Role of Multidrug Resistance Protein 3 in Antifungal-Induced Cholestasis

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ABSTRACT
Drug-induced liver injury is an important clinical entity resulting in a considerable number of hospitalizations. While drug-induced cholestasis due to the inhibition of the bile salt export pump (BSEP) is well investigated, only limited information on the interaction of drugs with multidrug resistance protein 3 (MDR3) exists and its role in the pathogenesis of drug-induced cholestasis is poorly understood. Therefore, we aimed to study the interaction of drugs with MDR3 and the effect of drugs on canalicular lipid secretion in a newly established polarized cell line system that serves as a model of canalicular lipid secretion. LLC-PK₁ cells were stably transfected with human Na⁺-taurocholate cotransporting polypeptide, BSEP, MDR3, and ABCG5/G8 and grown in the Transwell system. Apical phospholipid secretion and taurocholate transport were assayed to investigate the effect of selected drugs on MDR3-mediated phospholipid secretion as well as inhibition of BSEP. The established cell line displayed vectorial bile salt transport and specific phosphatidylcholine secretion into the apical compartment. The antifungal azoles, posaconazole, itraconazole, and ketoconazole, significantly inhibited MDR3-mediated phosphatidylcholine secretion. In contrast, amoxicillin clavulanate and troglitazone did not interfere with MDR3 activity. Drugs interfering with MDR3 activity did not display a parallel inhibition of BSEP. Our in vitro model for MDR3-mediated phospholipid secretion facilitates parallel screening for MDR3 and BSEP inhibitors. Our data demonstrate that the cholestatic potential of certain drugs may be aggravated by simultaneous inhibition of BSEP and MDR3.

Introduction
Bile, which is formed by hepatocytes and modified by cholangiocytes and the gallbladder, is important for the digestion and absorption of fat and the elimination of poorly water-soluble endo- and xenobiotics (Pollheimer et al., 2014). Primary bile is formed at the canalicular membrane of hepatocytes in a process that requires the interplay of several ATP-binding cassette transporters. In addition to small solutes, the main components of primary bile are bile salt (BS), phosphatidylcholine (PC), and cholesterol (Boyer, 2013). The BS export pump (BSEP, protein name; ABCB11, gene name) mediates ATP-dependent transport of BS from hepatocytes into the canaliculus. The multidrug resistance protein 3 (MDR3, ABCB4) translocates PC from the cytoplasmic to the outer leaflet of the canalicular membrane. ABCG5/G8 (ABCG5/ABCG8) (also called sterolin) facilitates the release of cholesterol from the canalicular membrane. These three ATP-binding cassette transporters work in concert for biliary lipid secretion (Small, 2003): BS in the canaliculus facilitates the release of PC from the outer leaflet and forms PC-BS-mixed micelles, which are crucial for the solubilization of biliary cholesterol. In addition, PC-BS-mixed micelles reduce the toxic activity of BS against the bile ducts (Trauner et al., 2008). Therefore, the proper and coordinated functioning of BSEP and MDR3 is critical for mixed micelle formation, and a misbalance in the activity of these transporters can lead to a decreased solubility of biliary cholesterol and consequently to gallstone formation.

In humans, the key role of canalicular ATP-binding cassette transporters in bile formation has been worked out by the clinical characterization of patients harboring mutations in the corresponding genes (Boyer, 2010). For example, studies of patients with inherited cholestatic liver diseases revealed that there are no backup systems for BSEP and MDR3 in the canaliculus. In addition, a functional impairment of either transporter may lead to acquired cholestatic liver disease, such as intrahepatic cholestasis of pregnancy (Pauli-Magnus et al., 2010). Furthermore, inhibition of BSEP by drugs is well established as a cause of acquired cholestatic liver disease in susceptible patients (Stieger, 2010; Bhamidimarri and Schiff, 2013). In contrast, reports of drug-induced liver injury (DILI) as a consequence of MDR3 inhibition by drugs are to the best of our knowledge very rare.

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ABBREVIATIONS: BNMG, LLC-PK₁ cell line transfected with BSEP, NTCP, MDR3 and ABCG5/G8; BS, bile salts; BSEP, bile salt export pump; C₄-NBD-PC, 1-palmitoyl-2-[6-[7-nitro-2-1,3-benzoxadiazol-4-yl]amino]hexanoyl]-sn-glycero-3-phosphocholine; DILI, drug-induced liver injury; DMEM, Dulbecco’s modified Eagle’s medium; HPTLC, high-performance thin-layer chromatography; MDR, multidrug resistance protein; NTCP, Na⁺-taurocholate cotransporting polypeptide; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PCR, polymerase chain reaction; TBS-T, Tris-buffered saline/Tween; TLC, thin-layer chromatography; WT, wild type.
While its incidence is unknown (Björnsson, 2014), DILI is a common cause for withdrawal or labeling changes of drugs after their approval, and therefore is a critical issue in drug discovery and development (Cheng et al., 2011; Corsini and Bortolini, 2013). Among DILI, cholestatic and mixed cholestatic hepatocellular injuries are often severe manifestations of drug toxicity (Padda et al., 2011). In order to predict and understand the mechanisms underlying drug hepatotoxicity, in vivo and in vitro model systems are used. A functional assessment of BSEP in isolated membrane vesicles obtained from cells overexpressing BSEP has become routine since the cloning of rat BSEP (Stieger, 2010), and studies with BSEP-containing vesicles are now widely used to either explain cholestatic events retrospectively (de Lima Toccafondo Vieira et al., 2014) or to investigate the cholestatic potential of new chemical entities during drug development (Thompson et al., 2012). Because lipids are practically insoluble in aqueous solution, functional assays for the characterization of MDR3-mediated PC release are less frequently used. Such assays often only assess the release of PC without detailed analysis of phospholipids species (Morita et al., 2007; Yoshikado et al., 2011). Alternatively, phospholipid secretion has been studied by using radioactively labeled choline, which limits the analysis to PC only (Groen et al., 2011). Using such assays, unspecified effects of BS added to nonpolar cell assay systems cannot be excluded.

