Expression and functional activity of P-glycoprotein in cultured hepatocytes from *Oncorhynchus mykiss*

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**Sturm, Armin, Christina Ziemann, Karen I. Hirsch-Ernst, and Helmut Segner.** Expression and functional activity of P-glycoprotein in cultured hepatocytes from *Oncorhynchus mykiss*. Am J Physiol Regulatory Integrative Comp Physiol 281: R1119–R1126, 2001.—P-glycoproteins encoded by multidrug resistance 1 (*mdr1*) genes are ATP-dependent transporters located in the plasma membrane that mediate the extrusion of hydrophobic compounds from the cell. Using cultured isolated rainbow trout hepatocytes, we characterized an *mdr1*-like transport mechanism of the teleost liver. Immunoblots with the monoclonal antibody C219, which recognizes a conserved epitope of P-glycoproteins, revealed the presence of immunoreactive protein(s) of 165 kDa in trout liver and cultured hepatocytes. In trout liver sections, the immunohistochemistry with C219 stained bile canicular structures. Compounds known to interfere with *mdr1*-dependent transport (verapamil, vinblastine, doxorubicin, cyclosporin A, and vanadate) all increased the accumulation of rhodamine 123 by hepatocytes. Verapamil, vinblastine, and cyclosporin A decreased the efflux of rhodamine 123 from hepatocytes preloaded with rhodamine 123. By contrast, the substrate of the canicular cation transporter tetraethylammonium and the inhibitor of the multidrug resistance-associated protein MK571 had no effect on rhodamine 123 transport. The results demonstrate the presence of an *mdr1*-like transport system in the teleost liver and suggest its function in biliary excretion.

**EXCRETION VIA BILE CONSTITUTES** an important mechanism in the elimination of xenobiotics, particularly for organic compounds exceeding a molecular mass of 400 Da (1). In fish, the significance of the biliary route in the excretion of hydrophobic chemicals is well documented (25, 33). For instance, after exposure of fish to effluents, the total bile concentrations of chlorophenolic compounds and/or their metabolites exceeded their blood plasma concentrations by $10^2$–$10^3$, indicating an effective hepatocellular concentration step (33). Similar concentration gradients of xenobiotics are found between blood and bile in mammals (1). Bile is formed at the bile canaliculi, i.e., tubular structures that form the most proximal part of the biliary tree. The membranes surrounding the canaliculi are specialized (apical) poles of adjacent hepatocytes that contain carrier proteins for transport of chemicals from hepatocytes into bile (1, 34).

Recently, molecular biological approaches have been employed to identify and characterize different carrier proteins present in the canicular plasma membrane of the mammalian hepatocyte (reviewed in Refs. 22, 34). Several canicular transport proteins belong to the ATP-binding cassette (ABC) protein superfamily (22). One group among these ATP-dependent proteins are the P-glycoproteins (P-gps), membrane transporters that are encoded by the highly conserved multidrug resistance (MDR) gene family (3, 17). Two genes encoding P-gps are known in humans (*MDR1* and *MDR2*, Ref. 35), whereas three have been described in rodents (*mdr1α, mdr1b*, and *mdr2;* Ref. 20). *Mdr1* genes can confer multidrug resistance in transfection studies with full-length cDNAs (10, 43) and encode P-gps that mediate the ATP-dependent extrusion of a broad spectrum of hydrophobic chemicals. Overexpression of *mdr1*-type P-gps in certain tumors is associated with their resistance to a broad spectrum of structurally and functionally unrelated hydrophobic drugs (multidrug resistance) (3, 17). In contrast to *mdr1* genes, *mdr2* genes code for phospholipid transporters and are not related to multidrug resistance or decreased drug accumulation (39). In addition to their occurrence in tumor cells, *mdr* genes are also expressed in normal mammalian tissues (12, 41, 42). The *mdr1* genes are expressed at high levels in adrenal cortex, renal proximal tubules, the canicular pole of hepatocytes, small and large intestinal mucosal cells, and pancreatic ductules (12, 41). Lower levels of expression of MDR1 exist in other tissues, including the capillary endothelial cells of the brain and testis (42). MDR2 is predominantly expressed in the canicular pole of hepatocytes and shows only a minor extrahepatic expression (39).

