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Sorafenib Hepatobiliary Disposition: Mechanisms of Hepatic Uptake and Disposition of Generated Metabolites

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ABSTRACT

Sorafenib is an orally active tyrosine kinase inhibitor used in the treatment of renal and hepatocellular carcinoma. This study was designed to establish whether transport proteins are involved in the hepatic uptake of sorafenib and to determine the extent of biliary excretion of sorafenib and its metabolites in human hepatocytes. Initial uptake was assessed in freshly isolated, suspended human hepatocytes in the presence of inhibitors and modulators. [^{14}C]Sorafenib (1 μM) uptake at 4°C was reduced by about 61–63% of the uptake at 37°C, suggesting a high degree of passive diffusion. Hepatocyte uptake of [^{14}C]sorafenib was not Na^+ dependent or influenced by the organic anion transporter 2 inhibitor ketoprofen. However, initial [^{14}C]sorafenib hepatocyte uptake was reduced by 46 and 30% compared with control values in the presence of the organic anion transporting polypeptide inhibitor rifamycin SV and the organic cation transporter (OCT) inhibitor decynium 22, respectively. [^{14}C]Sorafenib (0.5–5 μM) uptake was significantly higher in hOCT1-transfected Chinese hamster ovary cells compared with mock cells, and inhibited by the general OCT inhibitor, 1-methyl-4-phenylpryidinium. OCT1-mediated uptake was saturable with a Michaelis-Menten constant of 3.80 ± 2.53 μM and a V_{max} of 116 ± 42 pmol/mg/min. The biliary excretion index and in vitro biliary clearance of sorafenib (1 μM) in sandwich-cultured human hepatocytes were low (~11% and 11 ml/min/kg, respectively). Results suggest that sorafenib uptake in human hepatocytes occurs via passive diffusion, by OCT1, and by organic anion transporting polypeptide(s). Sorafenib undergoes modest biliary excretion, predominantly as a glucuronide conjugate(s).

Introduction

Sorafenib (Fig. 1), an orally active multikinase inhibitor, blocks tumor cell proliferation by targeting Raf/mitogen activated protein kinase/extracellular signal-regulated kinase and exerts an antiangiogenic effect by targeting vascular endothelial growth factor receptor-1/-2/-3 and platelet-derived growth factor receptor-β tyrosine kinases (Wilhelm et al., 2004). Sorafenib is approved for the treatment of renal and hepatocellular carcinomas and has demonstrated activity toward other malignancies (Ratain et al., 2006; Miller et al., 2009).

After oral administration of [^{14}C]sorafenib to healthy volunteers, approximately 77% of a 100-mg oral dose was excreted in feces (51% as parent), and 19% of the dose was excreted in urine as glucuronidated metabolites; approximately 17% of circulating radioactivity in plasma was in the form of sorafenib N-oxide (Lathia et al., 2006). Sorafenib oxidative metabolism is mediated by CYP3A4 yielding the N-oxide sorafenib metabolite. Sorafenib also undergoes glucuronidation by the uridine diphosphate-glucuronosyl-transferase (UGT1A9) pathway (Fig. 1) (Lathia et al., 2006). Peak plasma concentrations of sorafenib occur within 2 to 3 hours after a single oral dose (Strumberg et al., 2005); secondary peaks in the plasma concentration-time profile have been attributed to enterohepatic recirculation of sorafenib after cleavage of the glucuronide conjugate or reduction of the N-oxide in the gastrointestinal tract (Lathia et al., 2006). High interpatient variability in the C_{max} and the area under the concentration-time profile (AUC) in human plasma of sorafenib and the primary metabolite, sorafenib N-oxide have been reported after multiple oral doses of sorafenib (Strumberg et al., 2007; Miller et al., 2009). Variability in pharmacokinetics can be caused by interindividual differences in the metabolizing enzymes or the transport proteins, which also are subject to polymorphisms. Understanding the mechanisms of hepatic uptake and the extent of biliary excretion of sorafenib is particularly important in patients with unresectable hepatocellular carcinoma, where the target site of sorafenib is the liver.

Transport proteins can play an important role in the clearance of drugs from hepatic sinusoidal blood and the excretion of the parent drug and/or metabolite(s) across the apical membrane into the bile

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ABBREVIATIONS: AUC, area under the concentration-time profile; BCA, bicinchoninic acid; BCRP, breast cancer resistance protein; BEI, biliary excretion index; CHO, Chinese hamster ovary cells; $Cl_{biliary}$, in vitro biliary clearance; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; K_m, Michaelis-Menten constant; MPP^+, 1-methyl-4-phenylpryidinium; MRP2, multidrug resistance-associated protein 2; NTCP, Na^+-taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; P-gp, P-glycoprotein; TEA, tetraethylammonium.

