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# Functional characterization of rainbow trout (*Oncorhynchus mykiss*) Abcg2a (Bcrp) transporter

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ABSTRACT

ABCG2 (BCRP – breast cancer resistance protein) belongs to the ATP-binding cassette (ABC) superfamily. It plays an important role in the disposition and elimination of xeno- and endobiotics and/or their metabolites in mammals. Likewise, the protective role of ABC transporters, including Abcg2, has been reported for aquatic organisms. In our previous study we have cloned the full gene sequence of rainbow trout (*Oncorhynchus mykiss*) Abcg2a and showed its high expression in liver and primary hepatocytes. Based on those insights, the main goal of this study was to perform a detailed functional characterization of trout Abcg2a using insect ovary cells (*Spodoptera frugiperda*, Sf9) as a heterologous expression system. Membrane vesicles preparations from Sf9 cells were used for the ATPase assay determinations and basic biochemical properties of fish Abcg2a versus human ABCG2 have been compared. A series of 39 physiologically and/or environmentally relevant substances was then tested on interaction with trout Abcg2a and human ABCG2. Correlation analysis reveals highly similar pattern of activation and inhibition. Significant activation of trout Abcg2a ATPase was observed for prazosin, doxorubicine, sildenafil, furosemid, propranolol, fenofibrate and pheophorbide. Pesticides showed either a weak activation (malathione) or strong (endosulfan) to weak (chlorpyrifos, fenoxycarb, DDE) inhibition of trout Abcg2a ATPase while the highest activation was obtained for benzo(a)pyrene, curcumine and testosterone. In conclusion, data from this study offer the first characterization of fish Abcg2a, reveal potent interactors among physiologically or environmentally relevant substances and point to similarities regarding strengths and interactor preferences between human ABCG2 and fish Abcg2a.



## 1. Introduction

The human ABCG2 (BCRP – breast cancer resistance protein) belongs to the ATP-binding cassette (ABC) transporters superfamily and plays an important role in the elimination of xenobiotics. Together with the P-glycoprotein (Pgp; ABCB1) and members of the MRP (ABCC) subfamily, ABCG2 is a major mediator of the multidrug resistance (MDR) phenotype in tumor cells. Unlike ABCB1 or ABCCs, however, ABCG2 is a 72-kDa half-transporter, consisting of one nucleotide binding domain (NBD) and one membrane spanning domain (TMD) containing 6 α-helices (Wakabayashi et al., 2006). Upon dimerization, two ABCG2 monomers form the functional transporter that is similar to structure and transport mechanism of ABCB1 (Ni et al., 2010; Rosenberg et al., 2015). The binding of a substrate from the inner membrane leaflet or the cytoplasm results in conformational changes, activation and dimerization of two NBDs. Dimerization causes structural changes leading to the outward facing conformation and release of the substrate in the extracellular space. Finally, ATP hydrolysis resets the protein in the inward facing conformation making it ready for a new catalytic cycle (Rosenberg et al., 2015).

Through the described mechanism ABCG2 transports a wide range of structurally diverse xenobiotics, including both positively and negatively charged molecules of relatively high molecular mass and amphiphilic character (Basseville et al., 2014). Similar to ABCB1, ABCG2 is involved in efflux and resistance to many unmodified anticancer drugs (mitoxantrone, topotecan, irinotecan, etoposide, flavopiridol, methotrexate) as well as other therapeutics (statins, sulfasalazine, nitrofurantoin, cimetidine, imatinib). Contrary to ABCB1 and more similar to ABCCs, ABCG2 mediates transport of many conjugated endo- and xeno- biotics (Basseville et al., 2014; Chen et al., 2016). However, unlike ABCCs which are mainly involved in the transport of glutathione and glucuronide conjugates, ABCG2 exhibits preference toward sulfate conjugates (Álvarez et al., 2011; van de Wetering and Sapthu, 2012). Recent findings also support the role of ABCG2 in the cellular homeostasis of porphyrins and related compounds, and consequently in oxidative stress response (Krishnamurthy and Schuetz, 2011).

ABCG2 is expressed in the apical membranes of polarized epithelial cells of different organs involved in absorption (small intestine),

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distribution (placenta and blood brain barrier) and elimination (liver) (Fetsch et al., 2006). Taken together, the tissue distribution, cellular localization, and substrates' specificity of ABCG2 all indicate that this transporter plays a major role in the disposition and elimination of xeno- and endobiotics and/or their metabolites in mammals, together with ABCB1 and ABCC2.

In the context of environmental toxicology, protective role of ABC proteins in aquatic organisms was first recognized in early 1990s, revealing the presence of the Pgp (ABCB) family of transporters in marine and freshwater bivalves. Since then, the presence of the ABCB1 and ABCC types of efflux transporters has been demonstrated in many aquatic species (Bard, 2000; Sturm and Segner, 2005; Luckenbach et al., 2014). Most of the studies have been focused on identification of ABCs at the transcript, protein and/or functional levels, using functional in vivo studies or studies in permanent cell lines or in primary fish cell cultures (reviewed in Luckenbach et al., 2014). Furthermore, those studies essentially utilized multitransporter systems that express many transport proteins with overlapping substrate specificities. Consequently, such systems do not enable a detailed and reliable characterization of single ABC transporter(s). Yet, despite the presumable role of these transporters in defense against natural and man-made substances in aquatic organisms, with the exception of the study done by Fischer et al. (2013) on zebrafish Abcb4, no study has been focused on thorough functional characterization of ABC transporters in heterologous expression systems.

Insect ovary cells (*Spodoptera frugiperda*, Sf9) are the expression system often used in studying mammalian ABC transporters (Meyer et al., 1994; Trometer and Falson, 2010). Due to the high protein expression, membrane vesicles preparation from Sf9 cells overexpressing the target ABC transporter are used for ATPase assays or vesicular transport assays directed to the determination of substrate and inhibitor specificity of ABC transporters, including transporters from the ABCG family (Özvegy et al., 2001; Müller et al., 2006; Pozza et al., 2010). In our previous study (Zaja et al., 2008) we have cloned the full gene sequence of rainbow trout (*Oncorhynchus mykiss*) Abcg2 and showed its high expression in trout liver and primary hepatocytes as well as in other trout tissues. However, recent availability of the full genome data from multiple fish species reveals that there are two Abcg2 genes (*abcg2a* and *abcg2d*) present in the trout genome, and four predicted Abcg2 genes in the zebrafish genome, contrary to mammals that have only one ABCG2 protein. Multiple alignment of our trout sequence with mammalian ABCG2 and zebrafish Abcg2 proteins has revealed a high degree of similarity to mammalian ABCG2 and zebrafish Abcg2a, respectively. Therefore, based on those initial insights, the main goal of the present study was to perform a detailed functional characterization of trout Abcg2a using Sf9 insect cells as a heterologous expression system. Furthermore, basic biochemical properties and substrates' and/or inhibitors' affinities of trout Abcg2a versus human ABCG2 expressed in Sf9 insect cells have been compared as well.