Different reports have demonstrated the presence of P-gp(s) in teleost fish. In immunohistochemical stud-
ies, the distribution of conserved P-gp epitopes among tissues in fish resembled that in mammals (18). In Western analyses of liver extracts from teleost fish, a mammalian P-gp antibody recognized a band of 170 kDa, the approximate molecular mass of mammalian P-gps (9). Partial coding sequences of two genes showing high homology to mdr genes have been identified in a pleuronectid teleost (5). Functional studies of P-gp-like proteins in fish, however, have been restricted to the teleost kidney, in which the presence of a P-gp-like mechanism of the cell-to-lumen transport of hydrophobic compounds has been demonstrated (29, 36, 40). No studies are available to date, however, concerning the involvement of P-gp(s) in liver transport in fish.

The aim of this study was to characterize hepatic P-glycoprotein in teleost fish, using rainbow trout (Oncorhynchus mykiss) as a model. By immunochemical methods employing antibodies directed against conserved P-gp epitopes, the presence of P-pg(s) was shown in trout liver and cultured hepatocytes. To characterize hepatic P-gp(s) in trout on a functional level, we used isolated hepatocytes from trout, in which we studied the accumulation and efflux of rhodamine 123 (Rh123), a fluorescent substrate for mammalian mdr1-type P-gp, in the presence and absence of inhibitors of mdr1 proteins and other hepatic transport systems. To our knowledge, this is the first functional investigation of hepatic P-gp in fish. Such research appears important to gain fundamental insights into the mechanisms of hepatobiliary excretion in lower vertebrates and the function and regulation of their P-gp(s). Moreover, the study of hepatic P-gp(s) in fish has implications for the use of fish as surrogate species in toxicological testing.

Isolation and primary culture of trout hepatocytes. Hepatocytes were isolated as described by Mommsen et al. (31). In brief, after anesthetization with ethyl-4-aminobenzoate and the injection of 200 U of heparin, trout were ventrally opened and the liver was perfused in situ via the intestinal vein. The liver was perfused for 10 min with solution 1 (in mM: 137 NaCl, 5.4 KCl, 0.81 MgSO4, 0.44 KH2PO4, 0.33 Na2HPO4, 5.0 NaHCO3, and 10 HEPES, pH 7.63), followed by 25 min of perfusion with solution 2 [0.008% collagenase D and 1% defatted BSA dissolved in solution 1], followed by 5 min of perfusion with solution 1. Perfusion solutions were brought to 15°C and bubbled with air before use. After perfusion, the liver was removed and minced in ice-cold solution 3 (1% defatted BSA and 1.5 mM CaCl2 in solution 1). The resulting suspension was filtered through a set of nylon screens (250 μm, 100 μm, and 50 μm). Cells were collected by centrifugation (3 min, 50 × g). The pellet was washed three times with ice-cold solution 3. After the last washing step, cells were resuspended in modified M199 (Sigma-M-3274, supplemented to final concentrations of 3.5 mM HEPES, 4.1 mM NaHCO3, 3.4 mM CaCl2, 2 mM MgCl2, 10 U/ml ampicillin, and 10 μg/ml streptomycin). Cell viability was assessed by trypan blue exclusion and was at least 80%. The hepatocytes were seeded at a density of 0.34 × 106 viable cells/cm2 in modified M199 containing 5% FCS onto culture dishes previously coated with Matrigel (0.1 mg/ml protein applied at 110 μl per cm2 of culture dish surface). Hepatocytes were allowed to attach for at least 12 h in an incubator set at 15°C, and subsequently the medium was replaced with fresh, serum-free modified M199. Further changes of the medium were performed daily.

