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Role of Hepatic Transporters in the Disposition and Hepatotoxicity of a HER2 Tyrosine Kinase Inhibitor CP-724,714

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CP-724,714, a potent and selective orally active HER2 tyrosine kinase inhibitor, was discontinued from clinical development due to unexpected hepatotoxicity in cancer patients. Based on the clinical manifestation of the toxicity, CP-724,714 likely exerted its hepatotoxicity via both hepatocellular injury and hepatobiliary cholestatic mechanisms. The direct cytotoxic effect, hepatobiliary disposition of CP-724,714, and its inhibition of active canalicular transport of bile constituents were evaluated in established human hepatocyte models and *in vitro* transporter systems. CP-724,714 exhibited direct cytotoxicity using human hepatocyte imaging assay technology with mitochondria identified as a candidate organelle for its off-target toxicity. Additionally, CP-724,714 was rapidly taken up into human hepatocytes, partially via an active transport process, with an uptake clearance approximately fourfold higher than efflux clearance. The major human hepatic uptake transporter, OATP1B1, and efflux transporters, multidrug resistance protein 1 (MDR1) and breast cancer resistance protein, were involved in hepatobiliary clearance of CP-724,714. Furthermore, CP-724,714 displayed a concentration-dependent inhibition of cholyl-lysyl fluorescein and taurocholate (TC) efflux into canaliculi in cryopreserved and fresh cultured human hepatocytes, respectively. Likewise, CP-724,714 inhibited TC transport in membrane vesicles expressing human bile salt export pump with an IC_{50} of 16μM. Finally, CP-724,714 inhibited the major efflux transporter in bile canaliculi, MDR1, with an IC_{50} of ~28μM. These results suggest that inhibition of hepatic efflux transporters contributed to hepatic accumulation of drug and bile constituents leading to hepatocellular injury and hepatobiliary cholestasis. This study provides likely explanations for clinically observed adverse liver effects of CP-724,714.

Key Words: **CP-724,714; hepatocytes; hepatic transporters; hepatotoxicity; mechanism.**

CP-724,714 (Fig. 1) is a potent and selective orally active small molecule inhibitor of human epidermal growth factor receptor-2 (erbB2/HER2/neu) and was discontinued from clinical development for the treatment of erbB2-overexpressing tumors due to serious hepatotoxicities including jaundice reported in patients. During preclinical studies, CP-724,714 demonstrated dose-dependent antitumor activities in a number of murine models with human tumor xenografts (Jani *et al.*, 2007) and was generally well tolerated in animals. The principal toxicities of CP-724,714 observed in rats and dogs considered potentially adverse in humans were in the intestinal tract and liver. In rats, the liver effects included hepatocellular necrosis, Kupffer cell hypertrophy, increased serum hepatic transaminases (aspartate transaminase [AST], alanine transaminase [ALT]), and increased total bilirubin. In dogs, cholestasis and increases in AST, ALT, alkaline phosphatase (ALP), γ-glutamyltranspeptidase (GGT), total bilirubin, and bile acids were observed. All these effects in rats and dogs occurred at systemic exposures greater than anticipated to be achieved in clinical trials (Guo *et al.*, 2008; Munster *et al.*, 2007).

Based on preclinical safety results, a starting dose (250 mg QD) at less than 10% of the dose associated with severe irreversible toxicities in rodents and dogs was selected for clinical safety evaluation in patients with advanced malignant solid tumors overexpressing HER2. Surprisingly, CP-724,714 displayed dose-limiting toxicities including reversible cholestatic liver dysfunction in patients with elevated ALT, AST, GGT, and hyperbilirubinemia (HB) (Munster *et al.*, 2007). The incidence and severity of the toxicities were generally dose dependent: grade 3 hepatotoxicity (>3- to 10-fold increase of total bilirubin and/or >5- to 20-fold increase of transaminases) was reported in patients from 25% at 250 mg QD to 40% at 400 mg BID with a median onset of 15 days. Additionally, elevation of liver function tests significantly correlated to the steady-state systemic exposures of CP-724,714 (Guo *et al.*, 2008), suggesting that the parent molecule played a key role in the toxicity. Evaluation of human plasma samples collected around T_{max} from the treated subjects with HB also revealed that the parent compound, CP-724,714, remained to be the main peak, while no remarkable

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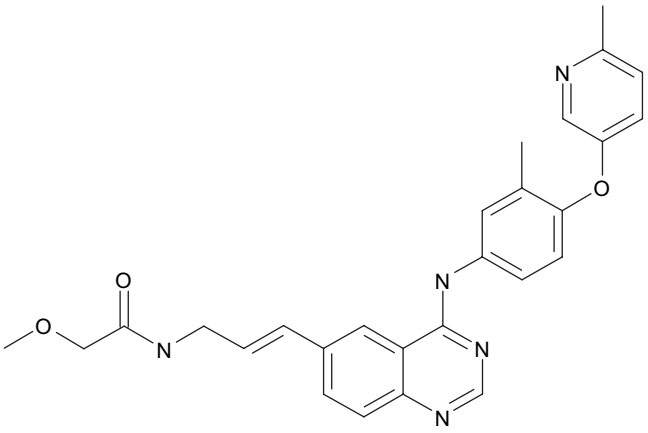

FIG. 1. Chemical structure of CP-724,714.

metabolites were identified from any of the evaluated subjects based on chromatographic evaluations. Drug-induced liver toxicity continues to be one of the major challenges for drug safety and is mainly characterized by hepatocellular injury with elevated serum transaminases and cholestatic liver damage with increased serum ALP, GGT, and bilirubin (Pauli-Magnus and Meier, 2006). Based on the clinical findings of CP-724,714, we hypothesized that the parent molecule likely exerted direct liver toxicity by both hepatocellular injury and hepatobiliary cholestatic mechanisms.

