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Detection and functional characterization of Pgp1 (ABCB1) and MRP3 (ABCC3) efflux transporters in the PLHC-1 fish hepatoma cell line

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Abstract

The PLHC-1 hepatoma cell line derived from topminnow (*Poeciliopsis lucida*) is one of the most frequently used fish cell lines in aquatic ecotoxicology. These cells have been well characterized regarding the presence of phase I and phase II enzymes involved in the metabolism of xenobiotics. However, the presence of the ABC transport proteins possibly involved in the MultiXenobiotic Resistance (MXR) mechanism as phase III of cellular detoxification has never been described in the PLHC-1 cells. The main goal of this study was the detection and functional characterization of toxicologically relevant xenobiotic efflux transporters from ABCB and ABCC subfamily in the PLHC-1 cells. Using specific primer pairs two PCR products 1769 and 1023 bp in length were successfully cloned and sequenced. Subsequent multiple alignment and phylogenetic analysis showed that these sequences share a high degree of homology with the P-glycoprotein (Pgp1; ABCB1) and the MRP3 (ABCC3). Functional experiments with fluorescent model substrates and specific inhibitors were used to verify that transport activities of Pgp- and MRP-related proteins are indeed present in PLHC-1 cells. Accumulation or efflux/retention rates of rhodamine 123, calcein-AM or monochlorbimane were time- and concentration-dependent. Cyclosporine A, MK571, verapamil, reversine 205, indomethacine and probenecid were used as specific inhibitors of Pgp1 and/or MRPs transport activities, resulting in a dose dependent inhibition of related transport activities in PLHC-1 cells. Similar to mammalian systems, the obtained IC_{50} values were in the lower micromolar range. Taken together these data demonstrate that: (1) the PLHC-1 cells do express a functional MXR mechanism mediated by toxicologically relevant ABC efflux transporters; and (2) the presence of all three critical phases of cellular detoxification additionally affirms the PLHC-1 cells as a reliable *in vitro* model in aquatic toxicology.

Keywords: Fish; PLHC-1 cells; ABC transport proteins; MXR; P-glycoprotein; MRP

1. Introduction

Fish liver cells are frequently used models in studying detoxification mechanisms of different xenobiotics in aquatic ecotoxicology (Fent, 2001; Schirmer, 2006). Although primary fish hepatocytes are advantageous in terms of resemblance to the *in vivo* situation, liver cell lines are easier to manipulate and offer the possibility of long term culture, resulting in reduced variability in comparison to primary hepatocytes. One of the most commonly used cell lines in aquatic ecotoxicology is the PLHC-1 hepatoma cell line derived from topminnow (*Poeciliopsis lucida*). These cells posses xenobiotic-metabolizing capacity and contain aryl hydrocarbon (Ah) receptor (Hahn et al., 1993). The induction of CYP1A as a result of exposure to polycyclic aromatic hydrocarbons (PAHs) in PLHC-1 cell line has been widely used for identification and evaluation of toxic potential of PAHs in complex environmental samples (Fent and Bätscher, 2000). The presence of biotransformation enzyme system also allows for testing of genotoxic potential of indirect carcinogens. Other effects such as lipid peroxidation, metallothionein induction and cytotoxicity (MTT, LDH or NRU assay) were successfully measured in these cells (Rau et al., 2004; Schlenk and Rice, 1998; Babich et al., 1991; Caminada et al., 2006). Although PLHC-1 cells are well characterized to the presence of phase I (i.e. CYP1A), and to lesser extent to phase II enzymes (GSTs, UGTs), the role of xenobiotic efflux transporters possibly involved in the Multi-Xenobiotic Resistance (MXR; Kurelec, 1992) mechanism as phase III of cellular detoxification has never been evaluated in these cells.

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The MXR system is mediated through the activity of transmembrane transport proteins belonging to the ATP-binding cassette (ABC) protein superfamily. Among numerous ABC proteins identified in humans (presently 48 genes organized into 7 subfamilies; http://www.nutrigene.4t.com/humanabc.htm), the data obtained using different animal models indicate that P-glycoprotein (ABCB1), MRP1 (ABCC1), MRP2 (ABCC2), MRP3 (ABCC3) and BCRP (ABCG2) are toxicologically the most important (Leslie et al., 2005). Through binding and hydrolyzing ATP these proteins actively transport a wide variety of xeno- and endobiotics out of the cell, lowering the toxic potential of parent compounds or their metabolites. Therefore, the primary consequence of this simultaneous transport of many structurally and chemically different substances is the phenomenon called multidrug or MultiXenobiotic Resistance.

Although there are some overlaps in structure and substrate specificity, there are major differences between Pgp1 and MRP1-3 in the transport mechanisms and the types of substrates they transport. The Pgp substrates are moderately hydrophobic, amphipathic, neutral or positively charged planar organic molecules of low molecular weight with basic nitrogen atom (Litman et al., 2001). Besides, it is known that Pgp is the primary active transporter involved in the efflux of unmodified xenobiotics. The substrates of proteins from the MRP subfamily are direct products of phases I and II metabolism. Due to this fact they are predominantly present in the form of glutathione, glucuronate or sulphate water soluble conjugates. In general, through excreting highly hydrophobic compounds phase III efflux transporters represent an integral part of cellular detoxification system, having considerable role in overall disposition, bioavailability and potential toxicity of xenobiotics (Xu et al., 2005; Wakabayashi et al., 2006).

Phase I and II enzymes are identified and well characterized in fish, pointing to the presence of similar mechanisms of detoxification of hydrophobic compounds as the ones found in higher vertebrates (Livingstone, 1998). The presence of the MXR system and related ABC efflux proteins has been identified in several fish species. Using polyclonal antibody C-219 the Pgp-like proteins have been detected in marine fish species, e.g. killifish (*Fundulus heteroclitus*) and freshwater fish species, e.g. rainbow trout (*Oncorhynchus mykiss*) (Cooper et al., 1999; Sturm et al., 2001). Pgp1 related partial or full length gene sequences are known for turbot (*Scophthalamus maximus*), flounder (*Platichthys flesus*) and zebrafish (*Danio rerio*) (Tutundjian et al., 2002; Williams et al., 2003; Bresolina et al., 2005). The studies reporting the presence of MRP-related proteins or genes in fish are scarcer. A full MRP2 gene sequence has been reported for small skate (*Raja erinacea*; Cai et al., 2003) and a partial MRP-like gene sequence has been obtained for the red mullet (*Mullus barbatus*; Sauerborn et al., 2004). A MRP2 related gene from the rainbow trout liver has been recently obtained in our laboratory. In addition, the large zebrafish genome sequencing project recently resulted in vast amount of sequences data related to ABC genes in this species (http://www.ensembl.org/Danio_rerio/index.html). Newly finished analysis of these data confirmed that these genes are highly conserved and that 77% of all human ABC genes have a zebrafish ortholog (Annilo et al., 2006).

All these data suggest that Pgp1 and MRP-like transporters in fish probably play a similar role in the xenobiotic detoxification as in mammals. Based on this hypothesis the aim of this study was the detection of Pgp1 and MRP-like genes and the functional characterization of related transport activities in the PLHC-1 fish hepatoma cell line. The sequences of high homology to mammalian and fish Pgp (ABCB1) and MRP3 (ABCC3) genes were identified and corresponding transport activities were confirmed using model substrates and inhibitors.

2. Materials and methods

2.1. Chemicals

Cyclosporin A (CA), rhodamine 123 (Rh123), monochlorbimane (MCB), verapamil (VER), reversin 205 (REV205), indomethacine (IND), probenecid (PRB), thiazolyl blue tetrazolium bromide (MTT), Triton X-100, dimethyl sulphoxide (DMSO), phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS), pyruvate and HEPES were purchased from Sigma, St. Louis, MO, USA. Minimum essential medium (MEM) and fetal bovine serum (FBS) were obtained from Gibco (Karlsruhe, Germany). MK571 was obtained form Cayman Chemicals Co. (Michigan, OR, USA). Calcein-AM (Ca-AM) was purchased from Molecular Probes (Eugene, OR, USA). Ethanol, isopropanol and all other chemicals used were of the highest analytical grade available and purchased from the Kemika, Zagreb, Croatia.

