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Human Organic Anion-Transporting Polypeptide OATP-A (SLC21A3) Acts in Concert with P-Glycoprotein and Multidrug Resistance Protein 2 in the Vectorial Transport of Saquinavir in Hep G2 Cells

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Abstract: Saquinavir mesylate (SQV) is the first-in-class and prototypical HIV protease inhibitor (PI) used in the treatment of HIV infection. SQV undergoes extensive hepatic metabolism and intestinal and bile secretion, and has poor and variable oral bioavailability. In previous studies, our group and others have described the interactions between SQV and absorptive and secretory efflux transporters such as MRP1, MRP2, and P-gp. However, the potential role of absorptive influx transporters such as OATP-A (SLC21A3) has not yet been reported for SQV. In the study presented here, the role of OATP-A in the influx transport of SQV was studied using a hepatic cell model, Hep G2, and *Xenopus laevis* oocytes overexpressing human OATP-A. In Hep G2 cells, SQV transport was found to be (i) concentration-dependent and saturable, (ii) temperature-sensitive, and (iii) proton (pH)- and sodium-independent. While GF120918, a specific inhibitor of P-gp, and MK571, a MRP transporter family inhibitor, significantly enhanced SQV uptake, estrone 3-sulfate, a substrate of OATP-A, significantly inhibited SQV uptake by Hep G2 cells. The observation that inhibitors of P-gp, MRP, or OATP-A have opposite effects on SQV uptake in polarized Hep G2 cells is consistent with their functions as hepatic efflux or influx transporters. In *X. laevis* oocytes into which OATP-A cRNA had been injected, the level of uptake of SQV was significantly greater than the level of uptake by oocytes into which water had been injected and was concentration-dependent and saturable ($K_m = 36.4 \pm 21.8\ \mu M$). This is the first report showing that SQV influx transport is directly facilitated by OATP-A. Given the wide body distribution of OATP-A, the current results suggest a potentially important role for OATP-A in the absorption and disposition of SQV *in vivo*. The data also suggest that in human hepatocytes basolaterally located OATP-A (influx transporter) may act in concert with apically located P-gp and/or MRP2 (efflux transporters) for the vectorial transport and excretion of SQV into bile.

Keywords: Saquinavir mesylate; organic anion-transporting polypeptide; sodium taurocholate cotransporting polypeptide; organic cation transporter; P-glycoprotein; canalicular multispecific organic transport

Introduction

Saquinavir mesylate is the first HIV protease inhibitor approved for use in patients in the United States. However, SQV absorption is highly variable as indicated by AUC coefficients of variation that are ≥30%. Two marketed SQV capsule formulations have mean oral bioavailabilities that range from 4 to 16%.[1] Even though first-pass intestinal hepatic metabolism by cytochrome P-450 monooxygenases (CYP) has been shown to be a major determinant in the oral

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clearance of SQV,[2,3] it is more frequently recognized that membrane transporters may have an equally important role in determining the oral absorption and disposition properties of drugs, including SQV.[4] Investigations have demonstrated that SQV is a substrate for the P-glycoprotein (P-gp, ABCB1) efflux pump, and correlated its transport by P-gp with its reduced bioavailability and limited CNS penetration.[4] Recently, we provided direct evidence that SQV is transported by multidrug resistance-associated protein (MRP1, ABCC1) and canalicular multispecific organic anion transporter (MRP2, ABCC2).[5] In a clinical study, after the intravenous administration of [^{14}C]SQV, 81 and 3% of the radioactivity was recovered in feces and urine, respectively, within 5 days of the treatment,[6] suggesting potential roles for biliary and intestinal secretion. The higher level of SQV excretion into the feces suggests that membrane transporters play a far more important role than simple passive diffusion in determining SQV bioavailability and disposition. Moreover, there is evidence suggesting that SQV shows significant drug−drug interactions at the membrane transporter level.[7,8]

