Cytochrome P450 inhibitory properties of common efflux transporter inhibitors.

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Abbreviations: ABC-transporter, ATP-binding cassette transporter; ACN, acetonitrile; BCRP, breast cancer resistance protein; BSEP, Bile salt efflux pump; CL, clearance; CL_int, intrinsic clearance; CYP, Cytochrome P450; DDI, drug-drug interaction; DMSO, dimethyl-sulfoxide; Em, emission wavelength; EMA, European Medicines Agency; Ex, excitation wavelength; fu, fraction unbound, free fraction; IC50, inhibitory concentration 50%; KHL, Krebs-Henseleit buffer; LC-MS/MS, liquid chromatography – tandem mass spectroscopy; MRP, multidrug resistance associated protein; NTCP, Na^+–taurocholate cotransporting protein; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; P-gp, P-glycoprotein; US FDA, US Food and Drug Administration
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 fourteen commonly used P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP), and Multidrug-Resistance associated Protein (MRP) inhibitors as inhibitors of Cytochrome P450 (CYP) 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 CYP-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 CYP enzymes were not strongly inhibited except a few cases of CYP2D6 inhibition. MRP and BCRP inhibitors showed limited CYP inhibition. Some inhibitors showed less CYP 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 CYP inhibitor in hepatocytes but only showed weak inhibition in microsomes. Quinidine shows an extensive cellular uptake, which may potentiate intracellular CYP inhibition. Elacridar is described as a potent and selective P-gp inhibitor and displayed modest CYP 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.
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 P450s (CYPs) (Elsby et al., 2012). ATP-binding cassette (ABC) efflux transporters such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multi-drug resistance associated protein 2 (MRP2) are expressed in several organs with barrier function (e.g. the intestines, the blood-brain barrier, and the placenta), but are also expressed in the human liver, in the apical membrane facing the bile canaliculae (Klaassen and Aleksunes, 2010). In the liver these transporters have an important role in excreting drugs and metabolites from the hepatocyte into the bile (Giacomini et al., 2010). Liver P-gp has a central role in the elimination and pharmacokinetics of many drugs and is often working in concert with CYP3A4 as exemplified by erythromycin metabolism, while BCRP is central to e.g. rosuvastatin biliary efflux (Kurnik et al., 2006; Kitamura et al., 2008) There is a significant overlap in tissue localization and substrate selectivity between CYP3A4 and P-gp (Zhang and Benet, 2001).

To investigate whether the disposition of a compound is influenced by ABC efflux transporters, model inhibitors of individual transporters are frequently employed (recommendations have been published by both the US Food and Drug Administration and the European Medicines Agency (US FDA, 2006; 2012, EMA, 2012)). Many transporter studies are performed in cell lines such as Caco-2, MDCK or HEK cells that do not express significant levels of CYP enzymes. To obtain reliable estimates of efflux transporter contribution to drug disposition in more complex systems such as hepatocytes, selective inhibitors not affecting other involved transporters or enzymes are needed. However, data on the CYP 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 was considered a selective inhibitor of the multidrug resistance associated protein family (MRP) but is now described to be almost as potent an inhibitor of the efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) (Matsson et al., 2009). Elacridar (formerly known as GF120918) 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 CYP drug metabolizing enzymes (Treiber et al., 2007, Parkinson, 2001; Zhang and Benet, 1995). 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 CYP enzymes, liver microsomes and isolated hepatocytes (Giacomini et al., 2010; Soars et al., 2009). Only the last of these include 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 CYPs and it is therefore preferable to study CYP inhibition both in microsomes and isolated hepatocytes (Brown et al., 2007, 2010).
The lack of information on the CYP inhibitory potential of efflux transporter inhibitors is an obstacle to experimental design when studying DDIs. We therefore investigated the CYP inhibition potential of several inhibitors, focusing on P-gp, MRP and BCRP inhibitors recommended for in vitro use by the US Food and Drug Administration (US FDA, 2006; Ozawa et al., 2004). Some of these inhibitors are also suitable for in vivo studies (US FDA, 2012) Their CYP inhibitory effects where tested in human recombinant CYPs (isoforms 3A4, 2D6, 1A2, 2C9, and 2C19), human liver microsomes, and isolated cryopreserved human hepatocytes using several CYP-selective probe substrates and the often used P-gp probe loperamide (Kalvass et al., 2013).
Material and methods

