terminus and intracellular or extracellular C terminus (Zhang and Wright, 2009; Zhang et al., 2012). There are three functional MATE proteins in humans (MATE1, MATE2 and MATE2-K) (Otsuka et al., 2005; Masuda et al., 2006), two in mice (MATE1a and MATE2) (Hiasa et al., 2006; Hiasa et al., 2007; Kobara et al., 2008), rats (Mate1 and Mate2) (Ohta et al., 2006; Hiasa et al., 2007), and rabbits (Mate1 and Mate2-K) (Zhang et al., 2007).

In our previous work we have shown that zebrafish (*Danio rerio*) has 6 *mate* genes grouped in two clusters of 3 genes each that probably arose through teleost specific whole genome duplication (Loncar et al., 2016). Zebrafish *mate* clusters are co-orthologous to human *MATEs*, but 1:1 cluster to gene orthology could not be determined since zebrafish *mates* are more similar within the group than with either of human *MATEs*. Mates are expressed in developing zebrafish embryos and in the tissues (kidney, liver, intestine, brain, gonads, gills and eye) of adult individuals where they interact with both physiological (steroid hormones, thiamine) and xenobiotic (pharmaceuticals, pesticides) compounds (Loncar et al., 2016). Although zebrafish is an emerging model in both clinical and in environmental pollution research, studies on interaction of zebrafish Mates with xenobiotics are still scarce. Therefore, in this study we aimed at testing a wide range of xenobiotic substances

(industrial chemicals, pesticides, and pharmaceuticals) for their interaction with Mate7 as potentially ecotoxicologically important fish efflux transporter. *Mate7* was shown to be present in embryos and in adult zebrafish likewise, with notably high expression of *mate7* transcripts in kidney and brain of adult fish, as well as in the early embryonic stages. Furthermore, it has been shown to transport multiple cationic dyes, and interact both with endo- and xenobiotics (Loncar et al., 2016). To efficiently address the main goal of the study, we have firstly developed a stable Mate7 over-expressing HEK293 Flp-In cell line. Next, to obtain a high throughput screening (HTS) assay a previously developed cellular uptake protocol was further modified. A series of 89 substances, selected on the basis of their occurrence in surface waters and/or reported interaction with mammalian Mates (Fent et al., 2006; Murray et al., 2010; Ivanyuk et al., 2017), were then screened for interaction with zebrafish Mate7. Compounds that showed significant interaction potency, determined as inhibition of the model fluorescent substrate (DAPI) uptake, were further characterized for the type of interaction, i.e. whether they are substrates or inhibitors of zebrafish Mate7. Finally, interaction specificities of mammalian versus zebrafish Mate7 were discussed.

Materials and Methods

Chemicals

All tested compounds, model fluorescent substrates and interactors alike, were purchased from Sigma-Aldrich (Taufkirchen, Germany) or Alfa Aesar (Ward Hill, MA, USA) unless stated otherwise.

Cloning and development of the Mate7-expressing cell line

A full-length zebrafish *mate7* sequence (NM_001302254.1) was obtained from zebrafish cDNA originating from male kidney by polymerase chain reaction using high fidelity Phusion DNA polymerase (Finnzymes, Vantaa, Finland) and specifically designed primers with HindIII and EcoRI restriction sites in the forward, and NotI and SmaI restriction sites in the reverse primer. An amplified DNA fragment was cloned into a pcDNA3.1/His vector (Thermo Fisher Scientific, Waltham, USA) linearized with combination of restriction enzymes HindIII/NotI that also excised the His tag sequence from the vector. The sequence was verified by DNA sequencing using automated capillary electrophoresis (ABI PRISM® 3100-Avant Genetic Analyzer) at the

Ruđer Bošković Institute DNA Service (Zagreb, Croatia). Three gene clones were sequenced and compared to the reported gene sequences from the NCBI and ENSEMBL databases. The verified *mate7* sequence was used for further subcloning.

A stable expression of Mate7 in genetically engineered HEK293 Flp-In cells (Thermo Fisher Scientific, Waltham, USA) was achieved using targeted integration of the *mate7* sequence subcloned into integration vector pcDNA5/FRT (Thermo Fisher Scientific, Waltham, USA) with combination of restriction enzymes HindIII/XhoI. pcDNA5/FRT/Mate7 constructs were specifically targeted into the genome of the Flp-In^{TM}-293 cells following the manufacturer's instructions. In short, 375 ng of the pcDNA5/FRT/Mate7 construct was co-transfected with the helper plasmid pOG44 in a 1:9 ratio (3,375 ng of DNA) with polyethylenimine (PEI) as transfection reagent (PEI:DNA = 1:1) in the cells seeded 48 h before transfection in a 6-well plate at concentration 7.5 x 10^5 cells/well. After 48 h, the cells were transferred (without trypsinization) to a 25 cm^2 cell culture flask and grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum. Cells were left to attach overnight. Hygromycin B was added in the morning to the final concentration of 100 µg/ml. The medium was changed every 3-4 days and hygromycin B selection continued until single colonies appeared (after approximately 25-30 days). Finally, Mate7 stable transfectants were functionally verified by determination of the uptake rate of model fluorescent substrates in comparison to mock-transfected cells.

Cellular uptake experiments

The cellular uptake experiments with FlpIn/Mock and FlpIn/drM7 cells were performed in 96-well plates. The cells were seeded in high glucose DMEM supplemented with 10% FBS, at density sufficient to reach confluence after 48 h cultivation (75,000 cells/well) after which the uptake assay was performed as follows. The medium was aspired and 100 µL (for inhibition assays) or 125 µL (for dye uptake determinations) of Na-based transport buffer (145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose and 5 mM Hepes or 10 mM Tris, pH 7.4 or 8.4) was added. For determination of the uptake of fluorescent model substrates, 25 µL of dye solution was added to the reaction mixture to reach the desired final concentration. In the case of DAPI and ASP+, real time uptake kinetics were then monitored for 5-10 minutes. As uptake kinetics of Rh123, amiloride and berberine cannot be measured in real time, 5 min uptake accumulation was determined after the uptake

112 of dye was stopped by rapid washing (2x) with ice cold PBS, followed by the cells lysis with 0.1% SDS for 30
113 min.
114 For intracellular acidification experiments, the cells were incubated for 20 minutes with 100 µL/well of 20 mM
115 NH_4Cl which was aspired prior to dye uptake measurement carried out as described above. Fluorescent signals
116 for various model substrates were read at following excitation and emission wavelengths: DAPI, 360/460 nm;
117 ASP+, 450/590 nm; Rh123, 485/530 nm; berberine, 355/540 nm; amiloride, 366/418 nm. The uptake into
118 mock-transfected cells that received empty vector was subtracted to obtain transporter-specific uptake.
119 The calibration curve for DAPI was obtained in the presence of a cell matrix or pure DNA generating similar
120 curves. Calibration curves for all other substrates were generated in 0.1% SDS and in the cell matrix dissolved
121 in 0.1% SDS, except for ASP+ where cell matrix without detergent was used. Resulting linear calibration curves
122 were the same in the SDS (where applied) and in the dissolved cell matrix. Using the obtained calibration
123 curves, corresponding uptake rates (uptake velocities) were expressed as pmol of substrate per mg of protein
124 per minute. Total cell protein content was determined according to the Bradford method.
125 Inhibition assays using DAPI or ASP+ as fluorescent substrates were performed in the same transport medium
126 as for the substrate uptake experiments. The applied concentrations of both DAPI and ASP+ were 2 µM. Taking
127 into consideration their solubility in water, the compounds tested for interaction were initially tested at
128 concentration of 100 µM unless stated otherwise. When interacting compounds showed inhibition of DAPI
129 uptake above 30%, which was set as an arbitrary threshold that indicates significant interaction, their
130 concentration-response curves were obtained, and related IC_{50} values were determined. Compounds with IC_{50}
131 of < 1 µM were designated as very strong (VSt) interactors, IC_{50} of 1-29 µM indicated strong (St) interaction
132 strength, interactors with IC_{50} of 30-99 µM were designated as moderate interactors, IC_{50} of 100-999 µM was
133 classified as weak interaction strength, and interactors with IC_{50} above 1,000 µM were considered as very
134 weak interacting substances.
135 To determine type of interaction for Mate7 interacting compounds, shift in K_m and V_{max} values for DAPI in the
136 presence of interacting compound (at the concentration equal to the IC_{50} value of the compound) at varying
137 concentrations of DAPI was measured. Experiments done in triplicate were repeated at least three times and

the uptake into mock cells was subtracted to obtain the transporter specific uptake. DAPI uptake followed Michaelis-Menten type kinetics, where K_m increase and no change in transport rate in presence of an interactor indicated competitive inhibition (i.e. the substance is identified as a substrate). Contrary to that pattern, no change in K_m and V_{max} decrease indicated non-competitive inhibition. Substrate affinity (K_m) was considered to be very high for K_m < 1 µM, high for K_m 1-29 µM, moderate for K_m 30-99 µM, and low for K_m > 100 µM.