In addition to intestinal transporters, hepatic transporters may also play a critical role in the pharmacokinetics of SQV. Human hepatocytes are polarized into a sinusoidal (basolateral) membrane facing the blood circulation and canalicular (apical) membrane facing the bile duct. Many efflux drug transporters, such as MRP2 and MDR1, are strictly localized to the apical side, while influx transporters, such as NTCP (sodium taurocholate cotransporting polypeptide, SLC10A1), OCT1 (SLC22A1), OATP-A (OATP1, SLC21A3), OATP-C (LST-1, OATP2, SLC21A6), and OATP8 (SLC21A8), are only expressed at the basolateral side of hepatocytes.[9,10] Since P-gp and MRP2 transport SQV and are located on the apical domain of hepatocytes and there is significant biliary excretion of SQV, the identity of a concerted SQV influx transporter(s) located on the basolateral domain of hepatocytes is of major interest and importance. The influx transporters at the basolateral side of human hepatocytes are classified into two major groups: Na^+-dependent, e.g., NTCP, and Na^+-independent transporters, e.g., OCT1, OATPs.[11−13] Among them, NTCP appears to have high substrate specificity; bile salts and their derivatives are the only known substrates.[14] Therefore, it is very unlikely that NTCP will be involved in SQV transport. Even though OCT1 does not transport SQV,[15] OATP-A and -C may be involved in SQV transport.[7,8] Although their names imply that they are transporters of organic anions, OATPs can also transport bulky organic cations (i.e., type II organic cations). While recently published data show that SQV can inhibit OATP-A-mediated fexofenadine and OATP-C-mediated estrodial 17β-glucuronide uptake,[7,8] it is not yet clear whether SQV can be transported by OATP-A and/or OATP-C. In this report, we provide in vitro evidence showing the uptake of SQV by OATP-A and the efflux transport by P-gp and MRP2 in the human Hep G2 hepatoma cell line. The kinetics of SQV uptake by OATP-A are also characterized by using *Xenopus laevis* oocytes into which OATP-A cRNA had been injected. Therefore, we have identified a basolateral transporter in hepatocytes that acts in concert with apical P-gp and MRP2 transporters to actively secrete SQV into the bile. Furthermore, the current results establish a scientific basis for further studies aimed at determining the physiological importance of OATP-A in other critical areas such as the brain, a major HIV sanctuary site.

Experimental Section

Materials. SQV and [^{14}C]SQV (20.3 mCi/mmol) were provided by Roche Laboratories (Nutley, NJ). GF120918 and MK-571 were provided by GlaxoSmithKline, Inc. (Research Triangle Park, NC) and Merck Laboratories, Inc. (Whitehouse, NJ), respectively. Superscript II reverse transcriptase, Taq DNA polymerase, TRIzol reagent, and Hank's balanced

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salt solution were purchased from Invitrogen (Carlsbad, CA). The BCA Protein Assay Kit and SuperSignal West Femto Maximum Sensitivity Substrate were purchased from Pierce (Rockford, IL). Human liver first-strand cDNA is from Multiple Tissue cDNA (MTC) Panels purchased from Clontech (Palo Alto, CA). Total tissue proteins from human liver, small intestine, and brain parietal lobe were purchased from Biochain (Hayward, CA). *X. laevis* oocytes into which water and human OATP-A (SLC21A3) cRNA had been injected and modifed Barth's solution were obtained from BD Gentest (Woburn, MA). The anti-OATP-A neat antiserum was purchased from Alpha Diagnostic International (San Antonio, TX). Hep G2 cells were kindly provided by T. A.-N. Kong from Rutgers University. All other chemicals were purchased from Sigma Chemicals or Fisher Scientific.

Cell Culture. Hep G2 cells were cultured in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate (90%), fetal bovine serum (10%), 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. *Xenopus* oocytes into which transporter cRNA or water had been injected were cultured in modified Barth's solution at 16 °C before the uptake studies were carried out.

Detection of Transporter Expression by RT-PCR. Hep G2 cells at 100% confluence were rinsed twice with 1× PBS, and total RNA was isolated from cells by using TRIzol according to the manufacturer's instructions (Invitrogen). One microgram of total RNA was reverse-transcribed to first-strand cDNA with Superscript II reverse transcriptase at 42 °C for 1 h. Hep G2 or human liver first-strand cDNA was amplified with Taq DNA polymerase. The primers for transporters were designed on the basis of the published sequences from NCBI-UniGene. The designed primers were as follows: OATP-A (GenBank entry NM_005075), 5′-GAT CCT GTG TGT GGA AAC AAT GGC-3′ (sense) and 5′-TGT AAT CCC ACA CCA AGG GAC TTC-3′ (antisense), product size of 302 bp; OATP-C (GenBank entry NM_006446), 5′-ACG TAG AGC AAC AGT ATG GTC AGC-3′ (sense) and 5′-CAT AGG TCA TGG TTA GTC CGG CAA-3′ (antisense), product size of 243 bp; OATP8 (GenBank entry AJ251506), 5′-CAA CAG TAC GGT CAG TCT GCA TCT-3′ (sense) and 5′-CAG ACT GGT TCC CAC TGA CTT TCA-3′ (antisense), product size of 329 bp; MDR1 (GenBank entry AF016535), 5′-ATC AGC AGC CCA CAT CAT C-3′ (sense) and 5′-CCA TCA AGC AGC ACT TTC C-3′ (antisense), product size of 285 bp; MRP2 (GenBank entry U49248), 5′-TGG AGT CTA CGG AGC TCT GGG-3′ (sense) and 5′-TGC TGG TGC TCA AAG GCA CGG-3′ (antisense), product size of 468 bp; NTCP (GenBank entry NM_003049), 5′-CCG GCT GAA GAA CAT TGA GGC ACT GG-3′ (sense) and 5′-AGG GAG GAG GTG GCA ATC AAG AGT GG-3′ (antisense), product size of 434 bp. The PCR conditions were as follows: 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 2 min for 35 cycles. PCR products were run on 1.5% agarose gels containing ethidium bromide and photographed on a UV box.