Reagents

Human recombinant enzymes were obtained from BD Gentest (BD Biosciences, San José, CA, USA) (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 bio-bank and a pool from seven individuals of mixed gender was used. Cryopreserved human hepatocytes (6-donor pool, mixed gender and lot OZL, SQI, REL, and AGR) were purchased from Celsis In Vitro Technologies (Baltimore, MD, USA). Probe substrates and inhibitors were all purchased from Sigma-Aldrich (St. Louis, MO, USA) except nelfinavir, ritonavir, and saquinavir, which were obtained from Toronto Research Chemicals (Toronto, ON, Canada). Cofactor NADPH, dimethyl-sulfoxide (DMSO), and Krebs-Henseleit buffer (KHL) were from Sigma-Aldrich.

Screening of CYP inhibitory effect

The CYP inhibitory effects of commonly used P-gp, MRP, and BCRP inhibitors (cyclosporin A, elacridar (GF120918), ketoconazole, nelfinavir, quinidine, reserpine, ritonavir, saquinavir, tacrolimus, verapamil, MK-571, probenicid, Fumitremorgin C (FTC), and gefitinib) were determined using recombinant human enzymes in an automated assay. Five CYP isoforms and their fluorescent probe substrates (used at 1 μM) were included: CYP1A2 (3-Cyano-7-ethoxycoumarin; excitation wavelength (Ex): 410 nm, emission wavelength (Em): 460 nm), CYP2C9 (7-Metoxy-4-(trifluoromethyl) coumarin; Ex: 410, Em: 538), CYP2C19 (3-Cyano-7-ethoxycoumarin; Ex: 410, Em: 460), CYP2D6 (3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin; Ex: 390, Em: 460) and CYP3A4 (Dibenzylfluorescin, Ex: 485 nm, Em: 535 nm). All probe substrates were
used at concentrations near or below their Km with the relevant CYP 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 to 500 μM with a final DMSO concentration of 0.2%. The reaction was initiated by adding NADPH after a 10 min pre-incubation (37°C, 5% CO2). After 55 min incubation (37°C, 5% CO2) 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-Naphtoflavone (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 NADPH or with DMSO alone, without inhibitors were run in triplicate. The effect of the inhibitors was calculated as the percentage inhibition of CYP probe substrate metabolism compared to 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 if inhibitors were fluorescent themselves or displayed large quenching of probe substrate fluorescence, neither was the case for the tested inhibitors. IC50 values were calculated by non-linear regression using the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

Enzymatic activity for the five CYPS in the pools of microsomes and cryopreserved hepatocytes was determined as published previously using a cocktail of selective substrates (Floby et al., 2009; Sohlenius-Sternbeck et al., 2010). CYP activities were determined to be close to long-time lab 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 CYP 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 CYP metabolism, were selected as test compounds. The incubation times were determined in pilot experiments (data not shown) and were set to 5 min for loperamide and 30 min 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 5 concentrations between 0.5 - 20 μM (0.5 - 100 μM for reserpine and quinidine). For the microsome 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 pre-incubated for 10 min at 37° C in 5 % CO₂. To start the metabolism NADPH (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 bupivacain (10 nM final concentration). Experiments were run in duplicate at three separate occasions. Controls without NADPH were included. Samples were processed and analyzed by liquid chromatography – tandem mass spectroscopy (LC-MS/MS) as described below.

The cryopreserved hepatocytes, a pool of six individuals (three male and three female), were thawed as recommended by the manufacturer and resuspended in Krebs-Henseleit buffer supplemented with 10 mM Hepes (Gibco), pH 7.4 (KHL) to a
concentration of $1.42 \times 10^6$ 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 microsome experiments) and hepatocytes were pre-incubated for 15 min ($37^\circ$ C, 5 % CO$_2$). The reactions were initiated by adding hepatocyte suspension (100 000 cells/well) to the compound plates. After incubation ($37^\circ$C, 5% CO$_2$) the reactions were terminated by adding two volumes of ice-cold acetonitrile containing the internal standard bupivacain (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 1200 x g 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% ACN. 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 ($10^6$ / ml) were incubated with test compound at a concentration of 1 µM with the addition of 0.1% DMSO. At intervals ranging from 0 and 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 two volumes of an ice cold stop solution of acetonitrile (ACN) with 200 nM warfarin (internal standard) and processed as described above. To study metabolism of the compound in the incubations aliquots of cells and media were removed and lysed without prior centrifugation. Intrinsic clearances, $\text{CL}_{\text{int, uptake}}$ and $\text{CL}_{\text{int, metabolism}}$ were calculated as described (Soars et al., 2007).
Fraction unbound (fu) in hepatocyte and microsome incubations

To compare IC50 values for CYP inhibition in microsomes and hepatocytes the influence of binding in the two experimental systems was taken into account.