## 2. Materials and methods

### 2.1. Chemicals

Triton X-100, Dimethyl Sulphoxide (DMSO), phosphate buffered saline without $Ca^{2+}$ and $Mg^{2+}$ (PBS), pyruvate, HEPES, and all compounds tested for their interaction with trout and human Abcg2/ABCG2 (shown on Fig. 7) were purchased from Sigma, St. Louis, MO, USA. The exceptions were MK571 (obtained from Cayman Chemicals Co., Michigan, OR, USA) and calcein-AM (Ca-AM; purchased from Molecular Probes, Eugene, OR, USA). Dulbecco's modified Eagle medium with F12 nutrient mixture (DMEM/F12), Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Karlsrhue, Germany). Ethanol, isopropanol and all other chemicals used were of the highest analytical grade available and purchased from Kemika, Zagreb, Croatia.

### 2.2. Generation of recombinant plasmids

Designation of gene and protein names used throughout the text is based on the Zebrafish Nomenclature Guidelines:
(https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines); e.g., fish: *shh*/Shh, human: *SHH*/SHH. After sequence verification, trout *abcg2a* was subcloned from pGEM T-easy vector into baculovirus transfer vector pAcHLT (BD Biosciences, Heidelberg, Germany) using *Eco*RI/*Not*I restriction digestion. Subsequently, the same *abcg2a* clone was subcloned into pcDNA3.1-His(+) vector for transfection of human embryonic kidney cell line (HEK293T) using the same restriction enzymes, and to pEGFP vector using EcoRI/*Apa*I digestion to produce recombinant Abcg2a protein with GFP tag fused to the N-terminus. Human *ABCG2* was kindly provided by Dr. Özvegy (Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary) in pcDNA3.1(−) vector and was subcloned into pAcHLT and pcDNA3.1-His (+) vector using NotI/*Kpn*I and EcoRI/*Eco*RV restriction enzymes, respectively.

### 2.3. Cell culture and transfection

Sf9 insect cells were cultured in TNM-FH medium with 10% FBS. Using the lipofectin reagent cells were co-transfected with recombinant pAcHLT vector containing human *ABCG2* or trout *abcg2a* and baculoviral DNA (BD Biosciences). First baculoviral stock was amplified in two rounds and final baculoviral titer was determined by endpoint dilution assay. Final transfection was made with 2, 4 or 8 multiplicity of infection (MOI) units and after 3 days the cells were collected for membrane preparation.

HEK293T cells were cultured in DMEM high glucose medium with 10% FBS. Cells were transiently transfected with recombinant pcDNA3.1-His(+) plasmids carrying human *ABCG2* or trout *abcg2a*. Polyethylenimine (PEI) was used as transfection reagent in 1:1 ratio to plasmid DNA. After 48 h cells were collected for membrane preparation.

### 2.4. Membrane vesicles preparation, Western blotting and fluorescence detection

Plasma membrane vesicles were prepared as described by Cornwell et al. (1986). The cells were scraped, washed once in PBS, resuspended and homogenized in TEMP buffer (50 mM Tris, 50 mM manitol, 2 mM EGTA, 2 mM β-mercaptoethanol, 10 μg/ml leupeptin, 8 μg/ml aprotinin, 0.5 mM PMSF). The cell lysate was centrifuged (1000 ×g, 10 min) and layered on 35% sucrose. After centrifugation at 16,000 ×*g* for 30 min in swinging rotor, the layer formed at the top of 35% sucrose was collected, diluted in sample buffer (10 mM Tris, 250 mM sucrose, pH 7.5) and centrifuged at 100,000 ×*g* for 2 h. The pellet containing plasma membrane vesicles was resuspended in 0.5 ml of sample buffer and homogenized by passing the suspension 10 times through a 27-gauge needle with a syringe, and then incubated on ice for 30 min to obtain membrane vesicles. Total proteins were determined by the Lowry method (Lowry et al., 1951). Twenty micrograms of protein per lane were separated by electrophoresis in 7.5% sodium dodecyl sulfate polyacrylamide gel. The proteins were then transferred to polyvinylidene difluoride membrane by semidry blotting. After blocking and washing steps the membranes were incubated overnight with the anti-polyhistidin antibody (Sigma-Aldrich, Taufkirchen, Germany). Goat anti-mouse IgG-HRP was used as secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were visualized using Opti-4CN Substrate Kit (Bio-Rad).

For fluorescence detection, HEK293T cells were grown on glass coverslips in 24-well culture plates. Twenty four hours after transfection with pEGFP vector carrying *ABCG2* or *abcg2a* clone, cells were mounted with Fluoromount, (Sigma-Aldrich, Taufkirchen, Germany) and GFP fluorescence was detected using confocal microscope Leica TCS SP2 AOBS (Leica Microsystems, Wetzlar, Germany).

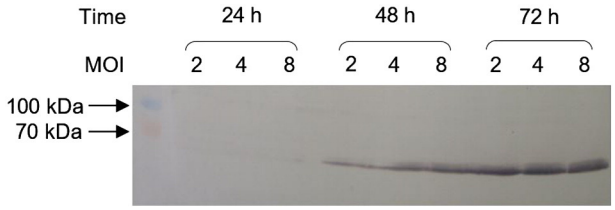


**Fig. 1.** The expression of rainbow trout Abcg2a in Sf9 cells. Sf9 insect cells were cultured and co-transfected with recombinant vector containing trout *abcg2a* and baculoviral DNA. Upon transfection done with 2, 4 or 8 multiplicity of infection (MOI) units, the isolation of plasma membrane vesicles was performed after 24, 48 or 72 h, and 20 μg of protein per lane were separated by electrophoresis. Trout Abcg2a was then visualized using primary anti-polyhistidin antibody and goat anti-mouse IgG-HRP secondary antibody, as described in Materials and methods section.

