Introduction

Drug disposition is influenced by the combined action of drug transporters and metabolizing enzymes in organs such as the intestine, liver, and kidney (Giacomini et al., 2010). Drug-drug interactions (DDIs) can be complex, involving both uptake and efflux transporters as well as metabolizing enzymes including cytochrome P450 (P450) (Elsby et al., 2012). ATP-binding cassette (ABC) efflux transporters such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug-resistance-associated protein (MRP) inhibitors as inhibitors of cytochrome P450 (P450) enzyme activities using recombinant enzymes. A subset of P-gp and/or CYP3A inhibitors were selected (cyclosporin A, elacridar, ketoconazole, quinidine, reserpine, and tacrolimus) for a comparison of P450 inhibition in human microsomes and hepatocytes. Most P-gp inhibitors showed CYP3A4 inhibition, with potencies often in a similar range as their P-gp inhibition, as well as less potent CYP2C19 inhibition. Other P450 enzymes were not strongly inhibited except a few cases of CYP2D6 inhibition. MRP and BCRP inhibitors showed limited P450 inhibition. Some inhibitors showed less P450 inhibition in human hepatocytes than human liver microsomes, for example, elacridar, probably due to differences in binding, permeability limitations, or active, P-gp mediated efflux of the inhibitor from the hepatocytes. Quinidine was a potent P450 inhibitor in hepatocytes but only showed weak inhibition in microsomes. Quinidine shows an extensive cellular uptake, which may potentiate intracellular P450 inhibition. Elacridar, described as a potent and selective P-gp inhibitor, displayed modest P450 inhibition in this study and is thus a useful model inhibitor to define the role of P-gp in drug disposition without interference with other processes.

Cytochrome P450 Inhibitory Properties of Common Efflux Transporter Inhibitors

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ABSTRACT

Drug transporter inhibitors are important tools to elucidate the contribution of transporters to drug disposition both in vitro and in vivo. These inhibitors are often unselective and affect several transporters as well as drug metabolizing enzymes, which can make experimental results difficult to interpret with confidence. We therefore tested 14 commonly used P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug-resistance associated protein (MRP) inhibitors as inhibitors of cytochrome P450 (P450) enzyme activities using recombinant enzymes. A subset of P-gp and/or CYP3A inhibitors were selected (cyclosporin A, elacridar, ketoconazole, quinidine, reserpine, and tacrolimus) for a comparison of P450 inhibition in human microsomes and hepatocytes. Most P-gp inhibitors showed CYP3A4 inhibition, with potencies often in a similar range as their P-gp inhibition, as well as less potent CYP2C19 inhibition. Other P450 enzymes were not strongly inhibited except a few cases of CYP2D6 inhibition. MRP and BCRP inhibitors showed limited P450 inhibition. Some inhibitors showed less P450 inhibition in human hepatocytes than human liver microsomes, for example, elacridar, probably due to differences in binding, permeability limitations, or active, P-gp mediated efflux of the inhibitor from the hepatocytes. Quinidine was a potent P450 inhibitor in hepatocytes but only showed weak inhibition in microsomes. Quinidine shows an extensive cellular uptake, which may potentiate intracellular P450 inhibition. Elacridar, described as a potent and selective P-gp inhibitor, displayed modest P450 inhibition in this study and is thus a useful model inhibitor to define the role of P-gp in drug disposition without interference with other processes.
affecting other involved transporters or enzymes are needed. However, data on the P450 and uptake transporter inhibitory potential of ABC efflux transporter inhibitors are often lacking, which can present an obstacle to experimental design and interpretation.

Many ABC efflux transporter inhibitors are not selective but interact with other members of the super family. MK-571 (3-[[3-[[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-[2-(dimethylcarbamoyl)ethyl]sulfanyl]propionic acid) was considered a selective inhibitor of the multidrug resistance-associated protein (MRP) family but is now described to be almost as potent an inhibitor of the efflux transporters P-gp and BCRP (Matsson et al., 2009). Elacridar (formerly known as GF120918, N-4-[2-[(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isouquinolinyl)ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide hydrochloride) was originally described to be P-gp selective but is today also recognized as a potent BCRP inhibitor (Matsson et al., 2009).

Additionally, several ABC efflux transporter inhibitors are known to also inhibit uptake transporters and P450 drug metabolizing enzymes (Parkinson, 2001; Zhang and Benet, 2001; Treiber et al., 2007). Cyclosporine A has often been used as a P-gp inhibitor but is also a potent inhibitor of CYP3A4 as well as organic anion transporting polypeptide (OATP) uptake transporters (Gertz et al., 2013).

