Potential Cholestatic Activity of Various Therapeutic Agents Assessed by Bile Canalicular Membrane Vesicles Isolated from Rats and Humans

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Summary: The active transport of solutes mediated by the bile salt export pump (BSEP/ABCB11) and multidrug resistance associated protein-2 (MRP2/ABCC2) are thought to involve bile acid-dependent and -independent bile formation, respectively. To evaluate the potential of therapeutic agents as inhibitors of such transporters on bile canalicular membranes, we examined the inhibition of the primary active transport of typical substrates by 15 drugs, clinically known to cause cholestasis in canalicular membrane vesicles. The inhibition by most of the compounds in rat canalicular membrane vesicles (CMVs) was minimal or observed at much higher concentrations than obtained in clinical situations. However, cloxacillin, cyclosporin A and midecamycin inhibited BSEP, and cyclosporin A and midecamycin inhibited MRP2 with an inhibition constant close to the clinical concentration. By comparing the inhibition potential between rat and human CMVs, the inhibition of BSEP- and MRP2-mediated transport by midecamycin and cyclosporin A was relatively similar whereas the inhibitory effect on BSEP-mediated transport by cloxacillin and glibenclamide was more marked in humans than in rats. These results suggest that the majority of cholestasis-inducing drugs have a minimal inhibitory effect on rat BSEP and MRP2 although species differences in inhibitory potential should be considered, especially in the case of BSEP.

Key words: cholestasis; biliary excretion; MRP2; BSEP

Introduction

Continuous bile formation is an important function of the mammalian liver. It is an osmotic secretory process that is driven by the concentration gradient of bile salts and other biliary constituents across the bile canaliculi. The transport of solutes from blood to bile is driven by several active transport systems in the plasma membranes of both the basolateral (sinusoidal) and apical (canalicular) surfaces of hepatocytes. The active transport of solutes across the latter membrane is thought to be the rate-limiting step in bile formation under physiological conditions.

Bile secretion has been traditionally divided into two components. One is the bile acid-dependent bile flow which is related to the excretion of bile salts mediated by the bile salt export pump (BSEP/ABCB11) in canaliculi. The other is the bile acid-independent bile flow, involving the passive excretion of inorganic electrolytes as well as the active excretion of glutathione (GSH) mediated by canalicular multispecific organic anion transporter/multidrug resistance associated protein-2 (MRP2/ABCC2).

Cholestasis may occur via interference with the unidirectional transport of biliary constituents across the canicular membrane because of hereditary or acquired impairment as a secondary consequence of structural damage to hepatocytes and the bile duct. Recently, hereditary defects of specific transporter molecules in canaliculi have been identified as playing a role in the pathogenesis of certain forms of cholestasis. Strautnieks et al. have reported that progressive familial intrahepatic cholestasis-2 (PFIC-2) is caused by a lack of BSEP. It has also been reported that a hereditary mutation of MRP2 leads to hyperbilirubinemia with cholestasis known as Dubin-Johnson syndrome.

In addition to these hereditary forms, cholestasis induced by drugs and other xenobiotic substances is a
common problem in clinical medicine. A great deal of effort has been devoted to investigate the mechanisms of cholestasis, although the pathogenesis of drug-induced cholestasis remains unknown. Recently, several cholestatic compounds have been found to interact with the export of bile acids and some organic anions at the canalicular ATP-dependent transporters including BSEP and MRP2. The immunosuppressant, cyclosporin A, has been found to inhibit both BSEP and MRP2 in vitro. Similar mechanisms involving transporters were recently postulated for rifamycin, rifampicin and glibenclamide. Therefore, it would be desirable in drug screening and evaluation processes to exclude any compounds which exhibit cholestatic potential by inhibiting transport across the bile canalicular membrane.