### 2.5. Cholesterol loading and ATPase activity assay

Cholesterol loading of Sf9 membrane vesicles was performed as described in Pál et al. (2007). Vesicles were loaded at indicated concentrations of cholesterol-RAMEB complex for 30 min at 37 °C. Afterwards vesicles were washed and resuspended in ATPase assay buffer (50 mM MOPS-Tris, pH 7.0, 50 mM KCl, 5 mM dithiothreitol, 2 mM EGTA, 2 mM sodium azide, 1 mM ouabain). ATPase activity was measured as described by Sarkadi et al. (1992) with minor modifications. Vesicles were diluted in ATPase assay buffer to 0.1 mg/ml and 40 μL were added per well of a 96-well plate. After 5 min preincubation at 37° with or without model activator, inhibitor, or tested compounds, reaction was started with addition of 10 μL of ATP. After indicated period of time, reaction was stopped by addition of 50 μL of 5% SDS solution per well. Amount of liberated inorganic phosphate was determined by subsequently adding 100 μL of mQ water, 60 μL of color reagent (1% ammonium-molybdate and 0,014% Na,K-tartrate in 2 N sulfuric acid) and 30 μL of freshly prepared 1% ascorbic acid. Absorbance was read at 625 nm after 30 min using microplate reader (Infinite M200, Tecan, Salzburg, Austria). ATPase activity was always measured without and in the presence of 1.2 mM orthovanadate to determine the orthovanadate sensitive ATPase activity.

### 2.6. Data analysis

Data are given as mean ± standard deviation and analyzed by *t*-test and one-way ANOVA ($p$-value <0.05). For the purpose of EC50 values calculation, the data were normalized and fitted to classical sigmoidal four parameters dose-response model:

$$y = b + (a-b)/\left(1 + 10^{((LogEC50-x)*h))}\right)$$

where $y$ is the response, $b$ represents the minimum of response, $a$ represents the maximum of response, $h$ is the shape parameter, and $x$ is the logarithm of inhibitor concentration. The EC50 value is the concentration of inhibitor that corresponds to 50% of maximal effect. The Michaelis-Menten parameters of maximal velocity ($V_m$) and ATP affinity ($K_m$) were obtained from plots of the ATPase activity as a function of ATP concentration by nonlinear regression using the following equation:

$$v = V_m * [S]/(K_m + [S])$$

where $v$ is ATPase activity, $V_m$ is maximal ATPase activity, $K_m$ is the Michaelis-Menten constant for the ATP and *[S]* substrate (ATP) concentration. All equations were fitted using PRISM 4.0 software (GraphPad Software Inc., San Diego, CA).

Statistically significant effects of tested substances on the ATPase stimulation or inhibition were identified by comparison of the obtained responses with baseline ATPase activities, using one-way analysis of variance (ANOVA) followed by Dunnett's test.

## 3. Results

### 3.1. Expression of proteins in Sf9 and HEK293T cells

Initially, we have tested the BXP-21 antibody against human ABCG2 and observed only weak cross-reactivity with trout Abcg2 (data not shown). Consequently, we expressed trout Abcg2a and human ABCG2 respectively, with N-terminal 6-His tag for an easier detection and

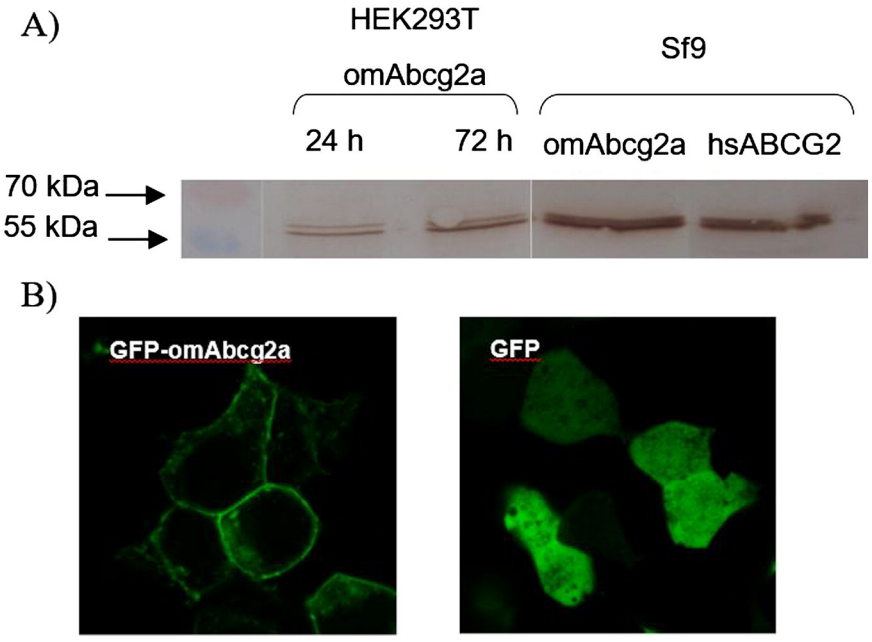


**Fig. 2.** (A) The expression of rainbow trout (omAbcg2a) and human (hsABCG2) transporters in HEK293T and Sf9 cells, respectively. The cells were cultured, transfected, and plasma membranes isolated after 24 and 72 h, respectively. Proteins (20 μg per lane) were separated by electrophoresis and visualized as described in Material and methods section. (B) Cell localization of rainbow trout Abcg2a after transfection of HEK293T cells with either recombinant plasmid carrying target gene in GFP-fusion vector (GFP-omAbcg2a; left micrograph) or with the empty vector (GFP; right micrograph).

better comparison of expression levels of the two proteins using anti-His antibody. Trout Abcg2a was efficiently expressed in Sf9 cells, showing molecular mass of approximately 60 kDa. (Fig. 1). The highest protein expression was observed after 72 h and using MOI of 8. This corresponds to the previously detected molecular mass of underglycosylated human ABCG2 expressed in Sf9 insect cells (Özvegy et al., 2001). When expressed in HEK293T cell line, trout Abcg2a has the same molecular mass as in Sf9 cells (Fig. 2A), and the protein was correctly localized in the membrane (Fig. 2B). Both trout Abcg2a and human ABCG2 showed characteristic double band already observed in previous studies.

### 3.2. ATPase assay optimization

ATPase assay is often used for characterization of human ABCG2. However, this assay has not been used for determination of non-mammalian Abcg2 orthologs thus far. Consequently, we have optimized the ATPase assay for the measurement of trout Abcg2a related ATPase activity. Firstly, we have found that 40 min incubation period, 5 μg/well of total proteins, and reaction temperature of 37 °C are optimal assay conditions for ATPase activity measurements (Fig. 3ABC). Secondly, since the important role of cholesterol for activity of human ABCG2 was previously demonstrated (Pál et al., 2007; Telbisz et al., 2007, 2014) we have performed the ATPase activity measurements in Sf9 membrane vesicles both loaded and not loaded with different amount of cholesterol. Although our data showed that cholesterol did modulate trout Abcg2a activity, similarly to reports previously shown for human ABCG2, the effect of cholesterol on trout transporter was weaker (Fig. 4.). The 2 mM concentration of cholesterol resulted in the highest response to the presence of model ABCG2 substrate sulfasalazine. Therefore, further screening of model substances for their interaction with ABCG2/Abcg2a was performed after loading the vesicles with 2 mM cholesterol-RAMEB complex.