2.2. Growth and treatment of cells

PLHC-1 cells (Hightower and Renfro, 1988) were obtained from the American Type Culture Collection (ATCC; LGC Promochem, Teddington, UK). The cells were grown at 30 °C MEM containing Earle's salts, nonessential amino acid, L-glutamine and 5% FBS as previously described (Hahn et al., 1996). One day prior to experiments the cells were seeded in 6-, 24- or 96-well plates in 5, 1 and 0.2 mL of medium per well, respectively. The seeding density was 3.5×10^5 cells/cm^2.

2.3. Cell viability assay

Cell viability was determined by the MTT reduction test adapted according to the Mosmann's procedure (Mosmann, 1983). After exposure of cells to a range of concentrations of tested chemicals for 2 and 24 h, the medium was removed and the cells were incubated for 3 h with 0.5 mg/mL MTT (50 μL/well) dissolved in MEM. The formazan salts were dissolved in isopropanol and the plates were read on a microplate reader Anthos HT-III (Asys Hitech GmbH, Eugendorf, Austria) at 570 nm using 750 nm as a reference wavelength. Viability was expressed as the percentage from corresponding control value. None of the inhibitors used in the transport activity assays reduced cell viability significantly in the tested concentration range (data not shown).

2.4. Detection of Pgp1 and MRP3 related gene sequences

Primer pairs for Pgp1 and MRP1/2 related genes were designed based on the available highly conserved regions among mammalian and fish ABCB1 and ABCC1/2 genes. The oligonucleotides were obtained from Invitrogen™. Total RNA was isolated from 3.5×10^6 cells using High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany). The quality of the RNA samples was analyzed by RNA 6000 Nano LabChip Kit (Agilent Technologies, Palo Alto, CA, USA).

Three micrograms of total RNA were then reverse transcribed using M-MuLV reverse transcriptase according to the manufacturer's instructions (RevertAid First Strand cDNA Synthesis Kit, Fermentas, Burlington, Ontario, Canada). PCR was performed in Biometra thermal Cycler (Goettingen, Germany). PCR reactions were conducted with 1 μL of cDNA in total volume of 50 μL. The cycling parameters were as follows: 2 min denaturation at 94 °C, 35 cycles of heat denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, polymerization at 72 °C for 60 s, and 7 min final extension at 72 °C. The aliquots of each reaction were separated by electrophoresis on 1.2% agarose gel in TAE buffer, the gels were stained with ethidium bromide and the PCR products were visualized under UV light. The bands of expected size were excised and eluted using MiniElute PCR Purification Kit (Qiagen, Hilden, Germany). The isolated fragments were cloned into the plasmid vector and introduced in *E. coli* using PCR Cloning Kit (Qiagen, Hilden, Germany). The plasmids were isolated from 3 mL of overnight culture using QIAprep Miniprep (Qiagen, Hilden, Germany). The inserts were sequenced on both strands by the VBC-Genomics (Vienna, Austria).

2.5. Functional detection and characterization of Pgp1 and MRP-like transport activities

For the purpose of functional detection of Pgp1- and MRP-like transport activities the cells were cultured for 24 h in 24-well culture plates in 1 mL of medium per well. Before the experiment the cells were washed in PBS and 250 μL of fresh medium containing variable concentrations of CA (dissolved in 96% EtOH), VER (in 96% EtOH), REV205 (DMSO), MK571 (DMSO), or PRB (in 1 M NaOH), was added to the wells. After a short (3–5 min) pre-incubation period with inhibitors the fluorescent Pgp1 and/or MRPs substrates (Ca-AM and Rh123; all dissolved in DMSO) were added at desired concentrations in 250 μL of fresh medium per well. The final concentrations of the solvents never exceeded 0.1%. The cells were then incubated for indicated periods of time. After the end of the incubation period the cells were washed two times in phosphate buffer saline (PBS) and finally lysed in 0.1% Triton X-100/PBS (250 μL/well). The fluorescence was measured using microplate reader (Fluorolite 1000, Dynatech, Chantilly, VA, USA) at 485 nm excitation and 530 nm emission wavelengths. The results were expressed as fluorescence units (FU) per 7×10^5 cells. When MCB was used as a substrate the cells were first loaded with 25 μM MCB for 20 min and then, after washing the cells in PBS, the inhibitors were added in desired concentrations in 400 μL of fresh medium. After the indicated periods of time the fluorescence of bimane–GS excreted in the medium was measured at 390 and 480 nm excitation and emission wavelengths, respectively (Cary Eclipse Microplate reader, Varian Inc., Palo Alto, CA, USA). The cells were than washed in PBS and lysed in the in 0.1% Triton X-100/PBS (250 μL/well) and the amount of retained bimane–GS was measured as well.

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 IC_{50} values calculation the data were fitted to classical sigmoidal four parameter dose–response model:

$$y = \frac{b + (a - b)}{1 + 10^{(\mathrm{Log}\,IC_{50} - x)h}}$$

where y is the response, b represents minimum of the response, a represents maximum of the response, h is shape parameter, x is logarithm of inhibitor concentration and IC_{50} is the concentration of inhibitor that corresponds to 50% of the maximal effect. All studies were performed in three to five independent experiments and results of typical experiments are shown in figures. All calculations were preformed using GraphPad Prism 4 for Windows.

Sequence manipulations, analysis and multiple alignments were done using BLAST Internet service, BioEdit and ClustalX software. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1.

3. Results

3.1. Detection of Pgp1 and MRP3 related gene sequences in PLHC-1 cell line

Based on known full and/or partial ABCB1 or ABCC1/2 sequences from mammalian and fish species, specific pairs of primers were designed. Using the primer pair (*forward*: AAGCTGCCAAGGAAGCCAA, *reverse*: GGGACACAATGCCGATCT) designed for detection of the Pgp1 gene a fragment of 1769 bp in length was obtained (Fig. 1A). Amplification with MRP1/2 specific primer pair (*forward*: CCGCTCTCCCATTTACTCCCATTTC, *reverse*: GAAACGGGTCCAGATTCATCC) led to obtaining a 1023 bp fragment (Fig. 1B). Sequencing of these PCR products and subsequent sequence comparison using BLASTX revealed that these sequences correspond to respective Pgp1 and MRP3 related sequences found in mammalian and fish species. Multiple alignments of these sequences are shown in Fig. 2A and B. The amino acid sequence deduced from the 1769 bp fragment is highly homologous to Pgp1 genes from mummichog (77%), zebrafish (64%) and human (54%). The amino acid sequence deduced from 1023 bp fragment shares a high degree of homology with MRP3 from other fish (takifugu 65% and pufferfish 62%) and mammals (human 48%). The results of phylogenetic analysis also indicate grouping of the identified sequences within ABCB and ABCC subfamily and reveal that these sequences are evolu-

tionary closest to Pgp1 and MRP3 found in other fish species (Fig. 2C).

3.2. Functional analysis of the Pgp1 and MRP-like transport activities in PLHC-1 cell line

Pgp1 and MRP-like transport activities were measured by determining the accumulation of three different model fluorescent substrates in the absence and presence of known inhibitors of related efflux transporters. Ca-AM is substrate for both Pgp1 and MRP1/2 (Holló et al., 1996). Rh123 was first considered a specific Pgp1 substrate but recent studies demonstrate that this dye is also a MRP1 substrate (Daoud et al., 2000). The third substrate used in this study was MCB. Similarly to Ca-AM this compound is nonfluorescent and readily taken up by the cells through simple diffusion. Inside the cells MCB becomes a substrate for glutathione-*S*-transferases which catalyze conjugation of MCB with glutathione (GSH). The resulting bimane–GS conjugate is highly fluorescent substrate of MRP subfamily, more specifically MRP1-4 (Bai et al., 2004; Lou et al., 2003).