Cytotoxicity assays

Cytotoxicity was determined using the MTT reduction assay adapted according to the Mosmann's procedure (Mosmann, 1983). The FlpIn/Mock and FlpIn/drM7 cells were seeded in 96-well plates and exposed to a range of concentrations of tested chemicals for 72 h, except for the rescue experiments were cells were exposed for 1 hour. Tested compounds were then removed, the cells washed in PBS and incubated for 72 h in DMEM supplemented with 10% FBS. Subsequently, the medium was removed and the cells were incubated for 3 h with 0.5 mg/ml MTT (100 µl/well) dissolved in DMEM. The formazan salts were dissolved in isopropanol (15 min with shaking) and the absorbance was read at 578 nm using 750 nm as a reference wavelength. Cytotoxicity was expressed as the percentage of product formed by mitochondrial activity with respect to the corresponding control (untreated cells) level.

Data analysis

All assays were performed in 3-5 independent experiments run in triplicates. Data shown on related figures represent mean ± standard errors (SE) or standard deviations (SD). All calculations were performed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA) as described below. The kinetic parameters, Km and Vmax values were calculated using the Michaelis-Menten equation (1):

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

where V is velocity (picomoles of substrate per milligram of proteins per minute), V_{max} is maximal velocity, [S] is substrate concentration and K_m is the Michaelis-Menten constant. The uptake into vector-transfected HEK293 cells was subtracted to obtain transporter-specific uptake.

For the purpose of IC_{50} calculations, data were fitted to the sigmoidal four-parameter dose-response model (variable slope), according to equation (2):

$$V = V_{min} + \frac{V_{max} - V_{min}}{1 + 10^{(logIC_{50} - A) \times h}} \quad (2)$$

where V is response, V_{min} represents minimum of response, V_{max} represents maximum of response, h is Hill slope parameter, IC_{50} is the concentration of inhibitor that corresponds to 50% of maximal effect, and A is the concentration of a tested compound.

Results

Kinetic parameters of Mate7 fluorescent substrates

Basic kinetic parameters were obtained for five fluorescent Mate7 substrates. The uptake of all five substrates followed the classical Michaelis-Menten kinetics and the obtained values (Table 1) are in good correlation with results obtained using the transient assay system (Loncar et al., 2016). However, only DAPI and ASP+ were selected as model fluorescent zebrafish Mate7 substrates for further experiments in this study as they enable real time uptake measurements more suitable for HTS requirements (Fig. 1).

Table 1. Kinetic parameters, K_m (µM) and V_{max} (pmol/mg protein/min) of the zebrafish Mate7 mediated uptake of fluorescent dyes. DAPI – 4',6-diamidino-2-phenylindole; ASP+ – 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide; Rh123 – rhodamine 123. Each value represents mean from at least three independent experiments with 95% confidence intervals (ci) stated. Related dose-response curves are shown in Figs. 1B and D, and Figs. S1A-C.

	K_m (μM)	95% ci	V_{max} (pmol/min/mg)	95% ci	N
DAPI	1.46	1.13-1.79	1092	1010-1174	27
ASP+	0.98	0.48-1.48	58.3	49.8-66.8	4
Rh123	0.29	0.14-0.45	8.14	6.70-9.58	3
Amiloride	3.02	0.99-5.06	20.9	16.7-25.0	4
Berberine	0.91	0.34-1.50	24.3	20.0-28.5	5

Probing the Mate7 substrate binding site

Kinetic experiments performed with varying concentrations of DAPI or ASP+ in the presence of other known fluorescent substrates showed that type of interaction in all combinations was a competitive inhibition (Figs. 2 and S2, and Tables 2 and S1). Accordingly, toxicity experiments (Table 3) clearly confirmed that Mate7 stable transfectant cells were more sensitive to toxic effects of the substrates applied than mock cells, due to the Mate7 active transport of respective substrates. Altogether, these data show that all dyes are transported through one and the same Mate7 binding site.

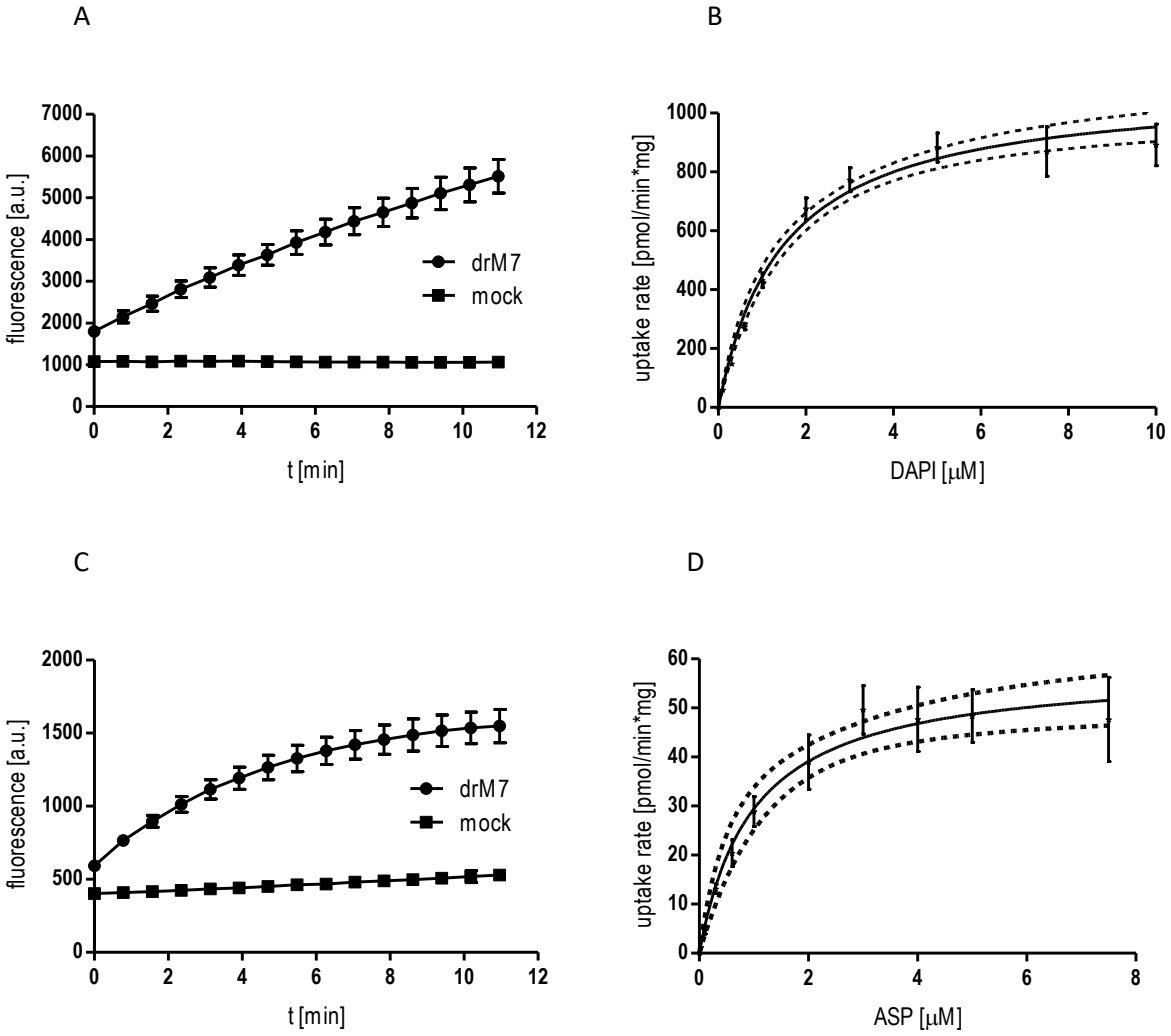


Figure 1. Michaelis-Menten kinetics of the uptake of fluorescent dyes 4',6-diamidino-2-phenylindole (DAPI), and 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP+) by zebrafish Mate7. **A –** time course of DAPI cellular accumulation; **B –** concentration dependence of DAPI uptake during 3-4 minutes of kinetic measurement; **C –** time course od ASP+ cellular accumulation; **D –** concentration dependence of ASP+ uptake during 3 minutes of kinetic measurement. The uptake into HEK293 Flp-In cells (mock cells) was subtracted to obtain transporter-specific uptake. Each value represents the mean + SE from at least three independent experiments.

