Zebrafish Oatp1d1 acts as cellular efflux transporter of the anionic herbicide bromoxynil

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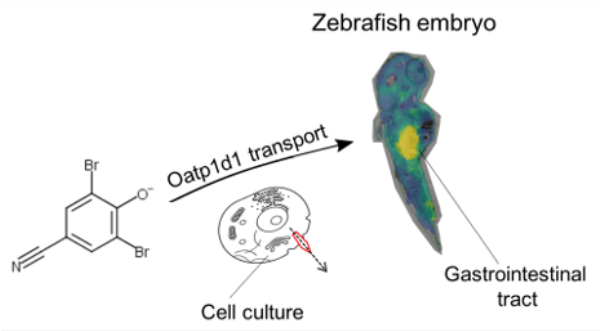
Abstract

Toxicokinetics of ionic compounds in the toxico-/pharmacological model zebrafish embryo (*Danio rerio*) depend on absorption, distribution, metabolism, and elimination (ADME) processes. Previous research indicated involvement of transport proteins in the toxicokinetics of the anionic pesticide bromoxynil in zebrafish embryos. We here explored the interaction of bromoxynil with the organic anion transporting polypeptide zebrafish Oatp1d1. Mass spectrometry imaging revealed accumulation of bromoxynil in the gastrointestinal tract of zebrafish embryos, a tissue known to express Oatp1d1. In contrast to the Oatp1d1 reference substrate bromosulfophthalein (BSP), which is actively taken up by transfected HEK293 cells overexpressing zebrafish Oatp1d1, those cells accumulated less bromoxynil than empty vector-transfected control cells. This indicates cellular efflux of bromoxynil by Oatp1d1. This was also seen for diclofenac but not for carbamazepine, examined for comparison. Correspondingly, internal concentrations of bromoxynil and diclofenac in the zebrafish embryo were increased when co-exposed with BSP, inhibiting the activities of various transporter proteins, including Oatp1d1. The effect of BSP on accumulation of bromoxynil and diclofenac was enhanced in further advanced embryo stages, indicating increased efflux activity in those stages. An action of Oatp1d1 as efflux transporter of ionic environmental compounds in zebrafish embryos should be considered in future toxicokinetics' assessments.

Graphical abstract



Introduction

The zebrafish embryo is widely used as an alternative model for chemical testing in toxicology and pharmacology.[1, 2] Acute toxicities of chemicals to zebrafish embryos and adult fish generally correlate to a high degree;[3] zebrafish embryos are therefore suggested as a replacement for adult fish in toxicity testing. However, fish embryo and adult stages differ to a large degree with regard to morphology, cell differentiation, and physiology, which are potential reasons for differences in the toxicokinetics (TK) of and sensitivity to chemicals. The main uptake route of chemicals from the water in embryos is *via* the body surface, whereas chemical uptake from the water proceeds *via* the gills in adult fish.[4] Chemicals with certain modes of action, such as neuroactive compounds, can show highly divergent toxicities to zebrafish embryo and adult fish life stages: In adult fish, the effect targets of neuroactive chemicals are the gills and the cardiovascular system, which in embryos are not yet fully developed. The embryo stages, therefore, are not affected by respiratory failure that in the adult stages is caused by such compounds.[5] An example for morphological differences that may cause differences in the toxicokinetics of chemicals is the yolk compartment, present in embryo but not in adult fish stages. Recently, we showed high accumulation of a range of organic neutral and ionic chemicals, including pharmaceuticals and pesticides, in the yolk of zebrafish embryos. From the overall amount taken up, up to 95% of the accumulated chemical were found in the yolk, depending on the compound and the embryonic stage. The results point to the importance of the yolk for bioaccumulation of certain chemicals in fish embryos.[6] Furthermore, chemicals targeting e.g. yolk utilization processes may cause embryo-specific effects.[7] Knowledge of the life stage-specific features and processes, such as organ differentiation and biochemical pathways, is therefore essential for the understanding of stage-specific TK and toxicity of chemicals.

In a previous study, the concentrations of the anionic bromoxynil were found to be similar in the embryonic body and the yolk compartments of zebrafish embryos within the first 48 h of exposure to the compound; thereafter, a larger proportion of bromoxynil accumulated in the embryonic body. This was in contrast to other neutral and cationic chemicals that accumulated to a higher degree in the yolk.[6] Furthermore, the uptake kinetics of bromoxynil over 96 h of exposure could not be explained by a kinetic model including also the parameters passive diffusion and barrier function of the aqueous

boundary layer.[6] This indicates that the uptake kinetics are majorly influenced by processes not considered in the prediction model. These could be biological processes, such as active protein-conferred chemical transport, enzymatic biotransformation, and/or developmental differentiation resulting in a morphological reorganization, such as, e.g., the opening of the gastrointestinal (GI) tract.[8]

The uptake of an ionic compound by an organism is often influenced by active cellular uptake or excretion processes. Enzymes/proteins related to ADME ("absorption, distribution, metabolism, and excretion") processes, i.e., metabolic enzymes or transport proteins, show high activities in tissues e.g. of the blood-brain barrier or the GI tract.[9, 10] It was shown that certain fluorescent dyes are effluxed from zebrafish embryos by the ATP-binding cassette (ABC) transporter Abcb4.[11] Furthermore, the TK of cocaine and meta-chlorophenylpiperazine, positively charged psychoactive substances, in zebrafish embryos were hypothesized to be influenced by transport proteins.[12] Indeed, a range of environmentally relevant compounds was shown to interact with the zebrafish organic anion transporters Oatp1d1[13] and Oat2a-e[14] from the SLC (solute carrier) superfamily in cellular tests.

Bromoxynil is a widely-used anionic pesticide, occurring as contaminant in European surface waters.[15] The bioaccumulation of certain ionic compounds was found to proceed very slowly,[16, 17] as we also previously observed for bromoxynil.[6] For assessing the acute toxicity of chemicals, it is essential to reach a steady-state of chemical uptake and elimination in an experimental organism within the experimental time frame in order not to underestimate toxicity. A large proportion of the chemicals registered under the European chemical regulation REACH is ionic.[18] For improving the ecotoxicological assessment of ionic chemicals we here aimed to elucidate biological parameters behind and thus the understanding of the uptake process of bromoxynil in zebrafish embryos as an example of a compound with the TK characteristics of a slowly bioaccumulating ionic chemical.

We (i) investigated the distribution of bromoxynil in the embryonic body in order to identify specific tissue areas of bromoxynil accumulation and (ii) explored the influence of active transport on the TK of bromoxynil, focusing on zebrafish Oatp1d1 (formerly Slco1d1) as a potentially relevant candidate transporter protein. In zebrafish embryo stages from 24 hours post fertilization (hpf) on, Oatp1d1 was previously shown to be expressed, indicating its presence and activity throughout development.[19] For

our study, we combined mass spectrometry (MS) imaging of zebrafish embryos, cellular transport assays, and uptake experiments with zebrafish embryos in the presence of bromosulfophthalein (BSP), a known substrate/inhibitor of several OATP/Oatp proteins.[20] We included diclofenac and carbamazepine in the experiments for comparison. Diclofenac, a substrate of Oatp1d1,[13] has a similar pKa (pKa=3.99[21]) as bromoxynil (pKa=3.75[22]) and > 99.9% are anionic at pH=7.4 (pH of the zebrafish embryo exposure medium). The uptake kinetics of diclofenac have also been shown to be slow in zebrafish embryos.[23] Carbamazepine was included as a neutral compound showing no or only minor interactions with Oatp1d1.[13]

Materials and methods

Experimental chemicals were: bromoxynil, BSP, [^{3}H]-BSP, carbamazepine, cyclosporin A (CsA), and diclofenac sodium salt. Details on these and other chemicals used are listed in the Supporting Information (section I).