Isolated canalicular membrane vesicles (CMVs) have been used to evaluate the transport systems on the membranes in vitro. As far as certain series of compounds which are reported to induce cholestasis are concerned, it has not been systemically examined yet whether or not transporter inhibition on bile canalicular membrane involves cholestasis. Therefore, in the present study, examining the possible involvement of transporter inhibition in drug-induced cholestasis, we have investigated the inhibitory effects of 15 compounds, which cause cholestasis in clinical situations, on both BSEP and MRP2 using CMVs. In addition, we have also evaluated the possibility of species differences in the inhibitory effects of several compounds between rats and humans.

Materials and Methods

Materials: Amoxycillin, chlorpromazine, clocxinilin, colchicine, cyclosporin A, doxycycline, glibenclamide, ketoconazole, midecamycin, minocycline, quinidine, sulpiride, tetracycline, ticlopidine, verapamil, ATP, AMP, creatine phosphate, and creatine phosphokinase were purchased from Sigma (St.Louis, MO). [3H]Taurocholate ([3H]TCA, 3.47 μCi/nmol) and [3H]glutathione (50.0 μCi/nmol) were purchased from New England Nuclear (Boston, MA). [3H]-(2,4-dinitrophenyl)-glutathione (DNP-SG, 50.0 μCi/nmol) was synthesized by the method described by Saxena and Henderson. All other chemicals were commercial products of analytical grade.

Isolation of CMVs from human and rat liver: The human liver sample (from a female Caucasian, aged 21, who died from head trauma) was provided by SRI International (Menlo Park, CA) and its shipment to SRI was coordinated by the National Disease Research Interchange (Philadelphia, PA). CMVs from one human and six male Sprague-Dawley rats (250–320 g, Japan SLC, Hamamatsu, Japan) were prepared as described previously. The purity of the prepared CMVs was checked by determining the activity of alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (γ-GTP). The activity of ALP was 536 ± 57 (CMVs) and 8.89 ± 0.61 nmol/min/mg protein (homogenate) for the rats (mean ± SE of 5 preparations, enrichment: 60.3 ± 6.4) and 848 ± 58 (CMVs) and 10.8 ± 0.8 nmol/min/mg protein (homogenate) for the human (mean ± SE of 3 preparations, enrichment: 78.6 ± 5.4). The activity of γ-GTP was 264 ± 61 (CMVs) and 5.43 ± 0.40 nmol/min/mg protein (homogenate) for the rats (enrichment: 48.5 ± 11.2) and 4196 ± 532 (CMVs) and 119 ± 7 nmol/min/mg protein (homogenate) for the human (enrichment: 35.2 ± 4.4). The activity of CMVs used in the present study was also checked by measuring the ATP-dependent uptake of standard substrates, [3H]TCA and [3H]DNP-SG. ATP-dependent uptake was obtained by subtraction of the uptake in the presence of AMP (ATP-independent) from that in the presence of ATP. The activities of CMVs used in the present study under control conditions were as follows. [3H]TCA (1 μM, incubation time 1 min at 37°C); ATP-dependent: 121 ± 24, ATP-independent: 4.78 ± 2.55 (rats), ATP-dependent: 14.4 ± 1.6, ATP-independent: 5.02 ± 1.53 μL/min/mg protein (human), respectively. [3H]DNP-SG (1 μM, incubation time 2 min at 37°C); ATP-dependent: 98.2 ± 12.7, ATP-independent: 3.55 ± 1.36 (rats), ATP-dependent: 5.81 ± 0.30, ATP-independent: 2.01 ± 0.28 μL/min/mg protein (human), respectively. A major part of uptake clearance of CMVs in the presence of ATP was ATP-dependent both in rats and human.

Uptake study by CMVs: The inhibition studies for BSEP and MRP2 using various organic anions were performed as follows: amoxycillin, chlorpromazine, clocxinilin, colchicine, cyclosporin A, doxycycline, glibenclamide, ketoconazole, midecamycin, minocycline, quinidine, sulpiride, tetracycline, ticlopidine and verapamil were dissolved in dimethyl sulfoxide and diluted with 10 mM Tris-250 mM sucrose buffer (pH 7.4). The final concentration of dimethyl sulfoxide in each assay mixture was 0.3% except for cyclosporin A (0.5%). The inhibition study was performed as reported previously.