We aimed to establish a model system for canalicular lipid secretion in a polarized model cell line displaying transcellular BS flux in order to investigate the impact of drugs on this process. We generated polar LLC-PK1 cell lines stably transfected with the Na\(^+\)-taurocholate cotransporting polypeptide (NTSP, SLC10A1), BSEP, MDR3, and ABCG5/G8. We found that antifungal azoles inhibit MDR3-mediated PC secretion, whereas other drugs associated with DILI, such as amoxicillin clavulanate or troglitazone did not interfere.

**Materials and Methods**

**Materials**

pIRESngeo3 and pIREShyg2 expression vectors were purchased from Clontech (Mountain View, CA). pSport1, pCRII-Topo, and the bicistronic vector pBudCE4.1 were purchased from Invitrogen (Carlsbad, CA). The pig kidney proximal tubule cell line LLC-PK1 and the human MDR3 cDNA clone (65706) were obtained from ATCC (Manassas, VA). The human ABCG5 and ABCG8 cDNAs were previously cloned (Noé et al., 2002). BSEP was cut out of pCRIITopo-ABCG8 with HindIII, blunt ending, and MluI and cloned downstream from its 5'-untranslated region. The human NTCP cDNA was previously cloned (Noé et al., 2002) or NTCP (Kullak-Ublick et al., 1997) have been previously described. The antisera against ABCG8 was raised as outlined in Stieger et al. (1994). The first 16 N-terminal amino acids of ABCG8 (Berge et al., 2000) were coupled C-terminally to keyhole limpet hemocyanin and used as antigen for ABCG8. Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 568 goat anti-mouse were purchased from ThermoFisher Scientific (Waltham, MA).

Nitrocellulose membranes and horseradish peroxidase conjugated anti-rabbit (RPN4301) and anti-mouse (NA931VS) secondary antibodies were purchased from GE Healthcare (Little Chalfont, United Kingdom). The protein bands were visualized with the Uptlight chemiluminescence reagent from Interchim (Montluçon, France). Restore PLUS Western Blot Stripping Buffer was purchased from ThermoFisher Scientific.

**Construction of Expression Plasmids.** Human BSEP (ABC11) cDNA was previously cloned (Noé et al., 2002). BSEP was cut out of pSport1-BSEP in three pieces with EcoRI and NotI and cloned via a three-fragment ligation into pIRESngeo3 via SalI and NotI. The resulting plasmid was named pIRESngeo3-BSEP. The human NTCP cDNA was previously cloned by Hagenbuch and Meier (1994). The NTCP cDNA was cut out of pSport1-NTCP with EcoRI and NotI and cloned into EcoRI/NotI-cut pIRESpuro2 (Clontech). The resulting plasmid was named pIRESpuro2-NTCP.

The MDR3 cDNA was cut out of the vector pJ3omega-MDR3 (ATCC) with HindIII and XbaI and first cloned into pSp. In a second step MDR3 was cut out of pSpRI-MDR3 with EcoRV and NotI and cloned into pIREShyg2. The resulting expression vector was named pIREShyg2-MDR3.

The human ABCG5 and ABCG8 cDNAs were obtained by PCR from human liver cDNA libraries by using the primers listed in Supplemental Table 1 and cloned into pIREShyg2 via NotI and SalI and cloned into pIREShyg2. The resulting expression vector was named pIREShyg2-MDR3.

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**Establishment of Stable Cells.** LLC-PK1 cells were sequentially transfected with pIRESngeo3-BSPE, pIRESpuro2-NTCP, pIREShyg2-MDR3, and pBudCE4.1-ABCG8-ABCG5 using either Effectene or jetPEI. After each transfection, stable transfectants were selected by subcloning individual colonies in the presence of the appropriate antibiotics followed by transfection with the next cDNA. Neomycin (700 µg/ml) was used for selection of colonies expressing BSEP. Puromycin (2 µg/ml) was used for NTCP, hygromycin B (500 µg/ml) was used for MDR3, and zeocin (100 µg/ml) was used for ABCG5/G8.
Cell Culture. LLC-PK1 cell lines, wild type (WT) and cells transfected with BSEP, NTCP, MDR3, ABCG5/G8 (BNMG), were cultured in high glucose DMEM supplemented with 5% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco) at 37°C in a 5% CO₂ humidified atmosphere. The medium of the multitransfected model cell line was supplemented additionally with 700 μg/ml geneticin sulfate G418, 400 μg/ml hygromycin B, 2 μg/ml puromycin, and 20 μg/ml zeocin. The cells were passaged by trypsinization twice per week once they reached 90% confluency.