First we determined the optimal accumulation time and substrate concentration in the presence and absence of specific inhibitors. In the case of Rh123 and Ca-AM the accumulation was linear up to 90 min (Fig. 3A and B). After that period

(A)

```
1    tttgagaccctggttggtgatcgagggactctacgtatgagcggaggacagaagcagaggattgcgattgctcgagctttggtccgcaaccccaaaatcc 100
      F  E  T  L  V  G  D  R  G  T  L  R  M  S  G  G  Q  K  Q  R  I  A  I  A  R  A  L  V  R  N  P  K  I

101  tcctactggacgaagccacgtctgcgctggacgctgagagcgagaccattgttcaagctgcactcgataaggtgcgactgggtcgtacaacactaattgt 200
     L  L  L  D  E  A  T  S  A  L  D  A  E  S  E  T  I  V  Q  A  A  L  D  K  V  R  L  G  R  T  T  L  I  V

201  ggctcaccgcctctcaacaatcagaaatgccgacgtgattgcaggcttccagaaaggtaaagttgtagagttgggcactcacagcgagctgatggcgaaa 300
       A  H  R  L  S  T  I  R  N  A  D  V  I  A  G  F  Q  K  G  K  V  V  E  L  G  T  H  S  E  L  M  A  K

301  cacggagtctaccacacgctggtcaccatgcagacctttcagaaagcagaggatgatgaggatgaaggagagttgtcaccaggtgagaaaagccccatga 400
      H  G  V  Y  H  T  L  V  T  M  Q  T  F  Q  K  A  E  D  D  E  D  E  G  E  L  S  P  G  E  K  S  P  M

401  aagaccccatgagtgagtctacgctgctgagaaggaagtctaccagaggatcctcgttcgctgcttcagctggagagaaaggagaaaaagaaaagggaaa 500
     K  D  P  M  S  E  S  T  L  L  R  R  K  S  T  R  G  S  S  F  A  A  S  A  G  E  K  G  E  K  E  K  G  K

501  gaatgatgaggacaaagcagaagaagaggaggacgtccccatggtgtcgttctttagggttctgcgcctcaatgcttctgagtggccttatattgtggtg 600
       N  D  E  D  K  A  E  E  E  E  D  V  P  M  V  S  F  F  R  V  L  R  L  N  A  S  E  W  P  Y  I  V  V

601  ggactgatttgtgccacaataaacggagccatacagcctctgtttgccgtcctcttctccaagattatcactgtgtttgcagagcagataaaatgttgtc 700
      G  L  I  C  A  T  I  N  G  A  I  Q  P  L  F  A  V  L  F  S  K  I  I  T  V  F  A  E  Q  I  K  C  C

701  agagaaagatcaaacttcttctcactcatgtttgtggccatcggagttggttgatttttcaccatgtttttacaggcattctgttctggaatatctggag 800
     Q  R  K  I  K  L  L  L  T  H  V  C  G  H  R  S  W  L  I  F  H  H  V  F  T  G  I  L  F  W  N  I  W  R

801  aattcttactttgagcaggcttgctgtcttcaagtccatgatgagacaagacctcgggtggtttgacagtcccaaaaacagtgtcgggcgctcaccaccc 900
       I  L  T  L  S  R  L  A  V  F  K  S  M  M  R  Q  D  L  G  W  F  D  S  P  K  N  S  V  G  R  S  P  P

901  gactggctacagacgcagcccaagtacaggggcgtcaggagttcgcctggcgacgttcgcccagaacatcgccaacctcgcaccggtgtgatcctggcgt 1000
      D  W  L  Q  T  Q  P  K  Y  R  G  V  R  S  S  P  G  D  V  R  P  E  H  R  Q  P  R  T  G  V  I  L  A

1001 ttgtgtacggctggagcacgctgctggttctggccgtggtgcccgtcattgcgctggccggagccgttcagatgaaaatgctcactggacatgcagccga 1100
     F  V  Y  G  W  S  T  L  L  V  L  A  V  V  P  V  I  A  L  A  G  A  V  Q  M  K  M  L  T  G  H  A  A  E

1101 agacaaaaaggagctggagaaagctggaaagatcgccacagaggccatagagaacatccgcactgttgcctctctcacaagagaaccaaaatttgagtct 1200
       D  K  K  E  L  E  K  A  G  K  I  A  T  E  A  I  E  N  I  R  T  V  A  S  L  T  R  E  P  K  F  E  S

1201 ttgtatcaggaaaatcttgtagttccttataagaactctcagaaaaaggcccatgtgtacggcttcaccttctccttctcccaggccatgatctacttcg 1300
      L  Y  Q  E  N  L  V  V  P  Y  K  N  S  Q  K  K  A  H  V  Y  G  F  T  F  S  F  S  Q  A  M  I  Y  F

1301 cctacgcagcctgttttcgattcggagcctggctcatcatagagggaaggatggatgtggagggagttttccttgtgatttctgcggttctgttcggagc 1400
     A  Y  A  A  C  F  R  F  G  A  W  L  I  I  E  G  R  M  D  V  E  G  V  F  L  V  I  S  A  V  L  F  G  A

1401 catggccgtcggcgaggccaactcctttgcaccaaactacgccaaggccaaaatgtccgcgtcccacctgctgatgctgctgaacaaagagcctgcgatc 1500
       M  A  V  G  E  A  N  S  F  A  P  N  Y  A  K  A  K  M  S  A  S  H  L  L  M  L  L  N  K  E  P  A  I

1501 gacaacctgtctgagcagggagacacgccggatatatttcatggtaacgtgagctttgaggatgtgaagtttaactatccgtcacggcccgacatcccaa 1600
      D  N  L  S  E  Q  G  D  T  P  D  I  F  H  G  N  V  S  F  E  D  V  K  F  N  Y  P  S  R  P  D  I  P

1601 tcttgcgaggcctgaatctgagcgtgaagaagggagaaactctggccctggtggggagcagcggctgtggaaagagcaccaccatccagctgctggagag 1700
     I  L  R  G  L  N  L  S  V  K  K  G  E  T  L  A  L  V  G  S  S  G  C  G  K  S  T  T  I  Q  L  L  E  R

1701 gttctacgaccccgagagagggccgagtggtgatggacaacatcgatgtcaaacagctaacattcgctg 1769
       F  Y  D  P  E  R  G  P  S  G  D  G  Q  H  R  C  Q  T  A  N  I  R
```

Fig. 1. Partial cDNA and deduced amino acid sequences of (A) Pgp1 (ABCB1) and (B) MRP3 (ABCC3) found in PLHC-1 cells. The nucleotide sequences have been submitted to the GenBank nucleotide database with accession numbers DQ842514 (ABCB1) and DQ842515 (ABCC3).