Table 2. Determination of the type of interaction with DAPI as substrate and fluorescent dyes as interactors. Kinetic parameters of DAPI uptake are given as apparent K_m ($K_{m,app}$) and apparent V_m ($V_{m,app}$) with 95% confidence intervals (ci). A representative example of at least three independent experiments, each done in triplicate, is shown. For the IC_{50} calculations data from at least three independent experiments were fitted to the sigmoidal four parameters dose-response model (variable slope) in the GraphPad Prism 5.

	IC_{50}[µM], n=3	95% ci	$K_{m,app}$ [µM]	95% ci	$V_{m,app}$ [pmol/min*mg]	95% ci	TOI
DAPI	NA	NA	0.96	0.59-1.33	963	852-1075	NA
ASP^+	7.10	5.88-8.48	3.41	1.79-5.01	948	729-1166	S
Rh123	0.23	0.22-0.25	1.75	1.40-2.10	820	758-885	S
Berberine	0.82	0.75-0.90	1.70	1.37-2.03	1007	937-1077	S

TOI – type of interaction; S – substrate; NA – not applicable

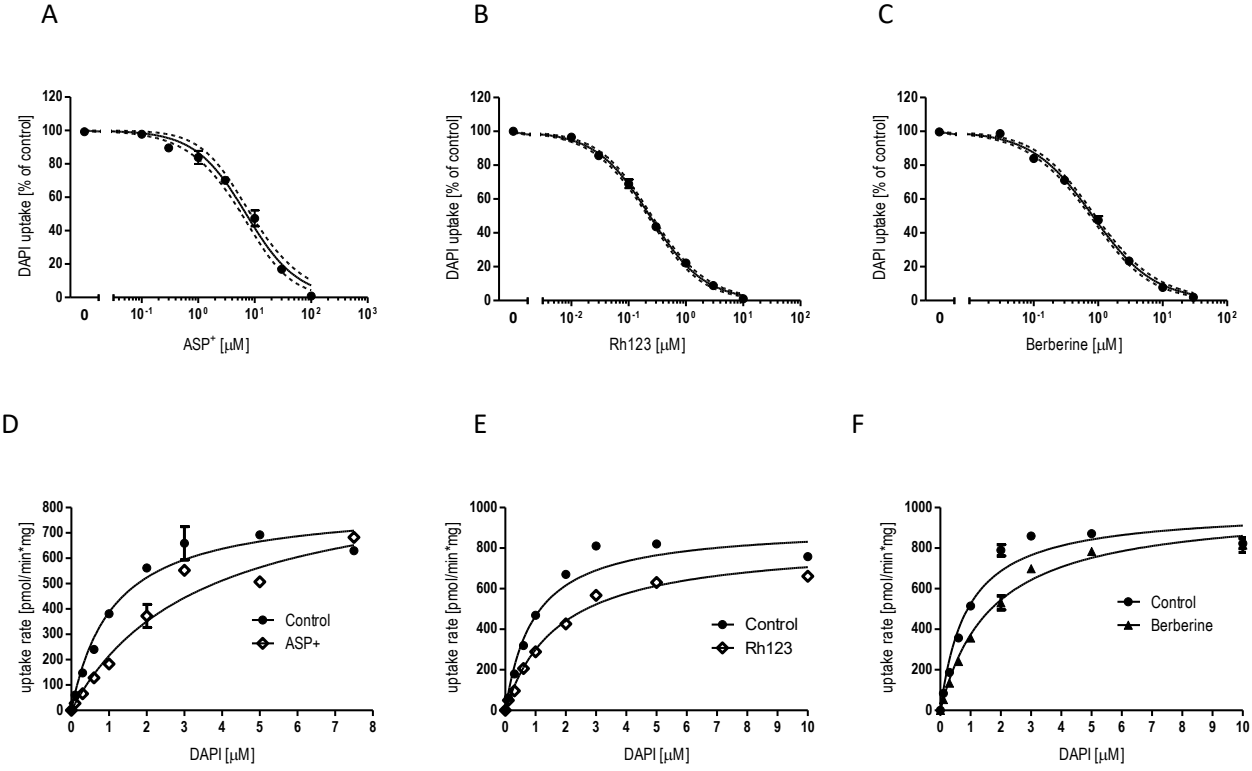


Figure 2. Concentration dependent inhibition (A-C) and determination of the type of interaction (D-F) of Mate7 mediated 4',6-diamidino-2-phenylindole (DAPI) uptake by fluorescent dyes ASP+ (A, D), Rh123 (B, E), and Berberine (C, F). The specific uptake of 2 µM DAPI is expressed as percentage relative to the DAPI uptake in the absence of an interactor (which is set to 100 %). Determination of the type of interaction was done through comparison of apparent kinetic parameters ($K_{m,app}$; $V_{m,app}$) of inhibited (IC_{50} value of respective fluorescent dye) versus uninhibited (control) reaction obtained by measuring uptake of increasing concentrations of DAPI. Each data point for A, B and C represents the mean ± SE from at least three independent experiments, while for D, E and F each data point is a mean ± SD from triplicate of a representative of at least three independent experiments.

Table 3. Determination of the type of interaction and sensitivity factors through modulation of cytotoxicity of Mate7 fluorescent substrates. Discrimination of the type of interaction (TOI) and calculation of sensitivity factors (SF) is based on the assumption that stably transfected Mate7 cells are more sensitive to toxic effects of the substrates applied, as determined by the MTT assay, in comparison to mock cells due to the Mate7 active transport of respective substrates inside of the cells. For the EC_{50} calculations, data from a representative experiment (done in triplicate) out of at least three independent experiments were fitted to the sigmoidal four-parameter dose-response model (variable slope) in GraphPad Prism version 5.

	mock		drM7			
	EC_{50} [µM]	95% ci	EC_{50} [µM]	95% ci	TOI	SF
ASP^+	7.57	5.92 - 9.67	2.30	1.84 - 2.86	S	3.3
Rh123	1.58	0.98-2.52	0.46	0.36-0.57	S	3.5
Berberine	16.5	7.34-36.9	2.95	1.29-6.71	S	5.6
DAPI	3.65	2.33-5.72	0.17	0.14-0.21	S	21.4

TOI – type of interaction; ci – confidence intervals; SF – sensitivity factor calculated as a ratio of EC_{50} values obtained for mock versus Mate7 transfected cells

Interaction of xenobiotic compounds with Mate7

A total of 89 chemicals were initially screened for interaction with zebrafish Mate7 (Fig. 3). Compounds were selected on the basis of their occurrence in surface waters, and/or reported interaction with mammalian Mates, and/or common usage in medical treatments. Substances (30 compounds) that showed DAPI uptake inhibition of at least 30% were further analysed to obtain IC_{50} values and to determine their type of interaction with zebrafish Mate7 (Table 4). Type of interaction is further supported with toxicity data, as summarized in Table 5.