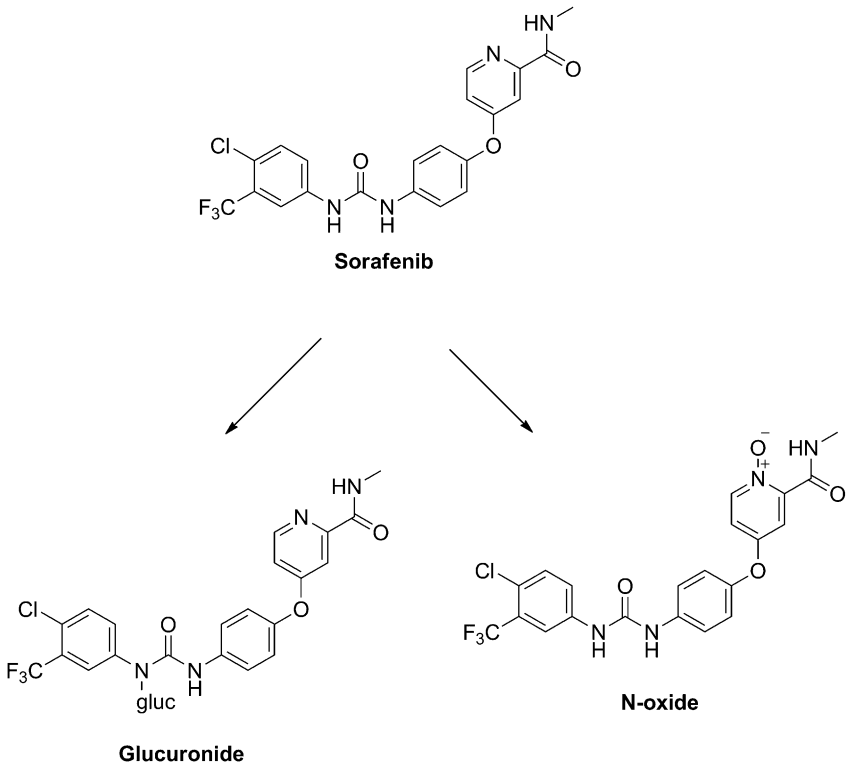

Fig. 1. Chemical structure of sorafenib and metabolic pathways.

canaliculus. The basolateral proteins that mediate the uptake of endogenous and exogenous compounds into hepatocytes include members of the solute carrier superfamily: Na^+-taurocholate cotransporting polypeptide (NTCP), organic anion transporters (OATs), organic cation transporters (OCTs), and organic anion transporting polypeptides (OATPs). NTCP, which is expressed exclusively in the liver, is Na^+ dependent and predominately accounts for the uptake of bile acids (Ho et al., 2004). Recently, a few drugs, such as rosuvastatin, have been reported to be NTCP substrates (Ho et al., 2006). The OATPs exhibit broad and overlapping substrate specificity and display an affinity for organic anions as well as some bulky cations and neutral steroids (Mikkaichi et al., 2004). In contrast to NTCP, the OATPs operate in an Na^+-independent manner and function as bidirectional carriers (Li et al., 2000; Briz et al., 2006; Mahagita et al., 2007). Three human isoforms— OATP1B1, 1B3, and 2B1—play a substantial role in the hepatic uptake of many endogenous and exogenous compounds, including bilirubin (Konig et al., 2000; Cui et al., 2001), fexofenadine (Cvetkovic et al., 1999), and many statins (Shitara and Sugiyama, 2006). OCTs are electrogenic uniporters that mediate primarily the transport of small cations, although the transport of anions and uncharged compounds has been reported (Koepsell et al., 2003). The OATs constitute a family of proteins that mediate transport of negatively charged endogenous and exogenous compounds in exchange for dicarboxylate ions. OATs are expressed predominantly in the kidney, although OAT2 has higher expression on the sinusoidal membrane of hepatocytes compared with the basolateral membrane of proximal kidney tubules (Sun et al., 2001). Transporters located in the apical membrane involved in the removal of drug and metabolite(s) into the bile canaliculus include P-glycoprotein (P-gp), multidrug resistance–associated protein (MRP2), breast cancer resistance protein (BCRP), and the bile salt export pump. Sorafenib is a weak substrate for P-gp, but the overall effect of this transporter on plasma concentrations is low based on in vivo results using mdr1a/1b(-/-) knockout mice (Gnoth et al., 2010).

The objective of the present study was to determine whether transport proteins are involved in the hepatic uptake of sorafenib and to determine the extent of biliary excretion of sorafenib and its metabolites in human hepatocytes. Initial uptake was assessed in freshly isolated human hepatocytes and in OCT1-transfected Chinese hamster ovary (CHO) cells. Sandwich-cultured human hepatocytes were used to determine the hepatobiliary disposition of sorafenib and metabolites.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), F-12 Nutrient Mixture, minimum essential medium nonessential amino acids solution (100×), L-glutamine, penicillin G-streptomycin solution (100×), gentamicin, and penicillin G-streptomycin-amphotericin B solution (100×) were purchased from Invitrogen (Carlsbad, CA). Rifamycin SV, ketoprofen, dexamethasone, TEA, 1,1-dimethyl-biguanide hydrochloride (metformin), fetal bovine serum, Triton X-100, HEPES, D-glucose, dexamethasone, trypsin-EDTA solution (1×), and Hanks' balanced salt solution (HBSS) modified with (H-1387) or without (H-4891) calcium chloride were obtained from Sigma-Aldrich (St. Louis, MO). Sorafenib tosylate (purity 99.7%), [^{14}C]sorafenib (3.09 MBq/mg radiochemical purity 99.2%), the internal standard [$^2H_3$, ^{15}N] sorafenib tosylate, and decynium 22 were provided by Bayer HealthCare AG (Wuppertal, Germany). [^{14}C]TEA (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{14}C]Biguanide (metformin, 110 mCi/mmol) was purchased from Moraveck Biochemicals (Brea, CA). ITS+ (insulin/transferrin/selenium) culture supplement was purchased from BD Biosciences Discovery Labware (Bedford, MA). Bicinchoninic acid (BCA) protein assay reagents and bovine serum albumin for the protein assay standard were purchased from Pierce Chemical Co. (Rockford, IL). Ultima Gold XR scintillation cocktail was purchased from PerkinElmer Life Sciences (Boston, MA). All other chemicals and reagents were of analytical grade and available from commercial sources.