(B)

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1    ctgcctcaggtggacaacatcgtggtgatggtggacctccgggtgtcagaaatgggctcctaccaagagttgctcaatcagaccggagccttcgcagagt 100
      L  P  Q  V  D  N  I  V  V  M  V  D  L  R  V  S  E  M  G  S  Y  Q  E  L  L  N  Q  T  G  A  F  A  E

101  ttctcaggaactatgctctggaagacgtcgtggaggaggaccaggccaccgaagagttaatagaagatgagttgttccccgatgacgtccttagcaacca 200
     F  L  R  N  Y  A  L  E  D  V  V  E  E  D  Q  A  T  E  E  L  I  E  D  E  L  F  P  D  D  V  L  S  N  H

201  ccacaccgacatggtggacaacgaacccatggttaatgaggcaaagaaaagttttatgagacagatcagcatcctgtcaggagacagcgagaacttccgg 300
       H  T  D  M  V  D  N  E  P  M  V  N  E  A  K  K  S  F  M  R  Q  I  S  I  L  S  G  D  S  E  N  F  R

301  tgccgctcggtcaggagacagagccagaagaagcaccaagaggcccaggagaagaaaccccaggaggtccagaaactcatccaggcagagacagcagaaa 400
      C  R  S  V  R  R  Q  S  Q  K  K  H  Q  E  A  Q  E  K  K  P  Q  E  V  Q  K  L  I  Q  A  E  T  A  E

401  caggccgggtaagaaccaaagtgttcctggaatacgcaaaggcagtgggactcgtgctgtcggtgatcatttgcttgctctacggctgccagagcgccgc 500
     T  G  R  V  R  T  K  V  F  L  E  Y  A  K  A  V  G  L  V  L  S  V  I  I  C  L  L  Y  G  C  Q  S  A  A

501  cgccattggcgccaacatctggctcagccattggaccagtgactcgttgacaaatcagaccaaggaaaacgttaacatgagggtgggggtgtacgcagcg 600
       A  I  G  A  N  I  W  L  S  H  W  T  S  D  S  L  T  N  Q  T  K  E  N  V  N  M  R  V  G  V  Y  A  A

601  ctgggcattgcccaaggtgtgctcattttggttaactgcctcttgtgccgggcctactgtatgctgcgggcggccaagctcacacaccggaacatgctgc 700
      L  G  I  A  Q  G  V  L  I  L  V  N  C  L  L  C  R  A  Y  C  M  L  R  A  A  K  L  T  H  R  N  M  L

701  aggggtcctgcgagccccgcaggccttcttcgagagcaccccgactgggcggttactgaaccgcttcagcaaagacgtggatgctatagactcccagat 800
     Q  G  V  L  R  A  P  Q  A  F  F  E  S  T  P  T  G  R  L  L  N  R  F  S  K  D  V  D  A  I  D  S  Q  I

801  ccccgataatattgacatatggatgcgcactttctggtacacactgaatgtgctgctcatatgctctgccctcaccccaatgttcctcatagtcatagcc 900
       P  D  N  I  D  I  W  M  R  T  F  W  Y  T  L  N  V  L  L  I  C  S  A  L  T  P  M  F  L  I  V  I  A

901  ccgttaatggtgttctattggtgggttcagagattttacgttgccacgtcgcggcagctgaagcgcctggagtcggttagccgctcccccatttactccc 1000
      P  L  M  V  F  Y  W  W  V  Q  R  F  Y  V  A  T  S  R  Q  L  K  R  L  E  S  V  S  R  S  P  I  Y  S

1001 atttcggggagacagtcaccggc 1023
     H  F  G  E  T  V  T  G
```

Fig. 1. (*Continued*).

a plateau is reached in the case of both substrates and with both inhibitors, 15 μM MK571 and 10 μM CA. In the absence of the inhibitors the accumulation rates were very low implying a high efficiency of Rh123 and Ca-AM efflux out of the cells. After an initial loading period of 20 min with MCB, the efflux of bimane–GS conjugate out of the cells was rapid. When the bimane–GS is measured in the medium in the absence of inhibitor, only 15 min is needed to reach the plateau and further increase in bimane–GS concentration is negligible during next 150 min. The presence of 15 μM MK571 delayed that period to 30 min (Fig. 3C). Similar kinetics is observed when the residual bimane–GS within the cells is measured. In the control cells the efflux of bimane–GS is complete within 30 min but 15 μM MK571 significantly prolonged that period to 90 min (Fig. 3C). Retention assays were found to be more sensitive and consistent in comparison to the efflux assay. Contrary to the efflux experiments where 1.5–2-fold inhibition is measured in the presence of 15 μM MK571, approximately 3-fold inhibition is measured using retention assay with the same concentration of MK571. Consequently, in all further experiments with MCB the effects of different inhibitors were measured using retention measurements after 20 min efflux period.

Accumulation/retention was concentration-dependent in the case of all three substrates (Fig. 4A–C). Concerning the standard deviations and the magnitude of response in the presence of inhibitors, 2.5, 0.25 and 25 μM concentrations of substrates were found to be optimal for the Rh123, Ca-AM and MCB, respectively. These concentrations were later used in the dose–response experiments.

Table 1
IC_{50} values and maximal accumulation (fold increase) of fluorescent substrates with model inhibitors used

Substrate	Parameter	Model inhibitor					
		CA	VER	REV205	MK571	PRB	IND
Rh123	IC_{50} (μM)	0.27	1.58	1.45	5.22	385.00	100.00[a]
	$Max_{accum.}$	2.60	1.90	2.40	3.42	2.10	3.72[a]
Ca-AM	IC_{50} (μM)	2.68	No effect	10.00[a]	5.80	184.92	55.90
	$Max_{accum.}$	6.30		1.80[a]	6.20	4.87	3.30
MCB	IC_{50} (μM)	6.35	No effect	No effect	11.70	21.50	200.00[a]
	$Max_{accum.}$	7.70			6.21	2.52	4.24[a]

[a] Not fitted values—the numbers represent maximal accumulation (fold increase) and concentration at which the maximal accumulation was achieved.

The effects of model transport inhibitors on the accumulation/retention of Rh123, CaAM and MCB are shown in Fig. 5A–C and estimated IC_{50} values and maximal fold inhibitions are presented in Table 1. All the inhibitors used led to the significant increase in the accumulation of Rh123 in a dose-dependent manner. CA was found to be the most potent inhibitor with IC_{50} value of 0.27 μM when Rh123 was used as substrate. VER and REV205 were of similar potency ($IC_{50} \sim 1.5$ μM) and the MK571 exhibited lower potency ($IC_{50} = 5.2$ μM). IC_{50} values obtained for PRB and IND were 10–100 times lower than the values obtained for other inhibitors. Maximal fold inhibition was similar for all the inhibitors used, ranging between two- and three-fold. When we used Ca-AM as substrate similar ranking in inhibitor potency was observed. IND and PRB were of significantly lower potency (IC_{50} values 185 and 55 μM, respectively) than the other inhibitors. CA was again the most potent one but its determined IC_{50} value of 2.7 μM was 10 times higher than in the experiments with Rh123. The IC_{50} for MK57 did not change significantly in comparison with experiments using Rh123 (5.8 μM). Contrary to what is expected VER

(A) **Pgp1 multiple alignment**

C-motif W - B

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PLHC-1    FETLVGDRGTLRMSGGQKQRIAIARALVRNPKILLLDEATSALDAESETIVQAALDKVRLGRTTLIVAHRLSTIRNADVI   80
Mummichog FETLVGDRGTQ-MSGGQKQRIAIARALVRKPKILLLDEATSALDAESETIVQAALDKVRQGRTTLIVAHCLSTIRNADVI  130
Zebrafish FETLVGDRGTQ-MSGGQKQRIAIARALVRNPKILLLDEATSALDAESETIVQAALDKVRLGRTTIVVAHRLSTIRNADVI  567
Human     FDTLVGERGAQ-LSGGQKQRIAIARALVRNPKILLLDEATSALDTESEAVVQVALDKARKGRTTIVIAHRLSTVRNADVI  598

PLHC-1    AGFQKGKVVELGTHSELMAKHGVYHTLVTMQTF-QKAEDDE-DEGELSPGEKSPMKDPMSESTLLRRKSTRGSSFAASAG  158
Mummichog AGLEKGKVVELGTHSELMEKKGVYHTLVTMQTF-QKADEGE-DEDNLSAGEKSPIHNNVIESPLLRRKSTRGSSFAASIG  208
Zebrafish AGFQNGEIVELGTHDELMERKGIYHSLVNMQMF-KSTEVAEEDSEEMTMDEKSPSVSSMNERTLFRQKSRSGS-------  639
Human     AGFDDGVIVEKGNHDELMKEKGIYFKLVTMQTAGNEVELENAADESKSEIDALEMSSNDSRSSLIRKRSTRRS-------  671

PLHC-1    EKGEKEKGKNDEDKAEEEEDVPMVSFFRVLRLNASEWPYIVVGLICATINGAIQPLFAVLFSKIITVFAEQIK-CCQRKI  237
Mummichog EKGDKKQEKEDEDKTEEDEDFPMVSIFKVLRLNASEWPYILVGLICATINGAIQPLFAVLFSKIITVFAEPDQ-TIIRQR  287
Zebrafish ----EKELKEEEKPTEEQEKVPNVSFLTVLKLNYPEWPYMVVGILCATINGGMQPAFAVIFSKIIAVFAEPDQ-NLVRQR  714
Human     VRGSQAQDRKLSTKEALDESIPPVSFWRIMKLNLTEWPYFVVGVFCAIINGGLQPAFAIIFSKIIGVFTRIDDPETKRQN  751

PLHC-1    KLLLTHVCGHRSWLIFHHVFTG-ILFWNIWRILTLS-RLAVFKSMMRQDLGWFDSPKNSVGRSPPDWLQTQPKYRGVR-S  314
Mummichog ANFFSLMFVVIGVVCFFTMFLQGFCFGKSGEVLTLKLRLGAFKSMLRQDLGWFDSPKNSVGALTTRLATDAAQVQGASGV  367
Zebrafish CDLYSLLFAGIGVLSFFTLFLQGFCFGKAGELLTMRLRFKAFNAMMRQDLAWYDDTKNSVGALTTRLAADTAQVQGATGV  794
Human     SNLFSLLFLALGIISFITFFLQGFTFGKAGEILTKRLRYMVFRSMLRQDVSWFDDPKNTTGALTTRLANDAAQVKGAIGS  831

PLHC-1    SPGDVRPEHRQPRTGVILAFVYGWS-TLLVLAVVPVIALAGAVQMKMLTGHAAEDKKELEKAGKIATEAIENIRTVASLT  393
Mummichog RLATFAQNIANLGTGVILAFVYGWELTLLILAVVPVIALAGAVQMKMLTGHAAEDKKELEKAGKIATEAIENIRTVASLT  447
Zebrafish RLATLAQNVANLGTAIVISFVYGWQLTLLILSIVPIMAVAGAIQMKLLAGHALKDKKELEQAGKIATEAIENVRTVVSLT  874
Human     RLAVITQNIANLGTGIIISFIYGWQLTLLLLAIVPIIAIAGVVEMKMLSGQALKDKKELEGSGKIATEAIENFRTVVSLT  911

PLHC-1    REPKFESLYQENLVVPYKNSQKKAHVYGFTFSFSQAMIYFAYAACFRFGAWLIIEGRMDVEGVFLVISAVLFGAMAVGEA  473
Mummichog REPKFESLYEENLVVPYKNSQKKAHVYGFTFSFSQAMIYFAYAACFRFGAWLIVEGRMDVEAVFLVISAVLFGAMAVGEA  527
Zebrafish RESKFESLYEENLIVPYKNAKKAHVFGLTFSFSQAMIYFAYAGCFKFGSWLIEQKLMTFEGVFLVISAVVYGAMAVGEA  954
Human     QEQKFEHMYAQSLQVPYRNSLRKAHIFGITFSFTQAMMYFSYAGCFRFGAYLVAHKLMSFEDVLLVFSAVVFGAMAVGQV  991

PLHC-1    NSFAPNYAKAKMSASHLLMLLNKEPAIDNLSEQGDTPDIFHGNVSFEDVKFNYPSRPDIPILRGLNLSVKKGETLALVGS  553
Mummichog NSFAPNYAKAKMSASHLMMLLNKEPEIDNLSERGESPDMFDGNVSFEDVKFNYPSRPDVPILRGLNLRVKKGETLALVGS  607
Zebrafish NSFTPNYAKAKMSASHVLMLINRAPAIDNSSEDGDKPDKFEGNVGFEHVYFKYPSRPDVPVLQGLKLRVKKGQTLALVGS 1034
Human     SSFAPDYAKAKISAAHIIMIIEKTPLIDSYSTEGLMPNTLEGNVTFGEVVFNYPTRPDIPVLQGLSLEVKKGQTLALVGS 1071

W - A
PLHC-1    SGCGKSTTIQLLERFYDPERG   574
Mummichog SGCGKSTTIQLLERFYDPRDG   628
Zebrafish SGCGKSTTIQLLERFYDPQQG  1055
Human     SGCGKSTVVQLLERFYDPLAG  1092
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Fig. 2. Multiple sequence alignment of deduced amino acid sequences of (A) Pgp1 and (B) Mrp3 with highly homologous sequences of ABCB1 and ABCC3 from other fish and mammalian species. Typical Pgp subdomains (Walker A and Walker B) and the signature region (C-motif) are additionally denoted on the Pgp1 sequence. Multiple sequence alignment was preformed using CLUSTAL W program. Phylogenetic tree (C) was based on the multiple alignment of closely related proteins from ABCC (MRP1, MRP2 and MRP3) and ABCB (Pgp1 and BSEP) subfamily found in fish, mammals and sea squirt (nonvertebrate chordate). The tree was generated using neighbor-joining method and the percentage concordance based on 1000 bootstrap iterations is shown at the nodes.

(B) **Mrp3 multiple alignment**