Although the mechanisms of drug-induced hepatotoxicities are not fully understood, it is well documented that modulations of hepatic transport processes could potentially result in clinically significant drug-transporter interactions, leading to hepatotoxicity (Kim, 2006; Shitara *et al*., 2006). A number of transporters expressed on the sinusoidal and bile canalicular membrane of human hepatocytes have been identified to be important in mediating the cellular uptake of endogenous and exogenous molecules into hepatocytes and efflux of these molecules into bile (Kim, 2006; Shitara *et al*., 2006). Organic anion transporting polypeptides, OATP1B1, OATP1B3, and OATP2B1, are predominant transporters responsible for the hepatic uptake of a variety of organic anionic compounds into hepatocytes (Mikkaichi *et al*., 2004). Whereas multidrug resistance associated protein 2 (MRP2), multidrug resistance protein 1 (MDR1), breast cancer resistance protein (BCRP), and bile salt export pump (BSEP) are the primary efflux transporters on the canalicular membrane mediating biliary excretion of endobiotics, xenobiotics, and/or their metabolites derived from phase II glucuronidation and sulfation into bile (Hirano *et al*., 2005; Shitara *et al*., 2006). These transporters play a critical role in determining hepatic exposure and clearance of xenobiotics and bile flow. Interference of their functional transport processes can lead to intracellular accumulation of xenobiotics and bile components, resulting in cell damage and cholestasis (Pauli-Magnus and Meier, 2006).

Owing to the observed hepatotoxicity in patients, we designed a panel of *in vitro* studies to evaluate the interactions of CP-724,714 with primary human hepatocytes and major hepatic transporters in particular to test our hypotheses and understand the mechanisms of CP-724,714–induced hepatotoxicity in patients. The objectives of our studies were (1) to investigate the direct cytotoxic effects of CP-724,714 on human hepatocytes; (2) to identify the major drug transporters involved in the hepatic uptake and hepatobiliary excretion of CP-724,714; and (3) to assess the potential for inhibition of hepatic efflux transporters by CP-724,714 leading to progressive drug/bile acid accumulation and hepatotoxicity.

MATERIALS AND METHODS

Materials. CP-724,714, a 2-methoxy-N-[3-[4-[3-methyl-4-[(6-methylpyridin-3-yloxy]phenylamino]quinazolin-6-yl]-E-allyl]acetamide (MW 469.55, chemical and isomeric purity > 99%), and [^{14}C]CP-724,714 (specific activity 96.8 μCi/mg) were synthesized at Pfizer Global Research and Development (Groton, CT). [^{3}H]estradiol 17β-D-glucuronide (specific activity 46.9 Ci/mmol) and [^{3}H]estrone 3-sulfate (specific activity 57.3 Ci/mmol) were purchased from Perkin Elmer (Boston, MA). [^{3}H]Bromosulfophthalein (BSP) (specific activity 17.5 Ci/mmol) was obtained from Hartmann Analytic (Braunschweig, Germany). Cholyl-lysyl fluorescein (CLF), tetramethyl rhodamine methyl ester (TMRM), Hank's balanced salt solution (HBSS), trypan blue, Hoechst 33342, Calcein AM, and HEPES buffer were purchased from Invitrogen (Carlsbad, CA). Taurocholate (TC), [^{3}H]TC (2 Ci/mmol) and [^{3}H]Midazolam (5 Ci/mmol) were purchased from NEN Life Sciences Product (Boston, MA). DMSO (dimethyl sulfoxide), trichostatin A, cyclosporine A, acyclovir, glyburide, BSP, and Dulbecco's Minimal Essential Medium were purchased from Sigma-Aldrich (Carlsbad, CA). Williams E medium (WEM) was purchased from either Sigma-Aldrich or Invitrogen. Biocoat 24- and 96-well collagen-I–coated plates, Biocoat poly-D-lysine–coated plates, Matrigel, and ITS+ (insulin, transferrin, selenous acid +linoleic acid) premix were purchased from BD Biosciences (Bedford, MA). Fetal bovine serum (FBS), penicillin-streptomycin, bovine insulin, dexamethasone, and L-glutamine were purchased from CellzDirect (Pittsboro, NC). DRAQ5 was purchased from Biostatus Limited (Leicestershire, UK). BCA protein assay kit was purchased from Pierce Chemical Company (Rockford, IL). The MDCK, human MDR1–transfected MDCK, and human MRP2–transfected MDCK cell lines were acquired from Prof. Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands), and the human BCRP–transfected MDCK cell line was constructed internally (Xiao *et al*., 2006). Human OATP1B1, OATP1B3, and OATP2B1 expressed in human embryonic kidney 293 (HEK 293) cells were obtained from Prof. Dietrich Keppler (DKFZ, Heidelberg, Germany).

Hepatocyte imaging assay technology. Cryopreserved human hepatocytes (lot Hu4000 from CellzDirect) were thawed, plated, overlaid with Matrigel, and cultured as described previously (Xu *et al*., 2008). After 3 days in culture and daily media changes, the cells were incubated with CP-724,714, initially solubilized in DMSO then diluted in WEM containing 5% FBS to a final DMSO concentration of 0.4%. After incubation for 24 h at 37°C, 5% CO_2, and 95% humidity, media were removed, and cells were stained by fluorescent probes in the same culturing medium without serum. The fluorescent probe used was TMRM (0.02μM, 1 h) to measure mitochondrial membrane potential and DRAQ5 (45μM, 30 min) as a counter-stain for nuclei. Automated live-cell multispectral image acquisition was performed on a Kinetic Scan Reader from Cellomics using a 20× objective and an XF93 filter (Xu *et al*., 2008). The relative amount of TMRM signal was quantified by ImagePro from MediaCybernetics (Bethesda, MD).

CLF imaging assay. Cryopreserved human hepatocytes (lot Hu4000 from CellzDirect) were thawed, plated, matrigel overlaid, and cultured as described previously (Xu *et al*., 2008). After 5 days in culture with daily media changes, the cells were incubated with CP-724,714, initially solubilized in DMSO then diluted in WEM containing 5% FBS to a final DMSO concentration of 0.4%. After incubation for 1 h at 37°C, 5% CO_2, and 95% humidity, media were removed and, cells were incubated with 5μM CLF and the test compound in WEM without serum for 15 min at 37°C with 5% CO_2 and 95% humidity. Dosing solutions were removed, and cells were incubated with 0.025% trypan blue in HBSS plus Ca^{2+}/Mg^{2+} for 1 min, to quench extracellular CLF signal. Trypan blue was removed, and cells were then washed twice with HBSS plus Ca^{2+}/Mg^{2+}. Automated live-cell image acquisition was performed on a Kinetic Scan Reader from Cellomics (Pittsburgh, PA) using a 20× objective and an XF100 filter, to capture CLF images with a fixed exposure time of 0.7 s. The nuclei were then counter-stained for 10 min with 10μg/ml Hoechst 33342. Fluorescent nuclei images were acquired using the Kinetic Scan Reader using a 20× objective and an XF93 filter, with a fixed exposure time of 0.2 s. The relative amount of CLF signal was quantified by ImagePro from MediaCybernetics.