Transport experiments were performed in 12-well Transwell plates having polycarbonate membrane inserts with a pore size of 3 μm. Transwell inserts were coated 1 day before seeding with 30 μl of a 25% (w/v) collagen R solution and dried at room temperature. Cells were seeded on Transwell membrane inserts at densities of 1.5 × 10⁵ to 2 × 10⁵ cells/insert. The medium in both compartments was replaced every 3 to 4 days. After 10 days, the integrity of the cell monolayers was assessed using Lucifer yellow. Both LLC-PK1 cell lines used for the experiments (WT and BNMG) were tested and found to be negative for mycoplasma. Caco-2 cells were cultured in high glucose DMEM (Gibco) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, and passaged twice per week once they reached 90% confluency.

Lucifer Yellow Monolayer Integrity Test. One day prior to transport experiments the integrity of the cell monolayers was tested using the fluorescent compound Lucifer yellow. Apical and basolateral media were removed and replaced by a 100 μM Lucifer yellow (Stewart, 1978) solution (in DMEM) in the basolateral/donor compartment and DMEM in the apical/acceptor compartment. The Transwell plates were slowly shaken and incubated for 1 hour at 37°C in the cell incubator. Next, 200 μl media aliquots were collected from the apical compartments and the concentration of Lucifer yellow was determined by measuring the fluorescence in a microplate reader Fluorescence Spectrometer Twinkle LB970 (Berthold Technologies, Bad Wildbad, Germany) at λex 430 nm and λem 535 nm (Stewart, 1981). The permeability of Lucifer yellow was calculated as percentage of Lucifer yellow in the receiver apical compartment compared with the control (filters only). Only monolayers with a permeability of less than 5% were selected for the experiments. After the integrity test, the medium was removed and replaced with standard culture medium.

Transport Studies.

Phosphatidylcholine eflux assay. The apical and basolateral culture media were removed and replaced by DMEM containing 1 mM taurocholate in the basolateral/donor compartment and DMEM supplemented with 50 mg/ml fatty acid-free bovine serum albumin in the apical/acceptor compartment. After 24 hours, the apical medium was collected and lipids were extracted according to the Bligh and Dyer (1959) method using a chloroform/methanol/water ratio of 2:1:0.8. Extracted lipids were loaded on HPTLC silica gel 60 plates with a concentrating zone by using an automated Camag TLC sampler AT54 (Muttenz, Switzerland) and separated by one-dimensional HPTLC or TLC as previously described (Gerloff et al., 1998). For the analysis of the phospholipid composition, plates were developed twice in a methylecetate, 1-propanol, chloroform, methanol, and 0.5% (v/v) potassium chloride solution [25:25:25:10:9 (v/v)]. After air drying, the plates were stained by dipping them in 3% (w/v) copper and 0.5% (w/v) potassium chloride solution [25:25:25:10:9 (v/v)]. After air drying, the plates were stained by dipping them in 3% (w/v) copper and 0.5% (w/v) potassium chloride solution [25:25:25:10:9 (v/v)]. The permeability of Lucifer yellow in the receiver apical compartment compared with the control (filters only). Only monolayers with a permeability of less than 5% were selected for the experiments. After the integrity test, the medium was removed and replaced with standard culture medium.

Protein Determination. All transport data were normalized to the monolayer's protein amount. The protein amount was determined with the bicinchoninic acid method (Smith et al., 1985) using the BCA Protein Assay Kit (Interchim).

Isolation of Cell Membranes. A total membrane fraction of WT, BNMG, and Caco-2 cells was isolated as previously described (Huber et al., 2007).

Solubilization of Cell Monolayers. Transwell filters were cut out of the insert and transferred to tubes on ice. Next, 200 μl 1% (w/v) Triton-X-100 was added to solubilize the cell monolayers. Tubes were centrifuged for 40 minutes at full speed and at 4°C in an Eppendorf Centrifuge 5417 R (Hamburg, Germany) to detach the cells from the filters.

Western Blotting. For Western blotting, total cell lysates of cells grown on standard dishes or on Transwell filters were solubilized in 1% (w/v) Triton-X-100, and 60–100 μg proteins were loaded per lane. Proteins were separated by 7.5% SDS-PAGE electrophoresis and transferred to nitrocellulose membranes according to standard procedures. Membranes were blocked 1 hour in 5% (w/v) milk-TBS-T buffer and probed for porcine MDR1 (1:100) and human MDR3 (1:600), BSEP (1:500), NTCP (1:1000) or ABCG5/G8 (1:1000) by incubation for 2 hours at room temperature with the appropriate antibody diluted in 5% (w/v) milk-TBS-T. After washing three times in TBS-T for 10 minutes, the blots were incubated for 1 hour at room temperature with the appropriate secondary antibody, either anti-mouse (1:3000) or anti-rabbit (1:30000) (Stieger et al., 1994). The protein bands were visualized using the Uplight chemiluminescence reagent. β-actin was used as the loading control and was detected using the PanActin antibody (1:1600). For stripping off antibodies, the blot was washed in TBS-T for 15 minutes and then incubated for 15 minutes in Restore PLUS Western Blot Stripping Buffer. Finally, the blot was washed again three times for 10 minutes in TBS-T.

Effect of drugs on MDR3 function. Experiments were started by replacing the apical medium with DMEM supplemented with 50 mg/ml fatty acid-free albumin and the basolateral medium with DMEM containing 12.5 μM C6-NBD-PC and 1 mM taurocholate. Drugs dissolved in dimethylsulfoxide (not exceeding 0.5% (v/v)) were added to both compartments. Drug concentrations were chosen by considering maximal total serum concentrations and in vitro cell cytotoxicity. After 24 hours, the apical medium was collected and the lipids were extracted. Fluorescence of the lipid-containing chloroform phase was measured in a microplate reader fluorescence spectrometer Twinkle LB970 (Berthold Technologies) (λex 485 nm, λem 535 nm). For quantification, standards of C6-NBD-PC dissolved in albumin-supplemented DMEM were extracted in parallel to the samples.