Western Blot Analysis. Twenty micrograms of human total tissue proteins was size fractionated via 4% stacking and 10% resolving SDS−PAGE. Immunoblotting was performed using a tanking blotting system from Bio-Rad and SuperSignal West Femto Maximum Sensitivity Substrate from Pierce. The primary antibody was diluted 1:1000 in 5% nonfat milk in PBS. The secondary antibody was HRP-conjugated goat anti-rabbit IgG (Sigma) used as a 1:5000 dilution.

SQV Uptake in Hep G2 Cells. Hep G2 cells were subcultured by trypsinization with 0.05% trypsin-EDTA and plated onto 48-well plates at a density of 2×10^5 cells/cm^2. Uptake of SQV was studied 1 day after confluence. Uptake was performed either in a NaCl medium (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose, and 10 mM HEPES, adjusted to pH 7.5 with Tris) or in a choline chloride medium in which NaCl had been replaced with 100 mM choline chloride. The osmolalities of both mediums were approximately 275 mOsm. The cells were prewashed in 3 × 0.5 mL of the respective uptake medium and then incubated at 4 or 37 °C in 0.2 mL of the same medium containing 3 μM (or the indicated concentrations) SQV spiked with [^{14}C]SQV. After the cells had been incubated for the indicated time period, the uptake medium was immediately removed from the wells, and the cells were washed with 3 × 0.5 mL of ice-cold uptake buffer. Cells were subsequently lysed with 150 μL of 0.2 N NaOH by pipetting up and down and then neutralized with 150 μL of 0.2 N HCl. A 270 μL aliquot was dissolved in 4 mL of scintillation fluid, and the radioactive activity was determined by scintillation counting. A 20 μL aliquot was removed for protein determination by using the BCA Protein Assay Kit purchased from Pierce. Uptake medium (pH 5.5 or 6.0) contained 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 12 mM $MgSO_4$, and 25 mM MES, adjusted to pH 5.5 or 6.0 with 0.2 M NaOH. Uptake medium (pH 6.5 or 7.4) contained 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 12 mM $MgSO_4$, and 25 mM HEPES, adjusted to pH 6.5 or 7.4 with 0.2 M NaOH. The osmolalities of the uptake media (pH 5.5−7.4) were approximately 290 mOsm. Since it was shown previously that CYP450 activity is significantly reduced in Hep G2 cells,[16] the potential role of SQV metabolism in biasing the current uptake results was ignored.

Inhibition Studies. Hep G2 cells were preincubated with 2 μM GF120918 or 5 μM MK571 for 20 min before the initiation of the uptake experiment. SQV uptake studies were carried out in the presence of inhibitors as described above. When estrone 3-sulfate was used as a putative inhibitor, the uptake of SQV was initiated in the presence of estrone 3-sulfate without preincubation (i.e., cis inhibition).

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SQV Uptake in Oocytes. The uptake studies with SQV were carried out 3 or 4 days after cRNA injection. The oocytes were preincubated for 30 min in medium A (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM MES, adjusted to pH 6.5 using 5 N NaOH) at room temperature before the initiation of the uptake experiment. The oocytes were then transferred to 700 μL of the uptake medium, which had the same constituents as medium A, in addition to 10 μM SQV spiked with [^{14}C]SQV as the substrate under investigation. After the incubation period, the uptake medium was removed and the oocytes were washed with 3 × 1 mL of ice-cold medium A. Each oocyte was digested in 150 μL of 10% SDS and assessed for radioactivity. The time course of SQV uptake by oocytes was studied over various time periods (from 30 to 180 min) to determine the optimal uptake time. For the investigation of the concentration dependence of SQV uptake, the oocytes were incubated in the presence of different concentrations of SQV (3−50 μM) spiked with [^{14}C]SQV. The uptake was stopped at the determined time point (90 min) as described above.

Statistical and Nonlinear Regression. The saturable uptake of SQV was analyzed by using the Michaelis−Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

where V and [S] are the initial uptake rate and SQV concentration, respectively. V_{max} and K_m represent the maximum uptake rate and Michaelis constant, respectively. The kinetic parameters were estimated using nonlinear least-squares regression analysis (Enzyme Kinetics, SigmaPlot 2001, SPSS Inc.). ANOVA was used for statistical comparisons with a significance level of $p < 0.05$.