Immunoblot analyses. Plasma membrane fractions of 300 mg of liver tissue or 30 × 106 hepatocytes per sample were isolated by sucrose gradient centrifugation according to Simpson et al. (38). To serve as a positive control, membrane fractions were prepared analogously from male bovine adrenal. The buffers used for homogenization and centrifugation contained 1 mM phenylmethylsulfonyl fluoride. Ten micrograms of protein per lane, determined according to Lowry et al. (27), were subjected to electrophoresis through 7.5% SDSPolyacrylamide gels (26). Proteins were then transferred to polyvinylidene difluoride membranes by semidry blotting (24) using a continuous buffer system (48 mM Tris, 39 mM glycine, 0.038% (wt/vol) SDS, and 15% (vol/vol) methanol, pH 9.0). P-gp was detected using the primary MAb C219 (15) or the primary polyclonal antibody PC03. PC03 was raised against a conserved sequence in the COOH-terminal cytoplasmic P-gp region (SALDTESEKVVQELDKAREG). Peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibodies, respectively, were used as the appropriate secondary antibodies. Protein bands were visualized by ECL using the ECL system. After densitometry, the staining intensity of the P-gp band obtained from a known amount of a bovine adrenal preparation.

Immunohistochemistry. Trout livers were fixed in Bouin’s fluid for 24 h, embedded in paraffin, sectioned, and deparaffinized. The following incubations took place in a moist chamber and were at room temperature except where noted otherwise. Between incubations, samples were rinsed four times with PBS-glycine (0.01 M potassium phosphate, 0.123 M NaCl, and 0.1 M glycine, pH 7.2). The samples were incubated for 30 min with 5% nonfat dry milk-PBS (0.01 M phosphate, 0.123 M NaCl, and 0.1 M glycine, pH 7.2). The samples were incubated overnight at 4°C with MAb C219 (2 μg/ml) in 5% nonfat dry milk-PBS. After rinsing, the tissue sections were incubated for 2 h with biotin-conjugated goat-anti-mouse IgG (1:400) in 5% nonfat dry milk-PBS. After rinsing, the tissue sections were incubated for 2 h with biotin-conjugated goat-anti-mouse IgG (1:400) in 5% nonfat dry milk-PBS. After rinsing, cell viability was assessed by trypan blue exclusion and was at least 80%. The hepatocytes were seeded at a density of 0.34 × 106 viable cells/cm2 in modified M199 containing 5% FCS onto culture dishes previously coated with Matrigel (0.1 mg/ml protein applied at 110 μl per cm2 of culture dish surface). Hepatocytes were allowed to attach for at least 12 h in an incubator set at 15°C, and subsequently the medium was replaced with fresh, serum-free modified M199. Further changes of the medium were performed daily.
dry milk-PBS. After rinsing, the samples were incubated for 3 h with horseradish peroxidase-coupled streptavidin (1:300) in PBS. Liver sections were then rinsed and treated with substrate (diaminobenzidine-H$_2$O$_2$ mixture) for 10 min, rinsed in tap water, and mounted.