```
PLHC-1 LPQVDNIVVMVDLRVSEMGSYQELLNQTGAFAEFLRNYALED---VVEEDQATEELIEDE--LFPDDVLSNHHTDMVDNE   75
  Fugu LPQVDNIMVLVEGRVSEMGSYQELL-QNGAFAEFLRNYSLED---IIEEDVITDEFEEEK--LFPDDALSN-HTDMVDNE  837
Pufferfish -------MVLVEGRVSEMGSYQELLNQNGAFAEFLRNYSLED---IIEEEMTTEEFEDEK--LFLDDALSN-HTDMVDNE   67
 Human LPQTDFIIVLADGQVSEMGPYPALLQRNGSFANFLCNYAPDEDQGHLEDSWTALEGAEDKEALLIEDTLSN-HTDLTDND  893

PLHC-1 PMVNEAKKSFMRQISILSGDSENFRCRSVRRQS--QKKHQEAQEKKPQEVQKLIQAETAETGRVRTKVFLEYAKAVGLVL   153
  Fugu PAINEEKRKFIRQISVISADGENARCRSVKRHACSQRKHAGMQEKKPQQTEKLIQAETTETGRVKTKVYLEYVKAVGPLL   917
Pufferfish PAINEEKRKFIRQISVISADGENVRSRSVRRHACSQRKHADMQDKKPQEMEKLIQAEATETGRVKMKVYLEYVKAVGPLL   147
 Human PVTYVVQKQFMRQLSALSSDGEGQGRPVPRRHLGPSEK---VQVTEAKADGALTQEEKAAIGTVELSVFWDYAKAVGLCT   970

PLHC-1 SVIICLLYGCQSAAAIGANIWLSHWTSDSLTNQTKENVNMRVGVYAALGIAQGVLILVNCLLCRAYCMLRAAKLTHRNML   233
  Fugu SVFICFLYGCQSAAAIGANIWLSQWTNDASTNQTQENINMRVGVYAALGLAQGILIMISSFTLAMGNIG-AARKLHHNLL   996
Pufferfish SVFICFLYGCQSAAAIGANIWLSVWTNDAATNQTQENVNMRVGVYAALGLAQGILIMISSFTLAMGNIG-AARKLHHNLL   226
 Human TLAICLLYVGQSAAAIGANVWLSAWTNDAMADSRQNNTSLRLGVYAALGILQGFLVMLAAMAMAAGGIQ-AARVLHQALL  1049