Results

Expression Profile of Major Liver Transporters in Hep G2 Cells. The expression of major hepatic transporters in Hep G2 cells was characterized by RT-PCR, using human liver cDNA as a positive control. Total RNA was extracted from Hep G2 cells and subjected to RT-PCR; human liver first-strand cDNA was subjected to PCR. Each PCR used cDNA-specific primers. As shown in Figure 1, of the six major hepatic transporters, only MDR1, MRP2, and OATP-A were detected, while NTCP, OATP-C, and OATP8 were absent in Hep G2 cells. The expression of all six major hepatic transporters was detected in human liver samples.

Time Course of SQV Uptake by Hep G2 Cells. Figure 2 depicts the uptake time course of 3 μM SQV at 4 and 37 °C. The rate of uptake at 37 °C was found to be linear for incubation for up to 10 min ($r^2 = 0.99$). Therefore, a 5 min incubation time period was chosen to determine the initial uptake rate in subsequent studies. The rate of uptake of SQV at 37 °C is significantly greater than that at 4 °C at every tested time point ($p < 0.05$), suggesting that membrane transporters may mediate the uptake of SQV by Hep G2 cells.

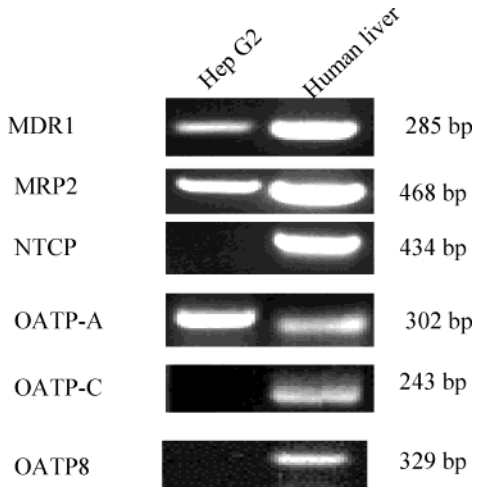

Figure 1. Expression profile of major hepatic uptake or efflux transporters in Hep G2 cells. RT-PCR was performed with total RNA isolated from Hep G2 cells, and PCR was performed with human liver first-strand cDNA. PCR products were size separated on a 1.2 or 3% agarose gel. The sizes of PCR products are shown next to the gel.

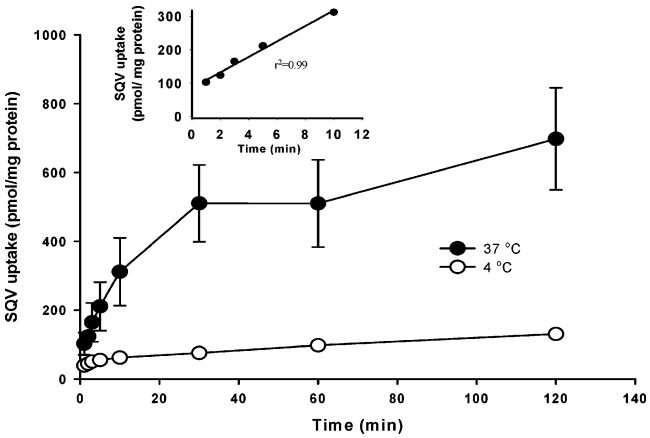

Figure 2. Uptake of SQV by Hep G2 cells as a function of time. Cells were incubated in HBSS buffer at 37 °C (●) or 4 °C (○) in the presence of 3 μM SQV (spiked with [^{14}C]SQV). Results are means ± the standard error of the mean of three separate uptake determinations. The rate of uptake of SQV at 37 °C is significantly greater than that at 4 °C at every tested time point ($p < 0.05$). The inset shows that the rate of SQV uptake at 37 °C within the first 10 min of incubation is linear ($r^2 = 0.99$).

Concentration Dependence of SQV Uptake by Hep G2 Cells. Figure 3 shows that the rate of SQV uptake at 37 °C was concentration-dependent and saturable, whereas the rate of uptake at 4 °C remained linear over the entire range of concentrations studied ($r^2 = 0.95$). The net rate of temperature-dependent uptake was obtained by subtracting the rate of SQV uptake at 4 °C from the rate of uptake at 37 °C. Kinetic analysis of net temperature-dependent uptake revealed an apparent Michaelis constant (K_m) of 94.6 ± 22.8 μM and a maximum velocity (V_{max}) of 10.7 ± 1.5 nmol (5 min)$^{-1}$ (mg of protein)$^{-1}$. The concentration dependence and saturability of SQV uptake by Hep G2 cells further suggested