**Accumulation and efflux of Rh123 and doxorubicin.** Trout hepatocytes cultured for 24 h after isolation were used in experiments on the accumulation or the efflux of the fluorescent substrates of mammalian mdr1-type P-gps, Rh123, and doxorubicin (11, 17). Verapamil, vinblastine, cyclosporin A, and doxorubicin were used as inhibitors of P-gp (17). Vannadate was used as an inhibitor of transport ATPases (37). TEA was used as a substrate of hepatic type I cation transport systems (34). The leukotriene receptor antagonist MK571 was used as an inhibitor of the multidrug resistance-associated protein (MRP) (14). Incubations took place at 15°C. In accumulation experiments, the medium of hepatocyte monolayers was replaced by serum-free modified M199 containing the indicated fluorescent P-gp substrate alone or in combination with the indicated inhibitor. After variable times of incubation, the medium was removed, the hepatocyte monolayer was washed with PBS, and the culture dish was stored at −20°C for later determination of intracellular levels of the respective P-gp substrate. In efflux experiments, the medium was replaced from hepatocyte monolayers and replaced by 5.25 µM Rh123 in serum-free modified M199. After 2 h of incubation, the medium was removed, and the cells were washed with PBS. One replicate per experiment was removed and stored at −20°C for the determination of the accumulated levels of intracellular Rh123 (“before efflux”). The remaining replicates were incubated for the indicated times with serum-free modified M199 alone or in combination with the indicated inhibitors. After incubation, the medium was removed and culture dishes were stored at −20°C for later determinations. For Rh123 determinations, cell culture plates were thawed and 2 ml n-butanol added to wells of 24- or 6-well plates to extract Rh123 and to precipitate protein. After 30 min of extraction, the Rh123 concentration in n-butanol was determined fluorometrically (excitation at 517 nm, emission at 532 nm). Doxorubicin determinations were carried out in an analogous way (excitation at 291 nm, emission at 582 nm). Intracellular levels of Rh123 or doxorubicin are reported as micrograms per 10$^6$ hepatocytes (visible cell numbers at the time of seeding).

**Statistics.** Data are given as means ± SE. Means were considered to be significantly different from the corresponding control mean when the probability value ($P$) was < 0.05 in the appropriate paired t-test or one-way repeated-measures analyses of variance (ANOVAs). These tests, matching observations on the same isolation of hepatocytes, were selected because systematic differences existed between isolations. After significant ANOVAs, comparisons to a control treatment were carried out using Dunnett test.

**RESULTS**

**Immunohistochemical detection of P-gp in trout liver and cultured hepatocytes.** In immunoblots, the polyclonal antibody PC03 and the MAb C219 recognized the same major band of a molecular mass of ~165 kDa in membrane fractions from trout liver and cultured isolated hepatocytes (Fig. 1). Both antibodies detected a protein of ~158 kDa in membrane fractions from male bovine adrenal, a tissue showing high levels of P-gp and used as a positive control (21). The 165-kDa immunoreactive protein(s) detected in trout liver and isolated hepatocytes thus most probably represent trout P-gp(s). Depending on species and tissue, the apparent molecular mass of P-gps may vary in the range between 130 and 180 kDa, reflecting differences in glycosylation (17). A minor band of 90 kDa was additionally recognized in trout liver membrane fractions by the polyclonal antibody PC03 (Fig. 1) and, at higher concentrations of sample, by the MAb C219 (not shown). The identity of the minor band is not known. It may represent a fragment of trout P-gp or an antigenetically related protein.

In trout liver paraffin sections, immunohistochemical staining with the antibody C219 specifically stained bile canalicular structures (Fig. 2). The dark staining product visualizes the bile canaliculi either as branch-like (longitudinal sectioning) or round structures (sagittal sectioning) that are localized within the double- or triple-layered hepatocyte tubuli.

**Accumulation and efflux studies with fluorescent substrates of mdr1-type P-gps.** To characterize trout hepatic P-gp(s) on a functional level, experiments were carried out with hepatocytes cultured for 24 h after isolation. The fluorescent dye Rh123, a known substrate of mdr1-type P-gps, was used as a positive control (21). A minor band of 90 kDa was additionally recognized in trout liver membrane fractions by the polyclonal antibody PC03 (Fig. 1) and, at higher concentrations of sample, by the MAb C219 (not shown). The identity of the minor band is not known. It may represent a fragment of trout P-gp or an antigenetically related protein.