Taurocholate transport assay. DMEM containing 10 μM taurocholate was prepared by dissolving α-taurocholate and unlabeled taurocholate in DMEM (0.2 μg/Ci/ml) and prewarmed in a water bath at 37°C. This medium was added either to the basolateral or the apical donor compartment in the Transwell system. After 10, 20, and 30 minutes, 50 μl aliquots were collected from the corresponding acceptor compartment. In order to assess the intracellular accumulation of taurocholate, 50 μl aliquots of the solubilized monolayers were used. Radioactivity in the aliquots was determined by liquid scintillation counting using a Packard Tri-Carb 2250CA liquid scintillation analyzer (Packard, IL, US).

Interaction of azoles with BSEP and NTCP. Overnight treatment of cell monolayers with 10 μM azoles or 4 μM PSC833 was followed by a taurocholate transport assay from the basolateral to the apical compartment. The cell monolayers were treated for a total of 24 hours with the drugs, which were added to both compartments.

Cytotoxicity Assay. Potential cytotoxicity of the drugs was checked by measuring lactate dehydrogenase release in the apical and basolateral medium and in the total cell lysate after 24 hours using a CytoTox96 Non-Radioactive Assay Kit (Promega, Madison, WI).

Protein Determination. All transport data were normalized to the monolayer’s protein amount. The protein amount was determined with the bicinchoninic acid method (Smith et al., 1985) using the BCA Protein Assay Kit (Interchim).

Isoflurane of Cell Membranes. A total membrane fraction of WT, BNMG, and Caco-2 cells was isolated as previously described (Huber et al., 2007).

Immunofluorescence. For immunostaining of BNMG and WT cells grown in the Transwell system, the filters were rinsed with phosphate-buffered saline (PBS), cut out of the insert with a scalpel and transferred into a 12-well plate. Immunostaining was performed according to Stieger et al. (1994). In brief, the monolayers were fixed for 20 minutes at room temperature in 4% (w/v) paraformaldehyde in PBS, quenched for 5 minutes with 0.25% (w/v) NH₄Cl in PBS followed by permeabilization for 10 minutes with 0.1% (w/v) saponin in PBS.
After blocking for 30 minutes with 2% (v/v) goat serum and 0.1% (w/v) saponin in PBS, the cells were incubated for 2 hours with primary antibodies diluted in PBS supplemented with 1% (w/v) bovine serum albumin and 0.1% (w/v) saponin for double staining of MDR3 (1:35) and NTCP (1:300) or BSEP (1:35) and α1-subunit of Na-K-ATPase (1:40). After three rinses for 10 minutes each, the secondary antibodies (1:300 each) were exposed to the specimens for 1 hour. After three rinses, the cells were mounted with the filters facing the slides, covered with Vectashield mounting medium [containing DAPI (4',6-Diamidin-2-phenylindol)] and cover-slipped in an airtight mode. Pictures were acquired by confocal laser scanning microscopy with a Leica SP8 system (Heerbrugg, Switzerland) and the files were processed with the ImageJ 1.47t software (National Institutes of Health, Bethesda, MD).

Quantitative Real-Time PCR Analysis. Total RNA was extracted from BNMG cells using TRIzol. DNAs were amplified from MDR3 and glyceraldehyde-3-phosphate dehydrogenase mRNA using the TaqMan master mix and human MDR3 primers (Hs00240956_m1) and porcine glyceraldehyde-3-phosphate dehydrogenase primers (Ss03374854_g1). The mRNA levels were measured using an Applied Biosystems ViiA7 Real Time PCR system (Life Technologies, Carlsbad, CA). The mRNA levels were expressed relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analysis. The data are expressed as mean ± S.D. The paired t test was used to evaluate the difference between absolute values of experimental and control samples. The one sample t test was used to evaluate the difference between experimental and control values of normalized data. The statistical analysis was carried out with GraphPad Prism (GraphPad Software Inc., San Diego, CA). Differences with P < 0.05 were considered as significant.

Results

Functional Characterization of the Model Cell Line. The LLC-PK₁ cell line stably transfected with NTCP, BSEP, MDR3, and ABCG5/G8 (termed BNMG cells) was tested for expression of the transporters by Western blotting. As shown in Fig. 1, NTCP, BSEP, MDR3, ABCG5 (with membranes from Caco-2 cells as the positive control), and ABCG8 were detected in BNMG cells, but not in WT cells.

To assess the functional polarity of BNMG cells, transcellular transport of the prototypic BS taurocholate was determined. Figure 2, A and B demonstrates that in BNMG cells the basolateral-to-apical transport rate of taurocholate is 56-fold higher than the reverse transport in BNMG cells, whereas no difference in the transport ratio was observed in WT cells. Figure 2C illustrates that the uptake of taurocholate is 3-fold higher in BNMG cells compared with WT cells and occurs exclusively at the basolateral membrane, demonstrating that NTCP is expressed at the basolateral membrane. These results indicate that NTCP and BSEP are functionally expressed in BNMG cells and mediate transcellular, vectorial transport of BS through the basolateral and apical membrane, as they do in human hepatocytes.