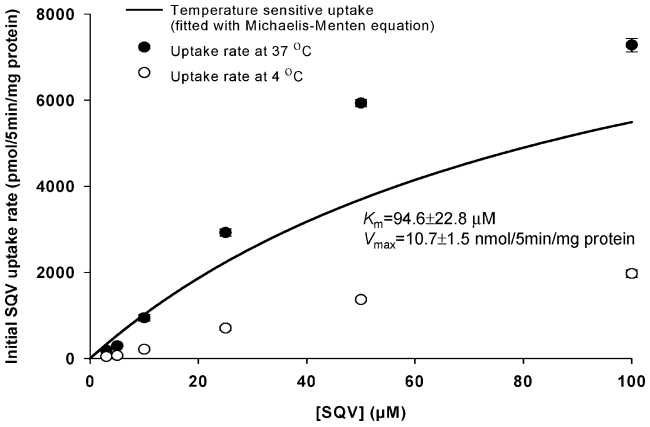

Figure 3. Kinetics of SQV uptake by Hep G2 cells. Cells were incubated in HBSS buffer at 37 °C (●) or 4 °C (○) in the presence of increasing concentrations of SQV (spiked with [^{14}C]SQV). The rate of uptake of SQV measured in the first 5 min is chosen as the initial uptake rate. The rate of SQV uptake measured at 4 °C is linear over the concentration range used in the study ($r^2 = 0.95$, fitted linear regression line not shown), and the rate of uptake measured at 4 °C was then subtracted from that measured at 37 °C, yielding net temperature-dependent uptake (—). Kinetic analysis of temperature-dependent uptake revealed an apparent Michaelis constant (K_m) of 94.6 ± 22.8 μM and a maximum velocity (V_{max}) of 10.7 ± 1.5 nmol (5 min)$^{-1}$ (mg of protein)$^{-1}$. Data are expressed as means ± the standard error of the mean of three individual uptake measurements. The net temperature-dependent uptake curve (—) was fitted by nonlinear regression analysis governed by the Michaelis−Menten equation.

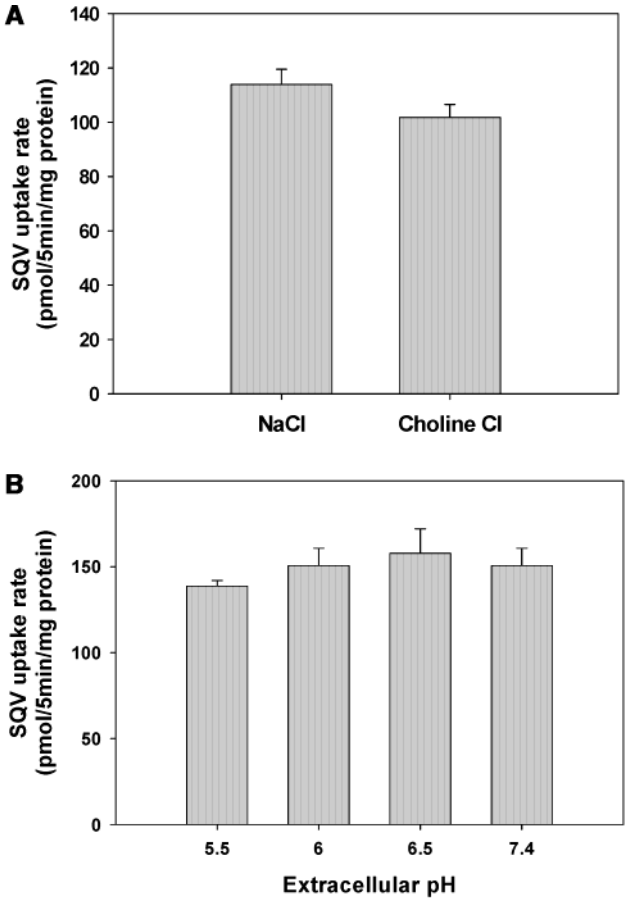

Figure 4. Sodium and proton independence of SQV uptake by Hep G2 cells. (A) The sodium dependence of SQV uptake was determined by incubating Hep G2 cells with 3 μM SQV (spiked with [^{14}C]SQV) in the presence or absence of Na^+ (Na^+ replaced with choline). The rate of SQV uptake within the first 5 min was measured. Data are expressed as means ± the standard error of the mean for two experiments performed in triplicate. (B) The pH of the uptake medium was adjusted to 5.5, 6, 6.5, and 7.4 separately, and then the rate of SQV uptake within the first 5 min was measured. Data are expressed as means ± the standard error of the mean for two experiments performed in triplicate.

that a transporter-mediated uptake is involved. The transporter-mediated uptake by Hep G2 cells presumably involves several uncharacterized endogenous transporters; therefore, the K_m is considered an apparent parameter.