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**Accumulation and efflux studies with fluorescent substrates of mdr1-type P-gps.** To characterize trout hepatic P-gp(s) on a functional level, experiments were carried out with hepatocytes cultured for 24 h after isolation. The fluorescent dye Rh123, a known substrate of mdr1-type P-gps (11), was employed to investigate an mdr1-like efflux mechanism in cultured trout hepatocytes. The calcium channel blocker verapamil and the immunosuppressive peptide cyclosporin A are known as chemosensitizers, i.e., compounds that inter-
fere with P-gp-dependent transport (13). The Vinca
alkaloid vinblastine and the anthracyclin doxorubicin
are anticancer drugs transported by mdr1-type P-gps
(3, 17) and, therefore, compete with other substrates.
Vanadate represents an inhibitor of ATPases (37),
including ATP-dependent transporters such as P-gp.
The time course of the accumulation of Rh123 by
tROUT hepatocytes was investigated using different
Rh123 concentrations either in the absence (control)
or the presence of verapamil or vinblastine (Fig. 3, A
and B). In the presence of 5.25 μM Rh123, the
accumulation of Rh123 by trout hepatocytes was
linear with time during the initial 90 min of incubation,
after which its rate decreased (Fig. 3A). Com-
pared with controls, the Rh123 accumulation by
hepatocytes cotreated with vinblastine was signifi-
cantly increased at all times of observation, whereas
the Rh123 accumulation by hepatocytes cotreated
with verapamil was significantly increased only after
180 and 300 min (Fig. 3A). Using linear regression,
we derived the initial rates of Rh123 (0–90 min) from
the experiment shown in Fig. 3A and similar exper-
miments using Rh123 concentrations of 0.53 and 1.58
μM (Fig. 3B). Vinblastine significantly increased the
initial rate of Rh123 accumulation with all Rh123
concentrations studied, whereas verapamil in-
creased Rh123 accumulation significantly only in
experiments with 0.52 μM Rh123, but not with 1.58
or 5.25 μM Rh123. The effects of further compounds
on the accumulation of Rh123 by trout hepatocytes
were investigated using a fixed incubation time of 2 h
(Fig. 4). Inclusion of doxorubicin or cyclosporin A
during the incubation of hepatocytes with 5.25 μM
Rh123 significantly increased intracellular Rh123
accumulation (Fig. 4). Similarly, the presence of 15
or 50 μM vanadate increased the accumulation of

![Fig. 2. Immunoreactivity of MAb C219 against P-gp(s) in rainbow
tROUT liver. C219 reacted with bile canalicular structures in rainbow
tROUT liver. Scale bar = 50 μM. Antibody staining was visualized by
the avidin-biotin-peroxidase method with diaminobenzidine as
chromagen.](#)
slight, nonsignificant increases of Rh123 accumulation (not shown). The organic cation TEA, a substrate for type I sinusoidal organic cation uptake systems and an electroneutral canalicular H+/organic cation antiporter (34), had no significant effect on Rh123 accumulation (Fig. 4). Moreover, the accumulation of the mdr1 substrate doxorubicin was studied in the absence (control) and the presence of verapamil and vinblastine (Fig. 5). Verapamil significantly increased doxorubicin accumulation by trout hepatocytes, whereas vinblastine caused only slight, nonsignificant changes.