The affinity of trout Abcg2a for ATP was slightly higher than affinity of human ABCG2, with respective $K_m$ values of 0.11 mM and 0.38 mM $K_m$ in the absence of cholesterol, respectively (Table 1). As expected, sulfasalazine (model activator) did not change the $K_m$ values for ATP, but it did significantly increase the $V_m$ (Table 1, Fig. 5). In the absence of cholesterol, sulfasalazine resulted in the more pronounced increase in the ATPase activity of human ABCG2 (from 13.8 to 18.4 mmol/min/$mg_{PROT}$) than of trout Abcg2 that only weakly responded to the presence of sulfasalazine (from 11.5 to 13.5 mmol/min/$mg_{PROT}$).

Upon cholesterol loading, $K_m$ values for ATP increased approximately 2–3 times, in the case of human ABCG2 and trout Abcg2a as well. In accordance with previous results that had demonstrated activation of human ABCG2 ATPase activity in response to cholesterol, we have

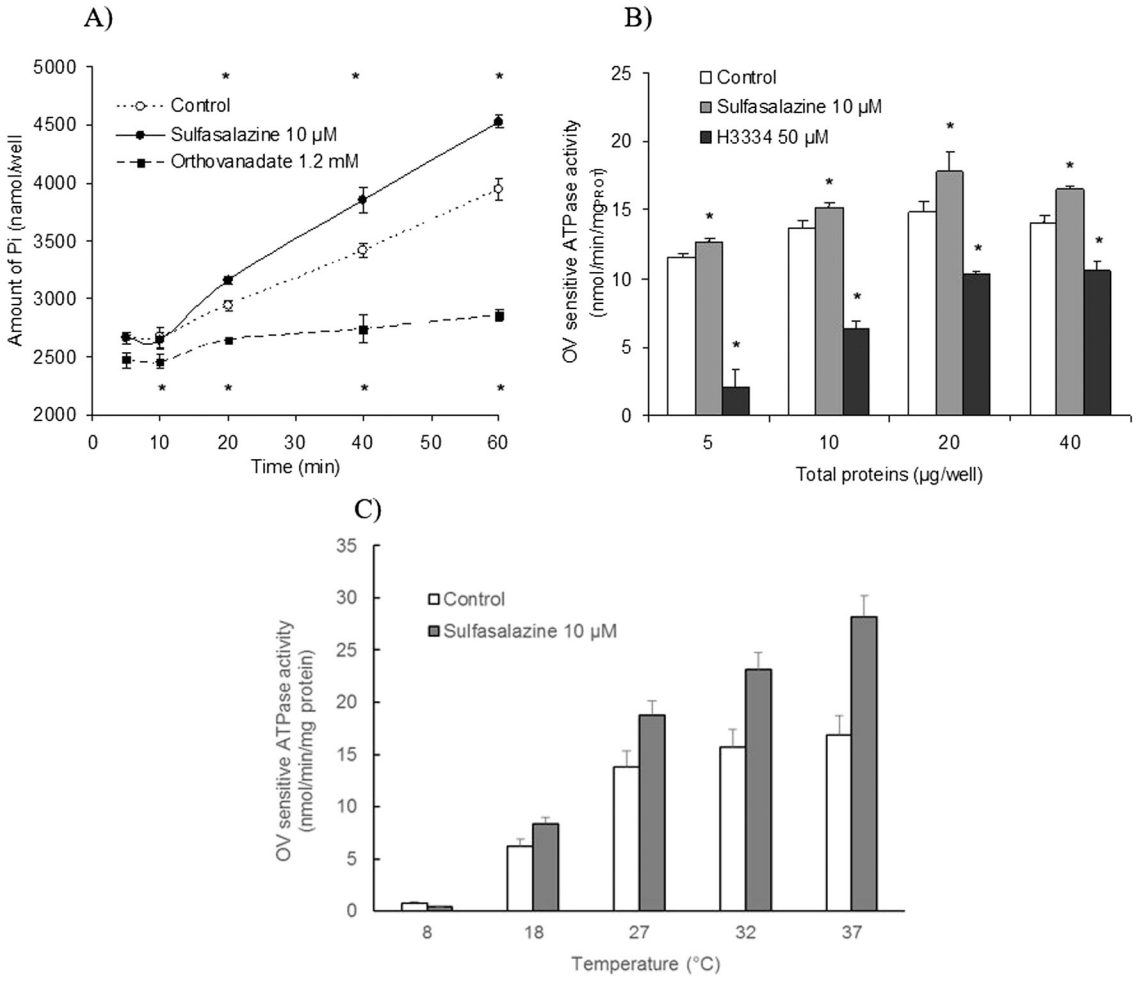


**Fig. 3.** Optimization of the ATPase assay for determination of trout Abcg2a ATPase activity. (A) Time dependent activation and inhibition of trout Abcg2a ATPase activity is shown, as determined and expressed as inorganic phosphate (Pi) release (nmol/well) without (control) and in the presence of either model activator sulfasalazine or ABC ATPase model inhibitor orthovanadate; (B) Effect of the total protein concentration on the orthovanadate sensitive trout Abcg2a ATPase activity in the presence of either model activator sulfasalazine or model inhibitor H3334; (C) Effect of the reaction temperature on the orthovanadate sensitive trout Abcg2a ATPase activity in the presence of sulfasalazine. Values are means ± SEM from three independent experiments. Statistically significant effects on ATPase stimulation or inhibition were identified by comparison of data with baseline (control) ATPase activities, respectively, using one-way analysis of variance (ANOVA), followed by Dunnett's test, and are indicated by asterisks ($P < 0.05$).