Experiments with zebrafish embryos

Fish were cultured and used according to German and European animal protection standards and approved by the Government of Saxony, Landesdirektion Leipzig, Germany (Aktenzeichen 75-9185.64). We used the UFZ-OBI/WIK zebrafish strain (generation F3-4), obtained originally from a local breeder and kept for several generations at the UFZ. Adult zebrafish were maintained and bred according to standard protocols.[24] Collection of eggs and culturing of the embryos were performed as described.[25] Three embryos were incubated in 6 mL exposure solution in a glass vial closed with a lid. The vials were horizontally agitated in an incubator at 26 °C with a light/dark cycle of 14:10 h.

Uptake kinetics experiments with zebrafish embryos

Each exposure experiment was set up in two independent exposures with embryos from separate egg batches (termed “exposure A/B”). Exposures A and B each consisted of 5 to 7 replicates with 3 embryos each. Each treatment was set up in ≥3 technical replicates to ensure sufficient numbers of intact embryos for the analyses (refer to Table S1). Blank controls (exposure solution without embryos) were run in parallel to each exposure and were used to monitor the compound's stability. The stability of BSP was determined *via* ultraviolet-visible spectroscopy (UVIKON 923, Bio-Tek Kontron Instruments, results

in Figure S2). Exposure experiments were performed with different exposure durations and with embryos at different developmental stages. Embryos were exposed to bromoxynil, diclofenac, and carbamazepine at nominal EC5 and EC25 (effect concentrations for 5% or 25% of the population), respectively, with two different concentrations of BSP. Nominal concentrations were 6.76 and 11.1 µM, respectively, for bromoxynil (refer to fish embryo toxicity (FET) test data in Figure S1b, regression curve for concentration dependence of "all effects"), 4.63 and 5.24 µM, respectively, for diclofenac,[26] and 231 and 259 µM, respectively, for carbamazepine.[27] These concentrations enabled robust detection of the test compounds in the tissue samples. BSP concentrations were set to 50 and 100 µM, respectively, corresponding to 20 and 40-times the Km-value from cell experiments (Figure S3). These BSP concentrations were in the sublethal range but caused reduced hatching (refer to the FET test data in Figure S1a)). Additionally, one exposure experiment with the combination of bromoxynil and diclofenac was conducted. Different exposure experiments were performed with different exposure durations and with embryos of different ages (refer to Table S1): *Exposure experiment 1* (A/B): start with embryos at 1±1 hpf, 96 h exposure; *Exposure experiment 2* (A/B): start with embryos at 1±1 hpf, 24 h exposure; *Exposure experiment 3* (A/B): start with embryos at 72±1 hpf, 24 h exposure.

If possible, the measurements of internal concentrations were performed with intact embryos; if not available in sufficient numbers, embryos with sublethal effects were also used. These included embryos with no blood circulation (nobc), decreased heartbeat frequency (hf), no pigmentation (nopig), or pericard edema (EdP). Embryos with lethal effects showing coagulation (k), no formations of somites (nosf), no detachment of tail (nodt), and no heartbeat (nohb) were not used for the measurements and discarded.

Zebrafish embryos at 1 ± 1 hpf (minimum 4-cell stage) or at 72 ± 1 hpf were transferred with 50 µL (1 hpf) or 100 µL (72 hpf) ISO-water into the exposure solution. At the end of each exposure, effects were recorded according to OECD 236[28] (see Tables 2, S5, S6, S7). Three pooled embryos were transferred to a Fast-Prep tube filled with glass beads, quickly washed twice with 1 mL ultrapure water, and upon removal of excess water tubes were snap-frozen in liquid N_2. Three replicates were taken per treatment. At the start and end of each exposure, aliquots from the exposure solutions were frozen until

analysis. For measurement details using ultraperformance liquid chromatography quadrupole-time-of-flight MS (UPLC-QToF-MS) see section I of the Supporting Information. The measured chemical concentrations in the exposure solutions are listed in Table S2.

Internal concentrations were calculated based on the volumes of whole embryos at the respective developmental stages.[6]

Details on the phenotypic assay are in section I of the Supporting Information.

MS imaging of zebrafish embryos

Laser ablation inductively coupled plasma MS (LA-ICP-MS) and matrix-assisted laser desorption/ionization coupled to a Fourier transform ion cyclotron resonance mass spectrometer (MALDI-FT-ICR-MS) were applied to analyze the spatial distribution of bromoxynil in zebrafish embryos. The examinations were performed upon exposure of the embryos to bromoxynil at 6.8 µM (≙EC5; Figure S1) for 72 and 96 h from 1 hpf. After the exposure, the embryos were washed twice with 1 mL ultrapure water. The techniques yield a spatial resolution of 25 to 50 µm; therefore, the identification of the tissue that had accumulated bromoxynil is limited to this resolution in our study.

LA-ICP-MS imaging of zebrafish embryos

Embryos were mounted on glass slides with NEG-50 Frozen Section Medium (Thermo Scientific) and dried at room temperature in a desiccator. LA-ICP-MS imaging measurements were carried out with a laser (193 nm ArF laser, Analyte G2, Teledyne CETAC Technologies, USA) coupled to a double-focusing sector field ICP-MS (Spectro, Ametek, Germany) with a Mattauch-Herzog geometry.[29] For LA-ICP-MS settings refer to the Supporting Information (section I). Background intensities of ^{79}Br in a zebrafish embryo exposed for 96 h to ISO-water are shown in Figure S11.

LA-ICP-MS imaging data were analyzed with Iolite 3.6 in Igor Pro 7.04. Exported data were overlaid with the optical microscopic image in MATLAB R2015b.

MALDI-FT-ICR-MS imaging sample preparation and measurements

For details on the procedure and parameter settings refer to Supporting Information (section I). Briefly, embryos were embedded in Richard-Allan Scientific NEG-50 Frozen Section Medium (Thermo

Scientific), frozen, and cut into sections (12 µm thickness) with a microtome (Cryo-Star HM 560, Microm International, Walldorf, Germany). Sections were mounted to ITO-coated glass slides (Bruker, Bremen, Germany). The matrix 9-aminoacridine was applied *via* sublimation. MALDI-MS imaging measurements were carried out with a MALDI ion source (335 nm, Smartbeam II, Bruker Daltonics, Bremen, Germany) coupled to an FT-ICR-MS (solarix XR 12T, Bruker Daltonics, Bremen, Germany). Bromoxynil was detected in negative ion mode as [M-H]- (275.84671 m/z ± 0.19313 mDa). Data were analyzed with SCiLs Lab 2017. Weak denoising, hotspot removal, and total ion count normalization were applied.

Cell experiments

For cloning of the *oatp1d1* cDNA encoding *Danio rerio* Oatp1d1 (drOatp1d1), generation of the drOatp1d1-overexpressing human embryonic kidney (HEK-OAtp1d1) cell line and cell culture conditions refer to the Supporting Information (section I).

Uptake and inhibition assays

Uptake and inhibition assays were conducted with two independent experiments (at least one day between experiments) with three replicates per treatment and experiment. For uptake and inhibition assays, 7 x 10^5 HEK-drOatp1d1 and the same amount of the respective control cells (HEK-Co; transfected with the empty vector pcDNA3.1(+) and selected under the same conditions) were seeded in poly-D-lysine coated 12-well plates (Sarstedt). The cells were cultured for 24 h and induced with 10 mM sodium butyrate for additional 24 h.