The predominant phospholipid in human bile is PC (Phillips, 1980; Alvaro et al., 1986). Therefore, we investigated the functional activity and selectivity of MDR3 in BNMG cells by chemically analyzing the lipids secreted into the apical compartment using HPTLC. To ensure the solubility of the secreted lipids in the medium, fatty acid–free albumin was added as a lipid acceptor to the apical compartment. However, the lipid acceptor albumin contains endogenous PC, requiring a correction of the concentration of PC determined. Densitometric quantification of PC demonstrates that BNMG cells (but not WT cells) specifically secrete PC, but no other phospholipids into the apical medium (Fig. 3, A and B). BNMG cells thus correctly recapitulate in vivo biliary phospholipid secretion. Having established the functional activity and selectivity of PC secretion in BNMG cells, we next tested the apical secretion of the fluorescent PC derivative C₆-NBD-PC, a known substrate for MDR3 (Smith et al., 2000). Figure 3C shows that C₆-NBD-PC is indeed secreted by MDR3. To test for the functional polarity of C₆-NBD-PC secretion, the transport of C₆-NBD-PC from the basolateral to the apical compartment was compared with the transport from the apical to the basolateral compartment. As previously demonstrated for taurocholate, practically no specific apical to basolateral secretion of C₆-NBD-PC could be observed (Supplemental Fig. 1). These data were complemented by immunohistochemical analysis of NTCP, BSEP, and MDR3 localization. The results in Fig. 4 show an apical expression of BSEP and MDR3, while NTCP is expressed at the basolateral membrane of BNMG cells, and labeling of WT cells does not exceed the background.

The small amount of C₆-NBD-PC observed in the apical medium of WT cells could have been MDR1 mediated because previous experiments (Smith et al., 2000) have shown that C₆-NBD-PC can be transported by MDR1. To rule out a significant role of MDR1 in apical C₆-NBD-PC secretion in our BNMG cells, we analyzed for the expression of endogenous porcine MDR1. Using the antibody C219—reported to cross react with pMDR1 (Crivellato et al., 1999) and known to recognize a common epitope on MDR1 and MDR3 (van Den Elsen et al., 1999)—practically no signal was observed in membranes isolated from WT cells, even after treatment of the cells with sodium butyrate (Fig. 5B). In contrast, a signal for MDR3 (140 kDa) was detected in membranes isolated from BNMG cells. Membranes isolated from Caco-2 cells were used as the positive control for MDR1 (Hosoya et al., 1996; Uchida
et al., 2015) and indeed displayed a band at the predicted size (170 kDa). Figure 5A shows the corresponding immunoblot using an MDR3-specific antibody known to not cross react with MDR1 (Scheffer et al., 2000), and hence not generating a signal in WT and Caco-2 cells. We also used an MDR1-specific antibody (Georges et al., 1990) known to cross react with pMDR1 (Lazarowski et al., 2005) and found only a faint signal above the background in WT and BNMG cells (data not shown). Combined, these data demonstrate that expression of pMDR1 in our cell lines is minimal and that the C6-NBD-PC secretion into the apical compartment is mediated by MDR3. The variable, small amounts of C6-NBD-PC released from WT cells into the basolateral compartment could be unspecifically absorbed by albumin (Supplemental Fig. 1).

In vivo, PC secretion requires canalicular BS secretion. The data presented in Fig. 3D demonstrate that our model cell line BNMG only secretes C6-NBD-PC in the presence of albumin and that a transcellular flux of taurocholate additionally increases C6-NBD-PC secretion. Moreover, Fig. 3D demonstrates that C6-NBD-PC secretion from BNMG cells critically depends on the presence of albumin as an acceptor of lipids in the apical medium.

**Validation of PC Secretion.** Smith et al. (2000) demonstrated that MDR3 is inhibited by verapamil and by PSC833. Figure 6 shows that MDR3-mediated secretion of C6-NBD-PC was reduced by 40% by verapamil and by 52% by PSC833. Hence, our model cell line BNMG is suitable for investigating the interaction of drugs with MDR3.

**Interaction of Antifungals with MDR3.** In patients, a cholestatic pattern of DILI is characterized by a predominantly elevated level of serum alkaline phosphatase (Aithal et al., 2011). In addition, inherited impairment of MDR3 function leads to an increase of serum γ-glutamyl transpeptidase (Jacquemin, 2012). Therefore, drugs reported to elicit an elevation of serum alkaline phosphatase and γ-glutamyl transpeptidase were studied in our in vitro model system. Based on clinical studies on DILI, many azoles have been associated with increased hepatic enzymes and hepatotoxicity (Gearhart, 1994; Somchit et al., 2004; Yoshikado et al., 2011). Several patients treated with itraconazole were reported to have increased serum alanine aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, and bilirubin, suggesting DILI with a mixed hepatocellular and cholestatic pattern (Lavrijsen et al., 1992; Yoshikado et al., 2011). Moreover, itraconazole has been reported to inhibit MDR3 (Yoshikado et al., 2011). Therefore, we studied the impact of different azoles on MDR3 function.

Figure 7A reveals that in addition to itraconazole, ketoconazole and posaconazole also inhibit MDR3 with the potency decreasing from posaconazole to itraconazole to ketoconazole. Fluconazole and voriconazole showed no inhibition of MDR3. Endogenous pMDR1 activity was not affected by itraconazole and ketoconazole, but was inhibited by about 50% in the presence of 10 μM posaconazole (Supplemental Fig. 2). In order to rule out interference of the azoles with MDR3 protein levels, the expression of MDR3 was compared with control BNMG cells by Western blotting. Figure 8A shows that itraconazole and posaconazole increased the protein levels of MDR3, while the other azoles had no effect on MDR3 protein. Figure 8B shows that the increase in MDR3 protein levels upon itraconazole treatment is time dependent, and hence unlikely due to unspecific effects of itraconazole. In contrast, BSEP (Fig. 8A) and NTCP (Supplemental Fig. 3) expression levels remained unchanged after treating BNMG cells with different azoles.