PLHC-1 QGVLRAPQAFFESTPTGRLLNRFSKDVDAIDSQIPDNIDIWMRTFWYTLNVLLICSALTPMFLIVIAPLMVFYWWVQRFY   313
  Fugu LNKLHTPQSFFDTTPIGRIINRFSKDIYVIDEALPATVLMLLGTVFVSLSTIIVIVSSTPIFLVVIVPLAFIYVFVQRFY  1076
Pufferfish LNKLHTPQSFFDTTPIGRIINRFSKDIYVIDEALPATVLMLLGTVFVSLSTIIVIVSSTPIFLVVIVPLAFIYVFVQRFY   306
 Human HNKIRSPQSFFDTTPSGRILNCFSKDIYVVDEVLAPVILMLLNSFFNAISTLVVIMASTPLFTVVILPLAVLYTLVQRFY  1129

PLHC-1 VATSRQLKRLESVSRSPIYSHFGETVTG   341
  Fugu VATSRQLKRLESVSRSPIYSHFSETVTG  1104
Pufferfish VATSRQLKRLESVSRSPIYSHFSETVTG   334
 Human AATSRQLKRLESVSRSPIYSHFSETVTG  1157
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Fig. 2. (*Continued*)

did not exhibit any inhibitory effect on Ca-AM accumulation. Similarly, the 10 μM concentration of REV205 resulted in relatively modest response causing 1.8-fold higher accumulation of Ca-AM than in the control cells, but a clear dose–response relationship was not observed and related IC_{50} could not be fitted. Considering the maximal level of inhibition it is noticeable that CA, MK571 and PRB resulted in two to three times higher levels of maximal inhibition when Ca-AM was used. Bimane–GS is a substrate specific only for MRP subfamily and Pgp1 specific inhibitor REV205 expectedly did not affect its retention within the cells. Although VER is a nonspecific inhibitor of both Pgp1 and MRP transport proteins in mammals, it did not modulate bimane–GS efflux in the PLHC-1 cells. As in the case with Rh123 and Ca-AM, PRB and IND were the least potent inhibitors resulting in poor dose–response relationships, and reliable IC_{50} values could not be calculated. CA and MK571 were potent modulators of bimane–GS efflux with respective IC_{50} values of 6.35 and 11.7 μM. The maximal levels of inhibition for these inhibitors were also high, resulting in eight-fold inhibition in the case of CA and six-fold inhibition with MK571.

4. Discussion

PLHC-1 is most frequently used cell line in studying phase I and II enzymes involved in the metabolism of xenobiotics in fish. In addition, this cell line is often used to evaluate environmental samples (soil, water or sediment extracts) for their cytotoxic properties and/or their potential to induce CYP1A. The primary goal of this study was to investigate whether the MXR mechanism as a third critical phase of cellular defense, mediated by toxicologically relevant ABC efflux transporters, is present in the PLHC-1 cells.

Two genes from the ABC transport family of proteins highly relevant for transport of xenobiotics in mammals were found in the PLHC-1 cells. Multiple alignment and phylogenetic analysis revealed a high degree of homology of detected sequences with those of ABCB1 and ABCC3 genes found in mammalian and fish species. To confirm the functional expression of the discovered genes, transport activity studies using different fluorescent substrates and wide range of inhibitors have been conducted. Time and inhibitor concentration-dependent accumulation of Rh123 and Ca-AM, well established substrates of mammalian Pgp1 (ABCB1), were observed in the PLHC-1 cells. Accumulation rates of both dyes were low in the control cells indicating high efflux rates. When model inhibitors were added, a concentration-dependent increase in substrate accumulation was observed (Figs. 3–5). CA was found to be the most potent inhibitor in the case of both substrates (Table 1). VER, a well characterized competitive substrate of human and rat Pgp1, inhibited the efflux of Rh123 but did not change the rate of Ca-AM accumulation. We observed similar phenomenon with primary rainbow trout hepatocytes (manuscript in preparation), probably reflecting group-specific differences concerning VER sensitivity between fish and mammalian Pgp1. CA and VER are not fully specific inhibitors of Ppg1 and at higher micromolar concentrations they are also known to inhibit transport proteins from the MRP (ABCC) subfamily (Haimeur et al., 2004). Therefore, REV205, a specific inhibitor of mammalian Pgp1, was used to additionally prove that functional Pgp1 is expressed in PLHC-1 cells. REV205 led to the increased accumulation of both substrates although clear

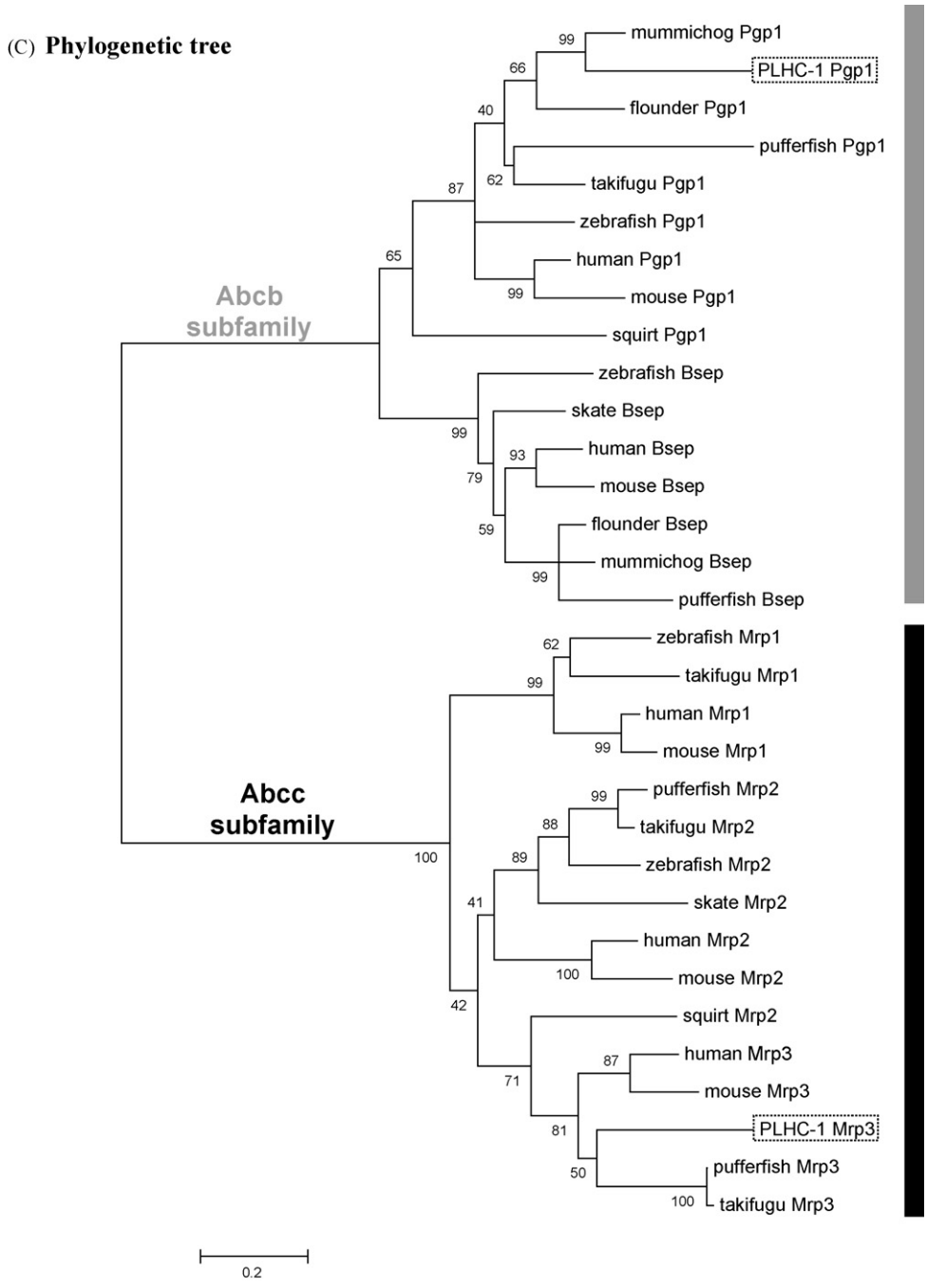

Fig. 2. (*Continued*).

concentration–response relation could not be established with Ca-AM (Fig. 5).