Sodium and Proton Independence of SQV Uptake by Hep G2 Cells. To further characterize the transporters involved in SQV uptake by Hep G2 cells, the effect of extracellular Na^+ on the uptake was investigated (Figure 4A). The rate of uptake of SQV by Hep G2 within the first 5 min was measured in the presence or absence of Na^+. The rate of SQV uptake is not significantly different whether the incubation buffer contains Na^+, indicating that SQV uptake by Hep G2 cells is Na^+-independent. Therefore, the involvement of the sodium-dependent transporter NTCP is ruled out. The effect of extracellular H^+ on the uptake of SQV by Hep G2 is shown in Figure 4B. The rate of uptake of SQV by Hep G2 cells within first 5 min was measured in uptake medium with the pH ranging from 5.5 to 7.4. There was not a significant difference in the rate of SQV uptake as a function of pH, indicating that SQV uptake by Hep G2 cells is proton-independent.

Effect of Inhibitors of P-gp, MRPs, and OATPs on the Intracellular Accumulation of SQV in Hep G2 Cells. To evaluate concerted transporters, the effects of several inhibitors were studied. Hep G2 cells were preincubated with 5 μM MK571, a specific inhibitor of MRP family transporters,[17] or 2 μM GF120918, a specific inhibitor of P-gp,[18] for 20 min. Uptake was initiated by incubating Hep G2 cells with 3 μM SQV in the presence or absence of MK571 or GF120918, and the rate was measured at various time points (10, 30, and 60 min). As shown in Figure 5A, inhibition of P-gp or MRPs significantly enhanced the intracellular accumulation of SQV at every tested time point ($p < 0.05$).

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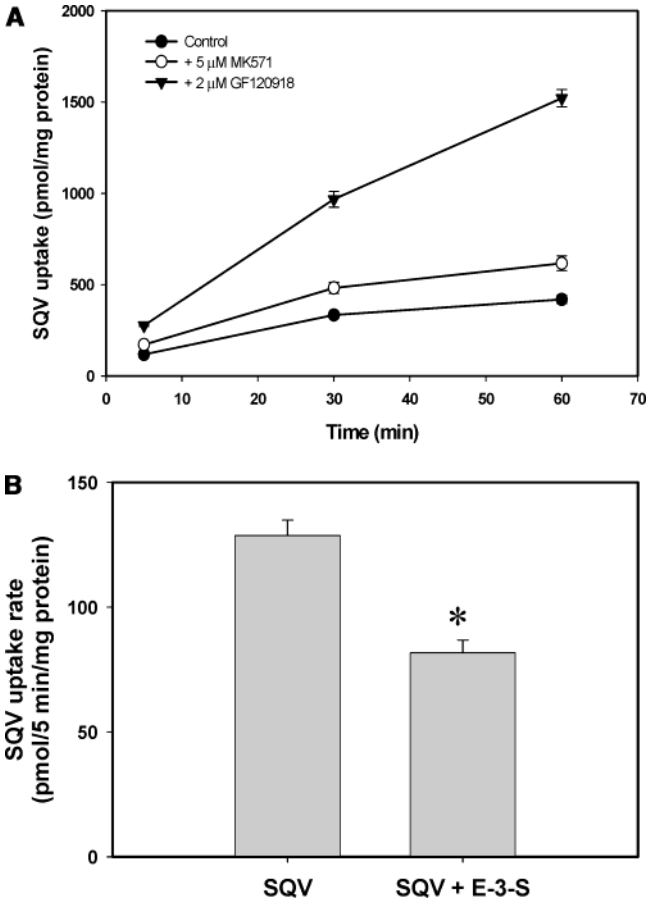

Figure 5. Effect of GF120918, MK571, and estrone 3-sulfate on the uptake of SQV by Hep G2 cells. (A) Effect of P-gp inhibitor (GF120918) and MRP inhibitor (MK571) on intracellular SQV accumulation in Hep G2 cells. Hep G2 cells were preincubated with 2 μM GF120918 or 5 μM MK571 for 20 min (inhibition groups) and then incubated with 3 μM SQV (spiked with [^{14}C]SQV) in the presence or absence of GF120918 or MK571. The level of intracellular SQV accumulation was measured at the time points shown in the figure. Data are expressed as means $\pm$ the standard error of the mean for two experiments performed in triplicate. The inhibition of P-gp or MRPs significantly enhanced the intracellular SQV accumulation at every tested time point ($p <$ 0.05). (B) Inhibitory effect of estrone 3-sulfate (10 μM) on the initial rate of uptake of SQV (3 μM) by Hep G2 cells. Data are expressed as means $\pm$ the standard error of the mean for two experiments performed in triplicate. The asterisk denotes a p of $<$0.05.