Data analysis: The data analysis was performed as reported previously. Briefly, the inhibition constants (K_i) for the uptake of TCA and DNP-SG by CMVs were obtained by fitting data to the following equations:

$$V_{(+I)} / V_{(-I)} = 1 / (1 + I_i / K_i)$$  \hspace{1cm} Eq (1)

where $V_{(+I)}$ and $V_{(-I)}$ represent the ATP-dependent transport velocity in the presence or absence of an inhibitor, respectively, and $I_i$ is the inhibitor concentration in the medium (μM). This equation was derived based on the assumption of competitive inhibition and the fact that the TCA and DNP-SG concentrations (1 μM) were much lower than the $K_m$ values (12.6 and 20.6 μM for TCA and 21.0 and 193 μM for DNP-SG in rats and humans, respectively).
humans, respectively). The fitting was performed by an iterative nonlinear least-squares method with a MULTI program, to obtain estimates of Ki. The input data were weighted as the reciprocal of the square of the observed values.

The clinically relevant concentrations of inhibitors were estimated from the following equation:

\[ f_u \cdot I_{in} \leq f_u \cdot (I_{max} + F_a \cdot Dose \cdot k_a / Q_h) \]  

Eq (2)

where \( f_u \) represents the plasma unbound fraction of the inhibitor. \( I_{max} \) is the reported value for the maximum concentration in the systemic circulation and \( I_{in} \) is the maximum inhibitor concentration at the inlet to the liver. \( F_a \) represents the absorbed fraction of the inhibitor, assumed to be 1. The doses of inhibitors administered were those given in publications. The term, \( k_a \), is the absorption rate constant and we chose 0.1 min \(^{-1} \) taking the maximum gastric emptying time (10 min) into consideration. \( Q_h \) represents the hepatic blood flow rate in humans (1610 mL/min).

**Statistical analysis:** The results are shown as mean ± SE for the number of determinations. Dunnett’s post-hoc test was used to determine the significance of differences between the mean of two groups, with \( P < 0.05 \) as the minimum levels of difference.

**Results**

**Compounds selected in the present study:** All the compounds (except colchicine) have been reported to produce clinical cholestasis. Cyclosporin A has been demonstrated to induce cholestasis in clinical situations, causing an increase in serum bilirubin and bile acids and a decrease in plasma bromosulfophthalein clearance. Chlorpromazine has been also shown to cause cholestatic jaundice with an incidence of 1–2% in clinical situations. Amoxycillin, clavulanic acid, doxycycline, glibenclamide, ketoconazole, midecamycin, minocycline, quinidine, sulpiride, tetracycline, ticlopidine and verapamil have been reported to cause cholestatic symptoms in clinical situations (at least 1–15 case reports each) as shown by liver function tests and/or liver biopsies. As for colchicine, no cholestasis has been described in clinical situations, although it reduces bile flow with leakage of lactate dehydrogenase into bile in rats and increases both serum glutamic acid oxaloacetic transaminase (SGOT) and alkaline phosphatase (ALP) in patients.

**Inhibition of ATP-dependent uptake of TCA and DNP-SG by CMVs:** The inhibitory effects of several compounds on the ATP-dependent uptake of TCA and DNP-SG by rat CMVs are shown in Fig. 1. The TCA uptake was inhibited by chlorpromazine, clavulanic acid, colchicine, doxycycline, glibenclamide, cyclosporin A, midecamycin, quinidine and verapamil (Fig. 1A, B), whereas chlorpromazine, colchicine, cyclosporin A, minocycline and midecamycin inhibited DNP-SG uptake in a concentration-dependent manner (Fig. 1C). The Ki values obtained are shown in Table 1. The Ki values for inhibition of TCA uptake by cyclosporin A and glibenclamide were similar to the previously published values (0.8 and 8.6 μM, respectively).