Hepatobiliary transport in sandwich-cultured human hepatocytes. A detailed protocol for assessing hepatobiliary transport of drugs in sandwich-cultured human hepatocytes was described previously (Bi *et al*., 2006; Kalgutkar *et al*., 2007). Briefly, cryopreserved hepatocytes (lot FEP obtained from Celsis/In Vitro Technologies, Baltimore, MD) were rinsed twice with 0.5 ml of either regular HBSS or Ca^{2+}/Mg^{2+}-free HBSS containing 1mM EGTA and then equilibrated in the same buffers for 10 min at 4°C or 37°C. [^{14}C]CP-724,714 (2μM) in regular HBSS was then added to both sets of cultures. [^{3}H]TC (1μM) was used as a positive control for active hepatic uptake and efflux, and midazolam (2μM) served as a reference compound for passive diffusion into hepatocytes. The protein concentration of cell lysates (lysed with Triton X-100) was determined using a BCA protein assay kit using bovine serum albumin as a standard following manufacturer's instruction. An average concentration of 0.4 mg/ml from multiple studies was used for calculations.

The equations used to calculate apparent uptake clearance ($Uptake_{app}$), apparent intrinsic biliary clearance ($CL_{bile,int,app}$) (Liu *et al*., 1999a), and biliary excretion index (BEI) (BEI was determined using B-CLEAR technology [Qualyst, Inc., Raleigh, NC]) (Liu *et al*., 1999b) over a 15-min interval are shown below. A 15-min interval was selected based on the approximate linear uptake of CP-724,714 in human hepatocyte lot FEP over 2, 10, and 15 min (data not shown). In the presence of Ca^{2+}/Mg^{2+}, the bile canaliculi remain intact (closed), whereas in the absence of Ca^{2+}/Mg^{2+}, the bile canaliculi tight junctions are disrupted (opened). Therefore, quantifying the accumulation of a test compound in the presence and absence of divalent cations allows one to determine the amount of test compound in the bile canaliculi.

$$\mathrm{Uptake}_{app} = \frac{\Delta\mathrm{Accumulation}_{(+Ca^{2+}/Mg^{2+},\,15-2\,\mathrm{min})}}{\Delta T_{(15-2\,\mathrm{min})} \cdot \mathrm{Concentration}_{(\mathrm{media})}}$$

$$CL_{bile,\,int,\,app} = \frac{\mathrm{Accumulation}_{(+Ca^{2+}/Mg^{2+})} - \mathrm{Accumulation}_{(Ca^{2+}/Mg^{2+}-\mathrm{free})}}{\mathrm{Incubation\,Time} \cdot \mathrm{Concentration}_{(\mathrm{media})}}$$

$$\mathrm{BEI} = \frac{\mathrm{Accumulation}_{(+Ca^{2+}/Mg^{2+})} - \mathrm{Accumulation}_{(+Ca^{2+}/Mg^{2+}-\mathrm{free})}}{\mathrm{Accumulation}_{(+Ca^{2+}/Mg^{2+})}} * 100$$

Hepatic OATP transporter inhibition assays. A similar assay condition for three major hepatic OATP substrate assays, OATP1B1, OATP1B3, and OATP2B1, was used as described previously (Kalgutkar *et al*., 2007). Transporter-transfected and wild-type HEK 293 cells were seeded onto 24-well poly-D-lysine–coated plates at a density of ~2.5×10^5 cells per well. After the cells were confluent, the cells were first washed three times with 1 ml uptake buffer. The transporter inhibition assay was initiated by incubation of the cells with 250 μl of probe substrate plus inhibitor for 5 min at 37°C. At the completion of the incubation, uptake was stopped by washing the cells three times with ice-cold buffer. The cells were then lyzed in 1% sodium dodecyl sulfate, and the accumulated radioactivity was determined. The probe substrates for the OATP1B1, OATP2B1, and OATP1B3 assays were 2μM [^{3}H]estradiol 17β-D-glucuronide, 5μM [^{3}H]estrone 3-sulfate, and 1μM [^{3}H]BSP, respectively. The concentration range of CP-724,714 tested in the inhibition assay was 1nM–100μM.

Human MDR1-MDCK, BCRP-MDCK, and MRP2-MDCK transwell assays. Human MDR1, BCRP, and MRP2 transporters transfected on the apical side of MDCK cells were employed in this study to measure A>B Papp and B>A Papp to assess the interactions of CP-724,714 with these transporters. The detailed assay protocols were described in our previous publications (Feng *et al*., 2008; Xiao *et al*., 2006). CP-724,714 concentrations in the MDCK efflux assays were measured using our previously published high-throughput liquid chromatography/mass spectrometry/mass spectrometry methodology (Xiao *et al*., 2006).

Transwell data analysis. The apparent permeability value (P_{app}) was calculated using equation 1 (Feng *et al*., 2008)

$$P_{app} = \frac{1}{\mathrm{Area} \times C_D(0)} \times \frac{dM_r}{dt}, \quad 1$$

where area is the surface area of the cell monolayer (0.0625 cm^2), $C_D(0)$ is the initial concentration of compound applied to the donor chamber, t is time, M_r is the mass of compound appearing in the receiver compartment as a function of time, and dM_r/dt is the flux of the compound across the cell monolayer. The efflux ratio (ER) was calculated using equation 2:

$$\mathrm{ER} = \frac{P_{app,B>A}}{P_{app,A>B}}, \quad 2$$

where A>B and B>A denote transport direction in which P_{app} was determined. The ratio of ratios (RR) was calculated using equation 3:

$$\mathrm{RR} = \frac{\mathrm{ER}_{transfected}}{\mathrm{ER}_{wt}}, \quad 3$$

where $ER_{transfected}$ is the ER determined in the transfected MDCK cells (MDR1-MDCK), and ER_{wt} is the ER determined in WT MDCK cells. The RR_{MDR1} denotes the ER in MDR1-MDCK–transfected cells compared with the ER in WT MDCK.