Suspended Hepatocyte Isolation and Uptake Studies. CellzDirect, Life Technologies (Research Triangle Park, NC), kindly provided freshly isolated human hepatocytes in suspension. Hepatocyte donors were a 60-year-old Caucasian woman and a 63-year-old Caucasian man with no recent history of smoking or alcohol use. Hepatocyte viability, as determined by trypan blue exclusion, was 89 and 90%. Cells were suspended in cold modified Hank's

buffer with 10 mM Tris/5 mM glucose (pH 7.4) or Na^+-free choline buffer (10 mM Tris, 5 mM glucose, 5.4 mM KCl, 1.8mM $CaCl_2$, 0.9 mM $MgSO_4$, 10 mM HEPES, and 137 mM choline; pH 7.4) and stored on ice before uptake studies were performed (Leslie et al., 2007). Isolated hepatocytes were suspended in the same buffer (1×10^6 cells/ml), placed on ice, and used immediately in experiments. Hepatocyte suspensions (1 ml; $n = 2$ livers, in triplicate) were preincubated in 16 × 100-mm glass test tubes at 37°C for 3 minutes; 0.1% dimethysulfoxide or chemical inhibitor was added 1 minute before [^{14}C]sorafenib (0.9 μM; 3.86 nCi; 0.9% methanol). The following concentrations of inhibitors were selected based on reported affinities for the given active transport processes: 20 μM rifamycin SV (OATP1B1, OATP1B3, and OATP2B1 inhibitor), 5 μM decynium 22 (OCT inhibitor), and 10 μM ketoprofen (OAT2 inhibitor). Aliquots (100 μl) of the suspension were removed at timed intervals (up to 2.5 minutes), placed in 0.4-ml polyethylene tubes, and centrifuged immediately through a top layer of silicone oil:mineral oil (82:18, v/v; 100 μl) into a bottom layer of 3 M KOH (50 μl). [^{14}C]Sorafenib in the cell pellet and supernatant were analyzed by liquid scintillation counting. Adherent fluid volume was estimated with [^{14}C]inulin as described previously (Baur et al., 1975). Protein concentrations for individual hepatocyte suspensions were determined with the BCA protein assay reagent kit (Pierce) as instructed by the manufacturer. Bovine serum albumin, as supplied by the manufacturer, was used as a standard (0.2–1 mg/ml).

Transport Studies in hOCT1-Expressing CHO Cells. Transport studies were carried out 5 days postseeding, as previously described (Ming et al., 2009). Briefly, stably transfected CHO cells were grown as monolayers in 24-well plates, and the medium was changed every other day. Cells were preincubated for 30 minutes at 37°C in transport buffer (HBSS with calcium chloride, 25 mM D-glucose, and 10 mM HEPES, pH 7.4). Experiments were initiated by replacement of the transport buffer with 0.4 ml of varying amounts of radiolabeled dose solutions in transport buffer. Initially, time-dependent experiments were conducted for up to 30 minutes to determine the linear uptake range (unpublished data). For concentration-dependent experiments, uptake was determined in the mock cells or CHO-OCT1 cells over a 10-minute period. Inhibition of OCT1-mediated uptake was performed in mock or CHO-OCT1 cells by concomitantly incubating 500 μM MPP^+ (1-methyl-4-phenylpryidinium) with the substrate [^{14}C]sorafenib. After incubation, dose solutions were aspirated and cells were washed four times with 4°C transport buffer. Cells were lysed with 500 μl of 0.1 N NaOH/0.1% SDS for 4 hours on an orbital shaker, and samples were analyzed by liquid scintillation counting. Data were normalized to protein concentration in each well, determined in duplicate aliquots using BCA protein assay reagent kit, as detailed above.

For estimation of Michaelis-Menten (K_m) parameters, OCT1-mediated uptake was determined as the difference in cell associated radioactivity in the hOCT1-transfected and mock cells at each substrate concentration. The K_m and V_{max} values were obtained by fitting the Michaelis-Menten equation $V = V_{max} \bullet [S]/(K_m + [S])$ to the data using WinNonlin v.5.2.1 (Pharsight, Mountain View, CA), where V represents the velocity of substrate transport, $[S]$ refers to the concentration of substrate, and K_m is defined as the concentration of substrate at the half-maximal transport rate (V_{max}).

Sandwich-Cultured Human Hepatocyte Studies. B-CLEAR-Human kits were purchased from Qualyst, Inc. (Research Triangle Park, NC). Human hepatocytes isolated from two different subjects (Table 1) were seeded at approximately 1.75×10^6 cells/well on six-well BioCoat plates in DMEM without phenol red supplemented with 2 mM L-glutamine, 1% (v/v) minimum essential medium nonessential amino acids, 100 units penicillin G sodium, 100 μg streptomycin sulfate, 1 μM dexamethasone, 5% (v/v) fetal bovine serum, and 10 μM insulin (day 0 of culture) and allowed to attach for 2–6 hours in a humidified incubator (95% O_2, 5% CO_2) at 37°C. After cell attachment, culture plates were swirled gently, and the culture medium was replaced with the same medium. Cells were overlaid 16–24 hours (day 1 of culture) after seeding with ice-cold Matrigel basement membrane matrix (0.25 mg/ml) in 2 ml/well cold serum-free DMEM containing 2 mM L-glutamine, 1% (v/v) minimum Eagle's medium nonessential amino acids, 100 units penicillin G sodium, 100 μg streptomycin sulfate, 1 μM dexamethasone, and 1% (v/v) ITS+ (insulin/transferrin/selenium). The culture medium was changed every 24 hours until experiments were performed on day 7 of culture.

Accumulation Studies. The method to determine substrate accumulation in sandwich-cultured hepatocytes has been described previously (Leslie et al., 2007; Wolf et al., 2008). Cells were incubated for 20 minutes at 37°C with 1.5 ml of sorafenib solution (1 and 10 μM). Medium samples were collected immediately, and hepatocytes were rinsed vigorously three times with 2 ml of ice-cold standard buffer after the incubation. Substrate uptake was corrected for nonspecific binding by subtracting uptake on blank six-well Biocoat plates overlaid with Matrigel. Data were normalized to protein concentration in each well, determined in duplicate with the BCA protein assay reagent kit. Because of incompatability of the protein assay with organic solvent, the average protein concentration for standard HBSS or Ca^{2+}-free HBSS incubations in the same liver preparation was used to normalize sorafenib content. Sorafenib-treated hepatocytes were stored immediately at −80°C until analysis. The cells were lysed with 1 ml of mobile phase containing internal standard, scraped off the plates and centrifuged at 10,000 × g for 5 minutes before analysis by liquid chromatography coupled with tandem mass spectrometry.