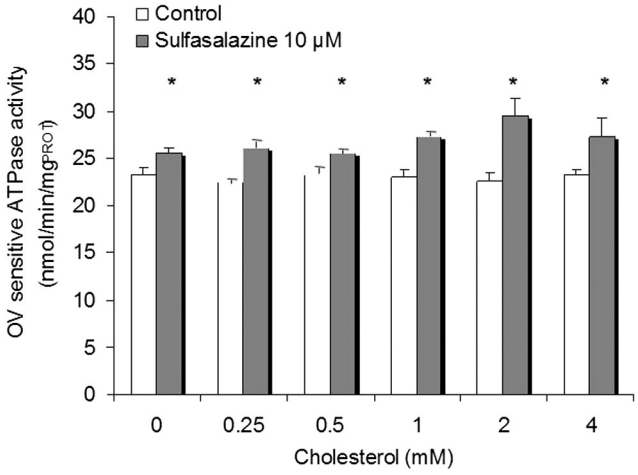


**Fig. 4.** The effect of cholesterol on trout Abcg2a ATPase activity. Sf9 membrane vesicles were loaded with different amount of cholesterol and resulting ATPase activity determined as described in Material and methods section. The values represent means ± standard deviations ($n = 3$). Statistically significant effects on ATPase stimulation were identified by comparison of data with baseline (control) ATPase activities and are indicated by asterisks ($P < 0.05$).

obtained significant increase in ATPase activity in the presence of sulfasalazine upon loading the membranes with cholesterol ($V_m$ of 27.5 mmol/min/$mg_{PROT}$). At the same time, baseline ATPase activity was approximately the same both in vesicles loaded with cholesterol and the ones not loaded with cholesterol, with corresponding $V_m$ values of 13.8 and 16 mmol/min/$mg_{PROT}$, respectively. Trout Abcg2a responded rather weakly to the cholesterol loading. Just as in the case of human ABCG2, baseline ATPase activity slightly increased in the presence of cholesterol (from 11.5 to 12.4 mmol/min/$mg_{PROT}$), and after loading with cholesterol addition of sulfasalazine resulted in only modest increase of the ATPase activity of trout Abcg2a (16.6 mmol/min/$mg_{PROT}$).

### 3.3. Functional characterization

Using the ATPase assay we have tested 39 compounds on their interaction with trout Abcg2a and human ABCG2, respectively. This series of potential interactors has been selected according to the available evidence on mammalian ABCG2 as well as on our recent insights on trout Abcg2a. The series encompasses various physiological and xenobiotic substances including numerous drugs and environmental contaminants. Model ABCG2/Abcg2a ATPase activator sulfasalazine and model inhibitor Hoechst 33342 showed similar EC50 values both in the case of human and trout transporters (Fig. 6AB).

However, human ABCG2 exhibited the higher activation of ATPase activity in the presence of model activators, over 2-fold in the presence of sulfasalazine. Trout Abcg2a resulted with only 1.4-fold activation of ATPase activity in the presence of sulfasalazine and 1.5-fold in the case of BaP, the most potent activator (Fig. 7). Among environmentally relevant compounds we have tested two polycyclic aromatic hydrocarbons, three metals and five pesticides. Apart from BaP that appeared to be the most potent activator, acetylaminofluorene resulted in a weak activation of ATPase activity at relatively high concentration. The same compound exhibited much stronger effect on human ABCG2, resulting in almost same activation as BaP (1.6-fold).

Pesticides were tested for their interaction with trout Abcg2a and most of them showed weak effect on ATPase activity at high concentrations. Only organochlorine pesticide endosulfan resulted in a significant inhibition of trout Abcg2a. Metals did not have any effect on either human ABCG2 or trout Abcg2a.

Despite the previously described limitation in identification of trout Abcg2a substrates, almost identical pattern of activation and inhibition of ATPase activity can still be observed for human ABCG2 and trout Abcg2a (Fig. 7). Besides differences observed for BaP and sulfasalazine potency, the prominent difference was observed for pravastatin. Pravastatin exhibited strong effect on human ABCG2, with 1.8-fold activation of the ATPase activity, while negligible effect was observed in the case of trout Abcg2a. Nevertheless, most of the compounds exhibited relatively similar pattern and the correlation analysis for the compounds that showed effect on both transporters resulted in statically significant correlation and clear linear relationship (Fig. 8).

## 4. Discussion

Considering the presence and function of Abcg2 in non-mammalian species, a growing body of evidence indicates its possible defensive role in aquatic organisms. Following initial studies that showed the presence of the Abcg2 in rainbow trout (Zaja et al., 2008; Lončar et al., 2010), identification of four Abcg2 genes in zebrafish genome (Annilo et al., 2006), or more recently, three Abcg genes in sea lamprey and Japanese lamprey (Ren et al., 2015), several recent studies have demonstrated up-regulation of the *abcg2* transcript in fish upon exposure to environmental contaminants. In 2012 de Cerio and colleagues reported up-regulation of the liver and down-regulation of the brain *abcg2*, respectively, upon exposure of juvenile thicklip grey mullets (*Chelon labrosus*) to perfluorooctane sulfonate (PFOS) and to Prestige-like heavy fuel oil. Likewise, the exposure of Nile tilapia to a prototypic environmental contaminant benzo(a)pyrene resulted in significant induction of the *abcg2* transcript in liver (Costa et al., 2012). Similar results were observed by Yuan et al. (2014) upon exposure of the early life stages of Chinese rare minnows (*Gobiocypris rarus*) to BaP.

However, since fish Abcg2 has not been studied in detail in suitable expression systems, the main goal of our study was to address interactor specificities of trout Abcg2a and to directly compare functional properties of related trout and human transporters. In order to do so we have

**Table 1**
$K_m$ and $V_m$ values for ATP of human ABCG2 and rainbow trout Abcg2a in the absence and presence of 2 mM cholesterol. $K_m$ and $V_m$ values were determined without and in the presence 10 μM sulfasalazine (SS) as an activator. Michaelis-Menten model was fitted and standard errors (SE) and correlation coefficients ($R^2$) are shown.

| | Human ABCG2 − cholesterol | | Human ABCG2 + 2 mM cholesterol | | Trout Abcg2a − cholesterol | | Trout Abcg2a + 2 mM cholesterol | |
|---|---|---|---|---|---|---|---|---|
| | Control | SS 10 μM | Control | SS 10 μM | Control | SS 10 μM | Control | SS 10 μM |
| *Parameters* | | | | | | | | |
| Km | 0.38 | 0.49 | 1.07 | 0.70 | 0.106 | 0.167 | 0.36 | 0.43 |
| Vm | 13.8 | 18.42 | 16 | 27.47 | 11.52 | 13.47 | 12.37 | 16.64 |
| *SE* | | | | | | | | |
| Km | 0.131 | 0.082 | 0.26 | 0.19 | 0.049 | 0.047 | 0.11 | 0.062 |
| Vm | 1.2 | 0.83 | 1.42 | 2.3 | 0.92 | 0.74 | 1.1 | 0.71 |
| $R^2$ | 0.86 | 0.97 | 0.94 | 0.93 | 0.67 | 0.89 | 0.83 | 0.96 |