Industrial chemicals

Very strong (TPenA), strong (TBA) and moderate (TPrA) interaction with zebrafish Mate7 was obtained for quaternary ammonium salts. TPenA is a strong non-competitive inhibitor (Table 4 and 5) while TPrA and TBA are moderate and strong competitive inhibitors with IC_{50} values of 60 µM and 6 µM, respectively.

Organotin compounds were shown to be strong to moderate non-competitive inhibitors with IC_{50} values of 60, 11 and 71 µM for triethyltin, tripropyltin and tributyltin, respectively (Table 4). Type of interaction for organotins was further supported with toxicity assay results, i.e. no difference in toxicity response has been obtained in transfected versus mock transfected cells upon exposure to organotins (Table 5). Bisphenol A showed moderate non-competitive inhibition of zebrafish Mate7 uptake. Selected antioxidant, phthalates, perfluorates and triazole did not show interaction with Mate7 at the chosen concentration of 100 µM under selected experimental conditions.

Pesticides

Tested pesticides either showed weak interaction (diazinon, atrazine,) or did not show any interaction at the selected 100 µM concentrations. Although atrazine showed stronger inhibition of DAPI uptake at 100 µM than diazinon, IC_{50} value for atrazine could not be obtained due to solubility issues at higher concentrations.

Pharmaceuticals

We have tested a wide range of pharmaceuticals (Figure 3; Table 4 and 5). Most of the tested analgesics, lipid regulators (except atorvastatin), NSAIDs, hormones (except estradiol) and antiviral agents did not show any interaction with Mate7 at the initial concentration of 100 µM. Acebutolol and propranolol (both beta blockers)

showed weak and strong interaction, respectively. They are both competitive inhibitors, together with ranitidine (H2 blocker), imipramine (antidepressant), DPH (H1 receptor antagonist), procainamide (antiarrhythmic drug), MPP+, pyrimethamine (antimalarial agent), and doxorubicin (antineoplastic agent). In the class of competitive inhibitors, propranolol (IC_{50} 20 μM), imipramine (IC_{50} 28 μM), doxorubicin (IC_{50} 14 μM) and pyrimethamine (IC_{50} 15 μM) showed strong interaction, while all the other competitive inhibitors showed moderate to weak interaction. All the other interactors – the antibiotic tetracycline; chemotherapeutics methotrexate and mitoxantrone; the cardiovascular agent sildenafil; the antidepressant fluoxetine; calcium channel blockers verapamil, quinidine, and diltiazem; the lipid regulator atorvastatin; the hormone estradiol; and the anti-parkinson agent pramipexole, inihibited DAPI uptake in the non-competitive fashion. Mitoxantrone, sildenafil, verapamil, quinidine, and pramipexole showed the strong interaction while tetracycline, fluoxetine and diltiazem elicited moderate interaction. Estradiol, atorvastatin, and methotrexate were shown to be weak non-competitive inhibitors.

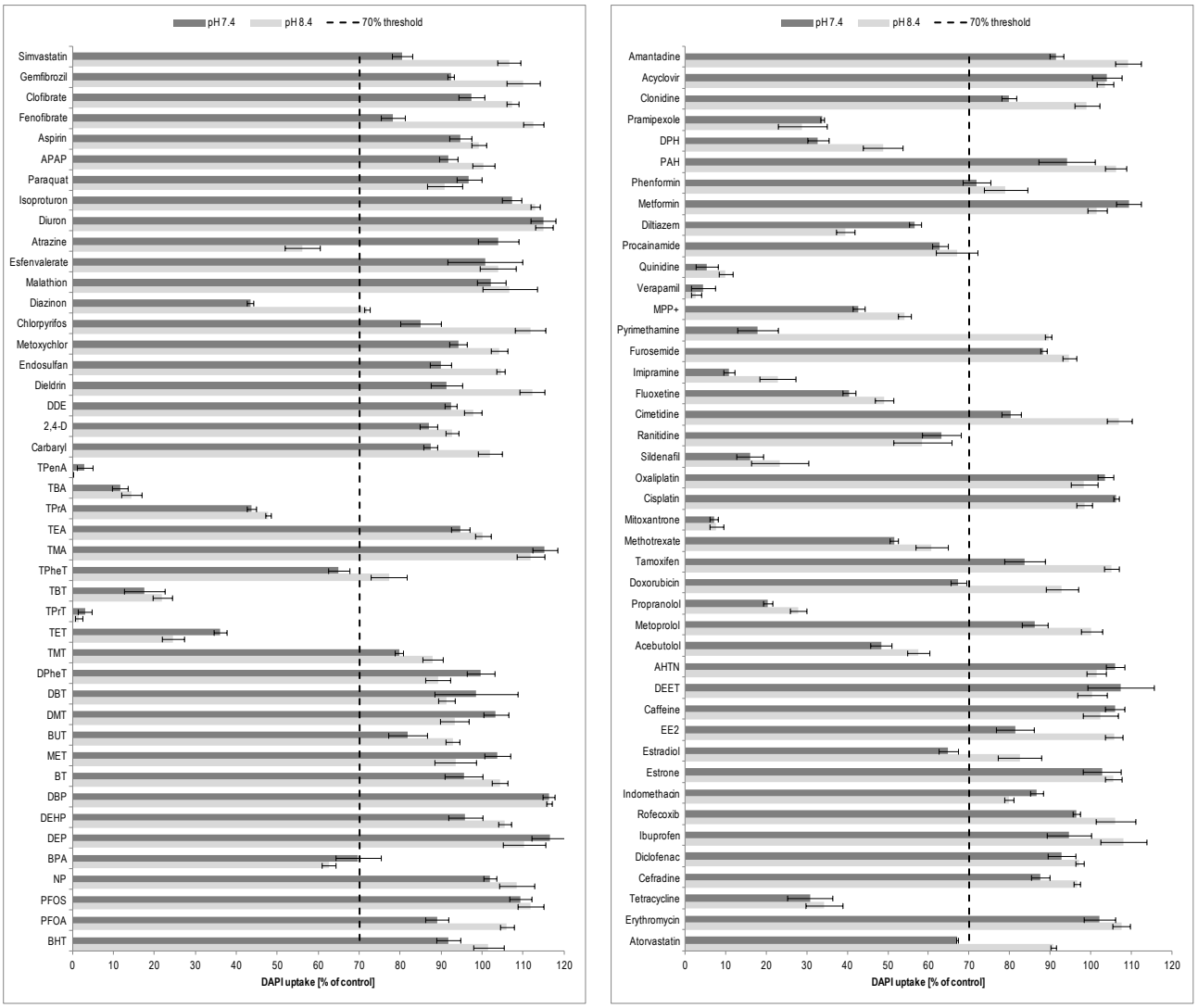


Figure 3. Interaction of zebrafish Mate7 with selected interactors. Data are expressed as percentage (%) of 4',6-diamidino-2-phenylindole (DAPI) uptake kinetics after co-incubation with each modulator (100 µM for all compounds, except for DDE, dieldrin, endosulfan, and methoxychlor which were set to 1 µM; doxorubicin, prazosin, BHT, DEHP, chlorpyrifos, esfenvalerate, tamoxifen and AHTN that were set to 10 µM; and gemfibrozil, simvastatin, and indomethacin that were set to 30 µM) relative to DAPI uptake kinetics in the absence of a modulator, which is set to 100%. Results obtained at both, pH 7.4 (dark bars) and at pH 8.4 (light bars) are shown. Dotted line represents the inhibition threshold set arbitrarily at 30%. Data represent mean ± SE from three to five independent experiments (n = 3–5). *Abbreviations used: acetaminophen (APAP), dichlorodiphenyldichloroethylene (DDE), 2, 4-dichlorophenoxyacetic acid (2, 4-D), tetrapentylammonium chloride (TPenA), tetrabutylammonium hydrogen sulphate (TBA), tetrapropylammonium chloride (TPrA), tetraethylammonium*

bicarbonate (TEA), tetramethylammonium chloride (TMA), triphenyltin chloride (TPheT), tri-n-butyltin chloride (TBT), tri-n-propyltin chloride (TPrT), triethyltin chloride (TET), Trimethyltin chloride (TMT), diphenyltin dichloride (DPheT), di-n-butyltin dichloride (DBT), dimethyltin dichloride (DMT), n-Butyltin trichloride (BUT), methyltin trichloride (MET), Benzotriazole (BT), Dibutyl phthalate (DBP), Bis(2-ethylhexyl)phthalate (DEHP), Diethyl phthalate (DEP), bisphenol A (BPA), Nonylphenol (NP), Perfluorooctanesulfonic acid (PFOS), Perfluorooctanoic acid (PFOA), Butylated hydroxytoluene (BHT), Diphenhydramine hydrochloride (DPH), p-aminohipuric acid (PAH), 1-methyl-4-phenylpyridinum iodide (MPP+), N,N-diethyl-meta-tolumide (DEET), 17 a-ethinyl estradiol (EE2), acetyl-hexamethyl-tetrahydro-naphthalene (AHTN).