Calcein AM MDR1 inhibition assay. The similar method was used as our previously disclosed protocol (Feng *et al*., 2008). The amount of efflux by MDR1 in MDR1-MDCK cells was calculated by (MDCK fluorescence − MDR1-MDCK fluorescence) for each treatment. The amount of inhibition was calculated by $(\text{the amount of efflux})_{untreated}$ − $(\text{the amount of efflux})_{treated}$. The percent of inhibition was calculated by $(\text{the amount of inhibition})_{treated}$/$(\text{the amount of efflux})_{untreated}$. Cyclosporin A was used as a positive control, and acyclovir was a negative control. IC_{50}s were determined from the percent of inhibition at each dose, using a data analysis program LabStats Excel Add-in (Nonclinical Statistics group, Pfizer Central Research, Sandwich, Kent, UK).

TC transport inhibition assay using sandwich-cultured human hepatocytes. Inhibition of TC transport in sandwich-cultured human hepatocytes with developed canaliculi was conducted according to the previously described protocol (Kostrubsky *et al*., 2003). Briefly, freshly prepared human hepatocytes were cultured for 72 h under the sandwich configuration in WEM to develop bile canaliculi. [^{3}H]TC (1μM) with increasing concentrations of CP-724,714 was incubated with hepatocytes for 15 min at 37°C. After stopping transport by removing buffer and washing cells, TC efflux from canalicular spaces was initiated by adding standard buffer or Ca/Mg^{2+}-free buffer for 10 min. Removal of Ca/Mg^{2+} from the incubation buffer opens the tight junctions and releases the bile acids that were accumulated therein. Aliquots of media were harvested and counted in a liquid scintillation counter. The difference in the amount of radioactivity between the two buffer conditions in the absence of test compound was defined as a 100% TC efflux into canaliculi. In the presence of an inhibitor, this difference became smaller and was used to calculate the percent inhibition of bile acid efflux. All values were normalized per amount of total cellular protein.

BSEP inhibition assay using membrane vesicles expressing BSEP. Inhibition of bile acids transport via BSEP was studied according to the protocol described previously (Kostrubsky *et al.*, 2006). The assay used recombinant baculovirus-infected Sf9 cell membrane vesicles expressing human BSEP from SOLVO Biotechnology. The assay was performed according to the manufacture's protocol with some modifications using glyburide as a positive control. Specifically, vesicles (50 μg) were added to the assay mix containing 10mM HEPES-Tris (pH 7.4), 0.1M KNO_3, 0.01M $Mg(NO_3)_2$, and 2μM of [^{3}H]TC in the presence or absence of tested drugs and preincubated for 7 min at 37°C. Transport was initiated with 4mM of ATP or AMP (background subtraction), and the mixtures were incubated for another 7 min at 37°C. Reactions were stopped with ice-cold wash buffer (10% 100mM Tris-HCl pH 7.4 and 10% 1M KNO_3). Membrane suspensions were then filtered and washed twice with wash buffer to remove substrate on the outside of the vesicles. After rapid filtration to separate the vesicles from the incubation solution, filters were counted in a scintillation counter. The results were expressed as percent of activity of TC transport in the presence of inhibitors relative to untreated control.

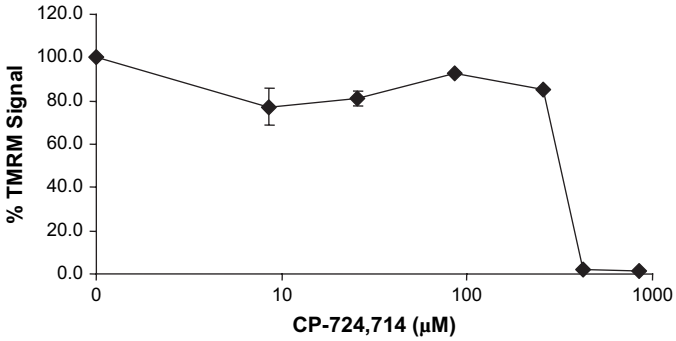

FIG. 2. The effect of CP-724,714 on mitochondrial membrane potential in sandwich-cultured primary human hepatocytes. The fluorescence intensity of TMRM was used to measure mitochondrial membrane potential. The error bars (SD) were used to display the experimental variability of duplicate samples at each concentration of CP-724,714 tested.

RESULTS

Toxicity of CP-724,714 on Primary Cultured Human Hepatocytes

We evaluated the effect of CP-724,714 on human hepatocytes using the recently characterized and validated hepatocyte imaging assay technology (HIAT). TMRM is a mitochondrial membrane potential dye that accumulates in actively respirating mitochondria, and its signal decreases in compromised mitochondria. This system exhibited a true positive rate of 50%–60% and a false-positive rate of <5% toward clinical drug-induced hepatotoxicity (Xu *et al.*, 2008). As shown in Figure 2, CP-724,714 reduced mitochondrial membrane potential, as measured by TMRM intensity, in overnight-treated hepatocyte cultures. The effect occurred precipitously between 255 and 426μM, with the lowest observed adverse effect level at 426μM. This is equivalent to 50-fold of the reported C_{max} of CP-724,714 in patients (4–5μg/ml or 8.5μM) (Munster *et al.*, 2007). The 50-fold C_{max} falls well within the concentration cut-off of 100-fold C_{max} for a "positive" test score for an orally dosed drug, which was previously validated across 300 diverse drug molecules (Xu *et al.*, 2008).