The mdr1 substrates vinblastine and doxorubicin and the chemosensitizers verapamil and cyclosporin A increased the accumulation of Rh123, suggesting the presence of a mdr1-like efflux mechanism in trout hepatocytes. The hypothesis that the observed increase in Rh123 accumulation reflects the inhibition of an active efflux mechanism was further examined in efflux experiments. Hepatocytes were first incubated with 5.25 μM Rh123 for 2 h. After washing with PBS, hepatocytes were further incubated in medium alone or medium containing different compounds known to interfere with mdr1-dependent transport (efflux incubation). Vinblastine significantly delayed the decrease in intracellular Rh123 levels and the complementary increase in extracellular Rh123 concentrations in the medium (Fig. 6). In similar experiments using a fixed efflux incubation period of 4 h, different chemicals were investigated concerning their effects on Rh123 retention by trout hepatocytes (Table 1). Verapamil, vinblastine, and cyclosporin A dose-dependently and significantly increased the intracellular levels of Rh123 retained by hepatocytes after 4 h of incubation in Rh123-free medium (Table 1). Doxorubicin effected only slight, nonsignificant increases in Rh123 retention. TEA and the inhibitor of the MRP MK571 had no apparent effects on Rh123 efflux (Table 1).
**Table 1. Effect of transport inhibitors on efflux of rhodamine 123 from rainbow trout hepatocytes**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Intracellular Rhodamine 123</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Efflux</td>
<td>5</td>
<td>0.295 ± 0.053†</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>0.193 ± 0.049</td>
</tr>
<tr>
<td>Verapamil, 2.5 μM</td>
<td>5</td>
<td>0.221 ± 0.045</td>
</tr>
<tr>
<td>Verapamil, 7.5 μM</td>
<td>5</td>
<td>0.236 ± 0.059#</td>
</tr>
<tr>
<td>Verapamil, 25 μM</td>
<td>5</td>
<td>0.264 ± 0.047†</td>
</tr>
<tr>
<td>Vinblastine, 7.0 μM</td>
<td>5</td>
<td>0.266 ± 0.043†</td>
</tr>
<tr>
<td>Vinblastine, 20 μM</td>
<td>5</td>
<td>0.275 ± 0.042†</td>
</tr>
<tr>
<td>Vinblastine, 60 μM</td>
<td>5</td>
<td>0.277 ± 0.043†</td>
</tr>
<tr>
<td>Before Efflux</td>
<td>6</td>
<td>0.215 ± 0.074*</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0.188 ± 0.071</td>
</tr>
<tr>
<td>Vinblastine, 20 μM</td>
<td>6</td>
<td>0.230 ± 0.084†</td>
</tr>
<tr>
<td>Cyclosporin A, 0.3 μM</td>
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<td>0.180 ± 0.079</td>
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<td>Cyclosporin A, 1.0 μM</td>
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<td>0.197 ± 0.079</td>
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<tr>
<td>Cyclosporin A, 3.0 μM</td>
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<td>0.214 ± 0.091*</td>
</tr>
<tr>
<td>Before Efflux</td>
<td>6</td>
<td>0.165 ± 0.030†</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0.125 ± 0.016</td>
</tr>
<tr>
<td>Vinblastine, 20 μM</td>
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<td>0.165 ± 0.030†</td>
</tr>
<tr>
<td>Doxorubicin, 1.0 μM</td>
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<td>0.136 ± 0.023</td>
</tr>
<tr>
<td>Doxorubicin, 3.0 μM</td>
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<td>0.134 ± 0.027</td>
</tr>
<tr>
<td>Doxorubicin, 10.0 μM</td>
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</tr>
<tr>
<td>Doxorubicin, 30.0 μM</td>
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<td>0.141 ± 0.029</td>
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<tr>
<td>Before Efflux</td>
<td>5</td>
<td>0.238 ± 0.032†</td>
</tr>
<tr>
<td>Control</td>
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<td>Control</td>
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<tr>
<td>Vinblastine, 20 μM</td>
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<td>0.268 ± 0.071†</td>
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<tr>
<td>TEA, 0.1 mM</td>
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<td>0.225 ± 0.080</td>
</tr>
<tr>
<td>TEA, 1.0 mM</td>
<td>5</td>
<td>0.219 ± 0.074</td>
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</tbody>
</table>

Values are means ± SE in μg/10⁶ cells; n, number of hepatocyte cultures from different fish. Hepatocytes were cultured for 24 h after isolation before use in the experiment. Cells were incubated for 2 h in medium containing 5.25 μM rhodamine 123 and washed with PBS. One triplicate cell culture per fish was removed at this stage for determination of intracellular rhodamine 123 levels (before efflux). The remaining cell cultures were incubated for 4 h with medium without (control) and with indicated chemicals in triplicate. After incubations, intracellular levels of rhodamine 123 were determined as described in MATERIALS AND METHODS. TEA, tetraethylammonium.