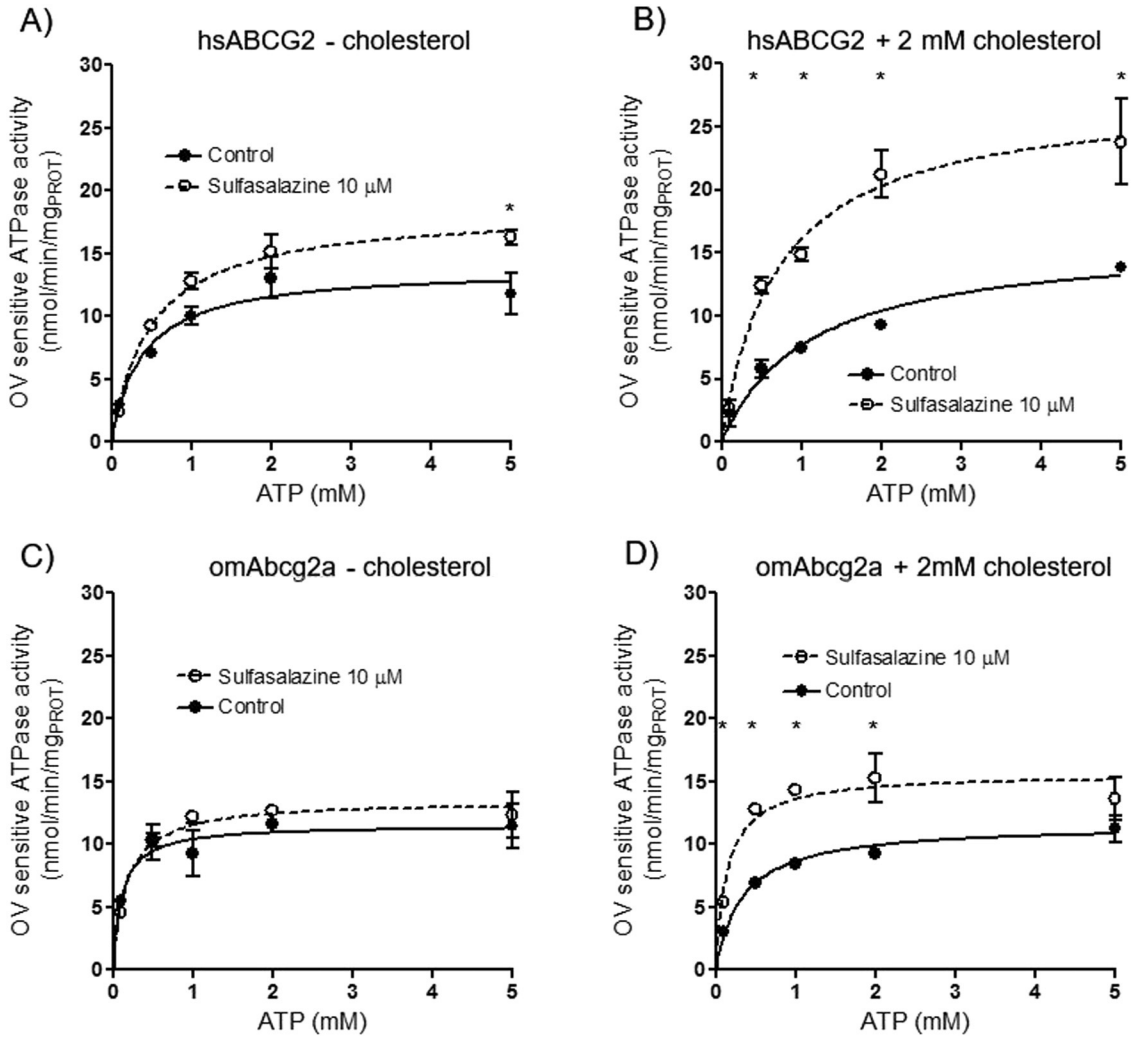


**Fig. 5.** The affinity of human ABCG2 or trout Abcg2a for ATP and its dependence on cholesterol. Human (hsABCG2 - AB) or trout (omAbcg2a - CD) ATPase activity was determined in corresponding Sf9 membrane vesicles in the presence of various ATP concentrations, with or without cholesterol and model substrate sulfasalazine, respectively. The values represent means ± standard deviations (n = 3). Statistically significant effects on ATPase stimulation were identified by comparison of data with baseline (control) ATPase activities and are indicated by asterisks ($P < 0.05$).

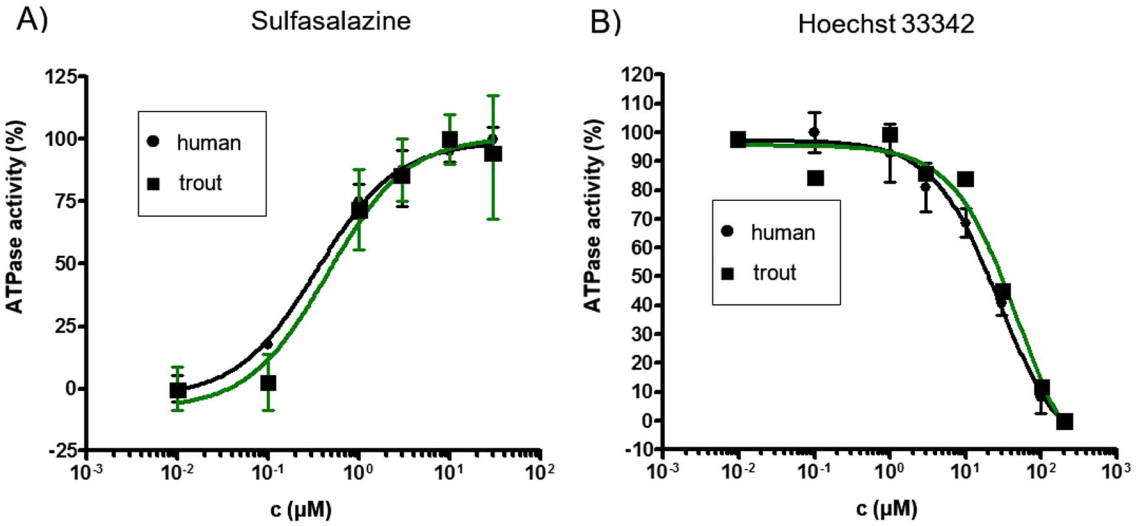


**Fig. 6.** Concentration dependent effect of the model ABCG2 substrate sulfasalazine (A) and model inhibitor Hoechst 33342 (B) on human ABCG2 and trout Abcg2a activity, respectively. ATPase activity is normalized and expressed as percentage of the basal ATPase activity set at 0% (sulfasalazine) or 100% (Hoechst 33342). The values represent means ± standard deviations (n = 3).