Prior to the uptake experiments, the cells were washed with prewarmed (37 °C) uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM K_2HPO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, 12.5 mM HEPES pH 7.3). The compounds bromoxynil, diclofenac, carbamazepine, and BSP were dissolved in the above-described uptake buffer at the desired concentrations, and the cells were incubated with this solution for 10 min as described previously.[30] Uptake of 0.01 µM radio-labeled BSP adjusted to a final concentration of 1 µM BSP served as a positive control for an Oatp1d1-mediated transport. Concentration-dependency studies to determine the K_m value of Oatp1d1-mediated BSP transport were performed with 1, 10, 20, and 30 µM BSP with 0.01 µM radio-labeled BSP adjusted to the respective concentration and used in

uptake experiments. Unlabeled BSP, as known substrate,[31] at 50 µM and 100 µM (20 and 40-times the K_m-value, Figure S3) was used as Oatp1d1 inhibitor. Bromoxynil, diclofenac, and labeled BSP were also applied together with the transport inhibitor CsA at 5 µM. Additionally, a treatment containing both bromoxynil and diclofenac at 10 or 100 µM each was set up. Table S3 gives an overview of the different chemical concentrations applied in the different experiments. Upon exposure, the cells were washed 3 times with ice-cold uptake buffer and lyzed with 80% methanol. The intracellular accumulation of the substrate was measured using UPLC-QToF-MS (section I Supporting Information) or by liquid scintillation counting. The protein concentration in each cell homogenate was determined as a reference point by the bicinchoninic acid assay (BCA Protein Assay Kit).

The Oatp1d1-mediated net transport was calculated as the difference of the accumulation in the Oatp1d1-expressing cells and the vector control cells.

Data analysis

Data were plotted with R 4.0.3. Permutation tests (Manly's, 5000 repetitions), which were carried out to test for statistical significance when two independent experiments were performed. The experiment and the treatment were taken as categorical variables. Manly's permutation test is a non-parametric analysis that allows to take independent experiments into account and does not require normality of the data (for more details and coding information refer to [32]).

Results

Spatial distribution of bromoxynil in zebrafish embryos

For investigations of the spatial distribution of bromoxynil in the embryonic body, imaging of tissue areas was performed with LA-ICP-MS and MALDI-FT-ICR-MS of zebrafish embryos exposed to bromoxynil only. Bromine signals detected with LA-ICP-MS occurred in tissues close to the head, the yolk, and along the embryonic tail, presumably the GI tract (Figure 1). The accumulation pattern of bromoxynil in the embryo as found with LA-ICP-MS was confirmed by MALDI-MS imaging results (Figure 2). The signal intensity of bromoxynil in the region close to the yolk is about twice as high as the signal intensity in the head region.

Cellular uptake experiments

The uptake of labeled BSP by the drOatp1d1-overexpressing cells was significantly enhanced compared to the uptake by the HEK-Co cells [mean uptake of 48.6 (drOatp1d1 cells) *vs.* 5.2 (control cells) pmol x mg protein^{-1} x min^{-1} upon exposure to 1 µM BSP, Figure 3]. In contrast, the amount of bromoxynil and diclofenac, taken up by drOatp1d1-expressing cells, was significantly lower in HEK-Co cells (Figure 3). At a concentration of 10 µM, the mean Oatp1d1-mediated efflux rate of bromoxynil and diclofenac was -5.1 and -5.6 pmol x mg protein^{-1} x min^{-1}, respectively, and 4- to 5-fold higher at the 10-fold higher exposure concentration (100 µM; Figure S4). No difference was seen between drOatp1d1-expressing and HEK-Co cells for carbamazepine, indicating no Oatp1d1-mediated transport of this compound under the applied experimental conditions (Figures 3 and S4).

For comparison of cellular results and zebrafish embryo exposures, 50 or 100 µM unlabeled BSP, as a competitive transport protein inhibitor (including Oatp1d1), was added in the experiment. The cellular net-uptake of the radio-labeled BSP at 1 µM was significantly lower ($p < 0.001$) in the presence of 50 or 100 µM unlabeled BSP than in the treatments containing 1 µM radio-labeled BSP only (Figure 4, Table 1). The net accumulation rate values in the treatments with 50 or 100 µM unlabeled BSP were close to zero because uptake of labeled BSP in the drOatp1d1- and HEK-Co-cells was not significantly different ($p > 0.05$).

Large standard deviations within and variations between replicate experiments in the treatments with the test compounds combined with BSP indicated considerable data variation (Table 1 and S4). In the experiments with bromoxynil and diclofenac, the results are therefore ambiguous and trends are not statistically significant (Figures 4 and S5). In the carbamazepine experiment, variation among replicate data is in comparison small (Figures 4 and S5). The net transport values for the treatments with BSP tend to be slightly lower than for the treatment without BSP. A statistically significant difference is seen between control and the 100 µM BSP treatments; however, the mean net transport values are rather low with -1.9 (Figure 4) and -9.1 pmol/mg protein/min (Figure S5).

Additionally, experiments were performed in which CsA, an inhibitor of different transport proteins including Oatp1d1, was applied in combination with labeled BSP, bromoxynil, and diclofenac,

respectively. While the net cellular uptake of labeled BSP was significantly reduced in the presence of CsA, no CsA effect was observed on the transport of bromoxynil and diclofenac (Figure S6).

Upon treatment of the cells with a mixture of bromoxynil and diclofenac, a decrease in the Oatp1d1-mediated export of the respective other compound was seen. Thus, in the mixture treatment net efflux of the compounds was less negative than when compounds were singly applied (Figure S7).

Toxicokinetic experiments with zebrafish embryos

Internal concentrations of test compounds were examined upon 96 h exposures (experiment 1). Observed toxicological effects in the treatments were: At the single compound exposures, no or delayed hatching was seen in BSP and carbamazepine treatments within the exposure period of 96 h (Table 2). Sublethal effects in embryos from bromoxynil (5-16% sublethal effects at EC5 and EC25) and diclofenac (5-43% sublethal effects at EC5 and EC25) treatments were missing heartbeat and blood circulation, tail deformation and the occurrence of edemas (Table 2). The addition of BSP at both 50 or 100 µM led to enhanced toxicity in the exposures with bromoxynil and diclofenac (Table 2). Observed effects were missing heartbeat, coagulation, no blood circulation, no hatching, reduced heartbeat rate and occurrence of edemas (Figure S8). In the 96 h exposures with diclofenac and bromoxynil at EC25 with BSP at 100 µM, 100% of the embryos were adversely affected; 100% of embryos were also affected in the exposure with diclofenac at EC5 with 100 µM BSP added. Due to high mortalities in the treatments, especially in the 96 h exposure with bromoxynil combined with BSP (25-75% mortalities at EC5 and 100% mortality at EC25 with 100 µM BSP), the number of samples for internal concentration measurements was considerably reduced, making interpretations difficult (Figure 5). The increased toxicity specific for bromoxynil but not BSP suggests that the internal bromoxynil concentration was increased when bromoxynil and BSP were combined in the exposure. This is also seen for diclofenac (Figure 5): we observed a significantly increased internal concentration of diclofenac with BSP addition. In the 96 h treatments with a combination of diclofenac and bromoxynil (both at EC5 and EC25, Table S7), the number of affected embryos was increased by about a factor of 2, and similar sublethal/lethal effects were observed as in the single compound exposures. The internal concentration measurements showed indications for an increased internal concentration of diclofenac and a decreased internal

concentration of bromoxynil (Figure S9). The only effect caused by carbamazepine and the combination of carbamazepine and BSP was reduced hatching after 96 h of exposure. Although data highly varied in the two replicate exposures, the number of embryos that did not hatch tended to be higher in the treatment with carbamazepine and BSP where the effect reached 100% effect in several replicates (Table 2). Furthermore, our results showed a significantly increased internal concentration of carbamazepine with BSP addition.