The fraction unbound in the hepatocyte incubations was predicted according to Kilford et al. (2008):

\[
\text{fu}_{\text{hep}} = \frac{1}{1 + 125 \times V_R \times 10^0.072 \times \log P / D^2 + 0.087 \times \log P / D - 1.126}
\]  

where \( V_R \) is the ratio between the cell volume and the incubation volume (0.005 at a cell concentration of \( 10^6 \) cells/ml).

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

\[
\text{fu}_{\text{mic}} = \frac{1}{1 + P \times 10^{0.072 \times \log P / D^2 + 0.067 \times \log P / D - 1.126}}
\]  

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

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 (\( 10^6 \) hepatocytes/ml) were homogenized by sonication. Each of the
inhibitors listed in Table 2 were 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 Inc., Rockford, IL, USA) at 37 °C for 4 h. All compounds were stable during the incubation. After dialysis samples were analyzed by LC-MS/MS and the fu was calculated.

**LC-MS/MS analysis**

Sample analysis was performed by LC-MS/MS using a Micromass Quattro Micro triple quadrupole (Micromass, Manchester, UK) 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, USA). A High Purity C18 5 μm 30 x 2.1 mm (Thermo Electron Corporation, Waltham, USA) 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 and bupivacain was used as internal standard. Other source parameters (e.g., collision energy, cone voltage, ion mode, molecular weight of parent and daughter etc.) were individually optimized for each compound (see Table 1). Quantification of each compound was achieved by comparison of the analyte/internal standard peak area ratios. A standard curve was included for each compound analysed 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-desmethyl-loperamide; and for quinidine, hydroxy-quinidine was used. Data are presented as means ± SD.
Statistical analysis

Data were analyzed using Graphpad Prism 5 software. IC50 determinations were performed utilizing non-linear regression. Statistical significance was tested using the two-tailed Student’s t-test. Probability values (P-values) are symbolized by: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, p-values of p < 0.05 were considered statistically significant.
Results

Cytochrome P450 inhibition by ABC-transporter inhibitors

The calculated IC50 values for each inhibitor for each human recombinant CYP 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: CYP IC50 below published IC50 values for the relevant transporter; Moderate: CYP IC50 from equal to tenfold higher than IC50 values reported for the ABC-transporter; Weak: CYP IC50 values more than tenfold 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 towards CYP3A4. Of these only MK-571 showed moderate CYP3A4 inhibition. CYP2C19 was the second most affected CYP but inhibitors were generally less potent against this isoform. The P-gp inhibitors elacridar and reserpine and the MRP inhibitor probenicid were exceptions to this general trend and were more potent inhibitors of CYP2C19 than CYP3A4. Reserpine was an extremely potent CYP2C19 inhibitor. Quinidine and reserpine were particularly powerful inhibitors of CYP2D6, while ritonavir and saquinavir were moderate inhibitors. In contrast CYP1A2 and CYP2C9 were only inhibited moderately by a few of the ABC-transporter inhibitors (CYP1A2, nelfinavir; CYP2C9, ritonavir, saquinavir and MK-571).

No correlation between CYP3A4 IC50 values measured in this study and P-gp literature IC50 values was found ($r^2=0.36$, $p=0.073$, data not shown). There was no correlation between P-gp and CYP2C19 IC50 values ($r^2 = 0.003$, $p= 0.89$).
CYP inhibition in human microsomes and cryopreserved hepatocytes

The inhibitory effects on CYP-mediated metabolism of the probe substrates loperamide and midazolam by the 6 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). IC50 values in Table 3 are based on the total concentration of inhibitor.