**DISCUSSION**

**Immunochemo evidence for the presence of P-gp(s) in trout liver.** Recent studies in teleost fish have demonstrated the presence of hepatic protein(s) that are immunoreactive to antibodies directed against conserved P-gp epitopes and that resemble mammalian P-gps with respect to their apparent molecular mass and their localization to the canalicular pole of the hepatocyte (9, 18). Our immunochemo observations on rainbow trout liver are in line with these reports on other species and, as do these studies (9, 18), strongly suggest the hepatic expression of P-gp(s) in teleost fish. However, the results obtained in this and other studies should be interpreted with caution, as the exact specificity of the used P-gp antibodies in fish is not known. The polyclonal antibody PC03 is raised against a conserved 26-amino acid sequence in the COOH-terminal cytoplasmic P-gp region (see MATERIALS AND METHODS), which includes the 7-amino acid epitope recognized by C219 (15). The epitope recognized by the MAb C219, an immunochemo probe used in this and other studies (9, 18), is present in all human and rodent P-gp genes known to date, including the mdr2 genes, the products of which are not related to hydrophobic drug transport. Moreover, C219 also recognizes a canalicular transport protein called “sister of P-gp” (7) that mediates the export of bile salts (16). Partial sequences of both P-gp and sister of P-gp genes have been identified in teleosts (5, 8). Hence, the possibility exists that multiple canalicular carrier proteins are detected by the antibodies C219 and PC03 in teleost liver. As long as more specific probes are lacking, antibodies directed against conserved epitopes such as C219 can yield basic information concerning the occurrence and distribution of P-gps in fish.

**Evidence for a mdr1-like efflux mechanism in trout hepatocytes.** Drug-resistant tumor cell lines overexpressing mdr1 genes exhibit a decreased accumulation and increased efflux of Rh123 and other fluorescent dyes compared with their drug-sensitive parental cell lines lacking mdr1 expression (4, 11). The decreased accumulation and increased efflux of fluorescent dyes in these cell lines can partly or completely be reversed by coadministration of compounds that interfere with mdr1-dependent transport (4, 11). Accordingly, assays, based on the observation of fluorescent dye accumulation/efflux and the effects of diagnostic compounds hereon, have been used to diagnose mdr1-type P-gp-dependent drug resistance in clinical tumor samples (11). The same approach has been employed to study the function of P-gps in cells from normal tissues, including isolated hepatocytes (6, 19).

In the present study, we examined the accumulation and efflux of Rh123 in cultured rainbow trout hepatocytes under the influence of different compounds that interfere with mdr1-type P-gp transport and are themselves substrates (17), namely the calcium channel blocker verapamil, the Vinca alkaloid vinblastine, the immunosuppressive peptide cyclosporin A, and the anthracyclin doxorubicin. In addition, vanadate, an inhibitor of ATPase transporters (37), was examined for its effects on Rh123 accumulation. In the mammalian hepatocyte, different transport proteins exist that are thought to contribute to drug transport (22). Hence, mechanisms of both xenobiotic uptake and secretion may have been underlying processes in our Rh123 accumulation and efflux experiments.

The uptake of highly hydrophobic cations, such as Rh123, at the hepatocyte’s sinusoidal membrane is assumed to occur mainly passively (34). The uptake of organic cations is further mediated by at least two transport proteins in sinusoidal membrane that preferably transport less hydrophobic cationic compounds (34). If the accumulation of Rh123 by trout hepatocytes involved transporter-mediated mechanisms, one would expect that other cationic compounds would (partly) inhibit Rh123 accumulation. The accumulation of Rh123 in this study, however, could not be decreased
by any of the investigated inhibitors/substrates of organic cation transport systems (Figs. 3 and 4). This argues against the significance of transporter-mediated uptake in Rh123 accumulation.