We next determined the IC50 value of the inhibition of C6-NBD-PC by posaconazole and found a value of 4.2 μM (Fig. 7B). Attempts to determine the IC50 value of itraconazole failed due to the cytotoxicity of this drug at higher concentrations (data not shown).
Interaction of DILI-Associated Drugs with MDR3.

We investigated various drugs for our in vitro studies: Amoxicillin clavulanate has been found to be the most common drug associated with DILI (Leise et al., 2014; Björnsson, 2015). Another drug linked to DILI is the atypical antipsychotic drug olanzapine (Devarbhavi et al., 2010), which was been shown to inhibit MDR1 (Wang et al., 2006). Because MDR1 is a close homolog to MDR3 (van der Bliek et al., 1988), it is conceivable that some MDR1 inhibitors may also modulate MDR3 by recognizing similar domains or surfaces (Morita et al., 2007). Troglitazone was a drug used for treatment of type II diabetes and was withdrawn from the market because of numerous reports of liver failure. Multiple mechanisms for troglitazone hepatotoxicity including mitochondrial injury and BSEP inhibition were proposed (Smith, 2003). Finally, octreotide is used to treat acromegaly, thyroid-stimulating hormone-secreting pituitary adenomas, and neuroendocrine tumors, and about half of the patients receiving octreotide have gallbladder stones (Dowling et al., 1992). Therefore, we tested all these drugs for potential inhibition of MDR3 in our in vitro model. Figure 9A demonstrates that, with the exception of olanzapine, none of the aforementioned drugs inhibited MDR3. Even olanzapine only showed a minor reduction of C₆-NBD-PC secretion. None of the drugs affected MDR3 expression (Supplemental Fig. 4).

Interaction of Cholagogues with MDR3.

Artichoke leaves extract (Cynara scolymus L.) is an old remedy against gastrointestinal and liver disorders in traditional medicine. It was reported to stimulate biliary secretion in humans (Kirchhoff et al., 1994; Matuschowski et al., 2005) and to have lipid-lowering properties (Adzet et al., 1987; Kraft, 1997; Wegener and Fintelmann, 1999). The active ingredients in artichoke leaves extract are cynarin and luteolin (Ben Salem et al., 2015). Similarly, boldo leaves extract (Peumus boldus Mol.) containing the active ingredient boldine, which is used against liver ailments in South America (Speisky and Cassels, 1994), was reported to stimulate choleretic (O’Brien et al., 2006; Zagorova et al., 2015). Therefore, we tested cynarin, luteolin, and boldine for potential stimulation of MDR3 activity, which would be beneficial to the bile ducts. As shown in Fig. 9B, all of the substances affected MDR3 activity or MDR3 protein expression levels (Supplemental Fig. 4) in our model system.

Effect of Azoles on BS transport.

Since inhibition of BSEP may contribute to DILI, we assessed the impact of azoles on taurocholate transport in BNMG cells. Figure 10A shows that ketoconazole, itraconazole, posaconazole, and...
PSC833 inhibit the apical secretion of taurocholate. Analysis of intracellularly retained taurocholate shows an accumulation in conditions where apical taurocholate secretion was impaired; indicating that the reduced transcellular BS flux resulted as a consequence of BSEP inhibition (Fig. 10B).

Discussion

In the present study, we successfully reconstructed canalicular lipid secretion in vitro by establishing stably transfected LLC-PK1 cells with key human transporters for bile formation. This model system recapitulates canalicular lipid secretion, and in conjunction with the Transwell system allows for efficient testing of drugs for interacting with canalicular lipid and BS secretion. The newly established model system has the following advantages: 1) direct experimental access to the basolateral and apical plasma membranes, which is not possible in hepatocytes in vivo; 2) the formation of a tight epithelium with the possibility to generate transcellular fluxes for BS and lipids, and hence the reconstitution of trans-hepatocellular fluxes of cholephilic compounds; 3) positive modulation of apical lipid secretion by taurocholate, and hence recapitulating the situation in (human) hepatocytes (Morita et al., 2007); and 4) the possibility to simultaneously assess the impact of drugs on BSEP-mediated BS and MDR3-mediated lipid secretion. The data presented here reveal inhibition of MDR3-mediated lipid secretion by several drugs. In addition, certain drugs altered the expression level of MDR3.

The diagnosis of DILI—in particular, idiosyncratic DILI—is challenging, which is in part related to the multitude of mechanisms leading to this condition (Aithal et al., 2011). DILI accounts for a considerable number of hospitalizations and liver failures with almost half of the cases presenting with either cholestatic or mixed cholestatic liver toxicity (de Lima Toccafondo Vieira and Tagliati, 2014). Canalicular BS secretion is one of the major driving forces of bile flow and inhibition of BSEP is a well-characterized mechanism of acquired cholestasis (Stieger, 2010), with the number of drugs inhibiting BSEP constantly growing (Dawson et al., 2012). An analysis of the Food and Drug Administration Adverse Event Reporting System database (http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Surveillance/AdverseDrugEffects/) revealed that antymycotics are involved in approximately 3% of all DILI cases (Raschi et al., 2014). Moreover, antifungal azoles have been associated with elevated levels of serum liver enzymes as well as liver injury occasionally leading to liver failure (Hann et al., 1993; Gearhart, 1994; Adriaenssens et al., 2001; Chang et al., 2007). Therefore, we
ular transfer of C6-NBD-PC in artificially prepared phospho-

This assumption is supported by the spontaneous intervesic-

NBD-PC is more water soluble than PC (Ruetz, 1998) and may

IC50

apical compartment in control transfected cells.