To receive an indication of whether effects and internal concentrations were dependent on the developmental stage we compared embryos from 24 h exposures that were started with embryos at 1 hpf (exposure experiment 2) and 72 hpf (exposure experiment 3), respectively. Nearly no phenotypic effects upon shorter exposures were observed for bromoxynil, diclofenac and carbamazepine (Tables S4 and S5). In embryos at 96 hpf, reduced movement was observed upon 24 h exposures to carbamazepine or the carbamazepine/BSP combination.

In the single compound exposures, the internal bromoxynil and diclofenac concentrations diverged in the embryos at the different developmental stages (Figure 5). Taking the volume change of the embryo body during development into account, uptake of bromoxynil was about 6.4- (exposure at EC5) and 6.8- (exposure at EC25) times higher in the more advanced than in the earlier developmental embryo stages. For diclofenac, uptake was 2.5- (exposure at EC5) and 3- (exposure at EC25) times higher in the more advanced developmental embryo stage. No difference between developmental stages was seen regarding the amount of accumulated carbamazepine. When BSP was also present in the exposure medium (Figure 5), internal concentrations of bromoxynil and carbamazepine upon 24 h exposures were increased, being more pronounced in the more advanced embryo stages. The co-presence of BSP in the diclofenac exposure did not significantly affect the internal diclofenac concentration at the different developmental stages (Figure 5).

Discussion

It has been previously suggested that the TK of ionic substances in zebrafish embryos are, beyond passive diffusion, majorly influenced by transport proteins.[6, 12] Our results confirm that cellular transporters may indeed play an important role in the TK of anions. In ecotoxicology, the focus so far

has been on ATP-binding cassette (ABC) efflux transporters.[33] However, the pharmacologically/toxicologically relevant cellular transporter system is complex[9] and studies e.g. with the SLC transporter zebrafish Oatp1d1 point to the potential ecotoxicological relevance also of other transporter types.[13] Based on previous findings that processes other than passive diffusion are likely involved in the uptake of bromoxynil by zebrafish embryos,[6] this study was aimed to examine the potential involvement of transporter proteins in the TK of bromoxynil. Our results for bromoxynil may eventually help to understand the TK of other anionic compounds showing slow uptake kinetics.

It needs to be considered that the LA-ICP-MS tissue imaging results (Figure 1) may not just represent the accumulation patterns of bromoxynil in fish embryo tissue but also of bromine-containing transformation products of bromoxynil. Thus, it has previously been shown that bromoxynil is metabolized in the zebrafish embryo to several transformation products that also contain bromine.[6] However, the MALDI-MS imaging results (Figure 2) confirmed that the parent compound bromoxynil accumulates in the GI tract.

Upon exposure of embryos to bromoxynil, bromoxynil signals were comparatively high in the GI tract (Figures 1 and 2). Based on the reported pH of the intestinal bulb in the GI tract of 5.0-5.4,[34] ion-trapping[18] as a potential reason for this bioaccumulation pattern of bromoxynil can be ruled out. It can be excluded that bromoxynil with a pKa of 3.75[22] will be increasingly ionized at the pH of the intestinal bulb compared to a pH of 7.55 in the embryo.[35] Also, the affinity of bromoxynil to certain biological molecules causing this bioaccumulation pattern, like it was found for pigments in the eye binding psychoactive drugs[12], seems unlikely, as MS imaging does not show an accumulation specifically around the eyes.

We considered the organic anion transporting polypeptide Oatp1d1 as a conceivable driver behind the accumulation pattern of bromoxynil in the zebrafish embryo for the following reasons: Oatp1d1 is expressed already in the zebrafish embryo[19] and various organs of adult zebrafish, with the highest transcript levels in the intestine.[31] The activity of this transporter inside the embryo was indicated by the finding that the fluorescent compound MC-LR-Texas red, found to act as a Oatp1d1 substrate, was taken up by the cells of the glomerulus of the pronephros.[33] As an SLC transporter, Oatp1d1 facilitates the

transport of substrates across the plasma membrane along a concentration gradient,[31] as seen for radio-labeled BSP used as a positive control compound: Compared to control cells, the uptake of labeled BSP was enhanced in Oatp1d1-overexpressing cells (Figure 3). For bromoxynil and diclofenac, however, a significantly lower accumulation in HEK-drOatp1d1 cells than in HEK-Co cells (Figure 3) indicated that Oatp1d1 also mediates cellular efflux.

In a previous study, the interaction of diclofenac and carbamazepine with zebrafish Oatp1d1 has been investigated with the fluorescent Oatp1d1 substrate lucifer yellow as a proxy for transporter activity[13]. These experiments indicate, in agreement with this study, the interaction of diclofenac but not of carbamazepine with Oatp1d1[13]. The assay with lucifer yellow is an indirect method, recording the intracellular change in fluorescence when a test compound is present. Hence, the assay does not indicate whether the transporter-interacting compound is taken up or effluxed from the cell; this could be specified by direct measurements of the test compounds, as performed here.

There is evidence that the transport mechanism of Oatps/OATPs is based on anion exchange: Cellular uptake of organic compounds can be coupled with the efflux of bicarbonate, glutathione, and/or glutathione-S-conjugates.[36,37] Whether the mechanism of a chemical's cellular efflux is based on co-transport with cellular ions, such as the ones above, or an exchange mechanism involving ions, e.g., contained in the medium, is not known and needs exploration. The action of an OATP protein as an efflux transporter has been observed before: The human renal transport protein OATP4C1 mediated cellular efflux of the compounds digoxin, asymmetric dimethylarginine (ADMA), L-arginine, and L-homoarginine.[38] The results in that study were obtained by direct measurements of the intra- and extracellular levels of radio-labeled compounds.[38]

There is no evidence for interaction of carbamazepine with zebrafish Oatp1d1 from the cellular tests with the fluorescent substrate dye Lucifer yellow[13] and when comparing the accumulation rates in HEK-Co and HEK-Oatp1d1 cells (Figure 3). However, accumulation of carbamazepine was reduced in the HEK-Oatp1d1 cells depending on the BSP concentration that was present (Figure 4). It may be assumed that stimulation of cellular efflux of carbamazepine by Oatp1d1 is caused by an allosteric effect shown before for other substrates and OATP proteins, e.g., OATP1B1 and OATP1B3.[39]

It can be assumed that also other cellular transporter types are involved in the TK of bromoxynil in the zebrafish embryo. BSP[30, 39] and especially CsA[40] inhibit a range of different transporters. Zebrafish embryos express a set of different transport proteins. Therefore, the transporter inhibiting action of BSP in the experiments with zebrafish embryos may not have been Oatp1d1-specific. For example, from the SLC superfamily Oatp2b1 is also present in zebrafish embryos.[19] Furthermore, diclofenac has been described as substrate of zebrafish Oat2a, Oat2b, and Oat2d in cell experiments,[14] potentially expressed in zebrafish embryos. Also, the interaction of test compounds with ABC efflux transporters, such as zebrafish Abcb4, is conceivable. Diclofenac, which in this study showed similar interaction with Oatp1d1 as bromoxynil, has been previously found to have a decreasing effect on both the basal and verapamil-stimulated ATPase activity of recombinant Abcb4.[41] This indicates that this compound acts as an inhibitor but not as a transported substrate of zebrafish Abcb4, which may not influence the TK of diclofenac. The effect of this protein on the TK of bromoxynil needs to be elucidated in the future.