Quinidine was an exception that for both probe substrates showed a statistically significant lowered IC50 values for midazolam (10-fold) and loperamide (20-fold) metabolism in hepatocytes compared to 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, that 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, fraction unbound in microsome and hepatocyte incubations was calculated using equations 1 and 2 for midazolam, loperamide, and the P-gp inhibitors listed in Table 3. 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 measuring binding it was found that for most compounds the predicted binding was underestimated by a factor of 2 or more. However, measured fu_{inc} was very similar in microsomes and hepatocytes for all compounds with the exception of elacridar that 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 loss method in suspensions of cryopreserved human hepatocytes. Elacridar showed no significant uptake by the cells (data not shown) while quinidine was subject to rapid uptake and substantial cellular accumulation. As can be seen in Fig. 1 hepatocyte uptake of quinidine was rapid and extensive, while metabolism was slow. Calculating intrinsic clearances for the two processes gave values of $\text{CL}_{\text{int, metabolism}} = 4.6 \ \mu\text{l/min/10}^6 \ \text{cells}$, and $\text{CL}_{\text{int, uptake}} = 130 \ \mu\text{l/min/10}^6 \ \text{cells}$ (calculated according to Soars et al., 2007, incorporating $f_u$ from Eqs 1 and 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 CYP inhibitors show substrate dependent effects and IC50 values should be interpreted with caution (Stresser et al., 2000). Quinidine and ketoconazole IC50 values listed in Table 2 are similar to data reported in the literature (Khojasteh et al., 2011). However, absolute CYP IC50 values could vary dependent on 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) 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 IC50s in a 0.5 – 5 μM range (Morrissey et al., 2012).

Ketoconazole and ritonavir are highly potent and selective for CYP3A4 and could be used to inhibit this CYP at concentrations below 1 μM, without affecting other CYP enzymes or transporters, as reported in the literature. Ritonavir is likely to interfere with OATP-mediated uptake at higher concentrations however, exhibiting IC50 values for many OATPs in the range 1-5 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 that 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 overlap between P-gp and CYP3A4 could find no correlation between IC50 values for the two proteins (Wandel et al., 1999; Kim et al., 1999). This latter finding was supported in this study where no correlation was seen between P-gp and CYP3A4 IC50 values. It was recently demonstrated that P-gp IC50 values shows very large intra-lab and intra-method 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 CYP inhibition. This is consistent with MRP-2 and BCRP not showing the same substrate overlap with a specific CYP as is exhibited by P-gp and CYP3A4. The exception to this was MK-571 that 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 (Mattson et al., 2009). MK-571 seems to fall within the CYP inhibition pattern exhibited by other P-gp inhibitors. Probenecid showed moderate CYP2C19 inhibition and is a more useful MRP inhibitor in this respect but its use might be hampered by its potent OAT1 and OAT3 inhibition (Ki: s of approximately 5 μM (Morrissey et al., 2012)). The two BCRP inhibitors tested, Fumitremorgin C and gefitinib, seems less problematic to use experimentally showing only weak CYP inhibition and no known potent interactions with other human transporters (Morrissey 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 while loperamide is metabolized by several enzymes; predominantly CYP3A4 and CYP2C8, but also CYP2B6 and CYP2D6 and (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 inhibition of CYP2C8.

Differences in inhibition between human liver microsomes and cryopreserved hepatocytes were generally small or showed somewhat higher IC50 values in hepatocytes. Calculated non-specific binding in the two systems was too similar to explain any of the differences seen. Measured non-specific binding was generally higher than the predicted values but showed little difference between the systems. Elacridar showed significantly higher binding in microsomes than in hepatocytes. This can however not explain the more potent CYP inhibition shown by elacridar in microsomes, as the increase in binding would lead to less potent CYP inhibition in microsomes than hepatocytes. Higher IC50 values in intact cells is probably due either to restrictions 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 (Bankstahl et al., 2013; Morrissey et al., 2012; Ozawa et al., 2004). We have recently shown that ABC efflux transporters, including P-gp, are present and active in human cryopreserved hepatocytes (Lundquist et al., unpublished). 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 CYP 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 (Morrissey et al., 2012; Ozawa et al., 2004).