The inhibition of MRPs does not enhance SQV accumulation as much as the inhibition of P-gp. To understand the contribution of OATPs to the uptake of SQV, Hep G2 cells were incubated with 3 μM SQV in the presence or absence of 10 μM estrone 3-sulfate (a typical substrate of OATPs) without preincubation (i.e., cis inhibition), and the initial rate of uptake of SQV was measured. Figure 5B shows that estrone 3-sulfate significantly inhibited SQV uptake, suggesting that one or more of the OATPs are involved in SQV uptake into Hep G2 cells.

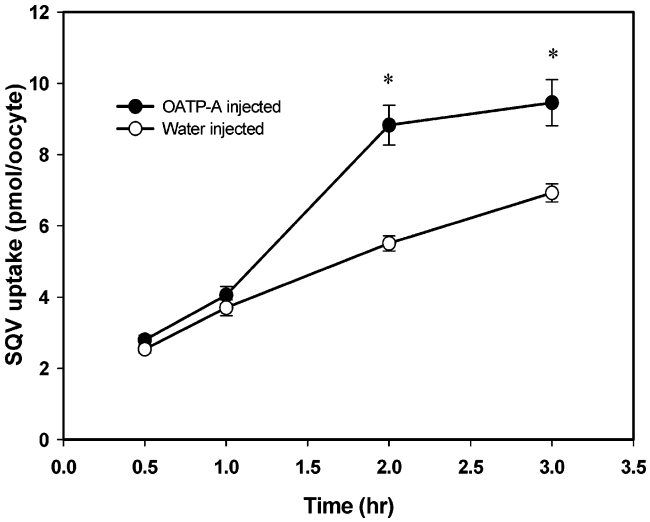

Figure 6. Time course of SQV uptake by *X. laevis* oocytes into which OATP-A cRNA had been injected. The rate of SQV uptake was measured in oocytes incubated for the indicated time periods in 10 μM SQV (spiked with [^{14}C]SQV). Each point represents the mean $\pm$ the standard error of the mean of 10−12 oocytes. Asterisks denote a p of $<$0.05. A representative of two experiments with similar results is shown.

Time Course of SQV Uptake by Oocytes into which OATP-A cRNA Had Been Injected. Given that Hep G2 cells only express OATP-A, the above results strongly suggested SQV uptake by OATP-A. Therefore, the oocyte model was used to confirm its involvement. The effect of the duration of the experiment on the uptake of SQV by oocytes into which OATP-A cRNA or water had been injected is shown in Figure 6. The rate of uptake by oocytes into which OATP-A cRNA had been injected was found to be significantly higher ($p < 0.05$) than that by oocytes into which water had been injected at 2 and 3 h. The rate of uptake of SQV by oocytes into which both cRNA and water had been injected increased as the incubation time was increased. The difference between the rate of uptake of SQV in oocytes into which OATP-A cRNA had been injected and control oocytes suggested that incubation for more than 1 h is needed to see the difference; therefore, an incubation time of 90 min was used in the subsequent oocyte studies.

Concentration Dependence of SQV Uptake by Oocytes into which OATP-A cRNA Had Been Injected. Figure 7 shows the concentration dependence and saturability of SQV uptake. The initial uptake rate was measured at concentrations ranging from 3 to 50 μM in oocytes into which both water and OATP-A cRNA had been injected. The SQV uptake rate was calculated from the difference between the uptake rate in oocytes into which OATP-A cRNA and water had been injected. The Michaelis constant (K_m) of SQV transport by OATP-A was estimated by nonlinear least-squares regression analysis and found to be 36.4 $\pm$ 21.8 μM, which is close to the reported value (64 μM) for a typical OATP-A substrate, taurocholic acid.[12]

Tissue Distribution of Human OATP-A. To elucidate the potential significance of OATP-A in SQV disposition, Western blot was used to examine the tissue distribution