Sample Analysis. Sorafenib and sorafenib N-oxide concentrations were determined by a liquid chromatography coupled with tandem mass spectrometry assay using a LTQ Orbitrap XL (Thermo Scientific, Bremen, Germany) coupled to an Agilent 1200 system (Agilent Technology, Waldbronn, Germany). Sorafenib and its metabolites were eluted from a Synergi Hydro RP 2.5-μm column (20 × 2 mm internal diameter; Phenomenex, Torrance, CA) using a mobile phase gradient at a flow rate of 0.3 ml/min (A: 0.05% formic acid in water; B: 0.05% formic acid in acetonitrile); 0 minutes 30% B, 5 minutes 60% B, 5.3 minutes 30% B. The column effluent was monitored using a LTQ Orbitrap XL (Thermo Scientific) by quantification of the exact mass of sorafenib, internal standard, sorafenib N-oxide, and sorafenib glucuronide. The calibration ranged from 1 ng/ml to 1000 ng/ml. The lower limit of quantification for sorafenib was 2 ng/ml and 1 ng/ml for sorafenib N-oxide.

Data Analysis. For accumulation studies in sandwich-cultured hepatocytes, the biliary excretion index (BEI, %) and in vitro biliary clearance (in vitro $Cl_{biliary}$) were calculated using B-CLEAR technology [Qualyst, Inc.; (Liu et al., 1999)]:

$$\text{BEI} = \frac{\text{Accumulation}_{\text{Cells+Bile}} - \text{Accumulation}_{\text{Cells}}}{\text{Accumulation}_{\text{Cells+Bile}}} \times 100$$

where substrate accumulation in the cells+bile compartments was determined in hepatocytes preincubated in standard buffer; cellular accumulation of substrate was determined in hepatocytes preincubated in Ca^{2+}-free HBSS.

$$\text{In Vitro } Cl_{\text{biliary}} = \frac{\text{Accumulation}_{\text{Cells+Bile}} - \text{Accumulation}_{\text{Cells}}}{AUC_{0-T}}$$

where $AUC_{0\text{-}T}$ was calculated using the log trapezoidal method; the theoretical dosing concentration was used for t = 0 and the final medium concentration for t = incubation time. In vitro $Cl_{biliary}$ values were scaled per kilogram of body weight using 0.948 (liver 1) and 1.35 (liver 2) mg of protein per well, assuming the following: 1 mg protein/1.75×10^6 cells, 107×10^6 hepatocytes per gram of human liver tissue, and 25.7 g of liver tissue per kg of body weight, as previously described (Davies and Morris, 1993).

Statistically significant differences in sorafenib uptake in transfected CHO cells were determined by a two-way analysis of variance followed by the Bonferroni post hoc test. The criterion for significance in all cases was $P < 0.05$.

TABLE 1

Demographics, BEI, and $Cl_{biliary}$ of [^{3}H]taurocholate in sandwich-cultured human hepatocytes

Donors had no history of tobacco or alcohol use or comedications; body mass index (BMI); sandwich-cultured hepatocytes were incubated with 1 μM [^{3}H]taurocholate (10 minutes). Results are presented as representative data from triplicate determinations in two livers.

Liver Donor Identification	Age	Gender	Race	BMI	Taurocholate	
					BEI %	In Vitro $Cl_{biliary}$
	yr			*kg/m^2*		*ml/min/kg*
Liver 1	44	Female	Caucasian	24	64.8	59.9
Liver 2	48	Female	Caucasian	21.7	62.6	32.4

Results

Uptake of Sorafenib in Suspended Human Hepatocytes. Initial uptake of [^{14}C]sorafenib into suspended human hepatocytes was linear up to about 1.5 minutes (Fig. 2, A and B). Uptake at 4°C was reduced by about 61–63% of the uptake at 37°C (Fig. 2, A–D). [^{14}C]Sorafenib uptake at all the time points sampled (Fig. 2, C and D) did not exhibit sodium dependence (average [^{14}C]sorafenib uptake was about 4, 13, and 14% greater than control values when sodium was replaced with choline in the uptake buffer at 0.5, 1.5, and 2.5 minutes, respectively), and it was not influenced significantly by ketoprofen (uptake was decreased by only 4% compared with control uptake). Initial [^{14}C]sorafenib uptake was reduced by 26, 46, and 42% of control values in the presence of the OATP inhibitor rifamycin SV at 0.5, 1.5, and 2.5 minutes, respectively, and reduced by 25, 30, and 39% of control values in the presence of the OCT inhibitor decynium 22 at 0.5, 1.5, and 2.5 minutes, respectively (Fig. 2, C and D; 2.5-minute data not shown).

Transport of Sorafenib in OCT1-Transfected CHO Cells. As expected, uptake of 10 μM [^{14}C]metformin (unpublished data) and 5 μM [^{14}C]TEA in OCT1-transfected CHO cells was increased around 7-fold compared with mock cells, confirming OCT1 function in these cells (Fig. 3A). The uptake of 5 μM [^{14}C]TEA was completely abated in the presence of 500 μM MPP^+ (Fig. 3A).