Hepatic Uptake and Excretion of CP-724,714 in Sandwich-Cultured Cryopreserved Human Hepatocytes

As illustrated in Figure 3, the accumulation of [^{14}C]CP-724,714 in hepatocytes occurred rapidly relative to that observed for the positive control, [^{3}H]TC. The apparent uptake clearance ($Uptake_{app}$) for [^{14}C]CP-724,714 was 22μl/min/mg protein, which was nearly twofold of that observed for [^{3}H]TC (12 μl/min/mg protein, Table 1); whereas the apparent intrinsic biliary clearance ($CL_{bile,int,app}$) of CP-724,714 was 5.0 μl/min/mg protein, similar to that of TC (5.2 μl/min/mg protein). Thus the estimated $Uptake_{app}$ of CP-724,714 was approximately four-fold greater than its $CL_{bile,int,app}$. The calculated BEI (or percent of drug in hepatocytes that was excreted into bile) at 15 min for CP-724,714 was low at 13%, compared to 44% for TC.

To further understand the mechanisms of hepatic uptake of CP-724,714 via either passive diffusion or active uptake process, the effect of temperature (4°C vs. 37°C) on the transport of CP-724,714 into hepatocytes was evaluated in this model. TC and midazolam served as reference compounds for active uptake and passive diffusion, respectively (Kalgutkar *et al.*, 2007). As expected, the uptake of midazolam into hepatocytes was independent of the temperature change from 37°C to 4°C, whereas TC accumulation decreased by ~96% at 4°C compared to that at 37°C indicating transporter-mediated uptake of TC (Fig. 4). Under these conditions, the uptake of CP-724,714 into hepatocytes at 4°C decreased by ~40% compared to that at 37°C, suggesting that ~40% of CP-724,714 uptake into hepatocytes is likely mediated through an active

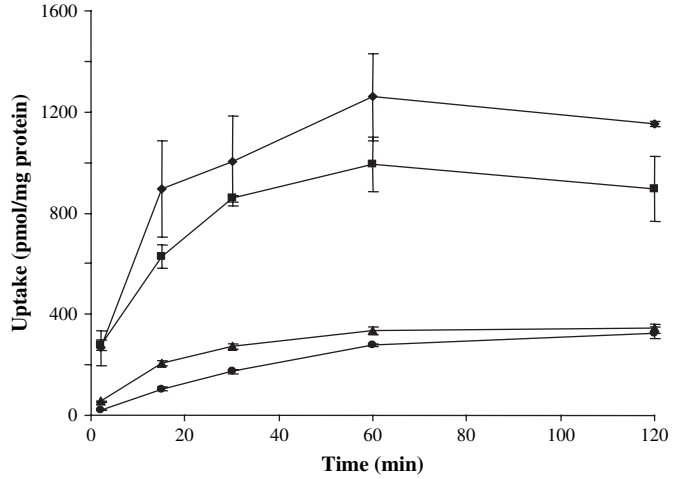

FIG. 3. Accumulation of CP-724,714 with Ca^{2+} (filled diamonds) and without Ca^{2+} (filled squares) in sandwich-cultured cryopreserved human hepatocytes. The assay was conducted using 2μM [^{14}C]CP-724,714, while 1μM [^{3}H]TC with Ca^{2+} (filled triangles) and without Ca^{2+} (filled circles) was used as a positive control. Data are mean ± SD to show the experimental variability of duplicate samples.

TABLE 1
Apparent uptake clearance, intrinsic biliary clearance, and BEI of [^{14}C]CP-724,714 and [^{3}H] TC in sandwich-cultured cryopreserved human hepatocytes

Compound	$Uptake_{app}$ (μl/min/mg protein)	$CL_{bile,int,app}$ (μl/min/mg protein)	BEI
CP-724,714 (2μM)	22 ± 7	5.0 ± 1.9	13 ± 3
TC (1μM)	12	5.2	44

The results for CP-724,714 are presented as the mean ± SD of three separate experiments run in duplicate. The result for TC is presented as the mean of two experiments run in duplicate.

carrier-mediated process, whereas the remaining ~60% of CP-724,714 uptake into hepatocytes is likely by passive diffusion.

Inhibition of CP-724,714 on Major Human Hepatic OATPs Uptake Transporters

CP-724,714 at concentrations between 1nM and 100μM was tested in OATP1B1-, OATP1B3-, or OATP2B1-transfected HEK 293 cells in the presence of their respective probe substrates. CP-724,714 demonstrated a concentration-dependent inhibition of OATP1B1-mediated uptake of its probe substrate, estradiol glucuronide, with an IC_{50} of 1.7μM (Fig. 5), whereas CP-724,714 did not show significant effects on OATP1B3- or OATP2B1-mediated transport (data not shown).

Interaction of CP-724,714 with Major Hepatic Efflux Transporters

The A>B Papp and B>A Papp for CP-724,714 in MDCK, MDR1-MDCK, BCRP-MDCK, and MRP2-MDCK cells are presented in Table 2 along with their calculated ERs, determined by B>A Papp divided by A>B Papp and RR, defined by $ER_{transfected}$ divided by ER_{MDCK}. The ERs for MDCK, MDR1-MDCK, BCRP-MDCK, and MRP2-MDCK cells were 4.0, 10, 9.2, and 2.6, respectively. The RRs for MDR1-MDCK, BCRP-MDCK, and MRP2-MDCK cells were 2.5, 2.3, and 0.65, respectively. RR is used to assess the potential of testing compound to be a substrate of these efflux transporters. A cut-off of 1.7 for RR_{MDR1}, 1.2 for RR_{BCRP}, and 1.5 for RR_{MRP2} has been established to classify compounds as a potential substrate for MDR1, BCRP, or MRP2 transporters, respectively (Feng *et al*., 2008; Xiao *et al*., 2006). Based on the cut-off values, CP-724,714 appeared to be a substrate of MDR1 and BCRP but not of MRP2.