When the relative ratio of the clinical concentration to the Ki value was calculated based on the maximum unbound concentration of each compound in circulating plasma (\( f_u \cdot I_{max} \)), the obtained ratios were less than 0.05 (no inhibition) except for the inhibition of TCA uptake by cloxacillin (Table 1). When the relative ratio of the clinical concentration to the Ki value was calculated based on the maximum unbound concentration at the inlet to the liver (\( f_u \cdot I_{in} \)), most of ratios were still close to 0.05 except for the inhibition of TCA uptake by cloxacillin, cyclosporin A and midecamycin and that of DNP-SG uptake by cyclosporin A and midecamycin (Table 1).

To obtain an insight into any species difference in such inhibition potential, the compounds with a ratio less than 0.014 were selected and their effect on the uptake of TCA and DNP-SG in human CMVs was examined (Fig. 2). Here, the chosen concentration of each compound was the Ki (half-inhibitory concentration) observed in rats. The TCA uptake in the presence of midecamycin and cyclosporin A was 51.3 and 29.0% of the control, showing inhibition comparable to that exhibited by rat CMVs, whereas that in the presence of cloxacillin and glibenclamide was much lower than 50%, suggesting greater inhibition in human CMVs (Fig. 2). Chlorpromazine, midecamycin and cyclosporin A exhibited slight or minimal inhibition (80–100% of control) of DNP-SG uptake in human CMVs (Fig. 2).

**Discussion**

Cholestasis caused by several therapeutic drugs has been suggested to involve the inhibition of transporters localized on the bile canalicular membranes. Bohme et al. have reported that cyclosporin A inhibits the ATP-dependent transport of TCA and leukotriene C4 (LTC4) with Ki values of 0.2 and 3.4 μM, respectively, in rat CMVs, and that the bile flow rate is reduced to 50% of baseline after cyclosporin A injection (25 mg/kg) in vivo in rats. Funk et al. have also demonstrated that troglitazone, an insulin-sensitizing drug for type 2 non-insulin-dependent diabetes mellitus, and its major metabolite, troglitazone sulfate, inhibit ATP-dependent TCA transport with a Ki of 1.3 and 0.23 μM, respectively, in rat CMVs and i.v. injection of troglitazone (25 mg/kg) increases plasma bile acid concentrations in vivo. Thus, at least some cholestatic agents inhibit the primary active transport mediated by BSEP and/or MRP2. This led us to systematically examine the inhibition potential of 15 compounds, reported to exhibit the
Fig. 1. Inhibition of the primary active transport of TCA and DNP-SG by various therapeutic agents in rat CMVs.
CMVs were incubated with 1 μM TCA (A and B, incubation time 1 min) or DNP-SG (C, incubation time 2 min) in the presence of the indicated concentration of each compound at 37°C, respectively. The ATP-dependent uptake was calculated by subtracting the uptake in the absence of ATP from that in its presence and shown as normalized values with respect to control values. Each data point represents the mean ± SE of 3 experiments from 6 preparations.

Table 1. Comparison of the inhibition constants (Kᵢ) of therapeutic drugs for BSEP and MRP2 with their clinical unbound concentrations