The methods routinely used to investigate hepatic DDIs include recombinant P450 enzymes, liver microsomes, and isolated hepatocytes (Soars et al., 2009; Giacomini et al., 2010). Only the last of these includes the step of distribution across a cellular membrane and the influence of drug transporters. It is now well established that active transport processes may influence the intracellular concentration of a compound (Giacomini et al., 2010; Klaassen and Aleksunes, 2010). The intracellular concentration of a compound can affect its potential to inhibit enzymes such as P450, and it is therefore preferable to study P450 inhibition both in microsomes and isolated hepatocytes (Brown et al., 2007, 2010).

The lack of information on the P450 inhibitory potential of efflux transporter inhibitors is an obstacle to experimental design when studying DDIs. We therefore investigated the P450 inhibition potential of several inhibitors, focusing on P-gp, MRP, and BCRP inhibitors recommended for in vitro use by the FDA (Ozawa et al., 2004; CDER, 2006). Some of these inhibitors are also suitable for in vivo studies (CDER, 2012). Their P450 inhibitory effects where tested in human recombinant P450 isoforms (3A4, 2D6, 1A2, 2C9, and 2C19), human liver microsomes, and isolated cryopreserved human hepatocytes using several P450-selective probe substrates and the often used P-gp probe loperamide (Kalvass et al., 2013).

**Materials and Methods**

**Reagents.** Human recombinant enzymes were obtained from BD Gentest (BD Biosciences, San José, CA) (CYP2D6) or were prepared in-house (CYP3A4, CYP2C9, CYP2A1, and CYP2C19) (Masimirembwa et al., 1999). Human liver microsomes were prepared in-house from a local biobank, and a pool from seven individuals of mixed gender was used. Cryopreserved human hepatocytes (six-donor pool, mixed gender and lot, OZL, SQI, REL, and AGR) were purchased from Celsis In Vitro Technologies (Baltimore, MD). Probe substrates and inhibitors were all purchased from Sigma-Aldrich (St. Louis, MO) except nelfinavir, ritonavir, and saquinavir, which were obtained from Toronto Research Chemicals (Toronto, ON, Canada). Cofactor NADPH, dimethylsulfoxide (DMSO), and Krebs-Henseleit buffer (KHL) were from Sigma-Aldrich.

**Screening of P450 Inhibitory Effect.** The P450 inhibitory effects of commonly used P-gp, MRP, and BCRP inhibitors—cyclosporin A, elacridar, ketoconazole, nelfinavir, quinidine, reserpine, ritonavir, saquinavir, tacrolimus, verapamil, MK-571, probenecid, fumitremorgin C (FTC), and gefitinib—were determined using recombinant human enzymes in an automated assay. Five P450 isoforms and their fluorescent probe substrates (used at 1 μM) were included: CYP1A2, substrate: [3-cyano-7-ethoxycoumarin; excitation wavelength (ex): 410 nm, emission wavelength (em): 460 nm]; CYP2C9, substrate: [7-methoxy-4-(trifluoromethyl) coumarin; ex: 410, em: 538]; CYP2C19, substrate: [3-cyano-7-ethoxycoumarin; ex: 410, em: 460]; CYP2D6, substrate: [3-(2-[N-diethyl-N-methylammonium]ethyl]-7-methoxy-4-methylcoumarin; ex: 390, em: 460]; and CYP3A4, substrate: [dibenzylfluorescein, ex: 485 nm, em: 535 nm]. All probe substrates were used at concentrations near or below their K<sub>m</sub>, with the relevant P450 to not mask any inhibition by saturation effects (Stresser et al., 2000; Makaji et al., 2010).