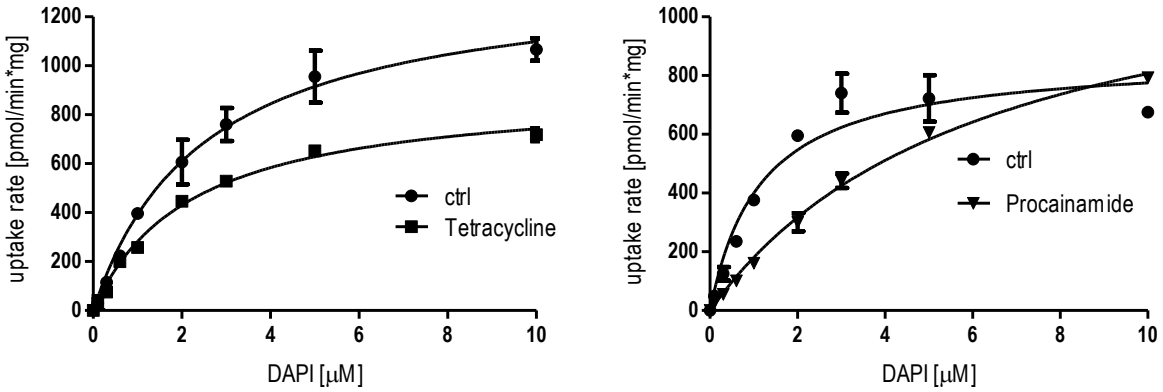


Figure 4. Michaelis-Menten kinetics experiment aimed at determination of the type of interaction. (A) example of an inhibitor (tetracycline), (B) example of a substrate (procainamide). Determination of the type of interaction was performed through comparison of apparent kinetic parameters ($K_{m,app}$; $V_{m,app}$) of inhibited reaction (IC_{50} value of respective fluorescent dye) versus uninhibited (control) obtained by measuring uptake of increasing concentrations of DAPI. Each data point represents mean ± SD from triplicate of a representative experiment out of at least three independent experiments.

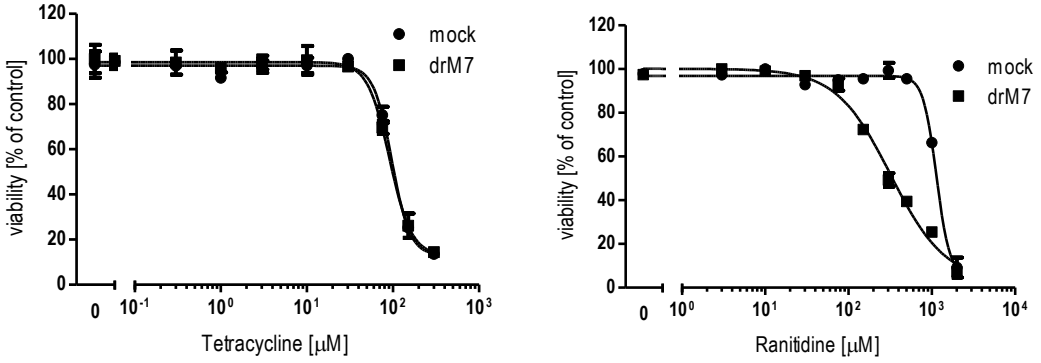
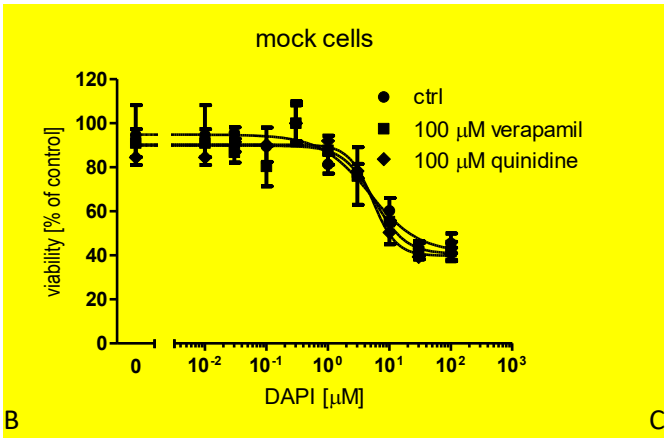


Figure 5. Cytotoxicity experiment aimed at determination of the type of interaction. (A) example of an inhibitor (tetracycline), (B) example of a substrate (ranitidine). Cytotoxicity modulation is based on the assumption that stable transfected Mate7 cells are more sensitive to toxic effects of the substrates applied, as determined by the MTT assay, in comparison to mock cells due to the Mate7 active transport of respective substrates inside of the cells. For the *EC50* calculations, data from a representative experiment (done in triplicate) out of at least three independent experiments were fitted to the sigmoidal four-parameter dose-response model (variable slope) in GraphPad Prism version 5.

Cytotoxicity modulation - rescue experiment

When Mate 7 cells were coexposed with model substrate i.e. confirmed toxicant (dapi) in combination with Mate 7 non-competitive inhibitor (verapamil or quinidine) they became 5-10 times more resistant compared to the cells incubated with toxicant only and without inhibitors. At the same time mock cells did not show notable increase in resistance (Fig. 6, Table S4).

A



B C

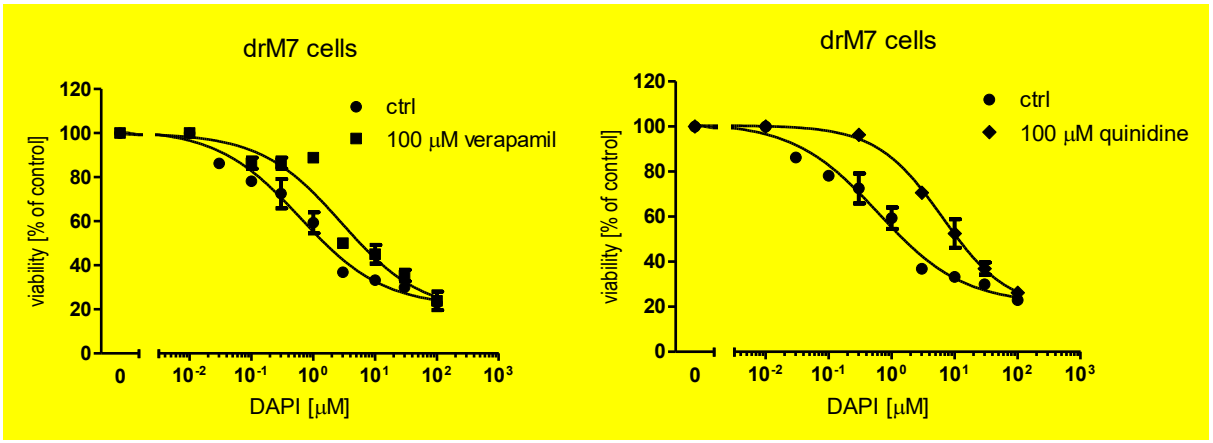


Figure 6. Cytotoxicity rescue experiment. (A) Mock cells exposed to substrate (dapi) with or without inhibitors for 1 hour and then left to grow for the next 71 hours. (B) Mate7 transfected cells exposed to substrate with or without 100 µM verapamil for 1 hour and then left to grow for the next 71 hours. (C) Mate7 transfected cells exposed to substrate with or without 100 µM quinidine for 1 hour and then left to grow for the next 71 hours. For the *EC50* calculations, data from a representative experiment (done in triplicate) out of at least three independent experiments were fitted to the sigmoidal four-parameter dose-response model (variable slope).