<table>
<thead>
<tr>
<th>compounds</th>
<th>Kᵢ,BSEP (μM)</th>
<th>Kᵢ,MRP2 (μM)</th>
<th>route</th>
<th>dose (mg)</th>
<th>fᵢ,u fᵢ,max (μM)</th>
<th>fᵢ,u fᵢ,a (μM)</th>
<th>References</th>
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<tr>
<td>amoxicillin</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>p.o.</td>
<td>500</td>
<td>11.2</td>
<td>71.9</td>
<td>18</td>
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<td>chlorpromazine</td>
<td>506</td>
<td>258</td>
<td>p.o.</td>
<td>800</td>
<td>0.0245</td>
<td>4.22</td>
<td>18, 19</td>
</tr>
<tr>
<td>cloxacillin</td>
<td>74.8</td>
<td>&gt; 1000</td>
<td>i.v.</td>
<td>1000</td>
<td>43.2</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>colchicine</td>
<td>539</td>
<td>975</td>
<td>p.o.</td>
<td>0.5</td>
<td>0.0120</td>
<td>0.0711</td>
<td>21, 22</td>
</tr>
<tr>
<td>cyclosporin A</td>
<td>1.09</td>
<td>1.96</td>
<td>p.o.</td>
<td>245</td>
<td>0.0501</td>
<td>0.936</td>
<td>18, 23</td>
</tr>
<tr>
<td>doxycycline</td>
<td>530</td>
<td>&gt; 1000</td>
<td>p.o.</td>
<td>100</td>
<td>0.898</td>
<td>3.32</td>
<td>18, 24</td>
</tr>
<tr>
<td>glibenclamide</td>
<td>5.29</td>
<td>&gt; 100</td>
<td>p.o.</td>
<td>25</td>
<td>0.0121</td>
<td>0.0750</td>
<td>25, 26</td>
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<tr>
<td>ketoconazole</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>p.o.</td>
<td>200</td>
<td>0.0601</td>
<td>0.294</td>
<td>18</td>
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<tr>
<td>midecamycin</td>
<td>154</td>
<td>140</td>
<td>p.o.</td>
<td>1200</td>
<td>1.98</td>
<td>79.8</td>
<td>27</td>
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<tr>
<td>minocycline</td>
<td>&gt; 1000</td>
<td>400</td>
<td>p.o.</td>
<td>100</td>
<td>0.874</td>
<td>3.39</td>
<td>24</td>
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<td>quinidine</td>
<td>542</td>
<td>&gt; 1000</td>
<td>p.o.</td>
<td>400</td>
<td>0.260</td>
<td>4.39</td>
<td>18, 28</td>
</tr>
<tr>
<td>sulpiride</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>i.m.</td>
<td>200</td>
<td>26.4</td>
<td></td>
<td>29, 30</td>
</tr>
<tr>
<td>tetracycline</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>p.o.</td>
<td>250</td>
<td>1.81</td>
<td>13.1</td>
<td>18</td>
</tr>
<tr>
<td>ticlopidine</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>p.o.</td>
<td>250</td>
<td>0.0311</td>
<td>1.07</td>
<td>18</td>
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<tr>
<td>verapamil</td>
<td>92.5</td>
<td>&gt; 1000</td>
<td>p.o.</td>
<td>80</td>
<td>0.0213</td>
<td>0.720</td>
<td>31, 32</td>
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</table>

* Kᵢ for BSEP was obtained from the inhibition of ATP-dependent uptake of TCA by rat CMVs.
* Kᵢ for MRP2 was obtained from the inhibition of ATP-dependent uptake of DNP-SG by rat CMVs.
* Maximum inhibitor concentration in circulating plasma.
* Maximum inhibitor concentration at the inlet to the liver was calculated from Eq (2).
cholestatic activity, on BSEP and/or MRP2 in present study.

Among the compounds examined, chlorpromazine, cloxacillin, colchicine, cyclosporin A, doxycycline, glibenclamide, midecamycin, quinidine and verapamil inhibited BSEP with a $K_i$ of 1.09–542 μM, whereas chlorpromazine, colchicine, cyclosporin A, midecamycin and minocycline inhibited MRP2 with a $K_i$ of 1.96–975 μM (Table 1). All the other compounds only minimally inhibit both transporters (Table 1). In addition, by comparing the obtained $K_i$ with $f_u \cdot I_{\max}$ and/or $f_u \cdot I_{\text{in}}$, for most of the compounds, except cloxacillin for BSEP, inhibition of the transporters seems to be observed at much higher concentrations than those seen in clinical situations. Thus, despite their cholestatic activity, most of the therapeutic drugs produce only minimal inhibition of BSEP and MRP2, implying that the inhibition by the metabolites of parent compounds, as in case of troglitazone, or other mechanisms for their cholestatic activity need to be considered.