DMSO stock solutions of the inhibitors were diluted in 50 mM phosphate buffer pH 7.4 to give final concentrations between 0.05 μM and 500 μM with a final DMSO concentration of 0.2%. The reaction was initiated by adding NAPDH after a 10-minute preincubation (37°C, 5% CO<sub>2</sub>). After 55 minutes’ incubation (37°C, 5% CO<sub>2</sub>) the remaining fluorescence was determined. The metabolic stability assay was run in duplicate at three separate occasions. Positive control reactions were run using well-known selective inhibitors at two concentrations: CYP1A2: α-naphthoflavone (0.5 μM and 0.05 μM); CYP2C9: sulfaphenazole (10 μM and 1 μM); CYP2C19: ticlopidine (10 μM and 1 μM); CYP2D6: quinidine (10 μM and 1 μM); and CYP3A4: ketoconazole (0.5 μM and 0.05 μM). Control incubations without NAPDH or with DMSO alone, without inhibitors were run in triplicate. The effect of the inhibitors was calculated as the percentage inhibition of P450 probe substrate metabolism compared with the control incubations without inhibitors, which were considered to have full fluorescent activity without quenching and 0 inhibition. Control incubations without NAPDH were analyzed to determine whether inhibitors were fluorescent themselves or displayed large quenching of probe substrate fluorescence, neither was the case for the tested inhibitors. IC<sub>50</sub> values were calculated by nonlinear regression using the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA).

Enzymatic activity for the five P450 isoforms in the pools of microsomes and cryopreserved hepatocytes was determined as published previously using a cocktail of selective substrates (Floby et al., 2009; Sohlenius-Stembeck et al., 2010). P450 activities were determined to be close to long-time laboratory averages.

**Effects of P-gp Inhibitors on Metabolism in Human Microsomes and Hepatocytes.** A subset of P-gp inhibitors (cyclosporin A, elacridar, ketoconazole, quinidine, reserpine, and tacrolimus) were tested in human liver microsomes and cryopreserved hepatocytes to investigate potential differences in inhibition in the two experimental systems. Nelfinavir, ritonavir, and saquinavir showed extensive P450 inhibition with fluorescent probes and were not tested further. Midazolam, a selective CYP3A substrate, and loperamide, a common probe for P-gp function exhibiting mixed P450 metabolism, were selected as test compounds. The incubation times were determined in pilot experiments (data not shown) and were set to 5 minutes for loperamide and 30 minutes for midazolam. The final concentrations of the test compounds were 1 μM. The P-gp inhibitors (elacridar, ketoconazole, cyclosporin A, tacrolimus, reserpine, and quinidine) were tested at five concentrations between 0.5 and 20 μM (0.5 and 100 μM for reserpine and quinidine). For the microscope assays, substrates and inhibitors were diluted in phosphate buffer 50 mM (pH 7.4) from DMSO stocks (DMSO concentration was fixed to 0.2% in all incubations). Microsomes (final concentration of 0.5 mg protein/ml), substrate, and inhibitors were preincubated for 10 minutes at 37°C in 5% CO<sub>2</sub>. To start the metabolism NAPDH (final concentration 1 mM) was added. The reactions were stopped after the appropriate incubation time by adding 100 μl ice-cold acetonitrile containing internal standard bupivacaine (10 nM final concentration). Experiments were run in duplicate at three separate occasions. Controls without NAPDH were included. Samples were processed and analyzed by liquid chromatography coupled with tandem mass spectroscopy (LC-MS/MS) as will be described.

The cryopreserved hepatocytes, a pool of six individuals (three male and three female), were thawed as recommended by the manufacturer and resuspended in KHL buffer supplemented with 10 mM Hepes (GIBCO), pH 7.4 (KHL) to a concentration of 1.42 × 10<sup>6</sup> cells/ml. The viability was determined using the trypan blue exclusion test (> 80% viability was accepted). Plates with substrates and inhibitors (final concentrations identical to the microscope experiments) and hepatocytes were preincubated for 15 minutes (37°C, 5% CO<sub>2</sub>). The reactions were initiated by adding hepatocyte suspension.
(100,000 cells/well) to the compound plates. After incubation (37°C, 5% CO₂), the reactions were terminated by adding 2 volumes of ice-cold acetonitrile containing the internal standard bupinovacaine (10 nM). Plates containing samples were incubated on ice for at least 10 minutes to ensure complete precipitation of protein. The plate contents were shaken and centrifuged at 1200g for 10 minutes. An aliquot of 200 μl supernatant from each well were transferred to a 96-well analysis plate. Before analysis by LC-MS/MS each fraction was diluted in KHL to 25% acetonitrile. Experiments were run in duplicate at three separate occasions. Controls without hepatocytes were included.