Discussion

In this study we analysed a wide range of environmentally relevant compounds regarding their interaction with zebrafish Mate7. Most of the tested compounds are widely used and their accumulation in surface water, sediment or soil could present a danger for the environment. To be able to efficiently identify zebrafish Mate7 interactors, and discern their strength and type of interaction, we have developed Mate7 stably over-

expressing HEK293 Flp-In cell line, and optimized a high throughput screening (HTS) assay based on a real time determination of cellular uptake of Mate7 fluorescent model substrates DAPI and ASP+.

To validate the assay we firstly verified that Mate7 indeed has only one binding site, i.e. that different substrates are going to be transported through the same active site. There have been conflicting reports concerning binding site of human MATEs. In reports from 2012 and 2013 (Astorga et al., 2012; Martinez-Guererro and Wright, 2013) the authors argue that substrates bind to different binding sites on MATE transporter surface. That would imply a complex binding region rather than a single binding site. Wittwer et al. (2013) explained the discrepancies between inhibition profiles of ASP+ and metformin transport in the same fashion, ascribing it to a substrate specific binding. However, in a report from 2016 (Martinez-Guererro et al., 2016) where extensive inhibition profiling of 400 compounds with four structurally distinct MATE substrates was conducted, the authors stated that no systematic influence of substrate structure on inhibitory efficacy or on the kinetic interaction of the inhibitor with the transporter (human MATE1) was found.

Therefore, to indirectly test the single binding site hypothesis we identified five fluorescent Mate7 substrates (Table 1) and selected DAPI and ASP+ as model substrates for kinetic profiling of remaining dyes (Tables 2 and S1, Figs. 2 and S2). Our kinetic determinations (summarized in Tables 2 and S1) indicate that, in agreement with Martinez-Guererro et al. report (2016) for human MATE1, zebrafish Mate7 also has a single binding site. This conclusion is further supported with (a) modulation of toxicity experiments performed with the aforementioned probes (Table 3); and (b) results of the inhibition profiling of the whole set of tested compounds for DAPI and ASP+ uptake inhibition that showed high correlation ($R = 0.82$, Figure S3). Therefore, we showed that there is only one zebrafish Mate7 binding site, and this finding enables discrimination of the type of interactors using Michaelis-Menten kinetics experiments based on the developed uptake assay protocol.

Secondly, it is important to note that MATEs are bidirectional transporters with direction of transport determined by pH gradient. This means that manipulation of the pH gradient can change the direction of transport from efflux to uptake. Most of the interaction studies done on MATEs were based on measurement of the uptake with gradient established either by intracellular acidification or extracellular alkalinisation

(Masuda et al., 2006; Tsuda et al., 2007; Tanihara et al., 2007; Grottker et al., 2011). Furthermore, it has been shown that some substrates including DAPI (Yasujima et al., 2010) and TEA (Otsuka et al., 2005; Ohta et al., 2006; Masuda et al., 2006) are transported even without pH gradient. Therefore we have compared transport rates for DAPI and ASP+ with and without intracellular acidification. Since significant differences in transport rate in favour of intracellular acidification were not observed (Figure S8), we decided to proceed without methodologically more complex intracellular acidification, but with two extracellular pHs (7.4 and 8.4, respectively) to be able to identify a broader scope of potential interactors.

We have tested more than 20 industrial chemicals most of which have not been checked for mammalian or teleost Mate interaction, and identified several potent interactors (Figure 3, Tables 4 and 5). Organotins are widely used industrial chemicals present in environment in nanomolar concentrations (Cole et al., 2015). Mihaljević et al. (2017) found organotins to be strong interactors of zebrafish Oct1 uptake transporter with IC_{50} values for DBT, TPrT and TBT in low µM range. We have observed similar interaction potency with Mate7 for TPrT (IC_{50} 11 µM), while TBT was less potent (IC_{50} 71 µM) and no interaction up to 100 µM was determined for DBT. It appears that organotins have higher affinity for Oct1 than for Mate7, as was further supported by our cytotoxicity modulation experiments which showed that organotins are not transported by Mate7 (Table 4 and 5; Fig S6 and S7).

Table 4. Type of interaction analysis with DAPI as substrate and set of chosen compounds as interactors. Kinetic parameters of DAPI uptake are given as apparent K_m ($K_{m,app}$) and apparent V_m ($V_{m,app}$) with 95% ci (confidence intervals). Data from a representative of at least three independent experiments, each done in triplicate, are shown. For the IC_{50} calculations data from at least three independent experiments were fitted to the sigmoidal four parameters dose-response model (variable slope) in the GraphPad Prism 5. Experiments were done at either pH 7.4 or 8.4, as indicated (control), and all the chemicals listed under their respective control values were tested at the same pH. Substances that showed strong or very strong interaction were typed in boldface.

compound	IC_{50} [µM]	95% ci	$K_{m,app}$ [µM]	95% ci	$V_{m,app}$ [pmol/min/mg]	95% ci	Interaction type/strength
Control, pH 8.4	-	-	*0.96*	*0.59-1.33*	*963*	*852-1075*	-
Bisphenol A	221	165-295	0.80	0.43-1.16	572	497-647	I / W
Atrazine	ND						
Diazinon	173	145-207	4.35	3.27-5.40	1549	1356-1741	S / W
Tetracycline	33.2	27.0-40.8	1.47	0.59-2.34	729	582-876	I / M
Pramipexole	20.8	17.9-24.2	1.80	1.11-2.47	549	473-623	I / St
Procainamide	268	214-336	6.28	5.21-7.35	1314	1194-1433	S / W
Acebutolol	134	114-157	4.87	3.60-6.13	1012	881-1141	S / W
Sildenafil	12.9	9.79-17.0	0.51	0.29-0.72	263	236-288	I / St
Ranitidine	146	127-169	8.30	4.26-12.2	1126	807-1446	S / W
Fluoxetine	57.9	42.0-79.7	0.87	0.39-1.34	299	243-354	I / M
Propranolol	19.7	17.4-22.3	3.46	2.32-4.58	1272	1086-1458	S / St
Methotrexate	80.5	71.9-90.2	1.31	0.93-1.68	354	321-386	I / M
MPP+	77.1	66.3-89.6	3.99	2.76-5.21	895	766-1022	S / M
Verapamil	3.05	2.43-3.82	0.82	0.61-1.00	508	474-541	I / St
Quinidine	6.03	4.85-7.50	1.23	0.83-1.62	645	579-709	I / St
Mitoxantrone	3.95	3.53-4.41	1.26	0.88-1.64	561	504-617	I / St
Imipramine	27.8	23.7-32.6	3.01	1.70-4.32	1025	834-1215	S / St
Diltiazem	50.9	39.0-66.4	0.65	0.39-0.91	369	328-409	I / M
DPH	61.4	55.1-68.5	4.01	3.16-4.82	1192	1077-1307	S / M
TPrA	59.9	52.0-69.1	2.96	1.84-4.06	789	664-915	S / M
TBA	6.14	5.00-7.54	2.09	1.48-2.68	912	811-1011	S / St
TPenA	0.59	0.53-0.65	1.62	1.40-1.83	472	450-493	I / VSt
TET	60.3	50.8-71.7	0.53	0.19-0.85	462	389-535	I / M
TPrT	11.0	9.26-13.0	0.76	0.11-1.40	462	306-617	I / St

TBT	70.7	57.7-86.7	0.17	0.00-0.60	160	60-258	I / M
Control, pH 7.4	-	-	*1.15*	*0.92-1.37*	*695*	*652-738*	-
Estradiol	258	198-337	1.23	1.01-1.45	512	482-543	I / W
Atorvastatin	117	99.3-137	1.22	0.79-1.65	523	460-586	I / W
Pyrimethamine	14.9	12.4-18.0	5.18	3.15-7.21	915	734-1097	S / St
TPheT	ND						
Doxorubicin	14.4	10.2-20.3	2.85	2.25-3.46	658	587-728	S / St