All of the investigated compounds known to interfere with mdr1-type P-gp-mediated transport, i.e., verapamil, vinblastine, cyclosporin A, doxorubicin, and vanadate, significantly increased the accumulation of Rh123 by trout hepatocytes (Figs. 3 and 4). Moreover, the accumulation of the mdr1 substrate daunorubicin by hepatocytes was increased by verapamil (Fig. 5). These observations are in accordance with the presence of a mdr1-like efflux mechanism in trout hepatocytes that is ATP dependent and sensitive to inhibition and/or competition. When different compounds were tested for their effects on the efflux of Rh123 from hepatocytes previously loaded with this dye, verapamil, vinblastine, and cyclosporin A acted as inhibitors (Table 1). This further supports the proposed presence of an mdr1-like efflux mechanism in trout hepatocytes that is probably involved in the biliary secretion of hydrophobic compounds.

In the mammalian hepatocyte, the different proteins involved in the active transport of hydrophobic compounds into bile partly overlap in substrate and inhibitor specificity. In addition to mdr1-type P-gp(s), members of the MRP subfamily are also ABC transporters associated with xenobiotic transport. The canalicular isoform of MRP has been shown to be identical to the functionally defined canalicular multispecific organic anion transporter (23). It has been speculated that MRP may also transport organic cations (28). Tumor cell lines showing high levels of MRP but lacking P-gp expression showed a decreased accumulation of the mdr1 substrates daunorubicin and Rh123 (2, 44) that was slightly sensitive to verapamil and cyclosporin A (2), suggesting that these compounds might also interact with MRP and possibly represent MRP substrates. Evidence for the presence of MRP-like proteins exists in elasmobranch fish (30). Because of the lack of effects of the MRP-inhibitor MK571 on Rh123 efflux observed in this study, it appears unlikely that MRP contributed to the mdr1-like mechanism in trout hepatocytes. In addition to the ABC proteins discussed above, an H+ /organic cation antiporter mechanism exists in the canalicular membrane (32). Of the compounds investigated in this report, TEA is a substrate of the canalicular H+ /organic cation antiporter mechanism (32). Because of the lack of effects of TEA on the accumulation and efflux of Rh123 in trout hepatocytes, it appears improbable that the H+ /organic cation antiporter interacted with Rh123 in our experiments.

The mdr1-like Rh123 efflux mechanism in trout hepatocytes characterized in the present report resembles a P-gp-like transport mechanism that was previously demonstrated in the teleost renal proximal tubule (29, 36, 40). Primary cultures of flounder proximal tubule epithelium mounted in Ussing chambers exhibited an active net secretion of the mdr1-type P-gp substrate daunomycin that was inhibited by verapamil, vinblastine, and cyclosporin A (40). The luminal daunomycin accumulation in primary cultured killifish renal tubules was sensitive to verapamil and cyclosporin A (29). Similarly, the secretion of a fluorescent cyclosporin analog in the same in vitro system could be blocked by verapamil, vinblastine, cyclosporin A, and other inhibitors/substrates of P-gp (35).

**Perspectives**

This study demonstrates the presence of a mdr1-like mechanism in cultured rainbow trout hepatocytes and suggests the functional activity of mdr1-type P-gp(s) in teleost liver. The molecular cloning of teleost P-gp genes, presently underway in different laboratories, will facilitate the determination of the molecular identity of the proteins involved in the canalicular secretion of hydrophobic compounds in fish. Together with such molecular studies, functional investigations are needed for a more thorough understanding of this process in fish. Such knowledge not only will significantly contribute to the understanding of liver physiology and its evolution within vertebrates but also appears highly relevant to predicting the effects of potentially toxic environmental contaminants on fish. The role of P-gp-like mechanisms in the biliary excretion may strongly influence xenobiotic body burdens in fish. Subsequent studies should therefore specifically address the question of which environmentally relevant xenobiotics interact with piscine P-gp(s). The hepatocyte model used in this report offers one experimental approach for such studies.

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**REFERENCES**