**Uptake Measurements in Hepatocytes.** To test whether the P-gp inhibitors are substrates of hepatic uptake transporters, uptake measurements in suspended cryopreserved human hepatocytes were performed using the media loss technique described by Soars et al. (2007). Briefly, hepatocytes (10⁶/ml) were incubated with test compound at a concentration of 1 μM with the addition of 0.1% DMSO. At intervals ranging from 0 to 15 seconds up to 90 minutes, aliquots were removed to a glass tube loaded into a bench-top centrifuge, and hepatocytes were pelleted by centrifugation. A sample of the buffer supernatant was removed and mixed with 2 volumes of an ice-cold stop solution of acetonitrile with 200 nM warfarin (internal standard) and processed as described previously. To study the metabolism of the compound in the incubations, aliquots of cells and media were removed and lysed without prior centrifugation. Intrinsic clearances, CLint,uptake and CLint, metabolism, were calculated as described by Soars et al. (2007).

**Fraction Unbound in Hepatocyte and Microsome Incubations.** To compare IC₅₀ values for P450 inhibition in microsomes and hepatocytes the influence of binding in the two experimental systems was taken into account. The fraction unbound (fu) in the hepatocyte incubations was predicted according to Kilford et al. (2008):

\[
fu_{\text{hep}} = \frac{1}{1 + 125 \times \frac{V_P}{10^{0.072 \times \log P + 0.067 \times \log D}} - 1.126}
\]

where \(V_P\) is the ratio between the cell volume and the incubation volume (0.005 at a cell concentration of 10⁶ cells/ml).

The fraction unbound in the microsome incubations was predicted according to Halifax and Houston (2006):

\[
fu_{\text{mic}} = \frac{1}{1 + P \times 10^{0.072 \times \log P + 0.067 \times \log D} - 1.126}
\]

where \(P\) is the microsomal protein concentration (0.5 mg/ml). In both eq. 1 and eq. 2, logP is used for bases and logd pH 7.4 for other ion classes. LogD and logP were calculated using a commercial package from Advanced Chemistry Development (Toronto, Canada).

The fraction unbound in microsome and hepatocyte incubations was also determined experimentally using the method published by Mateus et al. (2013). Briefly, suspensions of human liver microsomes (0.5 μg/ml) and human cryopreserved hepatocytes (10⁶ hepatocytes/ml) were homogenized by sonication. Each of the inhibitors listed in Table 2 was added to the homogenates at the highest and lowest concentration used. Samples were dialyzed against KHL in a Rapid Equilibrium Dialysis device (Thermo Fischer Scientific, Rockford, IL) at 37°C for 4 hours. All compounds were stable during the incubation. After dialysis samples were analyzed by LC-MS/MS and the fu was calculated.

**LC-MS/MS/MS Analysis.** Sample analysis was performed by LC-MS/MS using a Micromass Quattro Micro triple quadrupole (Micromass, Manchester, United Kingdom) coupled to a gradient pump composed of two Shimadzu LC-10AD VP isocratic pumps (Shimadzu Corporation, Kyoto, Japan) and a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland). The software MassLynx (controlling the LC-system and mass spectrometer), which includes QuanLynx (quantification) and QuanOptimize (MS/MS optimization) was used (Waters Corporation, Milford, MA). A high purity C₁₈ 5 μm 30 x 2.1 mm (Thermo Electron Corporation, Waltham, MA) analytical column was used. Chromatography was performed using a generic gradient at a flow rate of 0.4 ml/min. The mobile phase consisted of solvent (A) 2% acetonitrile in 0.1% (v/v) acetic acid in water and (B) 80% acetonitrile in 0.1% (v/v) acetic acid in water. The injection volume was 20 μl. Other source parameters (e.g., collision energy, cone voltage, ion mode, molecular weight of parent and daughter, etc.) were individually optimized for each compound (Table 1).

Quantification of each compound was achieved by comparison of theanalyte/internal standard peak area ratios. A standard curve was included for each compound analyzed, spanning a concentration range from twice the initial experimental concentration to below the limit of quantification. Standard curves were not available for metabolites. For midazolam, 1'-hydroxylation was used as the probe reaction to monitor metabolism; for loperamide, N-desethyl-loperamide; and for quinidine, hydroxy-quinidine was used. Data are presented as mean ± S.D.

**Statistical Analysis.** Data were analyzed using GraphPad Prism 5 software. The IC₅₀ determinations were performed using nonlinear regression. Statistical significance was tested using the two-tailed Student’s t test. P values are symbolized by *P < 0.05, **P < 0.01, and ***P < 0.001. P < 0.05 was considered statistically significant.