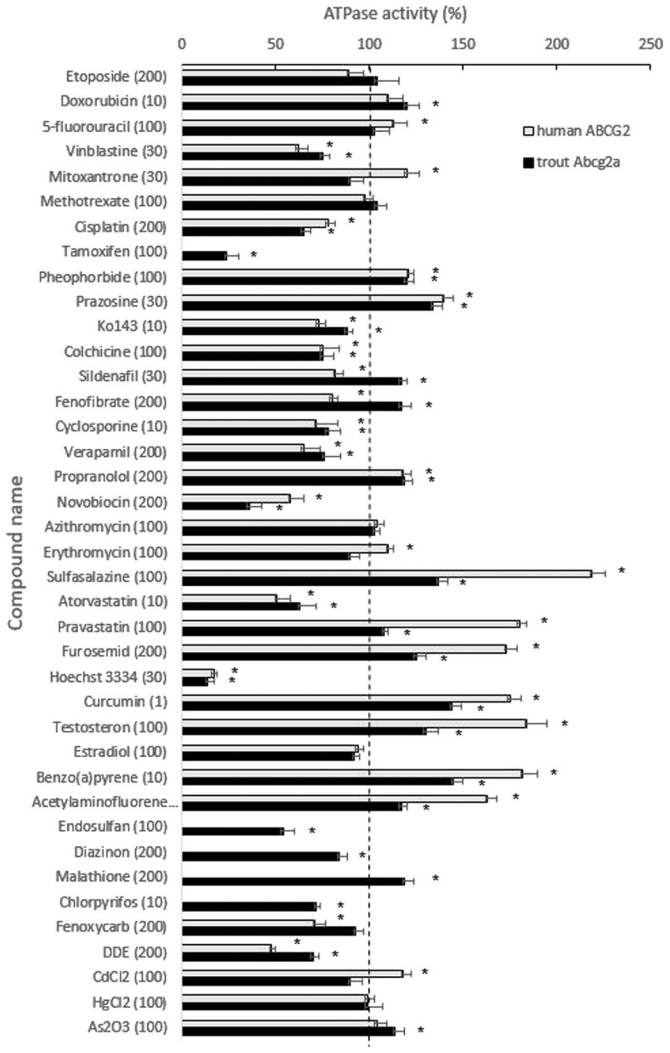


**Fig. 7.** Interaction of compounds with trout Abcg2a and human ABCG2 as determined using the determination of related ATPase activities. ATPase activity is normalized and expressed as percentage of the basal ATPase activity set at 100%. All compounds were tested within the wide concentration range (1 to 300 μM, depending on the solubility, and final concentrations of solvent (DMSO) never exceeding 0.5%), and only the highest responses of tested compounds are shown (related concentrations expressed in μM are shown in parentheses next to the names of compounds). The values represent means ± standard deviations (n = 3). Statistically significant effects on ATPase stimulation or inhibition were identified by comparison of data with basal ATPase activity and are indicated by asterisks ($P < 0.05$).

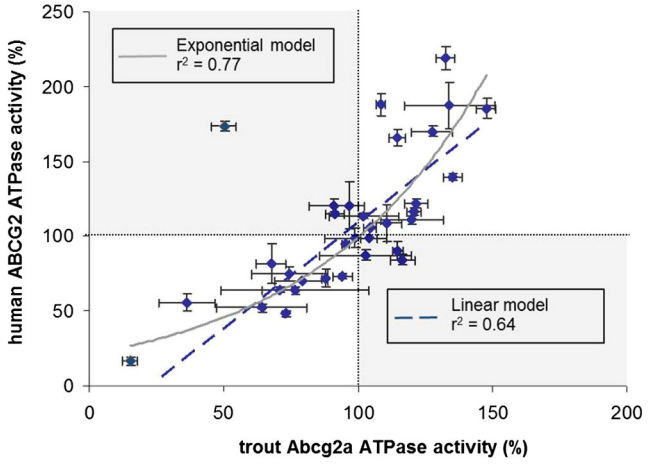


**Fig. 8.** Correlation analysis for the compounds that showed interaction with both trout Abcg2a and human ABCG2, as determined by the corresponding ATPase assays. Presented are correlation curves and coefficients ($r^2$) calculated using the exponential (solid line) and linear model (dashed line), respectively. Shaded quadrants represent the areas of opposite results obtained for the two transporters while white quadrants indicate the same direction of change (activation or inhibition) of related ATPase activities. Data are means ± SDs of triplicate determinations.

expressed the trout Abcg2a in Sf9 insect cells, a well-known experimental system often used in studying mammalian ABC transporters (Meyer et al., 1994; Trometer and Falson, 2010). Membrane vesicles preparation from Sf9 cells overexpressing trout or human transporter have then been used for the ATPase assay directed to determination of interactor specificities of trout Abcg2a versus human ABCG2.

Yet, there are potential disadvantages of the Sf9 expression system as well. Typical drawbacks are the lack of proper protein glycosylation and low cholesterol content in the insect cell membranes. N-glycosylation is often important for proper protein folding and membrane targeting. Nevertheless, several previous studies have shown that the lack of glycosylation does not affect the function and/or localization of human ABCG2 in insect cells (Diop and Hrycyna, 2005; Mohrmann et al., 2005). In our study, trout Abcg2a expressed in HEK293T and in Sf9 cells, respectively, showed the same molecular mass, unlike human ABCG2 which appeared to be fully glycosylated in HEK293 cells (Gupta et al., 2004).

Furthermore, it has been shown that inherently low cholesterol content in insect cell membranes strongly affects the ABCG2 function resulting in high ATPase baseline activity and consequently weak ATPase activation in the presence of a substrate. However, loading the vesicle with cholesterol using cyclodextrin carrier significantly increases the activation effect of the substrates on the ABCG2 ATPase activity and consequently the sensitivity of the assay (Pál et al., 2007; Telbisz et al., 2007, 2014). The same effect on the transporter ATPase activity in the presence of model ABCG2 activator sulfasalazine has been observed in our study, although the cholesterol effect was less pronounced for trout Abcg2a in comparison to the human transporter (Fig. 4.). However, the stimulating mechanism of cholesterol on ABCG2 function is still unclear. As has been discussed by leading authors in the field (Pál et al., 2007; Telbisz et al., 2014) there are several possible hypotheses. First and the most obvious explanation may be that cholesterol is an ABCG2 substrate by itself, although no inhibition of the human ABCG2 transport has been observed. Alternatively, cholesterol may act as an allosteric regulator for ABCG2-function. Finally, it is known that cholesterol forms separate domains (rafts or caveolae) in mammalian membranes, and several reports have found that ABC transporters are dominantly localized in those regions. Therefore, the cholesterol sensitivity of the trout Abcg2a might be an indirect mechanistic consequence of a preferably raft/caveolar localization of the transporter.