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 and coworkers that CYP inhibition was amplified by active uptake in hepatocytes for low clearance compounds but not for more rapidly metabolized substances (Brown et al., 2010). This fits well with the $CL_{int, \text{metabolism}}$ measured for quinidine in hepatocytes, 4.6 $\mu$l/min/10^6 cells (in vivo human $CL_{4 \text{ ml/min/kg}}$ (Obach et al., 1997)). It is therefore likely that the amplification of quinidine CYP inhibition by active uptake is even larger in vivo or in fresh hepatocytes since many uptake transporters, including OATPs and OCTs, are active but down regulated in human cryopreserved hepatocytes (Lundquist et al., unpublished). 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 CYP inhibition when selecting an inhibitor for P-gp (or other ABC-transporters) inhibition experiments. Cyclosporin A has IC50 values for human OATP1B1 and OATP1B3 in a clinically relevant range between 0.2 to 0.5 and 0.5 to 0.8 $\mu$M, respectively, below the IC50 for P-gp of 1.3 $\mu$M (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 little CYP interactions, but 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, ketoconazole can be used. In addition to its superior selectivity for MRPs probenicid showed much less CYP inhibition than MK-571, making it the MRP inhibitor of choice. Fumitremorgin C and gefitinib, finally, should be easy to use for BCRP inhibition with little risk of inhibiting CYP-mediated drug metabolism.
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Footnotes

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Figure legends

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)
Table 1: Test compound optimized settings for LC-MS/MS analysis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transition m/z</th>
<th>Ion source mode</th>
<th>Cone voltage [V]</th>
<th>Collision energy [eV]</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>Bupivacain</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. Cytochrome P450 inhibition by common ABC transporter inhibitors.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Transporter inhibited\textsuperscript{c}</th>
<th>Transporter IC50\textsuperscript{c}</th>
<th>CYP Inhibition (recombinant enzymes)</th>
<th>Other transporters inhibited\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(μM)</td>
<td>CYP3A4 IC50</td>
<td>(within 10-fold of main ABC transporter IC50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(μM)</td>
<td>CYP1A2 IC50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(μM)</td>
<td>CYP2C9 IC50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(μM)</td>
<td>CYP2C19 IC50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(μM)</td>
<td>CYP2D6 IC50</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin A\textsuperscript{a}</td>
<td>P-gp</td>
<td>1.3\textsuperscript{e}</td>
<td>11.7±1 (P)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Elacridar\textsuperscript{a}</td>
<td>P-gp</td>
<td>0.3\textsuperscript{e}</td>
<td>4.9±1.4 (W)</td>
<td>16±7 (W)</td>
</tr>
<tr>
<td>Ketoconazole\textsuperscript{a}</td>
<td>P-gp</td>
<td>1.2\textsuperscript{e}</td>
<td>&lt;0.02 (P)</td>
<td>30±4.7 (W)</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>P-gp</td>
<td>1.4\textsuperscript{e}</td>
<td>1.37±1.2 (P)</td>
<td>6.6±0.9 (M)</td>
</tr>
<tr>
<td>Quinidine\textsuperscript{a}</td>
<td>P-gp</td>
<td>2.2\textsuperscript{e}</td>
<td>4.5±0.6 (M)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Reserpine</td>
<td>P-gp</td>
<td>1.4 \textsuperscript{e, f, t}</td>
<td>55.9±4.2 (W)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Ritonavir\textsuperscript{a}</td>
<td>P-gp</td>
<td>3.8\textsuperscript{e}</td>
<td>0.05±0.04 (P)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>P-gp</td>
<td>6.5\textsuperscript{e}</td>
<td>0.5±0.16 (P)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Substance</td>
<td>Transporter</td>
<td>IC50 (M) or IC50 (W)</td>
<td>IC50 (M) or IC50 (W)</td>
<td>IC50 (M) or IC50 (W)</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------</td>
<td>----------------------</td>
<td>----------------------</td>
<td>----------------------</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>10</td>
<td>11.3±1.4 (M)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Probenidic</td>
<td>MRP</td>
<td>42\textsuperscript{c}</td>
<td>&gt;280 (W)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Fumitremorgin C</td>
<td>BCRP</td>
<td>1.3\textsuperscript{c}</td>
<td>ND (W)</td>
<td>ND (W)</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>BCRP</td>
<td>0.4\textsuperscript{c}</td>
<td>39.8±0.2 (W)</td>
<td>ND (W)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}P, Potent: CYP IC50 below published IC50 values for the relevant transporter; M, Moderate: CYP IC50 from equal to tenfold higher than IC50 values reported for the ABC-transporter; W, Weak: CYP IC50 values at least tenfold higher than reported transporter IC50 values > <: IC50 value predicted outside the concentration range tested. ND: No inhibition detected within the concentration range tested. IC50 values are based on total concentration. Mean ± SE listed, n=3, for elacridar n=4. The experiment was performed on three separate occasions.