**Results**

**Cytochrome P450 Inhibition by ABC-Transporter Inhibitors.** The calculated IC₅₀ values for each inhibitor for each human recombinant P450 are listed in Table 2. (IC₅₀ values in Table 2 are based on the total concentration of inhibitor.) The inhibition seen was classified into three categories: potent, moderate, or weak. Potent was P450 IC₅₀ below published IC₅₀ values for the relevant transporter; moderate was P450 IC₅₀ from equal to 10-fold higher than IC₅₀ values reported for the ABC-transporter; and weak was P450 IC₅₀ values more than 10-fold higher than reported transporter IC₅₀ values.

As can be seen, all P-gp inhibitors except elacridar, reserpine, and verapamil were potent or moderate inhibitors of CYP3A4. Ketoconazole was the most potent CYP3A4 inhibitor with 50-fold more potent CYP3A4 than P-gp inhibition. MRP and BCRP inhibitors were less potent toward CYP3A4. Of these, only MK-571 showed moderate CYP3A4 inhibition.

**P450 Inhibition in Human Microsomes and Cryopreserved Hepatocytes.** The inhibitory effects on P450-mediated metabolism of the probe substrates loperamide and midazolam by the six P-gp inhibitors listed in Table 3 were studied in human microsomes and cryopreserved hepatocytes. For most inhibitors, the determined IC₅₀ was either similar in microsomes and hepatocytes (cyclosporin A, tacrolimus) or higher in hepatocytes (elacridar, ketoconazole, reserpine) (Table 3). The IC₅₀ values listed in Table 3 are based on the total concentration of the inhibitor.

Quinidine was an exception that for both probe substrates showed statistically significantly lowered IC₅₀ values for midazolam (10-fold) and loperamide (20-fold) metabolism in hepatocytes compared with microsomes (Table 3).

Inhibition by the tested P-gp inhibitors in liver microsomes and hepatocytes was generally similar for both probe substrates used with the exception of reserpine, and to some extent quinidine, which both inhibited loperamide metabolism more strongly than midazolam metabolism (significantly different IC₅₀ values depending on probe substrate, P < 0.01 and P < 0.001 for quinidine and reserpine, respectively, Table 3).

To test whether differences in binding in microsomes and hepatocytes could explain the differences in IC₅₀ values obtained, the fraction
unbound in microsome and hepatocyte incubations was calculated using eq. 1 and eq. 2 for midazolam, loperamide, and the P-gp inhibitors listed in Table 3. The fraction unbound was also determined experimentally. The predicted differences in binding between microsome and hepatocyte experiments amounted to less than 30% for all these compounds. When binding was measured it was found that binding predictions using equations 1 and 2 often underestimated the measured value with a factor of 2 or more. However, the measured fuinc was very similar in microsomes and hepatocytes for all compounds with the exception of elacridar, which showed a more than 2-fold difference in binding between the two experimental systems. The differences in binding between microsome and hepatocyte incubations are listed in Table 3.

**Hepatocyte Uptake of ABC-Transporter Inhibitors.** The uptake of two inhibitors, elacridar and quinidine, was measured by the media absorption and hepatocyte incubations are listed in Table 3. The fraction unbound was determined experimentally. Elacridar showed no significant uptake by the cells (data not shown) whereas quinidine was subject to rapid uptake and substantial cellular accumulation. As can be seen in Fig. 1, the hepatocyte uptake of quinidine was rapid and extensive, but metabolism was slow. Calculating intrinsic clearances for the two processes gave values of CLint, metabolism = 4.6 μl/min/10^6 cells, and CLint, uptake = 130 μl/min/10^6 cells (calculated according to Soars et al., 2007, incorporating fuinc from eq. 1 and eq. 2).

**Discussion**

When investigating potential drug-drug interactions in vitro or in vivo, selective inhibitors are needed that will affect the system investigated without unwanted effects on other components involved in the disposition of the drug. Many P450 inhibitors show substrate dependent effects and IC50 values should be interpreted with caution.