CP-724,714 was tested in the *in vitro* MDR1 inhibition assay at concentrations between 0.3μM and 100μM (Fig. 6), using

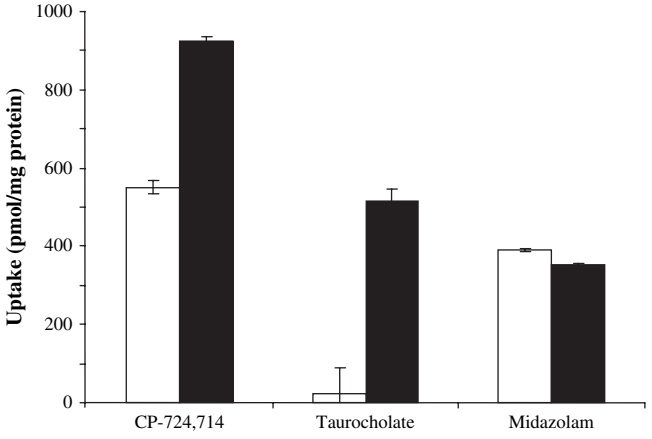

FIG. 4. The effect of temperature on the uptake of CP-724,714 in sandwich-cultured cryopreserved human hepatocytes (Lot FEP). The assay was conducted at 37°C versus 4°C for 10 min with 2μM [^{14}C]CP-724,714. Open bars represent uptake at 4°C, and solid bars represent uptake at 37°C. 1μM TC and 2μM midazolam were used as positive and negative controls, respectively, for active transport. Data are mean ± SD to show the experimental variability of duplicate samples.

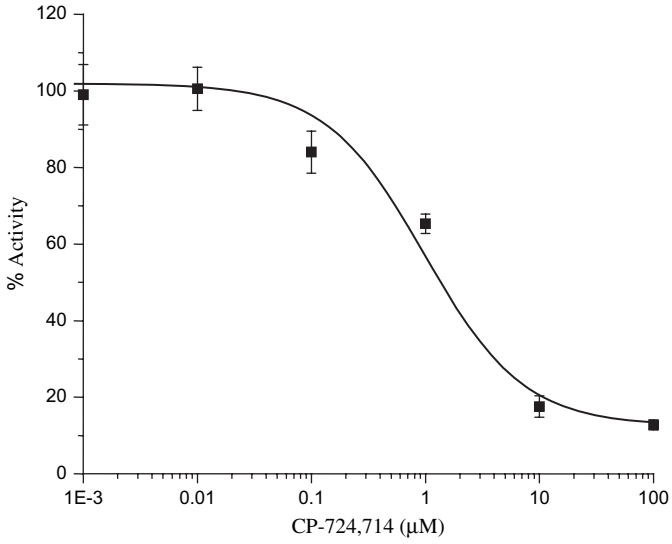

FIG. 5. Inhibition of OATP1B1 by CP-724,714. OATP1B1-transfected HEK cells were used, and the assay was conducted at 37°C for 5 min. The probe substrate for OATP1B1 inhibition assay was 2μM [^{3}H]estradiol 17β-D-glucuronide. Data are mean values ± SD for triplicate samples in 24-well plate. The estimated IC_{50} was 1.7μM.

TABLE 2
CP-724,714 (2μM) interactions with human biliary efflux transporters

	$P_{app} \times 10^{-6}$ (cm/sec) A→B	$P_{app} \times 10^{-6}$ (cm/sec) B→A	Efflux ratio (BA/AB)	Ratio of ratios ($ER_{transfected}$/ER_{MDCK})
Parental MDCK	8.4	34	4.0	NA
MDR1-MDCK	4.2	44	10	2.5
BCRP-MDCK	4.1	38	9.2	2.3
MRP2-MDCK	18	46	2.6	0.65

Results depicted are an average of replicate samples determinations from a single study. These studies were carried out twice with similar results, and one representative study is presented here.

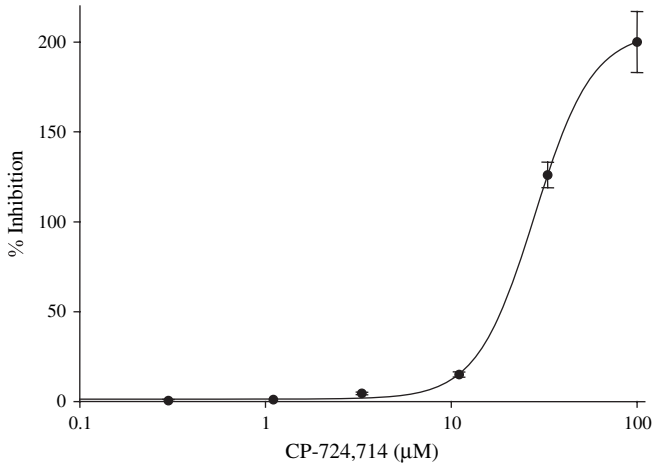

FIG. 6. Inhibition of MDR1 by CP-724,714. The assay was conducted in MDR1-transfected MDCK cells, and 2.5μM Calcein AM was used as an MDR1 substrate. The tested concentrations of CP-724,714 were between 0.3μM and 100μM in duplicate, and the estimated IC_{50} was ~28μM. Data are mean ± SD to show the experimental variability of duplicate samples. Cyclosporin A was used as a positive control and acyclovir as a negative control. The inhibition percentage higher than 100% was due to the inhibition of endogenous efflux transporters in MDR1-MDCK cells.

Calcein AM as a substrate. CP-724,714 inhibited MDR1-mediated transport with an estimated IC_{50} of 28μM.

Inhibition of Bile Salt Transport

The effect of CP-724,714 on bile salt transport was evaluated in three systems. First, CP-724,714 abolished the accumulation of a fluorescent bile acid analog, CLF, in the bile canaliculi space in cryopreserved human hepatocytes, in a concentration-dependent manner with an estimated IC_{50} of ~5μM (Fig. 7). Second, CP-724,714 demonstrated a concentration-dependent inhibition of [^{3}H]TC efflux into the canaliculi in sandwich-cultured fresh human hepatocytes prepared from two different donors (Fig. 8). Finally, inhibition of BSEP-mediated transport of TC was investigated using membrane vesicles expressing human BSEP. CP-724,714 exhibited a concentration-dependent inhibition of TC transport via BSEP with an IC_{50} of ~16μM (Fig. 9), which is consistent to what was observed in human hepatocytes.