\textsuperscript{b}Recommended for in vivo as well as in vitro use (US FDA 2012).

\textsuperscript{c}Transporter data are from University of Tokyo transporter database (TP-search; Ozawa et al., 2004), and the UCSF FDA TransPortal (Morrissey et al., 2012).

\textsuperscript{d}IC50 values from MDCK cells over expressing human transporters (Matsson et al., 2009).
*Caco-2 cell IC50 (US FDA, 2006)

*Ki value listed instead of IC50.
Table 3. Cytochrome P450 Inhibition by common ABC transporter inhibitor in human microsomes and cryopreserved hepatocytes.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition of loperamide metabolism\textsuperscript{a}</th>
<th>Inhibition of midazolam metabolism\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver Microsomes</td>
<td>Liver Cryopreserved Hepatocytes</td>
</tr>
<tr>
<td></td>
<td>IC50 ((\mu)M)\textsuperscript{c}</td>
<td>IC50 ((\mu)M)\textsuperscript{c}</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>15.3±1.2</td>
<td>26.3±1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elacridar</td>
<td>17.8±1.4</td>
<td>62.0±1.6*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.4±0.2</td>
<td>1.0±0.3*</td>
</tr>
<tr>
<td>Quinidine</td>
<td>116.4±2.6</td>
<td>4.8±1.3*</td>
</tr>
<tr>
<td>Reserpine</td>
<td>1.0±1.5</td>
<td>7.2±1.4*</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>6.3±1.3</td>
<td>10.7±1.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Measured binding\textsuperscript{d,e} Calculated binding\textsuperscript{d,f}

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Fu, SD (mic) Fu, SD (hep) Ratio (mic/hep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin A</td>
<td>0.02 0.01 0.02 0.01 1.1</td>
</tr>
</tbody>
</table>

\textsuperscript{d} Measured binding
\textsuperscript{e} Calculated binding
\textsuperscript{f} Measured binding
\textsuperscript{g} Calculated binding

\textsuperscript{c} IC50 (\(\mu\)M)

\textsuperscript{d} Measured binding
\textsuperscript{e} Calculated binding

\textsuperscript{f} Measured binding
\textsuperscript{g} Calculated binding

\textsuperscript{h} Measured binding
\textsuperscript{i} Calculated binding
<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean (μM)</th>
<th>SE</th>
<th>IC50 (μM)</th>
<th>Mean (μM)</th>
<th>SE</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elacridar</td>
<td>0.005</td>
<td>0.002</td>
<td>0.01**</td>
<td>0.01</td>
<td>0.4</td>
<td>0.28</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.33</td>
<td>0.06</td>
<td>0.47</td>
<td>0.09</td>
<td>0.7</td>
<td>0.84</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.83</td>
<td>0.05</td>
<td>0.91</td>
<td>0.02</td>
<td>0.9</td>
<td>0.81</td>
</tr>
<tr>
<td>Reserpine</td>
<td>0.17</td>
<td>0.02</td>
<td>0.19</td>
<td>0.02</td>
<td>0.9</td>
<td>0.50</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>0.17</td>
<td>0.02</td>
<td>0.18</td>
<td>0.03</td>
<td>0.9</td>
<td>0.84</td>
</tr>
<tr>
<td>Midazolam</td>
<td>0.30</td>
<td>0.02</td>
<td>0.36</td>
<td>0.04</td>
<td>0.8</td>
<td>0.16</td>
</tr>
<tr>
<td>Loperamide</td>
<td>0.74</td>
<td>0.01</td>
<td>0.80</td>
<td>0.01</td>
<td>0.9</td>
<td>0.54</td>
</tr>
</tbody>
</table>

*a Mean ± SE listed, n=3, for elacridar n=4. The experiment was performed on three separate occasions.

*b Statistically significant differences between values in cryopreserved hepatocytes and microsomes are indicated: * = p < 0.05; ** = p < 0.01.

*c IC50 values are based on total concentration.

*d Mic, microsome; hep, cryopreserved hepatocyte.

*e Fuinc in microsome and hepatocyte incubations was measured according to Mateus et al., 2013.

*f Fuinc in microsome and hepatocyte incubations was calculated according to Eqs. 1 and 2.