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transition</th>
<th>Ion Source Mode</th>
<th>Cone Voltage</th>
<th>Collision Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loperamide</td>
<td>477.15 → 266.05</td>
<td>Positive</td>
<td>19.00</td>
<td>22.00</td>
</tr>
<tr>
<td>Desmethyl-loperamide</td>
<td>463.07 → 252.12</td>
<td>Positive</td>
<td>30.00</td>
<td>28.00</td>
</tr>
<tr>
<td>Midazolam</td>
<td>326.01 → 291.17</td>
<td>Positive</td>
<td>46.00</td>
<td>22.00</td>
</tr>
<tr>
<td>Hydroxy-midazolam</td>
<td>341.98 → 168.03</td>
<td>Positive</td>
<td>37.00</td>
<td>34.00</td>
</tr>
<tr>
<td>Quinidine</td>
<td>325.05 → 81.05</td>
<td>Positive</td>
<td>30.00</td>
<td>34.00</td>
</tr>
<tr>
<td>Hydroxy-quinidine</td>
<td>341.10 → 184.19</td>
<td>Positive</td>
<td>30.00</td>
<td>34.00</td>
</tr>
<tr>
<td>Bupivacaine</td>
<td>289.16 → 140.19</td>
<td>Positive</td>
<td>37.00</td>
<td>16.00</td>
</tr>
<tr>
<td>Warfarin</td>
<td>308.90 → 163.01</td>
<td>Positive</td>
<td>18.00</td>
<td>16.00</td>
</tr>
</tbody>
</table>

*Both 1-OH and 4-OH hydroxymidazolam were detected.

### Table 2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Transporter Inhibited</th>
<th>Transporter</th>
<th>P450 Inhibition (Recombinant Enzymes)</th>
<th>Other Transporters Inhibited</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CYP3A4</td>
<td>CYP1A2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IC50 (μM)</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>P-gp</td>
<td>1.3</td>
<td>11.7 ± 1 (P)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Elacridar</td>
<td>P-gp</td>
<td>0.34</td>
<td>4.9 ± 1.4 (W)</td>
<td>16 ± 7 (W)</td>
</tr>
<tr>
<td>Ketocnazole</td>
<td>P-gp</td>
<td>1.2</td>
<td>&lt;0.02 (P)</td>
<td>30 ± 4.7 (W)</td>
</tr>
<tr>
<td>Neflinavir</td>
<td>P-gp</td>
<td>1.4</td>
<td>1.37 ± 1.2 (P)</td>
<td>6.6 ± 0.9 (M)</td>
</tr>
<tr>
<td>Quinidine</td>
<td>P-gp</td>
<td>2.2</td>
<td>4.5 ± 0.6 (M)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Reserpine</td>
<td>P-gp</td>
<td>1.4</td>
<td>55.9 ± 4.2 (W)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>P-gp</td>
<td>3.8</td>
<td>0.05 ± 0.04 (P)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>P-gp</td>
<td>6.5</td>
<td>0.5 ± 0.16 (P)</td>
<td>ND (W)</td>
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<tr>
<td>Tacrolimus</td>
<td>P-gp</td>
<td>0.74</td>
<td>1.05 ± 0.3 (M)</td>
<td>ND (W)</td>
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<tr>
<td>Verapamil</td>
<td>P-gp</td>
<td>2.1</td>
<td>31.6 ± 2.4 (W)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>MK-571</td>
<td>MRP</td>
<td>10d</td>
<td>11.3 ± 1.4 (M)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Probenecid</td>
<td>MRP</td>
<td>42</td>
<td>&gt;280 (W)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Fumitremorgin C</td>
<td>BCRP</td>
<td>1.3</td>
<td>ND (W)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Gefinitin</td>
<td>BCRP</td>
<td>0.4</td>
<td>39.8 ± 0.2 (W)</td>
<td>ND (W)</td>
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IC50 values are based on total concentration. Mean 6 S.E. listed, n = 3, for elacridar n = 4. The experiment was performed on three separate occasions.

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<td>16 ± 7 (W)</td>
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<tr>
<td>Ketocnazole</td>
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<td>ND (W)</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>P-gp</td>
<td>0.74</td>
<td>1.05 ± 0.3 (M)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Verapamil</td>
<td>P-gp</td>
<td>2.1</td>
<td>31.6 ± 2.4 (W)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>MK-571</td>
<td>MRP</td>
<td>10d</td>
<td>11.3 ± 1.4 (M)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Probenecid</td>
<td>MRP</td>
<td>42</td>
<td>&gt;280 (W)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Fumitremorgin C</td>
<td>BCRP</td>
<td>1.3</td>
<td>ND (W)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Gefinitin</td>
<td>BCRP</td>
<td>0.4</td>
<td>39.8 ± 0.2 (W)</td>
<td>ND (W)</td>
</tr>
</tbody>
</table>
(Stresser et al., 2000). Quinidine and ketoconazole IC$_{50}$ values listed in Table 2 are similar to data reported in the literature (Khojasteh et al., 2013). However, absolute P450 IC$_{50}$ values could vary depending on the substrates and experimental systems used.