399 ND – not determined; I – inhibitor; S – substrate; W – weak strength interaction; M – moderate strength;
400 St – strong interaction; VSt – very strong interaction
401

402 We also tested n-tetraalkylammonium compounds, a subgroup of quaternary ammonium compounds (QACs)
403 which are known model cation substances. However, TEA which is considered a model Mate(s) cationic
404 substrate did not show any interaction with zebrafish Mate7, in accordance with our previous data that
405 revealed TEA IC_{50} value in milimolar range. It has also been shown that TEA, TPrA and TBA are transported by
406 human OCT1 while TPenA inhibits transporters activity but is not transported itself (Zhang et al., 1999). This is
407 in line with our finding that TPrA and TBA are transported by Mate7 while TPenA is not.
408 In general, environmental concentrations of tested industrial compounds are at least 1,000 times lower than
409 concentrations that we experimentally found to inhibit zebrafish Mate7 transport function. That would imply
410 that industrial compounds that showed significant interaction with Mate7 are probably not environmentally
411 relevant. However, this assumption is valid only if bioaccumulation processes in organisms that are chronically
412 exposed to high concentrations of contaminants are not considered. E.g., although there is no evidence of
413 biomagnification of tributyltin in marine ecosystems, accumulation may occur, resulting in high tissue
414 concentrations in some organisms (Meador, 2000). Reported bioconcentration factors for TBT in freshwater
415 green algae, round crucian carp, tilapia and mollusks were 30,000 (Maguire et al., 1984), 5,000 (Tsuda et al.,
416 1986), 12,300 (Hongxia et al., 1998), and 17,000-350,000 (Gomez-Ariza et al., 2001), respectively (USDHHS,

2005). If these bioconcentration factors are taken into consideration, affinities of organotins for Mate7, determined in this study in lower µM range, are sufficient to modulate/disrupt the transporter's efflux activity.

Table 5. Determination of the type of interaction through modulation of cytotoxicity of Mate7 interactors. Type of interaction (TOI) discrimination and calculation of sensitivity factors (SF; calculated as a ratio of EC_{50} values obtained for mock versus Mate7 transfected cells) is based on the assumption that stable transfected Mate7 cells are more sensitive to toxic effects of the substrates applied, as determined by the MTT assay, in comparison to mock cells due to the Mate7 active transport of respective substrates inside of the cells. For the EC_{50} calculations, data from a representative experiment (done in triplicate) out of at least three independent experiments were fitted to the sigmoidal four-parameter dose-response model (variable slope) in GraphPad Prism version 5.

	mock		drM7			
compound	EC_{50} [µM]	95% ci	EC_{50} [µM]	95% ci	SF	TOI
Bisphenol A	112	101-124	106	62.8-178	1.1	I
Atrazine	ND					
Diazinon	not tox		not tox			-
Estradiol	20.7	18.5-23.2	25.7	22.6-29.2	0.8	I
Tetracycline	97.1	88.8-106	91.3	82.8-100	1.1	I
Pramipexole	893	833-957	844	480-1482	1.1	I
Procainamide	not tox		not tox			-
Acebutolol	not tox		not tox			-
Sildenafil	185	152-226	222	189-261	0.8	I
Ranitidine	1130	894-1427	322	250-414	3.5	S
Fluoxetine	24.0	20.5-28.1	26.5	24.4-28.5	0.9	I
Propranolol	not tox		not tox			-

Methotrexate	0.02	0.01-0.03	0.024	ND	0.8	I
Doxorubicin	2.56	2.23-2.94	1.13	1.00-1.27	2.3	S
Atorvastatin	3.68	3.07-4.42	4.55	3.83-5.42	0.8	I
MPP+	147	96.0-224	38.9	33.3-45.3	3.8	S
Pyrimethamine	not tox		not tox			-
Verapamil	95.0	80.5-112	70.1	55.9-87.7	1.4	I
Quinidine	77.9	72.2-84.0	73.0	53.6-99.2	1.1	I
Mitoxantrone	0.24	0.14-0.37	0.22	0.18-0.24	1.1	I
Imipramine	not tox		not tox			-
Diltiazem	53.7	44.9-64.1	42.6	23.4-77.2	1.3	I
DPH	not tox		not tox			-
TPrA	828	420-1634	115	83.6-158	7.2	S
TBA	211	138-323	40.1	30.8-52.1	5.3	S
TPenA	3.03	2.57-3.57	2.34	1.79-3.05	1.3	I
TET	7.89	7.18-8.69	6.26	5.22-7.50	1.3	I
TPrT	0.18	0.17-0.19	0.15	0.14-0.16	1.2	I
TBT	0.30	0.20-0.42	0.21	0.15-0.31	1.4	I
TPheT	ND					

ND – not determined due to the solubility issues; not tox – compound not toxic in the available solubility range; ci – confidence intervals; SF – sensitivity factor calculated as a ratio of EC_{50} values obtained for mock versus Mate7 transfected cells;
TOI – type of interaction

Among widely used pesticides, we screened 14 substances of different chemical composition and showed for the first time interaction of any vertebrate Mate transporter with the organophosphate diazinon, and the triazine atrazine, albeit with rather low affinity (Fig. 3, Tables 4 and 5).

436 Out of almost 50 screened pharmaceuticals, interaction with Mate7 was observed for 20. With exception of
437 interaction data for verapamil, quinidine and mpp+ as shown in our previous paper (Loncar et al., 2016), in
438 this study we report the first data on interaction of numerous pharmaceuticals with a fish Mate transporter.
439 Acebutulol, methotrexate, estradiol, atorvastatin and fluoxetine have not been shown to interact with Mates,
440 and herewith we suggest that Mate7 transports acebutolol, and is inhibited by the other four. The results
441 previously reported for procainamide and ranitidine are in line with our findings, while the result for the
442 pramipexole is in contradiction to our indication of its non-competitive inhibition of zebrafish Mate7 (Ivanyuk
443 et al., 2017). Tetracycline and verapamil inhibit zebrafish Mate7, however there is no indication of transport
444 which is similar to human MATE1. We showed that imipramine, propranolol, diphenhydramine,
445 pyrimethamine and doxorubicin are competitive inhibitors (substrates) while for human MATEs only inhibition
446 has been shown (Ivanyuk et al., 2017). Quinidine has been shown to be transported by human MATEs (Masuda
447 et al., 2006; Sato et al., 2008), and diltiazem was shown to interact with human MATEs (Tsuda et al., 2009).
448 Our results suggest that both are non-competitive inhibitors i.e. not transported by Mate7. Mitoxantrone and
449 sildenafil are non-competitive inhibitors of both zebrafish Mate7 and human MATE1 (Meyer zu Schwabedissen
450 et al., 2010; Wittwer et al., 2013). Likewise, we show that tetracycline, pramipexole, fluoxetine, methotrexate,
451 verapamil, estradiol, and atorvastatin all belong to the group of non-competitive inhibitors.
452 Similar to tested industrial substances, considering effective inhibitory concentrations of pharmaceuticals
453 found in our study, it is not clear whether inhibition of zebrafish Mate7 transport by these substances has any
454 physiological, pharmacological or environmental relevance? Although definitive answers to these questions
455 should be addressed by follow up *in vivo* studies preferably done with zebrafish Mate7 knockouts, our *in vitro*
456 experiments aimed at modulation of cytotoxicity offer some initial insights. As shown in Fig. 6, when stable
457 zebrafish Mate7 transfectants were coexposed with DAPI as the model toxic substance and Mate7 substrate
458 in combination with verapamil or quinidine as identified non-competitive inhibitors, a clear rescue from DAPI
459 cytotoxicity and shifts in related dose-response curves were observed in comparison to mock cells.
460 Consequently, as result of lower DAPI uptake transfected cells became 5-10 times more resistant compared

to the cells exposed to toxicant only and without inhibitors (Table S4). Therefore, these *in vitro* data point to possibility that modulation of the Mate7 transport function may also have toxicological relevance *in vivo*.