There is a more pronounced effect on the internal concentrations of bromoxynil and diclofenac by BSP in the more advanced developmental stages (Figure 5). This may additionally be seen as an indication of the involvement of cellular transport proteins, especially Oapt1d1, in the uptake of bromoxynil in zebrafish embryos. The increased expression of Oatp1d1 with progressing development of the embryo has been shown by Faltermann et al.[19] Thus, the more pronounced BSP effect on the internal bromoxynil and diclofenac concentrations in more advanced developmental stages correlates with increased expression levels of *slco1d1* (*oatp1d1*) transcripts (encoding Oatp1d1 protein), accompanied by higher transporter activity in the more advanced stages.[19] Along these lines, bromoxynil and diclofenac were taken up by tissue of developmentally more advanced embryos to a larger extent in the single compound exposures (i.e., without addition of BSP; Figure 5). This was most pronounced for bromoxynil, which showed an about 7-times increased uptake. When comparing 48 and 96 hpf stages, increased uptake in the more advanced developmental stage was also reported for paracetamol and diphenhydramine.[8, 16] The chorion as a potential chemical uptake barrier in the earlier stages was excluded for bromoxynil, paracetamol and diphenhydramine.[6, 8, 16] As the expression of Oatp1d1[19] and presumably also other transport proteins (e.g. Oatp2b1[19]) increases with the progression of zebrafish embryo development, respective transport activity may correspondingly be higher. However, other explanations for stage-

specific chemical uptake are conceivable. Thus, changes in the sorption capacity with the development of the GI tract have been suggested.[8]

The experimental concentrations of the test compounds applied here were three to four orders of magnitude above the levels of those compounds found in the environment.[15] The high experimental concentrations were necessary as concentrations needed to be (1) in the toxic range for assessing phenotypic effects of the test compounds in zebrafish embryos and (2) sufficiently high to detect the compounds in the embryo tissue for determination of the internal concentrations. Since the lower environmental concentrations are below the transporter capacity saturation, cellular transporters can be expected to impact the TK of the tested ionic compounds in the same manner as in this study.

The finding of the action of Oatp1d1 as an efflux transporter of bromoxynil in the cellular transport assays indicates that Oatp1d1 appears to constitute a mechanism keeping bromoxynil out of the body. This is supported by the finding that bromoxynil levels in the embryo tissue were higher when the compound was combined with BSP in the exposure (Figure 5), indicating that BSP blocks efflux - presumably by Oatp1d1 inhibition - of bromoxynil from the body. The action of Oatp1d1 as an efflux transporter of bromoxynil also coincides with comparatively high bromoxynil levels in the GI tract, a region of expression of Oatp1d1[31] (Figures 1, 2). Oatp1d1 may thus mediate the transport into the region of the lumen of the gut primordia, which in the functional intestine will lead to the elimination of bromoxynil *via* this route. The accumulation in the GI tract may thus be seen as a result of this elimination mechanism. Accumulation of bromoxynil in certain tissue areas in the embryonic body, potentially conferred by the activity of this transporter, may contribute to the comparatively high accumulation of this compound in the embryonic body.

By acting bi-directionally as cellular uptake and efflux transporter zebrafish Oatp1d1 may have a dual protective role.[38] By showing the bi-directional action of Oatp1d1 our results confirm that direct measurements of chemicals should be applied more commonly as they allow to determine the direction of translocation of compounds across cellular membranes. We show that a combined experimental approach investigating chemical interaction with relevant proteins in cell systems and bioaccumulation patterns and kinetics in the living zebrafish embryo can provide important insights into TK-relevant

processes. The insights in the TK of bromoxynil may also be relevant for other anionic compounds, e.g., bromoxynil, diclofenac, and clofibric acid possess similar pKa values and were shown to be taken up slowly into the embryo,[6, 23, 17] conceivably, due to active transport processes.

Our results, providing evidence that TK of the anion bromoxynil is cellular transporter-driven in zebrafish embryos, suggest that the activity of transport proteins in plasma membranes should be more considered in attempts to predict TK, particularly of ionic compounds. Furthermore, the following aspects should be addressed in future research:

- Investigations of the background of the bi-directional action of Oatp1d1.
- Investigations of the proteins' qualitative and quantitative effect on the TK of chemicals applying knock-down of expression of TK-related proteins in zebrafish embryos. Examination of the impact of morphological features and developmental processes, such as the opening of the GI tract[8] and the mouth,[42] and organ-specific parameters, such as the formation of the microbiome in the GI tract,[43] on TK.
- Examination of the consequences of the interaction of chemicals accumulated in the GI tract with the microbiome for TK and toxico-dynamics.

Supporting Information

Details on Materials and Methods; dose-response curves for bromoxynil and bromosulfophthalein (BSP); overview of conducted experiments; UV/vis absorbance BSP; BSP uptake curve into HEK-Oatp1d1 cells; additional cell results (100 µM incubation, addition with cyclosporin A, mixed incubation bromoxynil and diclofenac); phenotypic effects at the shorter exposures of zebrafish embryos; internal concentration of bromoxynil and diclofenac in mixed exposure; MS imaging with isotopic peaks of bromoxynil and on control embryos.

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Author contributions

Katharina Halbach, Till Luckenbach, and Jörg König conceptualized and designed the study. Katharina Halbach, Silke Aulhorn, and Marion Lecluse performed exposure experiments with zebrafish embryos. Silke Aulhorn analyzed the embryotoxicity data. Katharina Halbach performed the zebrafish embryo sample preparation for the measurements of chemicals levels in the tissue. Bettina Seiwert conducted the UPLC-QToF-MS analyses. Marion Lecluse performed the LA-ICP-MS and Sophia Leippe the MALDI-FT-ICR-MS imaging measurements with the help of Katharina Halbach, Stephan Wagner, and Oliver Jens Lechtenfeld. Jörg König was responsible for the study design of the cell experiments. Bettina Seiwert helped with the sample preparation of the cell samples. Katharina Halbach and Jörg König analyzed the data. Katharina Halbach, Jörg König, Thorsten Reemtsma, and Till Luckenbach discussed and interpreted the data. Katharina Halbach and Till Luckenbach wrote the manuscript; all other co-authors commented on the manuscript. All authors have approved the final version of the manuscript.

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Tables

Table 1. Oatp1d1-mediated net transport (difference of the accumulation in HEK-drOatp1d1 and HEK-co cells) of BSP (1 µM incubation) and bromoxynil, diclofenac and carbamazepine (10 µM incubation). The incubation time was 10 min. The data of two independent experiments (exposures A and B) are separately depicted; means ± SD of three replicates per concentration are shown. Significant differences between treatments with and without BSP are indicated by asterisks (Manly's permutation test, *** $p < 0.001$). The respective accumulation rates are reported in Figure 4.