Most of the tested P-gp inhibitors showed potent or moderate CYP3A4 as well as moderate CYP2C19 inhibition. However, for three compounds (elacridar, reserpine, and verapamil) the inhibition of P-gp was much more potent than the CYP3A4 inhibition. These should be possible to use experimentally for efficient P-gp inhibition without affecting CYP3A4 at concentrations in the low μM range. Reserpine is a potent inhibitor of both CYP2C19 and CYP2D6, which needs to be considered when designing experiments. The three inhibitors are also potent inhibitors of BCRP, BSEP, and OCT1, respectively, with IC$_{50}$ in the 0.5–5.0 μM range (Morrissey et al., 2012).

Ketoconazole and ritonavir are highly potent and selective for CYP3A4 and could be used to inhibit this P450 at concentrations below 1 μM without affecting other P450 enzymes or transporters, as reported in the literature. Ritonavir is likely to interfere with OATP-mediated uptake at higher concentrations, exhibiting IC$_{50}$ values for many OATPs in the range of 1 to 5 μM (Morrissey et al., 2012).

In addition to CYP3A4, most of the P-gp inhibitors also exhibited CYP2C19 inhibition. CYP2C9, CYP2D6, and CYP1A2 showed less inhibition by the P-gp inhibitors, suggesting that CYP3A4 and CYP2C19 are the major enzymes to consider when designing P-gp inhibition experiments. A notable exception was the P-gp inhibitors reserpine and quinidine, which potently inhibited CYP2D6.

It has been reported that P-gp and CYP3A4 show a large overlap in inhibitor and substrate recognition (Wacher et al., 1995; Kim et al., 1999; Wandel et al., 1999; Yasuda et al., 2002). However, studies on the overlap between P-gp and CYP3A4 could find no correlation between the IC$_{50}$ values for the two proteins (Kim et al., 1999; Wandel et al., 1999). This latter finding was supported in our study, where we found no correlation between the P-gp and CYP3A4 IC$_{50}$ values. It was recently demonstrated that P-gp IC$_{50}$ values show very large intralaboratory and intramethod variability, which might obscure a correlation between CYP3A4 and P-gp inhibitor potencies (Bentz et al., 2013).

The tested MRP and BCRP inhibitors showed weak P450 inhibition. This is consistent with MRP-2 and BCRP not showing the same substrate overlap with a specific P450 as is exhibited by P-gp and CYP3A4. The exception to this was MK-571, which showed moderate CYP3A4 (as well as CYP2C9 and CYP2C19) inhibition. It is now known that MK-571 is not selective for MRPs but shows similar inhibition of P-gp and BCRP (Matsson et al., 2009). MK-571 seems to fall within the P450 inhibition pattern exhibited by other P-gp inhibitors. Probenecid showed moderate CYP2C19 inhibition and is

![Fig. 1. Metabolism and uptake of quinidine in human cryopreserved hepatocytes. The P-gp inhibitor quinidine exhibited slow metabolism but rapid uptake into hepatocytes. (Mean ± SD, n = 3).](image-url)
cryopreserved hepatocytes were generally small or showed somewhat
bition of CYP2C8.
inhibition of loperamide metabolism may in addition point to an inhi-
and CYP2D6 (Kim et al., 2004). In agreement with earlier publications
much more potent inhibition of loperamide than midazolam
metabolism to similar extents. However, quinidine and reserpine showed
actions with other human transporters (Morrissey et al., 2012).
transporters keeping intracellular inhibitor concentrations low. In line
of compound intracellularly is masked by binding (Brown et al., 2007;
interacting with other human transporters (Morrisssey et al., 2012).
most inhibitors tested inhibited both loperamide and midazolam
metabolism to similar extents. However, quinidine and reserpine showed
much more potent inhibition of loperamide than midazolam
metabolism. This is most likely due to midazolam being metabolized by
CYP3A4 alone whereas loperamide is metabolized by several
enzymes—predominantly CYP3A4 and CYP2C8, but also CYP2B6
and CYP2D6 (Kim et al., 2004). In agreement with earlier publications
(Khojasteh et al., 2011), quinidine potently inhibited CYP2D6, but the
inhibition of loperamide metabolism may in addition point to an inhi-
bition of CYP2C8.