[^{14}C]Sorafenib uptake was significantly higher in OCT1-transfected CHO cells compared with mock cells over the concentration range examined (0.5–5 μM). OCT1-mediated uptake was saturable with a K_m of 3.80 ± 2.53 μM and V_{max} of 116 ± 42 pmol/mg/min. The OCT inhibitor, MPP^+, decreased [^{14}C]sorafenib uptake in OCT1-transfected and mock CHO cells, suggesting that other MPP^+ sensitive transport processes are involved in sorafenib uptake (Fig. 3B). MPP^+ reduced [^{14}C]sorafenib uptake in mock cells by ~49–63%, suggesting that passive diffusion plays a role in sorafenib uptake, consistent with data generated in suspended human hepatocytes at 4°C.

Hepatobiliary Dispostion of Sorafenib in Human Sandwich-Cultured Hepatocytes. The hepatobiliary disposition of [^{3}H]taurocholate and sorafenib was measured in human sandwich-cultured hepatocytes. After a 10-minute incubation with 1 μM [^{3}H]taurocholate, the BEI and in vitro $Cl_{biliary}$ for both livers (Table 1) were consistent with previous data generated in this model system. The cellular accumulation of sorafenib appeared to be dose dependent (Table 2). Sorafenib cellular accumulation was approximately 2 orders of magnitude greater than the primary metabolite sorafenib N-oxide after a 20-minute incubation at the 1 μM sorafenib dose, and greater than 1 order of magnitude at the 10 μM sorafenib dose (Table 2).

The BEI of sorafenib in sandwich-cultured human hepatocytes was low (~11%). The sorafenib in vitro $Cl_{biliary}$ was moderately low at 1 and 10 μM sorafenib (~11 ml/min/kg), ranging from approximately one-third to one-fifth of the taurocholate in vitro $Cl_{biliary}$ values in each of the liver donors (Tables 1 and 2). After a 20-minute incubation with either 1 or 10 μM sorafenib, sorafenib N-oxide concentrations were below the detection limit (<1 ng/ml) in medium, except for the 10 μM dose in hepatocytes prepared from the second liver; however, longer incubation times of 60 and 120 minutes resulted in slightly higher medium concentrations of sorafenib N-oxide (Fig. 4). The BEI of sorafenib glucuronide at the 1 μM dose was negligible for both liver donors at 20 minutes; sorafenib glucuronide was detected in medium at all the time points and increased with the longer incubation time. The biliary excretion of sorafenib glucuronide increased with incubation time (BEI = 0, 42, and 40% at 20, 60, and 120 minutes, respectively) (Fig. 4).

Discussion

Hepatic transport proteins are recognized increasingly as important determinants of the pharmacokinetics of many drugs, as well as key sites of drug-drug interactions (Soars et al., 2009). Genetic polymorphisms of uptake transport proteins also have been implicated in

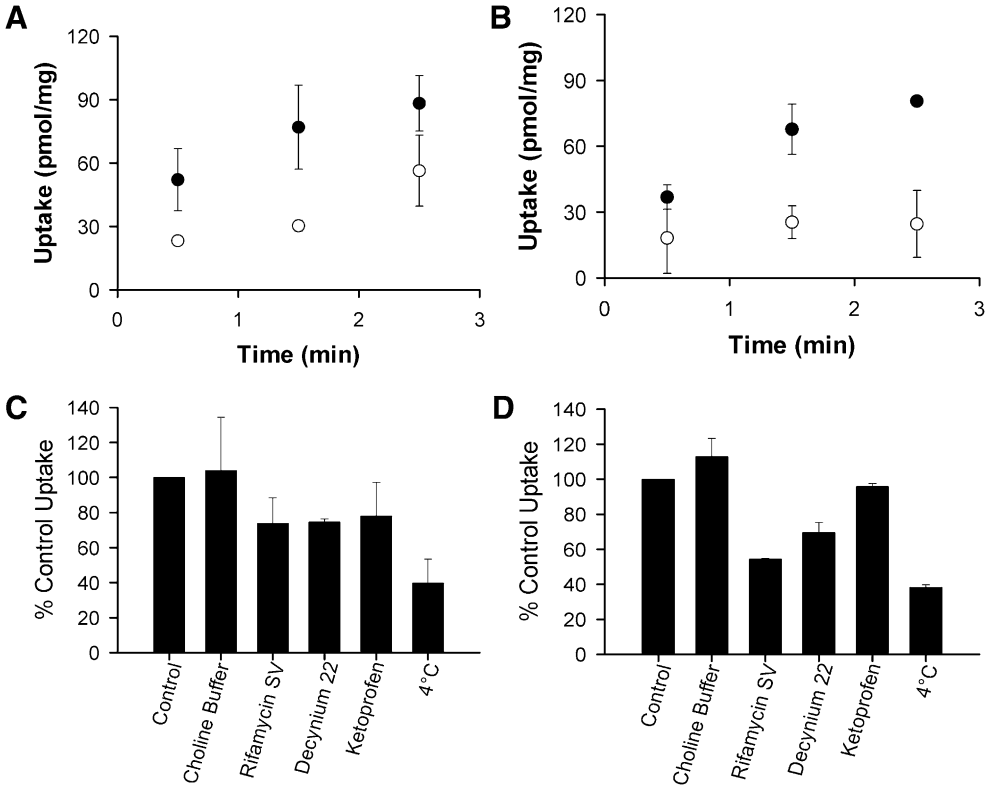

Fig. 2. Uptake of [^{14}C]sorafenib (0.9 μM) in suspended human hepatocytes from two separate donors (A and B) over 2.5 minutes. Hepatocytes were incubated at 37°C (●) or 4°C (○) in standard buffer (mean ± S.D.; n = 2 livers in triplicate). Initial uptake of [^{14}C]sorafenib (0.9 μM) at 0.5 minutes (C) and 1.5 minutes (D) in suspended human hepatocytes incubated at 37°C in standard buffer, unless otherwise specified, and in the presence of transport protein modulators, including choline buffer, inhibitors (rifamycin SV, decynium 22, and ketoprofen) and lower incubation temperature (n = 2 livers; mean percentage ± range).