Substance A	Concentration (substance A; µM)	Substance B	Concentration (substance B; µM)	Exposure	Net transport (pmol x mg protein^{-1} x min^{-1})	
					mean	SD
BSP	1	BSP	0	A	19.0	1.0
				B	7.0	0.8
BSP***	1	BSP	50	A	0.98	0.18
				B	0.60	0.10
BSP***	1	BSP	100	A	0.38	0.21
				B	0.37	0.16
Bromoxynil	10	BSP	0	A	-6.8	2.0
				B	-18.8	3.1
Bromoxynil	10	BSP	50	A	-7.6	1.0
				B	-5.7	1.1
Bromoxynil	10	BSP	100	A	-16.7	1.2
				B	-10.4	0.9
Diclofenac	10	BSP	0	A	3.4	4.4
				B	-41.3	5.0
Diclofenac	10	BSP	50	A	5.8	5.0
				B	-19.1	8.7
Diclofenac	10	BSP	100	A	-7.3	3.3
				B	-17.5	5.6
Carbamazepine	10	BSP	0	A	-0.42	0.30
				B	0.70	0.83
Carbamazepine	10	BSP	50	A	-0.092	0.099
				B	-0.91	1.5
Carbamazepine***	10	BSP	100	A	-1.6	0.14
				B	-2.2	0.57

Table 2. Toxicities of the test compounds for zebrafish embryos in 96 h exposures (experiment 1). Exposures were performed twice in independent experiments with embryos from different egg batches (exposures A and B). The term "total effects" comprises lethal and sublethal (phenotypic) effects. Embryos with the observed phenotypic effects are depicted in Figure S8. For measured concentrations of EC5 and EC25 values refer to Table S2. k: coagulation; mtail: tail deformation; nohb: no heartbeat; nobc: no blood circulation; EdP: edema pericard; hf: reduced heart rate; (beh: observed less movement, not quantitatively assessed)

Substance A (expos. conc.)	**Substance B (expos. conc.)**	**Mortality [%]**		**Total effects [%]**		**Observed effects**
		Exposure A	**Exposure B**	**Exposure A**	**Exposure B**	
Control		0	8	0	8	k
BSP (50 µM)		0	0	60	0	no hatching
BSP (100 µM)		0	0	39	0	no hatching
Bromoxynil (EC5)		0	0	0	7	mtail
Bromoxynil (EC5)	BSP (50 µM)	24	27	41	33	no hatching, nohb
Bromoxynil (EC5)	BSP (100 µM)	75	25	88	50	nohb, nobc
Bromoxynil (EC25)		5	17	10	33	nohb, nobc
Bromoxynil (EC25)	BSP (50 µM)	100	80	100	85	nohb, k
Bromoxynil (EC25)	BSP (100 µM)	100	100	100	100	nohb, k, no hatching
Diclofenac (EC5)		0	7	27	20	EdP, no hatching
Diclofenac (EC5)	BSP (50 µM)	7	7	93	93	nobc, EdP, no hatching, (beh)
Diclofenac (EC5)	BSP (100 µM)	13	20	100	100	nobc, EdP, hf, no hatching, (beh)
Diclofenac (EC25)		0	0	43	5	EdP
Diclofenac (EC25)	BSP (50 µM)	62	0	100	81	EdP, hf, nohb, no hatching
Diclofenac (EC25)	BSP (100 µM)	57	11	100	100	EdP, nohb, no hatching
Carbamazepine (EC5)		0	0	73	22	no hatching
Carbamazepine (EC5)	BSP (50 µM)	0	0	100	22	no hatching
Carbamazepine (EC5)	BSP (100 µM)	0	0	100	89	no hatching
Carbamazepine (EC25)		0	0	81	33	no hatching
Carbamazepine (EC25)	BSP (50 µM)	0	0	100	89	no hatching
Carbamazepine (EC25)	BSP (100 µM)	0	0	100	89	no hatching

Figures

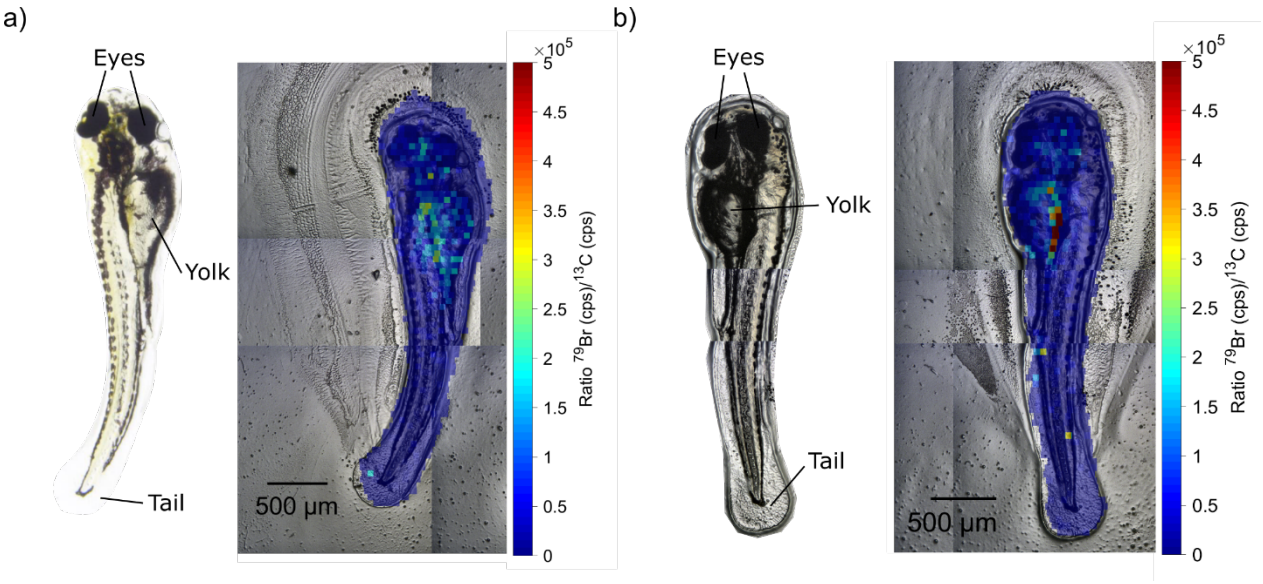

Figure 1. Tissue distribution of bromine (^{79}Br) in zebrafish embryos upon exposure to the compound bromoxynil (96 h exposure to EC5). Bright-field images of two individuals (a, b) at approximately 97 hpf are shown (on the left of each image) before the ablation. Images to the right show the bright-field images overlaid with the calorimetrically indicated tissue levels of ^{79}Br (normalized to tissue density represented by ^{13}C) obtained with LA-ICP-MS.