Differences in inhibition between human liver microsomes and
cryopreserved hepatocytes were generally small or showed somewhat
higher IC₅₀ values in hepatocytes. The calculated nonspecific binding
in the two systems was too similar to explain any of the differences
observed. Measured nonspecific binding was generally higher than the
predicted values but showed little difference between the systems.
Elacridar showed significantly higher binding in micromoles than in
hepatocytes. This cannot explain the more potent P450 inhibition
shown by elacridar in micromoles, as the increase in binding would
lead to less potent P450 inhibition in microsomes than hepatocytes.
The higher IC₅₀ values in intact cells is probably due either to re-
strictions of membrane permeability impeding entry of the inhibitor
into the cells or to active efflux of the inhibitor via P-gp or other efflux
transporters keeping intracellular inhibitor concentrations low. In
line with this assumption, elacridar showed small uptake in cryopreserved
human hepatocytes in suspension, possibly due to P-gp-mediated
efflux of the inhibitor. Many P-gp inhibitors, including elacridar, are
also substrates of the transporter (Ozawa et al., 2004; Morrisssey et al.,
2012; Bankstahl et al., 2013). We have recently shown that ABC
efflux transporters, including P-gp, are present and active in human
cryopreserved hepatocytes (Lundquist et al., 2014a). For inhibitors
that are efflux transporter substrates or display differences in binding
between microsome and hepatocyte incubations, the higher IC₅₀ Value
measured in cryopreserved hepatocytes might be more indicative of
their in vivo IC₅₀.

In contrast to other tested inhibitors, quinidine showed much more
potent P450 inhibition in hepatocytes than in human liver microsomes,
regardless of the probe substrate used. This could be due to cellular
accumulation of quinidine by active uptake, and the compound
was found to be rapidly taken up by human cryopreserved hepatocytes.
Quinidine is known to be a substrate of several OATPS and OCTs
(Ozawa et al., 2004; Morrisssey et al., 2012).

Uptake of an inhibitor in hepatocytes can have different consequences.
Often, it leads to no change in the inhibitor potency, as the accumulation
of compound intracellularly is masked by binding (Brown et al., 2007;
Grime et al., 2008). It has been shown by Brown et al. (2010) that P450
inhibition was amplified by active uptake in hepatocytes for low clearance
compounds but not for more rapidly metabolized substances. This fits
well with the CLₜot measured for quinidine in hepatocytes, 4.6 μM/min/
10⁶ cells (in vivo human CL 4 ml/min/kg (Obach et al., 1997)). It is
therefore likely that the amplification of quinidine P450 inhibition by
active uptake is even larger in vivo or in fresh hepatocytes because many
uptake transporters, including OATPs and OCTs, are active but down-
regulated in human cryopreserved hepatocytes (Lundquist et al., 2014b).
Down-regulation of OATPs in cryopreserved hepatocytes has also been
demonstrated by Badolo et al. (2011) and Kimoto et al. (2012).

Finally, it is not sufficient to consider ABC transporter and P450
inhibition when selecting an inhibitor for P-gp (or other ABC
transporter) inhibition experiments. Cyclosporin A has IC₅₀ values for
human OATP1B1 and OATP1B3 in a clinically relevant range
between 0.2 and 0.5, and 0.5 and 0.8 μM, respectively, below the IC₅₀
for P-gp of 1.3 μM (Shitara et al., 2003; Ho et al., 2006; Treiber et al.,
2007). The uptake of an OATP1B1 substrate—and, consequently, its
intracellular metabolism—will therefore be compromised before any
effects of P-gp inhibition can be detected.

To summarize, with regard to the inhibitors tested in this study,
elacridar would be the P-gp inhibitor of choice if CYP2C19 is not
involved in metabolism of the probe substrate. Alternatively, verapamil
shows a good profile with few P450 interactions, but it runs the risk of
interfering with OCT1-mediated uptake. If a combination of CYP3A4
and P-gp inhibition is advantageous—for example, in an experiment
on intestinal permeability in an Ussing chamber—ketocanoazole can
be used. In addition to its superior selectivity for MRPs, probenecid
showed much less P450 inhibition than MK-571, making it the MRP
inhibitor of choice. Fumitremorgin C and gefitinib should be easy to use
for BCRP inhibition, with little risk of inhibiting P450-mediated drug
metabolism.

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Participated in research design: Lundquist, Englund, Skogastierna, Projean.
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Contributed new reagents or analytic tools: Johansson.
Performed data analysis: Lundquist, Englund, Skogastierna, Johansson.
Wrote or contributed to the writing of the manuscript: Lundquist, Englund,
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