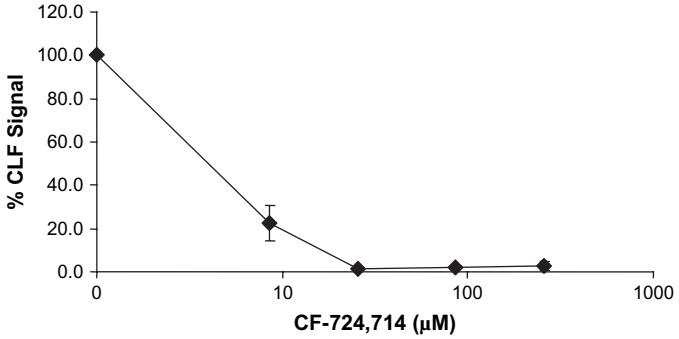

FIG. 7. Inhibition of CLF accumulation in bile canaliculi by CP-724,714 in sandwich-cultured human hepatocytes. Human hepatocytes were incubated with 5μM CLF and increasing concentrations of CP-724,714 for 15 min at 37°C. The accumulation of CLF signal in the bile canalicular space was imaged and quantified. Data are mean ± SD to show the experimental variability of duplicate samples. The estimated IC_{50} was ~5μM.

DISCUSSION

Hepatic toxicity remains one of the leading causes for discontinuation of preclinical and clinical candidates in drug development. CP-724,714 displayed unexpected dose-limiting toxicities including reversible cholestatic liver dysfunction with elevated ALT, AST, and/or GGT with or without HB during

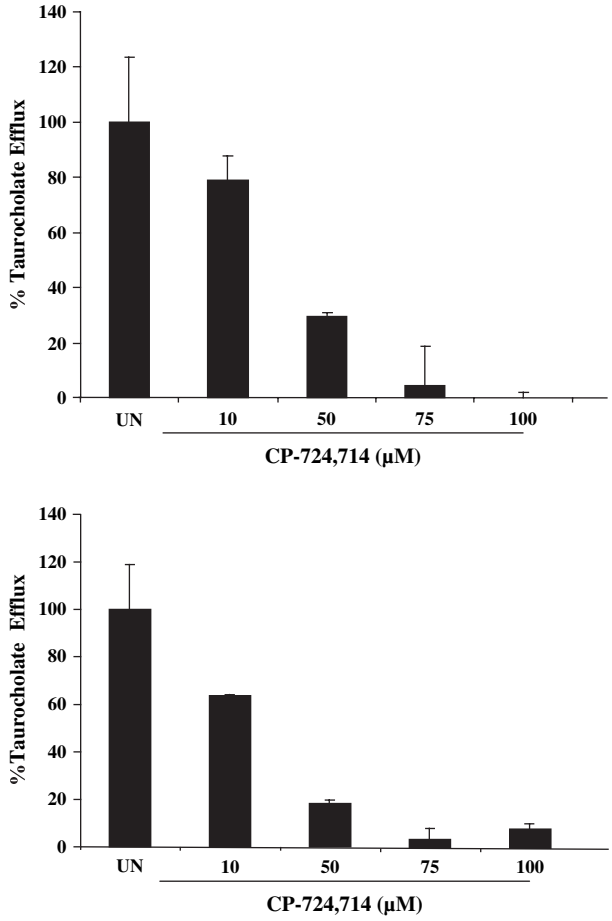

FIG. 8. Inhibition of TC efflux by CP-724,714 in sandwich-cultured human hepatocytes. Fresh human hepatocytes were incubated with 1μM [^{3}H]TC in the presence of increasing concentrations of CP-724,714 for 15 min at 37°C. After stopping transport by removing buffer and washing cells, TC efflux from canalicular spaces was initiated by adding standard or Ca/Mg^{2+}-free buffer for 10 min. Aliquots of media were harvested and counted in a liquid scintillation counter. Each value represents the mean of duplicate treatments of hepatocytes with the range indicated by the vertical bars. The two figures represent two studies conducted with cells prepared from two donors. UN, untreated cells.

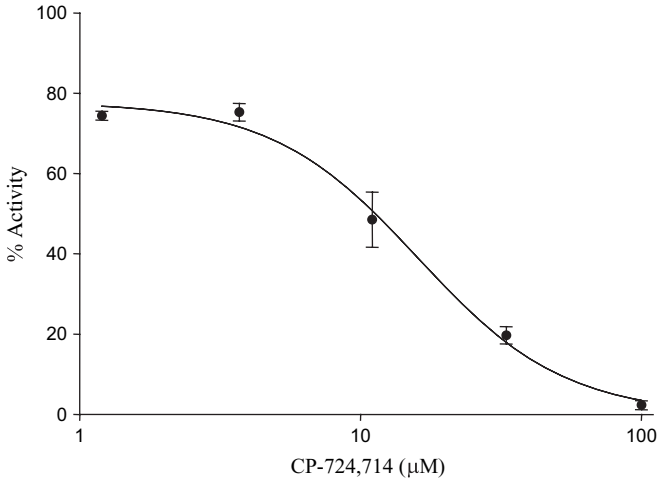

FIG. 9. CP-724,714–mediated inhibition of TC uptake by BSEP. The assay used membrane vesicle expressing BSEP. CP-724,714 was tested at 0, 1.2, 3.7, 11, 33, and 100μM, while [^{3}H]TC was used at 2μM. Data are mean values ± SD to show the experimental variability of duplicate samples. Estimated IC_{50} was 16μM. Glyburide was employed as a positive control in the BSEP vesicle inhibition assay and its IC_{50} was 2μM, as expected.

early dose escalation clinical trials. As the hepatotoxicity in patients significantly correlated to the steady state exposures of CP-724,714 and CP-724,714 remained to be the major analyte in patient plasma with HB, we hypothesized that the parent molecule likely played the key role in the observed hepatotoxicity. Thus, we first tested the direct cytotoxic effect of CP-724,714 on primary cultured human hepatocytes by employing a recently validated *in vitro* HIAT system (Xu *et al.*, 2008). CP-724,714 exhibited a concentration-dependent reduction and achieved complete inhibition of mitochondrial membrane potential in human hepatocytes at 426μM (50-fold C_{max}). This is well within the 100-fold C_{max} for a physiologically relevant cut-off established during the previous validation study, although it is worth to note that CP-724,714 was not part of the 300 compounds employed during the HIAT system validation. The results have suggested that a direct damage to mitochondria may have contributed to CP-724,714–induced hepatotoxicity especially since hepatic accumulation of CP-724,714 is likely due to its active uptake into hepatocytes and profound effect on efflux transporters.