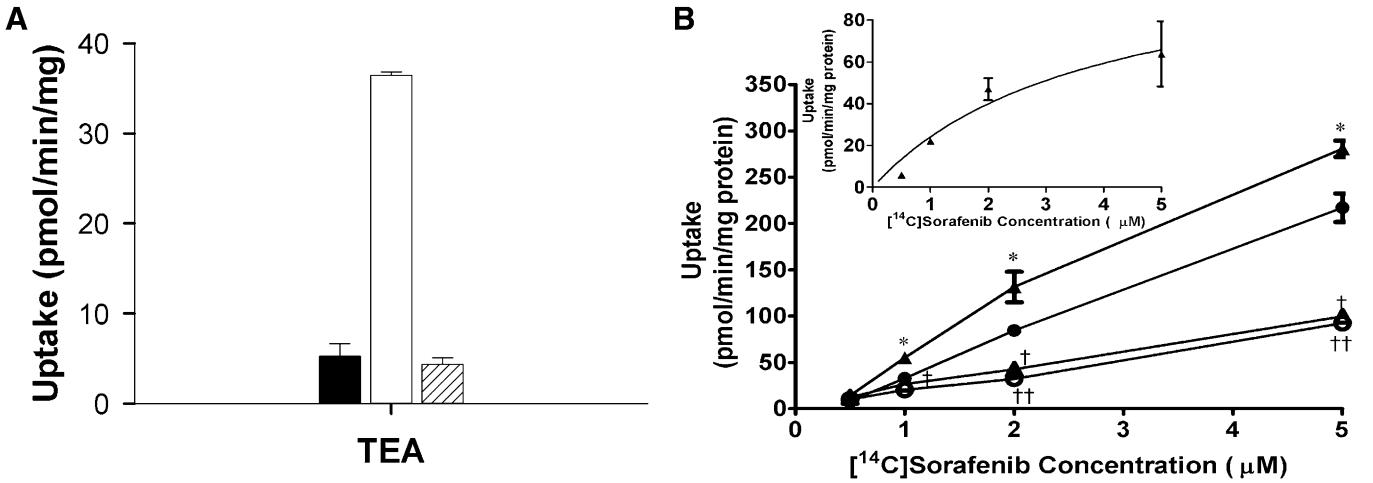

Fig. 3. Uptake of the positive control (A) [^{14}C]TEA (5 μM) was assessed in CHO-OCT1 cells in the absence (white bar) and presence (hatched bar) of 500 μM MPP$^+$ and mock cells (black bar). Uptake of (B) [^{14}C]sorafenib (0.5–5 μM) in CHO-hOCT1 cells. CHO-hOCT1 cells were incubated in the absence (▲) and presence (Δ) of 500 μM MPP$^+$, and mock cells in the absence (●) and presence (○) of 500 μM MPP$^+$ at 10 minutes. Inset: OCT1-mediated [^{14}C]sorafenib transport determined as the difference in uptake in CHO-hOCT1 and mock cells at each substrate concentration. The curve represents the best fit of the Michaelis-Menten equation to the OCT1-mediated uptake data. Data represent mean ± S.D. (n = 3); *$P < 0.001$ OCT1 versus mock; $^{\dagger}P < 0.001$ OCT1 versus OCT1+MPP$^+$; $^{\dagger\dagger}P < 0.001$ mock versus mock+MPP$^+$; when error bars are not visible, they are smaller than the symbol.

interindividual differences in the pharmacokinetics and pharmacodynamics of clinically relevant drugs such as the statins (Ieiri et al., 2009). In the present study, the transport proteins involved in the hepatic uptake of sorafenib were investigated, and the hepatobiliary disposition of sorafenib and metabolites was assessed.

Sorafenib is a very lipophilic compound (log D_7 = 5.16; predicted properties SciFinder Scholar, version 2007, CAS, Columbus, OH). The initial uptake of sorafenib in human hepatocytes was examined at 37°C versus 4°C to assess the contribution of passive diffusion to overall uptake. The initial uptake of [^{14}C]sorafenib at 4°C was reduced by 61 and 63% at 0.5 and 1.5 minutes, respectively, compared with 37°C, which suggests a high degree of passive diffusion (Fig. 2, A–D). The contribution of passive diffusion versus carrier mediated uptake remains unclear as a result of the effect of temperature on both processes. There was also a high degree of passive diffusion in CHO cells (Fig. 3B). Furthermore, greater than 54% of the sorafenib dose partitioned into human sandwich-cultured hepatocytes after a 20-minute incubation with 1 μM sorafenib based on the mass of drug remaining in the media at the end of the incubation period in relation to the initial dose (Table 2). These findings are in agreement with the reported high Papp in the absorptive direction of 16.4 ± 12.3 and 33.5 ± 16.1 × 10^{-6} cm/s for 0.1 and 1 μM sorafenib, respectively, determined in Caco-2 cells (Gnoth et al., 2010).

The active uptake of [^{14}C]sorafenib (0.9 μM) was investigated with transport protein modulators. Rifamycin SV (20 μM) was selected as an inhibitor of all the relevant human isoforms of OATP expressed in the liver: OATP1A2, OATP1B1, OATP1B3, and OATP2B1 (Vavricka et al., 2002). Decynium 22 (5 μM) was used as an OCT inhibitor (Zhang et al., 1997; Hayer-Zillgen et al., 2002), and OAT2 function was inhibited with ketoprofen (10 μM) (Morita et al., 2001; Ohtsuki et al., 2002). To assess Na$^+$-dependent transport by NTCP, choline-based buffer was substituted for Na$^+$-based buffer in suspended hepatocytes. The sensitivity of the transport proteins and specificity to the inhibitors rifamycin SV and decynium 22 were confirmed in the presence and absence of the model probe substrates [^{3}H]estradiol-17-β-D-glucuronide (OATP substrate) and [^{14}C]TEA (OCT substrate), as published previously (Swift et al., 2010). Sorafenib uptake at all time points sampled was sensitive to rifamycin SV and decynium 22,

TABLE 2

Accumulation, BEI, and $Cl_{biliary}$ of sorafenib or sorafenib N-oxide in sandwich-cultured human hepatocytes

Sandwich-cultured hepatocytes were incubated with 1 and 10 μM sorafenib for 20 minutes. Results are presented as mean ± S.D. from triplicate experiments from two livers.