It has been reported that Rh123 and Ca-AM are not solely Pgp1 substrates. Rh123 was shown to be the MRP1 (ABCC1) substrate and Ca-AM is most likely substrate for MRP1 and MRP2 (ABCC2) as well (Daoud et al., 2000; Holló et al., 1996). These findings from mammalian models were confirmed in our experiments with the PLHC-1 cell line. All of the established inhibitors of MRP mediated efflux in mammalian systems also led to marked increase in the accumulation of Ca-AM and Rh123 in PLHC-1 cells. Comparing the IC_{50} values, MK571 was 10–40 times more potent than PRB or IND, resembling results reported for mammalian models (Table 1). It is noticeable that CA, VER and REV205 (all Pgp1 inhibitors) significantly changed their inhibitory properties depending on the substrate used. All of these substrates are found to be more potent (lower IC_{50} values; Table 1) when Rh123 is used as substrate. On the contrary, MRP specific inhibitors did not significantly change inhibiting potential with different substrates. When maximal level of inhibition was considered significant differences were also observed between Rh123 and Ca-AM. CA, MK571 and PRB resulted in two- to three-fold higher maxi-

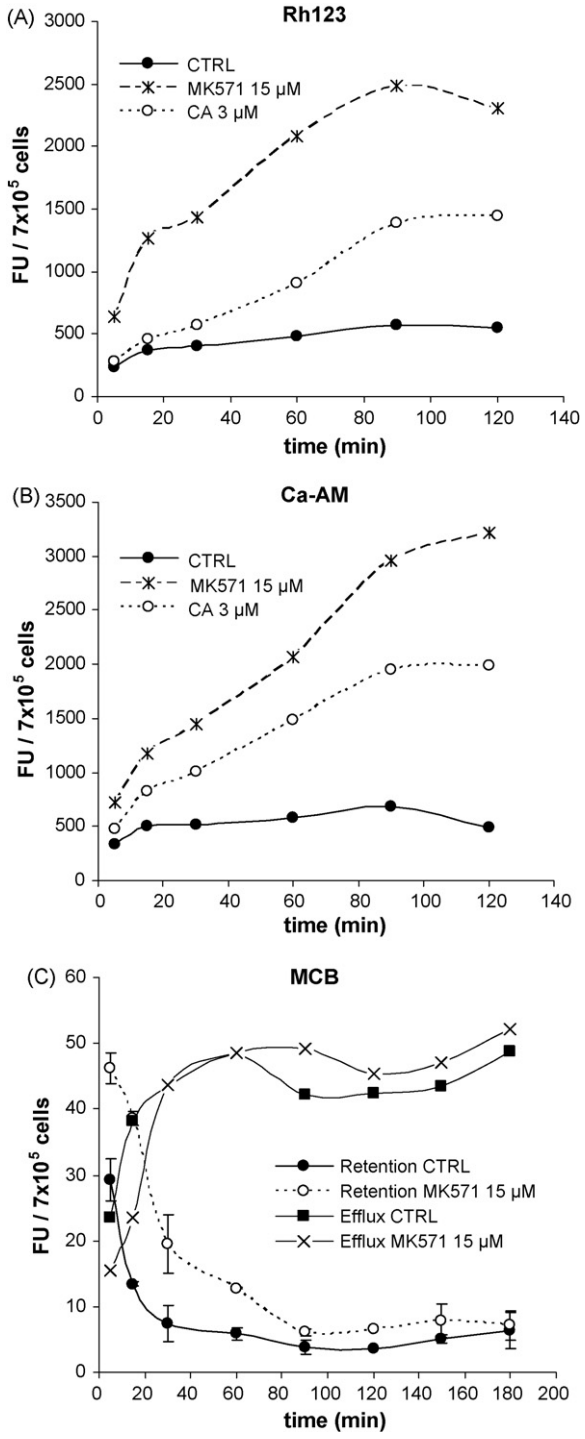

Fig. 3. Time dependency of either accumulation of rhodamine 123 (A) and calcein-AM (B) or of retention/efflux of monochlorbimane (C) in the PLHC-1 cells. The cells are exposed to model fluorescent substrates with or without addition of model inhibitors, as described in detail in Section 2. Data represents means ± standard deviations (unless S.D.s are less than 10% of response) of fluorescent units (FU) determined per 7×10^5 cells.

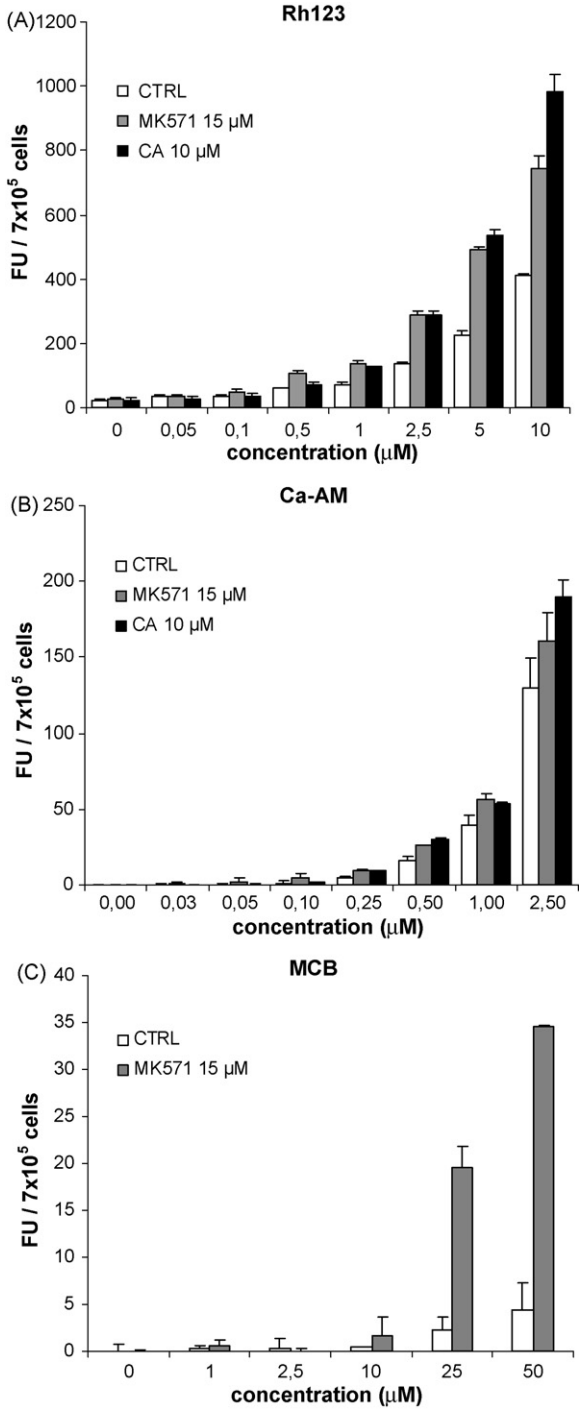

Fig. 4. Concentration-dependent accumulation of rhodamine 123 (A) and calcein-AM (B) or retention of monochlorbimane (C) in the PLHC-1 cells. The cells are exposed to various concentrations of model fluorescent substrates with or without addition of model inhibitors, as described in detail in Section 2. Data represents means ± standard deviations of fluorescent units (FU) determined per 7×10^5 cells.

mal level of inhibition when Ca-AM was used as a substrate (Table 1). The described differences in IC_{50} values and levels of maximal inhibition probably reflect differences in affinities of the Pgp1 and/or MRPs binding sites between Ca-AM and Rh123.