Yoshikado et al. (2011) also found efflux of14C-PC into the

lipid vesicles (Elvington and Nichols, 2007). Notably

inhibition of canalicular transporters, particularly MDR3, by

best of our knowledge, this is the first report of direct

interaction of five clinically used antifungal

azoles with MDR3: fluconazole, itraconazole, ketoconazole,

posaconazole, and voriconazole. We found that posaconazole,

itraconazole, and ketoconazole (in decreasing order) signifi-

cantly inhibited MDR3-mediated PC secretion and, in addition,

BSEP-mediated taurocholate secretion in our model cell

line. It is interesting that in the presence of albumin, not only

our BNMG cells, but also WT cells, secrete (a small but

measurable amount) of C6-NBD-PC, both into the apical (Fig

3C; Supplemental Fig. 1) and the basolateral compartments

(Supplemental Fig. 1). A possible interpretation is that C6-

NBD-PC is more water soluble than PC (Ruetz, 1998) and may

thus spontaneously partition into the aqueous compartments.

This assumption is supported by the spontaneous intervesic-

ular transfer of C6-NBD-PC in artificially prepared phospho-

lipid vesicles (Elvington and Nichols, 2007). Notably Yoshikado et al. (2011) also found efflux of 14C-PC into the apical compartment in control transfected cells.

Posaconazole strongly inhibited the activity of MDR3 (83%,

IC50 ~4.2 μM) and moderately inhibited BSEP (38%). To the

best of our knowledge, this is the first report of direct inhibition of canalicular transporters, particularly MDR3, by

posaconazole. Posaconazole-related elevation of liver enzymes or liver injury is rarely reported in clinics and most available data are in the context of clinical trials (Courtney et al., 2005; Girmenia, 2009; Moton et al., 2009). The apparent discrepancy between our findings and the current literature may be the following reasons: 1) posaconazole was approved in 2006 and thus released into the market about 20 years after ketocona-

zole and itraconazole; and 2) posaconazole has a rather narrow indication for the treatment of invasive fungal infections (e.g., aspergillosis and candidiasis) in patients refractory to other antifungals (second line therapy) or for the prophylaxis of antifungal infections in patients receiving chemotherapy or during immunosuppression after hematopoietic stem cell transplantation (Frampton and Scott, 2008). Therefore, the patient population exposed to posaconazole is limited and patients are often on polypharmacy, which generates difficul-
ties in associating a particular drug with a specific molecular pathway leading to the adverse event.

We found that in addition to posaconazole, itraconazole also significantly inhibited the activity of MDR3 (42%) and moderately that of BSEP (26%). A previous study reported almost complete inhibition of PC secretion at 1 μM itraconazole (Yoshikado et al., 2011). The discrepancy with our data may be related to distinct experimental setups. Yoshikado et al. (2011) used a transient transfection system and higher taurocholate concentration (3 mM), both of which may have had an impact on cell viability. They also preincubated their cells with itraconazole prior to the start of the efflux experi-

ment. Our in vitro findings with itraconazole are supported by numerous reports of hepatic liver injury in the literature (Hann et al., 1993; Hay, 1993; Chang et al., 2007), and in particular by reports on a cholestatic pattern of liver injury (Lavrijsen et al., 1992; Hann et al., 1993; Talwalkar et al., 1999; Yoshikado et al., 2011).

Interestingly, ketoconazole only weakly (20%) inhibited MDR3 but was the most potent BSEP inhibitor (67%) inhibition) in the present study. Previous studies are in line with our observed BSEP inhibition by ketoconazole (IC50 ~3 μM) (Dawson et al., 2012). Ketoconazole has been withdrawn from the European and Australian markets and underwent strict product relabeling in Canada and the United States due to

Fig. 6. Control for inhibition of MDR3-mediated C6-NBD-PC transport. BNMG monolayers were treated 24 hours with 10 μM verapamil or 4 μM PSC653, and apical C6-NBD-PC secretion in the presence of 1 mM taurocholate basolateral and 50 mg/ml albumin apical was assessed. Results are shown as mean ± S.D. of five independent experiments performed in triplicates; *P < 0.05 versus dimethylsulfoxide (DMSO).

Fig. 7. Interaction of azoles with MDR3. (A) Effect of azoles on C6-NBD-PC secretion. BNMG monolayers were treated 24 hours with 10 μM azoles in the presence of 1 mM taurocholate basolateral and 50 mg/ml albumin in the apical. Results are shown as mean ± S.D. of four independent experiments performed in duplicates to quadruplicates; *P < 0.05, **P < 0.01. (B) Determination of the IC50 value of posaconazole for MDR3. Using the described model, a dose-response curve for MDR3 inhibition by posaconazole was generated by assessing 12.5 μM C6-NBD-PC secretion in the presence of different concentrations of posaconazole and substracting the background (C6-NBD-PC secretion in treated WT). The percentual MDR3 activity was plotted against the logarithm of the concentration of inhibitor/posaconazole. An IC50 value of 4.2 μM (95% confidence interval, 3.08–5.76) was calculated using GraphPad Prism. Data represent the mean ± S.D. of two independent experiments performed in duplicates.
hepatotoxicity (Gupta et al., 2015). Although these adverse effects were mainly attributed to the inhibition of CYP3A isoforms by ketoconazole, emerging evidence (Morgan et al., 2010; Dawson et al., 2012) and our results suggest that inhibition of MDR3 and BSEP may contribute to ketoconazole-induced hepatotoxicity in susceptible patients, similar to what has been shown for troglitazone (Stieger, 2010).