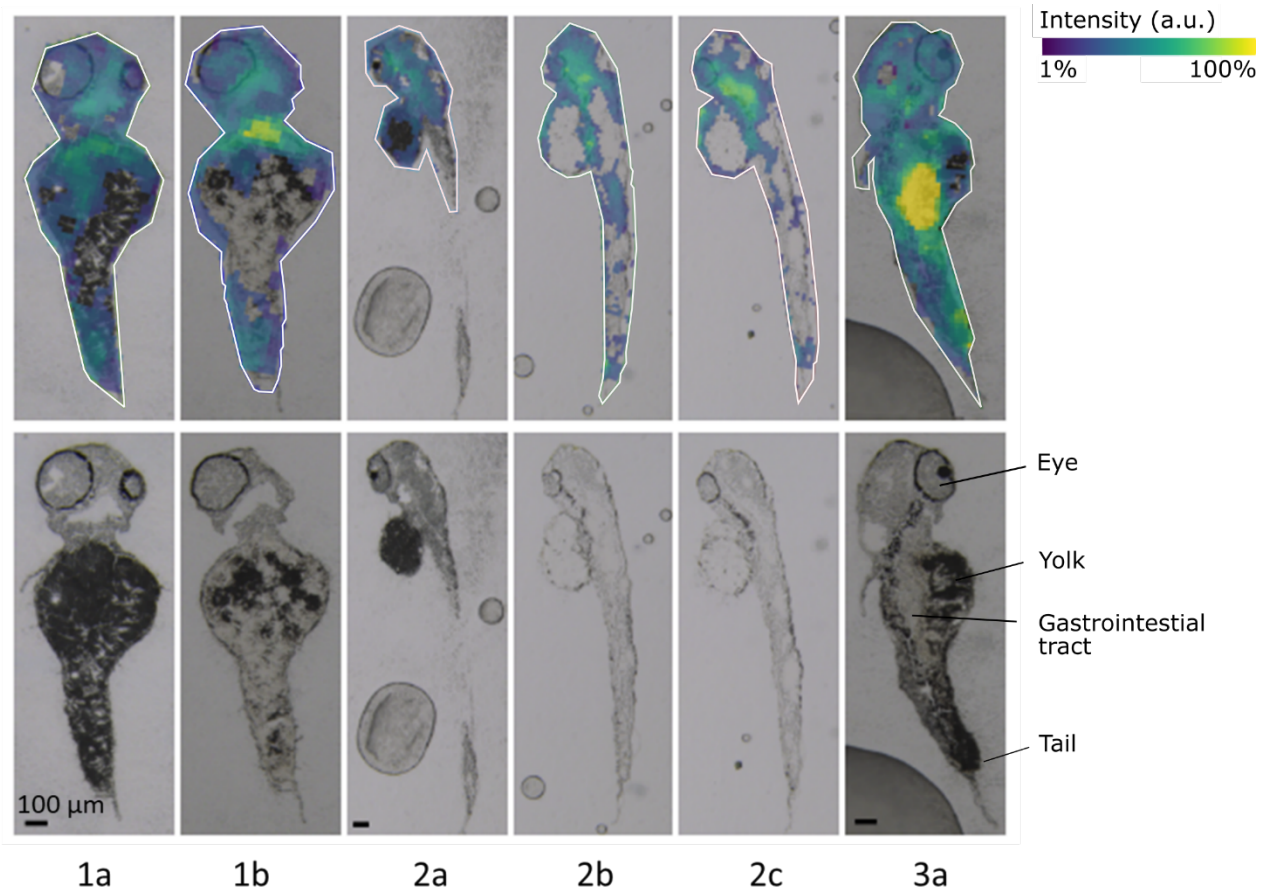

Figure 2. Tissue distribution of bromoxynil (upper row, 275.84671 m/z ± 0.19313 mDa) in exposed zebrafish embryos (1-3) upon exposure to the compound for 72 h (EC5). Images in the upper row are bright-field images (see lower row) overlaid with the color-coded intensity of bromoxynil. Different sections (a-c) of three different embryos (1-3) are shown. The white lines, surrounding the embryo tissue in the cryosections, mark the measured tissue areas. The scale bar is 100 µm. The color bar shows the range from low (purple) to high bromoxynil signal intensity (yellow).

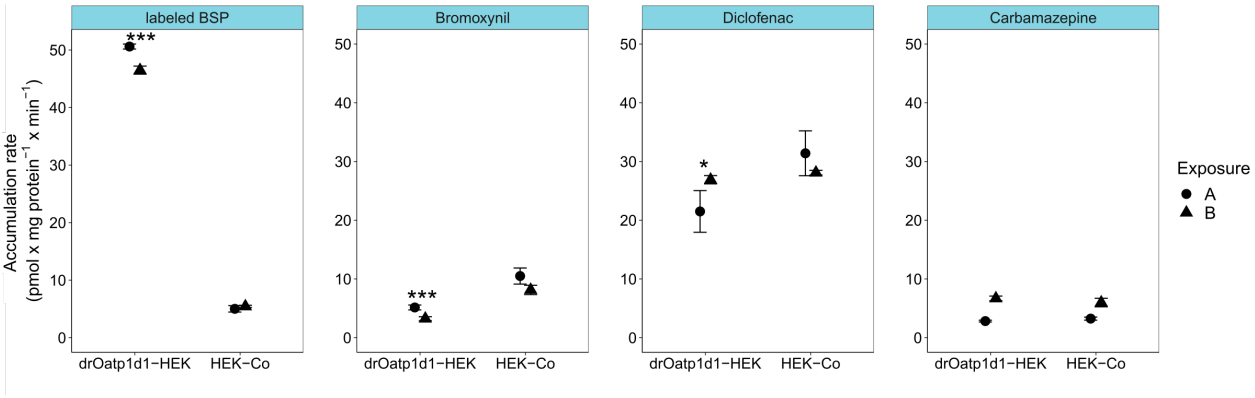

Figure 3. Accumulation rates of labeled BSP (1 µM), bromoxynil, diclofenac and carbamazepine (10 µM) in zebrafish Oatp1d1-expressing (HEK-drOatp1d1) and control cells (HEK-Co) cells. The incubation time was 10 min. The data of two independent experiments (exposures A and B) are separately depicted; means ± SD of three replicates per concentration are shown. Net transport values: 46 and 41 pmol x mg protein^{-1} x min^{-1} ([^{3}H]-BSP), -5.3 and -4.9 (bromoxynil), -9.9 and -9.1 (diclofenac), -0.4 and 0.7 (carbamazepine). Significant differences between accumulation rates in HEK-drOatp1d1 and HEK-co cells are indicated by asterisks (Manly's permutation test, * $p < 0.05$, *** $p < 0.001$). Please refer to Figure S4 for cellular accumulation data upon incubation to test compounds at 100 µM.