Liver Donor Identification	Compound	Medium Concentration	Accumulation		BEI %	In Vitro $Cl_{biliary}$
			Cells + Bile	Cells		
		pmol/ml	*pmol/ml*	*pmol/ml*		*ml/min/kg*
Liver 1	Sorafenib 1 μM	39.1 ± 2.3	1210 ± 230	1570 ± 70	0	NA
Liver 2		460 ± 16	917 ± 41	819 ± 23	11	11.1
Liver 1	Sorafenib 10 μM	475 ± 59	7200 ± 130	6760 ± 550	6	11.1
Liver 2		1600 ± 75	6430 ± 130	5760 ± 240	10	11.5
Liver 1	N-oxide (sorafenib 1 μM)	BLQ (<1.00)	9.89 ± 2.62	12.3 ± 0.5	0	NA
Liver 2		BLQ (<1.00)	6.91 ± 0.22	6.14 ± 0.22	11	NA
Liver 1	N-oxide (sorafenib 10 μM)	BLQ (<1.00)	80.8 ± 5.1	63.2 ± 12.9	22	NA
Liver 2		11.8 ± 4.6	361 ± 13	346 ± 27	4	NA

BLQ, below the limit of quantitation; NA, not applicable.

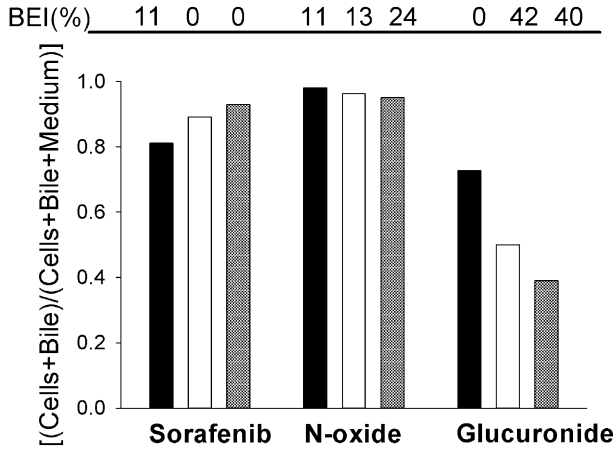

Fig. 4. Ratio of amount of sorafenib and formed metabolites in (cells + bile)/(cells + bile + medium) in day 7 sandwich-cultured human hepatocytes from liver 2 incubated with 1 μM sorafenib for 20 (solid bars), 60 (open bars), and 120 (hatched bars) minutes. The BEI was calculated after triplicate determination of substrate accumulation in cells and cells + bile. A standard was unavailable for sorafenib glucuronide, so the peak area under the curve divided by the internal standard area under the curve was used.

suggesting that the OATPs and OCTs are involved in the hepatic uptake this tyrosine kinase inhibitor (Fig. 2, C and D). The contribution of OATP1B1 and OATP1B3 to the hepatic uptake of sorafenib has been confirmed in vitro (Zimmerman et al., 2013). The involvement of OCT1 in sorafenib uptake was investigated further using a hOCT1-expressing CHO cell line. This finding contradicts work by Hu et al., who reported no appreciable uptake of sorafenib by *Xenopus laevis* oocytes expressing OCT1, OATP1A2, OATP1B1, or OATP1B3 (Hu et al., 2009). This apparent discrepancy could be explained by experimental differences. For example, in the present studies, sorafenib uptake into CHO cells was saturable after 10 minutes; Hu et al., incubated sorafenib with *X. laevis* oocytes for 1 hour, possibly masking the active uptake component. Furthermore, different in vitro model systems may yield conflicting data. For example, Agarwal et al. (2011) conclusively demonstrated the transport of sorafenib by Bcrp both in vitro and in vivo, in contrast to the data generated in LLC-PK1 cells transfected with BCRP (Hu et al., 2009; Agarwal et al., 2011). Imatinib, another tyrosine kinase inhibitor, is an OCT1 substrate in the human T-lymphoblastoid cell line CCRF-CEM (Thomas et al., 2004). Some substrate overlap exists between OCTs and OATPs, which have an affinity for numerous type II (bulky) cations such as N-methylquinine (van Montfoort et al., 1999). Furthermore, the class of tyrosine kinase inhibitors has been shown to inhibit metformin uptake in OCT-transfected cell lines (Minematsu and Giacomini, 2011).

Decynium 22 inhibition of sorafenib uptake in suspended human hepatocytes was likely due to OCT1 based on further studies demonstrating that OCT1-mediated uptake of sorafenib was significantly higher in OCT1-transfected CHO cells compared with mock cells over the concentration range examined (0.5–5 μM) (Fig. 3B). These results are the first to demonstrate that sorafenib is a substrate of OCT1 with a K_m of 3.8 μM. Interestingly, sorafenib uptake in nontransfected mock cells was inhibited partially by MPP^+, suggesting that other MPP^+-sensitive endogenous transport proteins in the CHO cell line are involved in sorafenib uptake. The unknown transport protein(s) that is/are sensitive to MPP^+ in mock CHO cells may be present in human hepatocytes. We cannot rule out the possibility that rifamycin SV inhibited the uptake of sorafenib by this unknown transport protein(s) in the suspended hepatocyte experiments.