Most members of the MRP subfamily of transport proteins function as organic anion transporters. It has been shown that these proteins extrude a variety of substrates including anticancer drugs and glutathione, glucuronide and sulphate conjugates of diverse compounds. The best characterized, most abundant and toxicologically most relevant MRPs in the liver

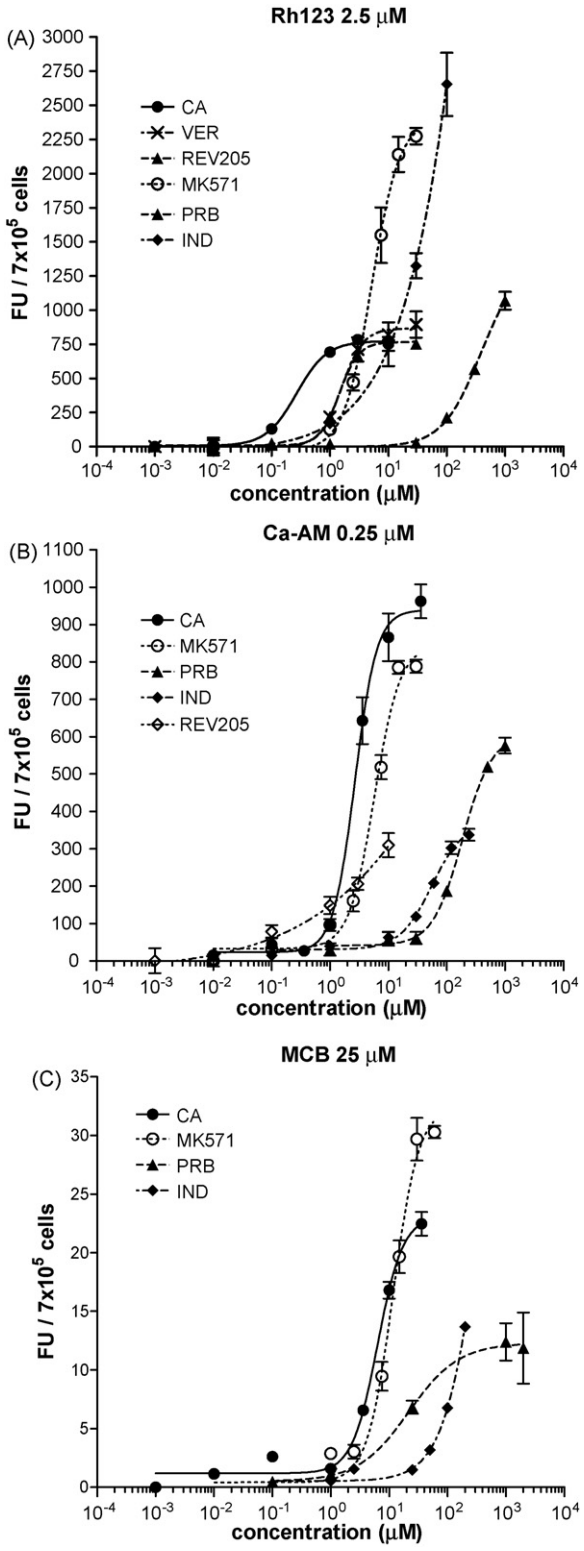

Fig. 5. The effects of model inhibitors on the accumulation of rhodamine 123 (A) and calcein-AM (B) or retention of monochlorbimane (C) in PLHC-1 cells. The cells are exposed to model fluorescent substrates with or without addition of various concentrations of model inhibitors, as described in detail in Section 2. Data represents means ± standard deviations of fluorescent units (FU) determined per 7×10^5 cells.

of mammals are MRP1 and MRP2 expressed in the basolateral and apical membranes of hepatocytes, respectively (Leslie et al., 2005). In order to additionally demonstrate that MRP-like activity is present in PLHC-1 cells we used nonfluorescent MCB which freely diffuses into cells where it becomes a substrate for glutathion-*S*-transferases and forms bimane–GS conjugate. This newly formed highly fluorescent metabolite is a substrate for different MRP transporters. Efficient efflux of bimane–GS out of the PLHC-1 cells was observed, resulting in almost complete clearance during 30 min of efflux (Fig. 3C). This efflux was highly sensitive to the presence of model MRP inhibitors. PRB, IND and MK571 were all found to significantly block the efflux of bimane–GS, with MK571 being the most potent among them (Fig. 5C; Table 1). CA was also shown to be very potent inhibitor with IC_{50} value and level of maximal inhibition similar to that found for MK571. These results confirm the previously observed nonspecificity of CA which, same as in mammalian cells, inhibits transport proteins from both ABCB and ABCC subfamily. REV205 did not modulate efflux of bimane–GS confirming its Pgp1 specificity and offering additional proof that two different transport activities, Pgp1 and MRP like, are truly present in PLHC-1 cells.

However, the presence of genes and/or transcripts of toxicologically highly relevant MRP1 or 2 efflux transporters was not observed in this study. As mentioned earlier, MRP1 and 2 are expressed in the normal liver tissue of mammalian and probably fish species. These transporters exhibit high affinity toward glutathione conjugates. Based on these facts and our initial experiments with MCB we have actually designed our primers towards detection of MRP1 and MRP2 related genes in PLHC-1 cells. We have used numerous different primer pairs designed at high homology regions of fish (zebrafish, pufferfish, mummichog, flounder, little skate) and mammalian (human and rat) MRP1 and 2 genes. Despite various PCR conditions tested we did not obtain any PCR product related to MRP1 or MRP2 genes. However, we successfully cloned the 1023 bp PCR product which was found to be highly homologous to MRP3 (ABCC3) genes found in pufferfish, tetraodon, rat and human (Figs. 1 and 2). MRP3 expression is usually highest in the adrenal glands and its level in normal liver tissue of mammals is usually low. However, it can be highly up-regulated in pathophysiological conditions where MRP2 is down-regulated (Donner and Keppler, 2001; Kruh and Belinsky, 2003). Together with kinetic properties (high Km and Vm constants) these findings suggest that MRP3 may be sort of a back-up mechanism when excretion of organic anions into bile *via* MRP2 is blocked. It is known that isolation of primary hepatocytes can dramatically change the pattern of expression of ABC efflux transporters probably due to loss of three-dimensional structure and hepatocyte depolarization (Luttringer et al., 2002). PLHC-1 cells were grown as a monolayer culture so it is possible that the immortalization of hepatocytes and depolarization signal during the initiation of the culture resulted in down-regulation of MRP2 and subsequent up-regulation of MRP3 through a compensatory mechanism. In addition, initial studies aimed at characterization of transport properties of cloned rat MRP3 showed that, in spite of the highest homologies between MRP1 and MRP3 among all proteins

from this subfamily (58% amino acid sequence identity), MRP3 is able to transport only glucoronide and not glutathione conjugates (Hirohashi et al., 1999). Later on, it was discovered that human MRP3 is able to transport glutathione conjugates (such as DPN-SG), reflecting surprising differences in substrate affinities between rat and human MRP3 (Kool et al., 1999). More recently, Akita et al. (2002) demonstrated that both rat and human MRP3s are able to transport GSH conjugates in addition to glucuronides. Consequently, it is likely that fish MRP3 is also able to transport GSH conjugates out of the cell and this hypothesis would explain high rate of bimane–GS efflux out of the PLHC-1 cells. Nevertheless, contribution of other transporters from the MRP subfamily (MRP1, MRP2 and/or MRP4) cannot be excluded and should be addressed in future research.

In conclusion, the results of this study demonstrate that besides the phases I and II enzyme activity the fish PLHC-1 cells do express toxicologically relevant ABC efflux transporters involved in detoxification and excretion of xenobiotics. Moreover, the fact that the PLHC-1 cells possess all three critical elements of cellular detoxification machinery additionally confirms this cell line as a reliable and useful *in vitro* model in (eco)toxicological research.

Acknowledgment

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