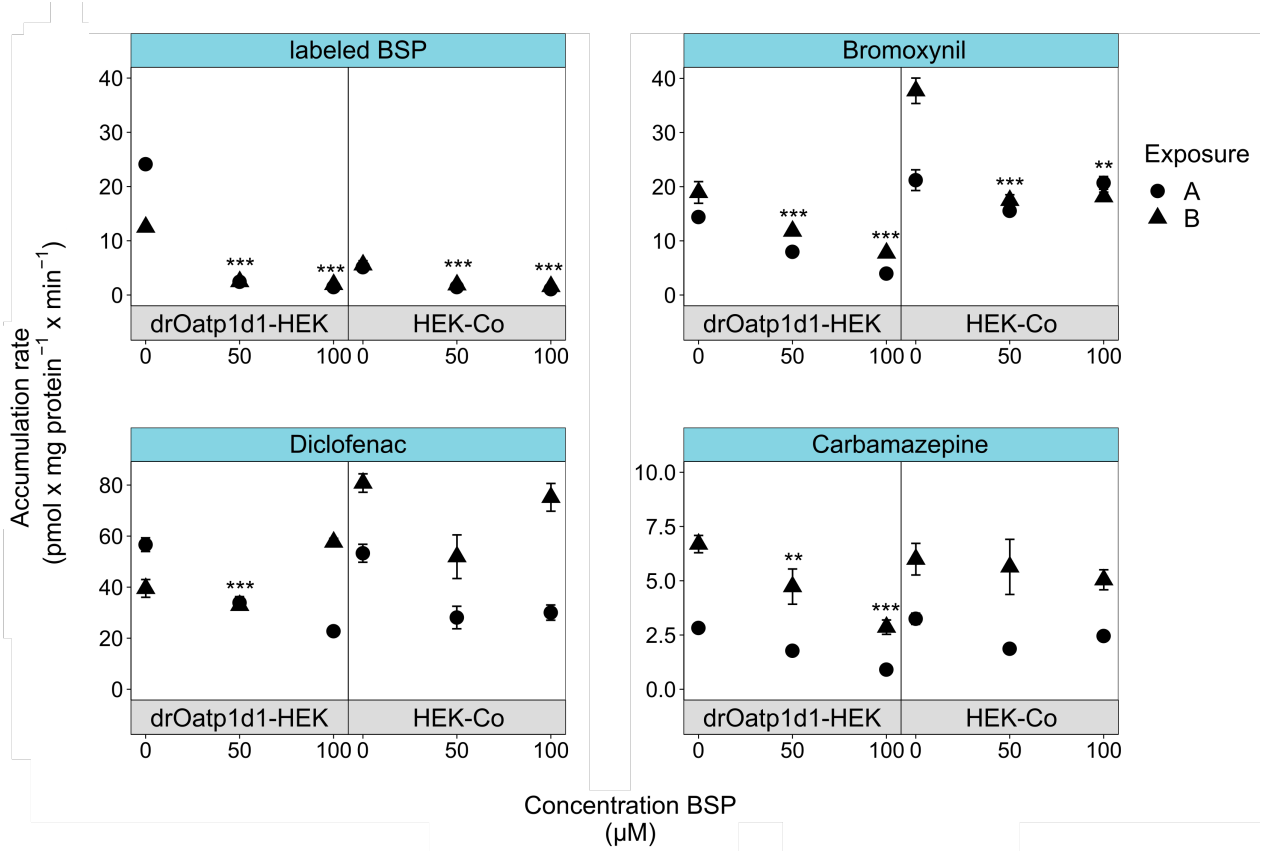

Figure 4. Accumulation of labeled BSP (1 µM incubation), bromoxynil, diclofenac and carbamazepine (10 µM incubations) in zebrafish Oatp1d1-expressing (HEK-drOatp1d1) and in control (HEK-Co) cells. The incubation time was 10 min. The data of two independent experiments (exposures A and B) are separately depicted; means ± SD of three replicates per concentration are shown. Significant differences between treatments with and without BSP are indicated by asterisks (Manly's permutation test, ** $p < 0.01$, *** $p < 0.001$). Please note different scales of the y-axes. The respective net transport values are reported in Table 1. Please refer to Figure S5 for accumulation rate data upon incubation to test compounds at 100 µM.

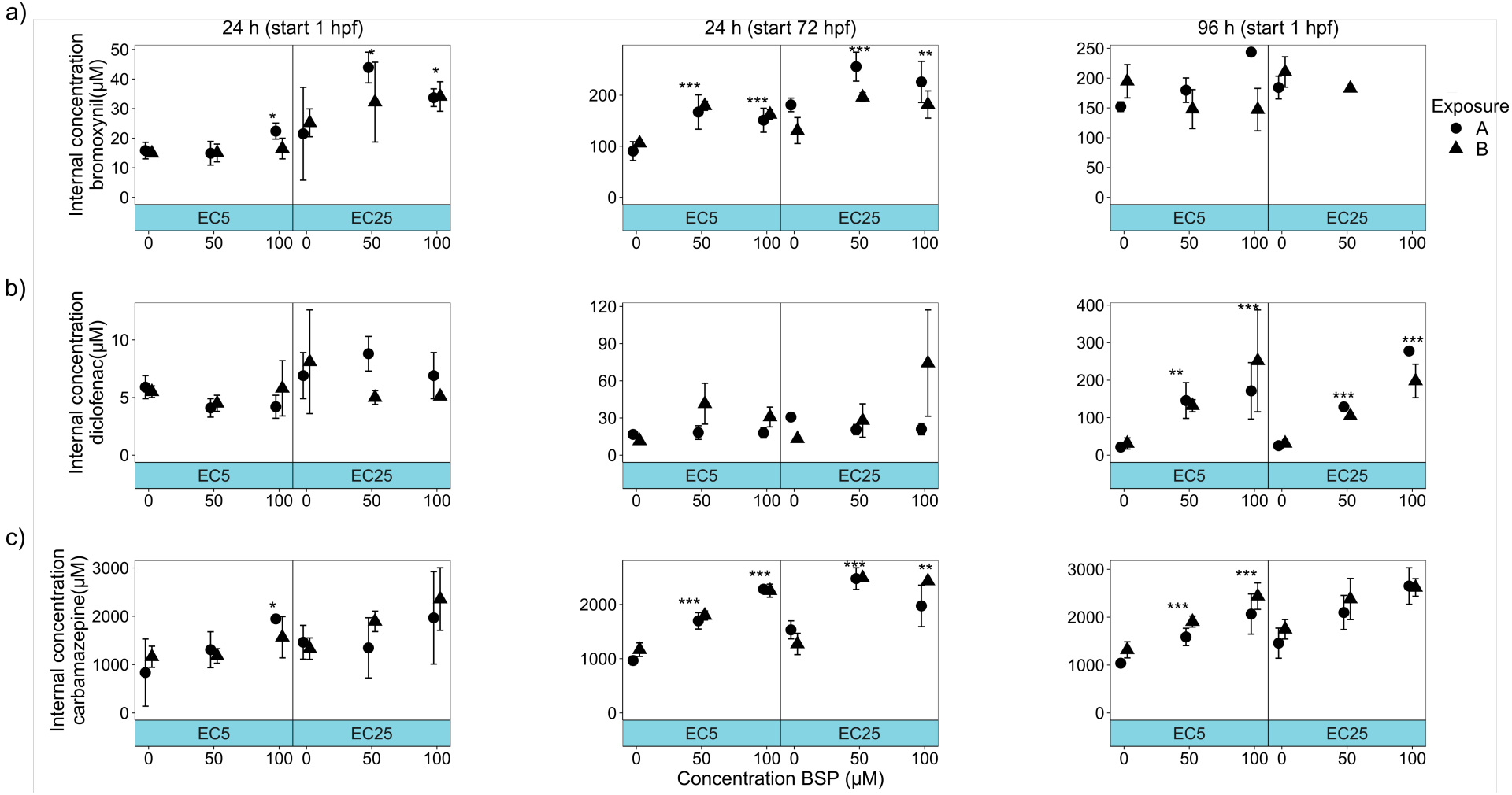
a)
b)
c)
24 h (start 1 hpf)
24 h (start 72 hpf)
96 h (start 1 hpf)
Internal concentration bromoxynil(µM)
Internal concentration diclofenac(µM)
Internal concentration carbamazepine(µM)
EC5
EC25
Exposure
A
B
Concentration BSP (µM)

Figure 5. Internal bromoxynil (a), diclofenac (b) and carbamazepine (c) concentrations in zebrafish embryos. The embryos were exposed to the compounds at EC5 or EC25 alone or in combination with 50 or 100 µM BSP for 96 h (experiment 1) and 24 h (experiment 2: starting at 1 hpf and experiment 3: starting at 72 hpf). Exposures were performed twice in independent experiments with embryos from different egg batches (exposures A and B). Data are shown as means (symbols) and standard deviations (bars) from three replicates; each replicate consists of the data from three pooled embryos. Significant differences between the data for treatments with 50 µM or 100 µM BSP and the respective treatments without BSP are indicated by asterisks (Manly's permutation test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Reduced numbers of replicates for 96 h exposure with bromoxynil (EC5 with 100 µM BSP: n=1 in Exp. A; EC25 with 50 µM BSP: n=1 in Exp B; n=0 for Exp. A with EC25 with 50 µM BSP and Exp. A+B EC25 with 100 µM BSP) and diclofenac (EC25 with 50 µM BSP: n=2 in Exp. A; EC25 with 100 BSP: n=1 in Exp. A) because of high mortality. Please note different scales of the y-axes.