To discuss in more quantitative terms the relationship between the inhibition of these transporters and the observed cholestasis, further information is needed. In particular, the inhibitory activity of these therapeutic drugs may exhibit a species difference between rats and humans. As a first step to obtain such information, the inhibition in human CMVs was also examined for the compounds showing inhibitory effects in rat CMVs. As far as the inhibition of DNP-SG transport was concerned, the inhibitory activity of the three compounds examined was relatively weak in humans, whereas the inhibition of TCA transport by cloxacillin and glibenclamide was more marked in humans than in rats (Fig. 2), suggesting a possible species difference for BSEP. We previously reported that the marker enzyme activities, enrichment factors, sideness and yields of CMVs were basically similar between rats and humans.7) Because these values in the present studies were almost identical to previous data,7) the species difference found in the present study was considered to be an intrinsic difference and independent of characterization. We also reported a difference in the $K_m$ for the primary active transport of organic anions and TCA between human and rat CMVs,7) supporting a possible species difference in the affinity of primary active transport systems. It has been shown that the homologies in the amino acid sequences of BSEP and MRP2 between rats and humans are about 82 and 78%, respectively.34,35) The homologies of active sites, which interact with inhibitors, are still unknown, however, and it is possible that these differences in amino acid sequence may result in a large difference in transporter inhibition. As for cloxacillin and glibenclamide, both compounds were reported to increase serum bilirubin and alkaline phosphatase, leading to clinical jaundice. In addition, liver biopsy specimens from jaundiced patients treated with these compounds revealed the cholestatic ability of these agents.36,37) Needless to say, to assess the degree of inhibition potential using human CMVs more precisely, a comparison between the $K_i$ values and clinically relevant concentrations is needed. Therefore, to examine the possible involvement of BSEP inhibition in their cholestatic activity, further quantitative analysis should be performed.

Considering that BSEP and MRP2 are localized on bile canalicular membranes, the estimation of the inhibition of these transporters should be performed using the hepatic unbound concentration rather than the plasma unbound concentration. Therefore, the concentra-

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**Fig. 2.** Inhibition of the primary active transport of TCA and DNP-SG by therapeutic agents in human CMVs.

CMVs were incubated with 1 μM TCA (A, incubation time 1 min) or DNP-SG (B, incubation time 2 min) in the presence of each compound at a concentration equal to the $K_i$ values obtained in rat CMVs. The ATP-dependent uptake was calculated by subtracting the uptake in the absence of ATP from that in its presence. Each data point represents the mean ± SE of 3 experiments from 1 preparation.
cytes should also be examined to improve the estimation of inhibition. Stieger et al. have reported that the cholestatic estrogen-metabolite, estradiol-17 β-glucuronide, inhibits BSEP function in CMVs from normal rat liver and BSEP/MPR2-coexpressing vesicles, but not in CMVs from MRP2-deficient GY rats or vesicles expressing only BSEP, suggesting that estradiol-17 β-glucuronide exhibits trans-inhibition of BSEP after its secretion into bile canaliculi by MRP2. This type of inhibition should also be considered in addition to cis-inhibition for a better understanding of the detailed mechanism governing transporter inhibition.

In conclusion, here we have examined the inhibition potential of a series of therapeutic drugs, producing clinical cholestasis, on BSEP and MRP2. Although most of the drugs have only a minimal inhibitory effect on rat BSEP and MRP2, inhibition of BSEP at clinically relevant concentrations of cloxacillin as well as its potent inhibition in human CMVs suggests the possible involvement of BSEP inhibition in cholestasis. Further quantitative analysis assessing the inhibition of human transporters is needed to clarify the importance of transporter inhibition in the cholestatic activity of other therapeutic compounds, including cyclosporin A, glibenclamide, and midecamycin.

References