Accordingly, some assumptions may be credibly formulated for the most potent and/or environmentally most relevant zebrafish Mate7 pharmaceutical interactors determined in this study. For example, although tetracyclines are persistent in aquatic environment, their maximal environmental concentrations are in ng/L range (Daghrir and Drogui, 2013) which is too low to excrete notable effect on Mate7. However, tetracycline is easily precipitated with cations as potassium, and accumulates in the sewage sludge and sediments (Daughton and Ternes, 1999). In addition, although data on tetracycline plasma concentrations in zebrafish are not available, maximal concentrations recorded in humans are in µM range which would be sufficient to modulate Mate7 activity. Sildenafil is a potent and selective inhibitor of cGMP-specific phosphodiesterase type 5 used to treat erectile dysfunction and pulmonary arterial hypertension. It has been shown to competitively inhibit ABCB1 and ABCG2 at clinically achievable concentrations and reverse the efflux of paclitaxel, mitoxantrone and methotrexate *in vitro* (Shi et al., 2011; Stacy et al., 2013). Wittwer et al. (2013) showed that 20 µM sildenafil inhibits human MATE1 transport of ASP+. In this study we demonstrated that sildenafil strongly inhibits Mate7 in non-competitive manner, implying it could impair Mate7 protective or physiological function.

Mitoxantrone is an antineoplastic agent that has been shown to interact with human MATE1 at IC_{50} values similar to those we determined for zebrafish Mate7 (Meyer zu Schwabedissen et al., 2010; Grottker et al., 2011). The peak plasma concentration reported *in vivo* for mitoxantrone is 0.6 µM (Brunton et al., 2008; Meyer zu Schwabedissen et al., 2010). It has also been shown to interact with zebrafish Oct1 uptake transporter (IC_{50} 85 µM). Mitoxantrone does not influence trout Abcg2a ATPase activity while it has been shown to be transported by human ABCB1 and ABCG2 (Sarkadi et al., 2006; Zaja et al., 2016).

Both human MATEs have been shown to transport pramipexole (Knop et al., 2015; Ivanyuk et al., 2017). Our results show that pramipexole (IC_{50} 21 µM) strongly inhibits zebrafish Mate7 which is also supported by toxicity data. There are several possible explanations for the observed discrepancy between zebrafish Mate7 and human MATEs. Tsuda et al. (2009) reported that pramipexole has much higher affinity for human MATE2-K

than for MATE1, and since zebrafish has at least 5 functional Mate transporters it could be that some of the other four, and not Mate7, transports pramipexole. Knop et al. (2015) showed that pramipexole uptake by human MATE1 is much lower than transcellular efflux by the same transporter. They speculate this could be due to the different affinity for MATE1 in extracellular versus intracellular space, i.e. the affinity is not the same on the both sides of membrane. Finally, it could be that the difference is species related.

Verapamil, calcium channel blocker used as antiarrhythmic agent, inhibits both human MATEs (Tanihara et al., 2007) and supposedly is being transported by MATE2-K (Masuda et al., 2006). It is a substrate of OCT2 and ABCB1 (Ivanyuk et al., 2017). Its peak *in vivo* concentration has been reported to be 0.55 µM (Brunton et al., 2008). It inhibits zebrafish Oct1 at IC_{50} of 14 µM (Mihaljevic et al., 2017), and our results indicate that verapamil is a strong inhibitor of zebrafish Mate7 (IC_{50} 3.0 µM).

Quinidine, a sodium and potassium channel blocker used as antiarrhythmic agent, is transported by both human MATE1 (Sato et al., 2008) and MATE2-K (Masuda et al., 2006). It is also transported by human ABCB1 and OCT2, but it does not interact with ABCG2 or ABCC2 (Ivanyuk et al., 2017). Zebrafish Oct1 (IC_{50} 140 µM) has 20 times lower affinity for quinidine than Mate7 (IC_{50} 6.0 µM). The peak plasma concentration of 3.7 µM (2.9 mg/l) has been reported for quinidine sulphate (Brunton et al., 2008). Acute ecotoxicology data for quinidine are available for several aquatic species, with EC_{50} values ranging from 8.3 - 274 mg/l (Webb, 2004). Our assays showed quinidine to be a potent non-competitive inhibitor of Mate7.

The second group of pharmaceutical substances are those identified in this study as zebrafish Mate7 substrates: propranolol, acebutolol, procainamide, ranitidine, imipramine, DPH, diazinon, MPP, doxorubicin, and pyrimethamine. Propranolol has been shown to strongly interact with human MATEs (IC_{50} 8 µM) (Astorga et al., 2012). It inhibits human ABCB1 activity but is not transported (Bachmakov et al., 2006). Stott et al. (2015) have shown using primary trout gill cell culture that efflux of propranolol or its metabolites is pH dependent and increases with extracellular acidification from pH 8 to pH 6. They used propranolol concentration that is close to environmentally reported values (4 nM or 1 µg/L), and actually speculated that the observed, pH dependent efflux, might be due to the activity of an exporter present in gill that is not a member of the ABC family. Mate7 present in the zebrafish gill tissue may explain active efflux of propranolol observed in the trout,

513 as we show here that propranolol is a strong competitive inhibitor (IC_{50} 19.7 µM), i.e. the substrate of zebrafish
514 Mate7. Unfortunately, the finding could not be further supported with toxicological experiment due to lack of
515 toxicity (MTT assay) in the range of specific transport.
516 Imipramine has been shown to interact with human MATEs with IC_{50} values between 40 and 200 µM (Tsuda
517 et al. 2009). Stott et al. (2015) showed that imipramine uptake in trout gill cells is mediated via carrier process.
518 Our results indicate that imipramine is transported by Mate7 with high affinity (IC_{50} = 28 µM). Maximal
519 recorded environmental concentration of imipramine is 0.4 pM (0.14 ng/l) (Stott et al., 2015), and in plasma
520 it reaches concentration of 0.6 µM (200 µg/l) (Brunton et al., 2008).
521 Doxorubicin is an anthracycline antibiotic used as chemotherapeutic agent. Its therapeutic dose could go up
522 to 1.75 µM (950 µg/l) (Brunton et al., 2008). Inhibition of human MATE1 has been shown (Wittwer et al., 2013),
523 but transport has not been verified. We show that doxorubicin is a high affinity competitive inhibitor (IC_{50} 14.4
524 µM), as is further supported by results of our toxicity modulation experiments (Table 5).
525 Pyrimethamine, a folic acid antagonist used as an antimalarial agent, has been reported as the potent inhibitor
526 of both human MATEs (Ito et al., 2010). Our results indicate that pyrimethamine is a high affinity competitive
527 inhibitor of zebrafish Mate7 (IC_{50} 15 µM). Clinical concentrations of pyrimethamine are in lower micromolar
528 range (2.3 µM) (Kusuhara et al., 2011) while in the environment maximal concentration of 0.2 nM (60 ng/l)
529 has been reported (Azzouz and Ballesteros, 2013).
530 In summary, in this study we developed an effective high throughput screening assay for identification and
531 initial characterization (substrates versus non-competitive inhibitors) of zebrafish Mate7 interactors,
532 demonstrated its usability by screening a wide range of environmentally relevant substances, and reported
533 the first data on identification of potent Mate7 inhibitors and/or substrates among tested substances.
534 Interaction with any vertebrate Mate transporter has been shown for the first time for a series of industrial
535 compounds, pesticides, and pharmaceuticals. Some of the inhibitors identified could be of environmental
536 concern. They may potentially impair normal Mate7 efflux function and thus decrease the fish defence
537 capacity against environmental contaminants, or interfere with transport of yet unidentified physiological
538 substrates.

In addition, based on the data obtained in this study it appears there are significant differences between zebrafish Mate7 and mammalian Mates' substrate preferences, a finding that should be taken into consideration when using zebrafish as a model organism in pharmacological and toxicokinetics studies. Finally, identifying drugs that may inhibit Mates is important for predicting drug-drug interactions that may lead to nephrotoxicity through increase in drug accumulation as a consequence of transporter(s) inhibition.

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Additional information

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