To investigate the hepatobiliary disposition of sorafenib, studies were performed in human sandwich-cultured hepatocytes. The dosing concentrations (1 and 10 μM) used in these studies were in the range of the reported total maximum plasma concentration (5 to 21 μM) after multiple oral doses of sorafenib (100–600 mg twice daily) (Strumberg et al., 2005), but higher than the expected unbound plasma concentration of sorafenib based on reported binding to plasma proteins (99.5% bound; package insert). Sorafenib, a P-gp and Bcrp substrate (Hu et al., 2009; Gnoth et al., 2010; Agarwal et al., 2011), exhibited a relatively low BEI (up to 11%; Table 2) and in vitro $Cl_{biliary}$ (up to 11.5 ml/min/kg), which is not surprising because of the extent of CYP3A4- and UGT1A9-mediated metabolism observed in vivo (Lathia et al., 2006). The model bile acid [^{3}H]taurocholate, which is generally considered to have a high hepatic clearance, was included as a system control in the two liver donors, but it also serves as a good reference point for compounds with high BEI (64.8 and 62.6%) and high in vitro $Cl_{biliary}$ (59.9 and 32.4 ml/min/kg) (Table 1).

Biotransformation of sorafenib to the N-oxide is mediated primarily by CYP3A4 (Lathia et al., 2006; Ghassabian et al., 2012). The low formation of sorafenib N-oxide in day 7 human sandwich-cultured hepatocytes may be due to lower cytochrome P450 enzyme activity after isolation and culture (Hoen et al., 2000; Boess et al., 2003). Dexamethasone is a prototypical cytochrome P450 inducer that is added to cell culture medium. In the present studies, dexamethasone concentrations in the culture medium were only 1 μM, which is much lower than the 10 μM or higher concentrations used in some human and rat sandwich-cultured hepatocyte studies to induce CYP3A4 and Cyp3A1/2 protein expression and increase activity of CYP3A4 and Cyp3A1/2, as measured by testosterone 6β-hydroxylation (LeCluyse et al., 1996). Sorafenib N-oxide is the primary circulating metabolite in human plasma (Lathia et al., 2006); concentrations of sorafenib N-oxide in medium, a surrogate for blood, increased with the longer incubation times. Although no glucuronide was detected in the bile of sandwich-cultured hepatocytes after a 20-minute incubation, sorafenib glucuronide was excreted into bile after incubation of hepatocytes with sorafenib for 60 and 120 minutes, as demonstrated with the higher BEI (40–42%) (Fig. 4). The increased formation and biliary excretion of sorafenib glucuronide after longer incubation times may partially explain the significant amount of parent drug recovered in feces after oral dosing [~77% of a 100 mg oral dose was excreted in feces, of which 51% was the parent drug (according to the package insert)]. Based on our results, we hypothesize that sorafenib glucuronide undergoes biliary excretion; a portion of the glucuronide conjugate is cleaved in the gastrointestinal tract; subsequently, generated sorafenib is reabsorbed. This hypothesis is supported by the clinical observation of secondary peaks in the sorafenib plasma concentration-time profile (Lathia et al., 2006). Sorafenib glucuronide also was detected in the medium of sandwich-cultured hepatocytes (Fig. 4), in agreement with the findings that glucuronidated metabolites of sorafenib are recovered in human urine after oral administration.

Sorafenib metabolites, specifically the glucuronide conjugates, require transport proteins for biliary excretion and basolateral efflux. As mentioned, sorafenib is a P-gp and BCRP substrate and may also be an MRP2 substrate (Shibayama et al., 2011), suggesting that these transport proteins may play a role in the biliary excretion of sorafenib and its metabolites. MRP2 is responsible for the biliary excretion of many glucuronide conjugates of drugs, as well as bilirubin conjugates (Kamisako et al., 1999), and may transport sorafenib glucuronide into bile. Clinically relevant drug interactions associated with impaired biliary clearance have been reported for digoxin with coadministration of the P-gp inhibitors quinidine, verapamil, and ritonavir (Fenner et al., 2009). Furthermore, it is well recognized that patients with liver

disease develop adaptive changes in transport protein expression; these adaptations protect the hepatocyte from the intracellular accumulation of toxic compounds such as bile acids. In fact, many patients with hepatocellular carcinoma develop cirrhosis, which results in the downregulation of basolateral uptake transport proteins often coupled with the upregulation of canalicular and basolateral efflux proteins (Zollner et al., 2003, 2007). This highlights the importance of understanding the mechanisms of sorafenib hepatobiliary disposition.

In conclusion, sorafenib uptake in suspended human hepatocytes, CHO cells and sandwich-cultured human hepatocytes was extensive. Uptake into human hepatocytes was temperature dependent and decreased approximately 61–63% at 4°C, suggesting a high degree of passive diffusion. The active portion of hepatic uptake was sensitive to rifamycin SV and decynium 22, suggesting the involvement of OATPs and OCT, respectively. OCT1-mediated uptake of sorafenib was confirmed in OCT1 overexpressing CHO cells. Sorafenib undergoes modest biliary excretion as the parent compound; biliary excretion of the major metabolites, N-oxide and glucuronide conjugate(s), is increased with incubation time in sandwich-cultured hepatocytes.

Authorship Contributions

Participated in research design: Swift, Proctor, Thakker, Gnoth, Radtke, Brouwer.

Conducted experiments: Swift, Lee, Han, Proctor.

Contributed new reagents or analytic tools: Swift, Thakker, Gnoth, Lang.

Performed data analysis: Swift, Nebot, Brouwer.

Wrote or contributed to the writing off the manuscript: Swift, Nebot, Gnoth, Brouwer.

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