The uptake of CP-724,714 into hepatocytes is partially due to active transport and likely via OATP1B1 as supported by the evidence that a more than twofold increase of CP-724,714 uptake was observed in OATP1B1-transfected HEK 293 cells over nontransfected HEK 293 cells at 1μM and 10μM of CP-724,714. No significant uptake was seen in OATP1B3- or OATP2B1-HEK 293 cells under the same experimental conditions. Therefore, it is likely that OATP1B1 in conjunction with MDR1 and BCRP mediates the carrier-mediated vectorial transport of CP-724,714 from portal vein to bile, which plays a key role in controlling the hepatocellular exposure and hepatobiliary excretion of CP-724,714 in humans.

The disposition of bilirubin and bile salts via hepatocellular transport has been well studied and reported extensively. Interruption of these processes can result in clinically significant HB and lead to cholestatic liver damage (Pauli-Magnus and Meier, 2006). Specifically, unconjugated bilirubin is removed from systemic circulation into hepatocytes in part via liver-specific OATP1B1 (Cui *et al.*, 2001). The bilirubin is then conjugated with glucuronide acids by a specific bilirubin-UDP-glucuronosyltransferase (UGT1A1) to form bilirubin-glucuronides and subsequently eliminated into bile via the biliary transporter MRP2 (Keitel *et al.*, 2000). Inhibition of OATP1B1 could potentially increase the levels of unconjugated bilirubin in the systemic circulation (Campbell *et al.*, 2004). Likewise, mutation or inhibition of MRP2 could result in elevated conjugated bilirubin concentration in hepatocytes (Büchler *et al.*, 1996), followed by eliminating conjugated bilirubin back to the circulation. Although CP-724,714 inhibits human OATP1B1 with an IC_{50} of 1.7μM, since it is highly bound to plasma proteins (>98%), the predicted maximal unbound concentration of CP-724,714 in human portal blood (0.2–0.3μM) (Yamano *et al.*, 2001) based on the reported human pharmacokinetics (C_{max} = 4–5 μg/ml; ka = 0.725/h) (Guo *et al.*, 2008) would be substantially lower than the estimated IC_{50}. Therefore, the chance of CP-724,714–induced HB via OATP1B1 inhibition is unlikely, which is consistent with the clinical observations that mostly conjugated bilirubin increased in patients.

As previously reported, compounds having a combination of the following factors have a greater potential for clinical hepatoxicity: (1) high molecular weight with expected biliary excretion of parent and metabolites, (2) high administrated doses with observed plasma concentration at >1 μg/ml, and (3) a concentration-dependent inhibition of bile acid transport *in vitro* (Kostrubsky *et al.*, 2006). CP-724,714 was rapidly absorbed in animals and humans following oral administration. In dogs, about 42% of the radioactivity was recovered in bile following oral administration of [^{14}C]CP-724,714, suggesting that hepatobiliary excretion represents the primary route of drug elimination. CP-724,714 was rapidly taken up into human hepatocytes and was predicted to be cleared into human bile in part via efflux transporters, MDR1 and BCRP. It has been reported that hepatotoxicity of xenobiotics is significantly enhanced in animals with obstructed bile flow (Klaassen, 1973). CP-724,714 demonstrated a concentration-dependent inhibition of CLF, a fluorescent bile acid analog, accumulation in bile canaliculi. In addition, the efflux of taurocholic acid into canaliculi was inhibited in fresh cultured human hepatocytes. Finally, the direct inhibitory effect of CP-724,714 on BSEP-mediated transport was confirmed in BSEP-expressing membrane vesicles with an IC_{50} of 16μM. Inhibition of BSEP has been well studied and reported to cause cholestasis with the increase of potentially harmful bile salts and/or bile acid in hepatocytes, leading to hepatic damage and HB in the clinic (Hofmann 2007; Thompson and Strautnieks, 2001; Vallejo

et al., 2006). This mechanism responsible for clinical hepatotoxicity of a number of therapeutics via inhibition of BSEP was previously investigated (Kostrubsky *et al*., 2003, 2006, 2007). Our results have collectively demonstrated that CP-724,714 inhibited bile excretion in three different *in vitro* systems.

Although the determined IC_{50}s for inhibition of bile salt (via BSEP) and CP-724,714 (*via* MDR1) excretion into bile are relatively higher than the observed systemic exposures of CP-724,714 in patients, significant hepatic accumulation of CP-724,714 is highly likely. In sandwich-cultured human hepatocytes, CP-724,714 was rapidly taken up into the hepatocytes with an overall mean apparent uptake clearance approximately fourfold higher than efflux clearance into bile, suggesting that CP-724,714 may accumulate within hepatocytes and result in an intracellular concentration much higher than portal concentration. Consistent with this potential for hepatic accumulation, a whole-body autoradioluminography study in rats indicated rapid distribution of [^{14}C]CP-724,714 into extravascular tissues with a liver/blood ratio of 3.8 based on the total area under the curve following a single oral dose administration. Additionally, the unbound fraction of CP-724,714 in human liver microsomes (~40%) is >20-fold of that in human plasma (<2%). Therefore, it is highly likely that CP-724,714 can achieve a much higher unbound concentration in the liver than portal exposures to inhibit the efflux of CP-724,714 and bile acids via MDR1 and BSEP efflux transporters, respectively, resulting in progressively increasing concentrations of CP-724,714 in hepatocytes with HB and hepatic damage (Byrne *et al*., 2002; Jansen *et al*., 1999; Keitel *et al*., 2000).

In summary, CP-724,714 has the propensity to decrease mitochondrial membrane potential in primary cultured human hepatocytes at elevated concentrations *in vitro* and lead to direct hepatocellular injury. In addition, CP-724,714 demonstrated uptake clearance approximately fourfold greater than its efflux clearance into bile. Finally, CP-724,714 inhibited MDR1 and BSEP transporters that contribute to CP-724,714 and bile acid elimination. Therefore, the clinically observed CP-724,714–induced dose-dependant hepatotoxicity in patients could be the result of active hepatic uptake combined with an inhibition of human hepatobiliary efflux transporters, leading to progressive hepatic accumulation of drug and bile salts, and liver damage. These studies highlighted the advantage of using a combination of *in vitro* molecular and cellular test systems helping to understand the clinical observation, thus paving the way for the ultimate goal of minimizing human drug toxicity.

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