Neither fluconazole and nor voriconazole affected MDR3-mediated PC secretion or BSEP activity in our model system. These results agree well with several comparative in vitro and epidemiologic studies suggesting that fluconazole is the safest azole (Somchit et al., 2002; Chang et al., 2007; Cronin and Chandrasekar, 2010). In contrast, voriconazole has been often associated with hepatotoxicity. However, its toxicity seems to correlate with CYP2C19 polymorphisms (Trubiano et al., 2015) and not with an inhibition of hepatobiliary transporters.

The various azoles affecting the canalicular transporters are structurally similar. Posaconazole and itraconazole both contain an extended piperazine-phenyl-triazole moiety, whereas ketoconazole contains a piperazine-phenyl moiety. In contrast, voriconazole and fluconazole are structurally different and did not inhibit MDR3. It is tempting to speculate that the piperazine-phenyl-triazole moiety is partly responsible for the interaction with MDR3.

Amoxicillin clavulanate is a drug causing liver injury with high frequencies (Björnsson, 2015). Therefore, we extended our studies to this drug. We also studied troglitazone, which has been withdrawn from the market due to severe liver toxicity (Dawson et al., 2012). Neither drug caused a decrease
in phospholipid secretion in our model, suggesting that MDR3 inhibition is not implicated in the mechanism of toxicity. These findings also suggest that our model cell line is suitable for studying the interaction of drugs with MDR3 function and does not generate MDR3 protein susceptible to pathophysiological irrelevant inhibitions, for example, as a consequence of a lipid and protein environment distinct from that of the canalicular membrane.

Finally, we found that itraconazole and posaconazole led to a marked time-dependent increase in MDR3 protein levels. No significant change in BSEP (Fig. 8A) or NTCP (Fig. 8B and Supplemental Fig. 3) protein levels was detected under the same condition. Since we used cDNAs to generate our model cell line, an upregulation of translation cannot be the reason for this observation. This interpretation is in line with the finding that the PPARα agonists fenofibrate and bezafibrate, both known to stimulate MDR3 promoter activity (Ghonem et al., 2014), did not lead to a change of MDR3 protein levels in our cell line (Fig. 8B). Also, we did not see an increase of mRNA for MDR3 (Fig. 7C), ruling out a stabilization of the mRNA by these two drugs. Since we used for Western blotting total cell fractions, we cannot quantitate expression changes of MDR3 in the apical membrane, but the increase in the protein may either be due to an increased translation rate or to stabilization of MDR3 protein in our cell line.

In conclusion, azoles, used to treat fungal infections, inhibit BSEP and MDR3 to varying extents. Of the various possible distinct molecular mechanisms leading to DILI (Stephens et al., 2014) the observed inhibition of MDR3 and BSEP described here can rationalize one mechanism leading to DILI caused by azoles in susceptible patients.

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Authorship Contributions
Participated in research design: Mahdi, Locher, Stieger.
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Performed data analysis: Mahdi, Synal-Hermanns, Yoker, Stieger.
Wrote or contributed to the writing of the manuscript: Mahdi, Synal-Hermanns, Yoker, Locher, Stieger.

Fig. 9. Effect of hepatotoxic drugs and cholangogues on MDR3-mediated PC secretion. Cell monolayers were treated 24 hours with the compounds. (A) Effect of 300 μM amoxicillin, 100 μM clavulanate, 100/300 μM amoxicillin clavulanate (co-amoxicillin), 25 μM octreotide, 10 μM olanzapine, and 10 μM troglitazone on C6-NBD-PC secretion. (B) Effect of 10 μM boldine, 100 μM cynarin, and 10 μM luteolin on C6-NBD-PC secretion. Results are shown as mean ± S.D. of three independent experiments performed in duplicates or triplicates. DMSO, dimethylsulfoxide.

Fig. 10. Interaction of azoles with transcellular taurocholate transport. Monolayers were treated with 10 μM azoles or 4 μM PSC833 for 24 hours. The transport assay was started by adding 10 μM taurocholate to the basolateral compartment. (A) Effect of azoles on taurocholate transport. After 30 minutes, the taurocholate transported to the apical compartment was assessed. The total taurocholate secreted in the apical compartment was normalized to the total amount of protein per monolayer. Drug-treated monolayers were normalized to control monolayers treated with dimethylsulfoxide (DMSO). Results are shown as mean ± S.D. of three independent experiments performed in duplicates; *P < 0.05 versus DMSO. (B) Intracellular accumulation of taurocholate as indicator for BSEP inhibition. After 2 hours, the monolayers were washed once with PBS and solubilized in 1% (w/v) Triton X-100 and the intracellular-retained taurocholate was assessed. Results were normalized to the protein’s amount. Drug-treated monolayers were normalized to DMSO-treated monolayers. Results are shown as mean ± S.D. of two independent experiments performed in duplicates.
MDR3 Inhibition by Antiinfectials

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