Finally, in most cases the profile of the drug-dependent ATPase activity determined in ATPase assays closely reflects the nature of the transporter-drug interaction: compounds may be classified as substrates, inhibitors, or may have no effect on the transporter. Still, in many cases the transported substrates cannot measurably increase the basal rate of ATPase activity which can be relatively high just like in the case of ABCG2. Besides that, both stimulation and inhibition may occur at increasing drug concentrations, and there are substances that simultaneously behave like ABCG2 substrates and related ATPase inhibitors, as is the case with fluorescent ABCG2 substrate Hoechst 33342 (Ozvegy et al., 2002; Pál et al., 2007). In our system, the maximal activation of trout Abcg2a ATPase activity, as determined in cholesterol loaded membrane vesicles, resulted in 1.5-fold activation in the presence of model activator sulfasalazine (Fig. 5D). Along with a solid reproducibility and robustness of the system, the obtained range allowed a reliable identification of trout Abcg2a interactors.

Consequently, our testing of 39 physiologically and/or environmentally relevant substances on their interaction with trout Abcg2a

revealed some potent interactors. Apart from the model activator sulfasalazine, a significant activation of trout Abcg2a ATPase was observed among various drugs like prazosin, doxorubicine, sildenafil, furosemid, propranolol, fenofibrate and pheophorbide, although at concentrations not environmentally relevant. Likewise, pesticides showed either a weak activation (malathione) or strong (endosulfan) to mostly weak (chlorpyrifos, fenoxycarb, DDE) inhibition of trout Abcg2a ATPase at high concentrations (Fig. 7).

The highest activation of trout Abcg2a ATPase was obtained for BaP, curcumine, and testosterone. BaP is a prototypical environmentally relevant polycyclic aromatic hydrocarbon (PAH) and our data support recent studies that point out to possible involvement of fish Abcg2-like proteins in response to PAHs. The increase in the abundance of Abcg2 transcripts in fish liver has been reported in response to exposure of mullets to Prestige-like heavy fuel oil containing PAHs (de Cerio et al., 2012), or more specific exposure of Nile tilapia and Chinese rare minnows to BaP, respectively (Costa et al., 2012; Yuan et al., 2014). This is further supported by recent in vitro study by Hessel et al. (2013) who has shown that apical transport of 1-glucuronide and 1-sulfate pyrene conjugates is mediated by human ABCG2 expressed in intestinal Caco-2 cells. Likewise, ABCG2 may be functionally regulated by the aryl hydrocarbon receptor (AhR) ligands, as has recently been shown by Halwachs et al. (2014) who demonstrated that exposure of MDCKII cells stably expressing mammalian (goat) ABCG2 caused a dose-dependent increase in EROD activity and *abcg2* gene expression upon exposure to AhR agonists like TCDD. Therefore, there is a possibility that rainbow trout Abcg2a has a role in efflux of PAHs and/or their metabolites in fish liver, which represents a toxicologically relevant hypothesis that should be thoroughly addressed in future research.

Curcumin was the second most potent activator of trout Abcg2a ATPase in our study, showing almost maximal detectable ATPase activation at the lowest concentration (1 μM) when compared to other tested substances (Fig. 7). Curcumin is a plant diarylheptanoid mainly used as a food additive, but has also been shown to exhibit a wide range of pharmacological activities. Importantly, during the process of studying its anti-cancer properties, curcumin was found to inhibit mammalian ABC transporters including ABCA1, ABCB1, ABCC1, and ABCG2 (Zhang et al., 2014), and even selectively kill multidrug-resistant cells that express the ABCG2 transporter (Rao et al., 2014). Thus, our data clearly show that curcumin expresses a significant interaction potential toward fish Abcg2a as well.

As for the testosterone effect on trout Abcg2a ATPase, our data are in accordance with studies that implicate the involvement of mammalian ABCG2 in the transport of sterols and natural steroids. Janvilisri and colleagues showed in 2003 stimulation of human ABCG2 ATPase activity by cholesterol, estradiol, progesterone and testosterone, implying that ABCG2 might have a role in the transport of sterols in human, in addition to its defense function directed at the transport of xenobiotics. Soon after, the inductive effect of testosterone on liver Abcg2 mRNA has been shown in rat and mice (Tanaka et al., 2004). And a more recent in vitro study done by Dankers et al. (2012) on interaction between ABCG2 and 12 steroid hormones using membrane vesicles of HEK293-ABCG2 cells has shown the efficient allosteric inhibition of ABCG2 transport by testosterone, estradiol, progesterone and androstenedione, pointing to the role of ABCG2 in steroid hormone regulation. Our data suggest that fish Abcg2a may have the same physiological role, apart from its putative function in cellular defense.

Finally, considering substrate/interactor specificities of human ABCG2 versus trout Abcg2a, our correlation analysis conducted for compounds that showed interaction with both trout and human transporter reveals highly similar pattern of activation and inhibition of ATPase activity (Fig. 8). Despite some significant differences between fish Abcg2a and human ABCG2 that are especially pronounced in the case of BaP, sulfasalazine and pravastatin potencies (Fig. 7), most of the compounds exhibited a relatively similar interaction pattern and the correlation analysis resulted in statistically significant correlation and clear linear relationship. Therefore, although there are some important and possibly (eco)toxiciologically relevant differences in substrate preferences between human ABCG2 and fish Abcg2a, it is reasonable to assume that the highly similar pattern of interaction does exist between the two transporters. And the obtained cross resistance pattern of trout Abcg2a probably represents a vital element of its putative defense function, a hypothesis that remains to be addressed in follow up in vivo studies.

In conclusion, data from this study offer the first thorough characterization of the fish Abcg2a efflux transporter in a heterologous expression system, reveal some potent interactors among physiologically or environmentally relevant substances, and point to both significant differences and overall similarities in interactor preferences and strengths between human ABCG2 and fish Abcg2a. Based on these initial insights further studies should be done in order to deepen our understanding of the significance of fish Abcg2a and its interactors in relation to probable ecotoxicological and physiological relevance of this transporter.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2016.07.005.

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