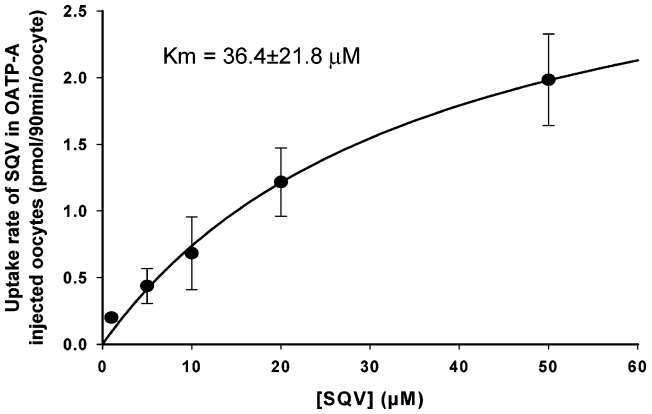

Figure 7. Concentration dependence of SQV uptake in *X. laevis* oocytes into which OATP-A cRNA had been injected. The rate of uptake of SQV was measured in the uptake medium (pH 6.5) containing various concentrations (1, 5, 10, 20, and 50 μM) of SQV (spiked with [^{14}C]SQV) at 25 °C for 90 min. The expressed transport due to the OATP-A cRNA injection was calculated as the difference between the rates of uptake in oocytes into which cRNA had been injected and oocytes into which water had been injected. The mean uptake value in oocytes into which water had been injected was subtracted from each value for oocytes into which cRNA had been injected to obtain the rate of uptake due to OATP-A activity. The difference was used to calculate the mean and standard error of the mean. Each point represents the mean ± the standard error of the mean of six to eight oocytes. A representative result from one of two experiments with similar outcomes is shown. The fitted line represents the carrier-mediated uptake estimated from the kinetic parameters analyzed with SigmaPlot.

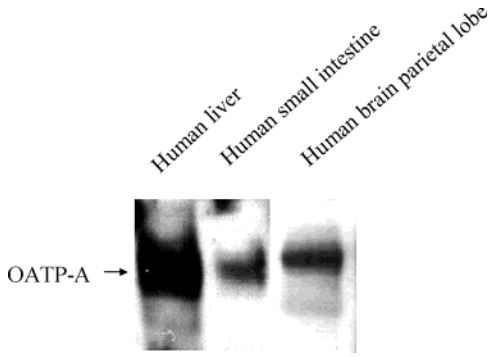

Figure 8. Expression of OATP-A in human small intestine, liver, and brain. The level of expression of OATP-A in human small intestine (i.e., SQV absorption site), liver (i.e., a major SQV elimination site), and the parietal lobe of the brain (i.e., a major HIV sanctuary site) was determined at protein levels. Human tissue total proteins (20 μg) were size fractionated by 10% SDS−PAGE and transferred to PVDF membrane. OATP-A protein was detected with a rabbit anti-OATP-A antibody.

pattern in humans. In this study, only human small intestine, liver, and brain were analyzed because they represent the major biopharmaceutical barriers for SQV absorption and elimination and a major HIV sanctuary site, respectively. As Figure 8 shows, at the protein level OATP-A was detected in human liver, small intestine, and the parietal lobe of the brain.

Discussion

SQV was the first HIV protease inhibitor approved for use in humans for the treatment of HIV infection and AIDS. Although this class of drugs shows satisfactory therapeutic efficacy in highly controlled clinical situations, their practical effectiveness is limited by low and variable bioavailability, poor penetration of HIV sanctuary sites such as the brain, and numerous drug−drug interactions.[1,4] SQV has the highest observed bioavailability variability among ritonavir, indinavir, and nelfinavir, and its low and variable bioavailability has been primarily attributed to metabolism by cytochromes P450.[19] While in vitro studies using human liver micosomes showed that the specific isoenzyme, CYP3A4, is responsible for more than 90% of the hepatic metabolism,[6,20] there is a growing body of evidence which strongly suggests that membrane transporters also determine the biopharmaceutical characteristics of SQV.[5,21]

These results show that the uptake of SQV by Hep G2 cells is sodium- and proton-independent, concentration-dependent and saturable, and temperature-dependent, suggesting the involvement of an energy-dependent mechanism. Kinetic analysis of temperature-dependent uptake revealed an apparent Michaelis constant (K_m) of 94.6 ± 22.8 μM and a maximum velocity (V_{max}) of 10.7 ± 1.5 nmol (5 min)$^{-1}$ (mg of protein)$^{-1}$. The uptake transporters in human liver can be classified into Na^+-dependent transporters represented by NTCP and Na^+-independent transporters represented by organic anion transport polypeptides (OATPs).[14,22] The expression of major hepatic transporters in the currently used Hep G2 cells was assessed by RT-PCR, and showed that MDR1, MRP2, and OATP-A are expressed at the mRNA level, while NTCP, OATP-C, and OATP8 were not detected. This finding is consistent with the results reported by others.[12,23−25] Estrone 3-sulfate is a typical substrate of the

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OATP family with reported Michaelis constants (K_m) ranging from 4.5 to 100 μM for various members;[26,27] therefore, it was used as a competitive inhibitor of OATP-A in this study. It was found that estrone 3-sulfate (at 10 μM) significantly ($p < 0.05$) inhibits the initial rate of uptake of 3 μM SQV by Hep G2 cells. Since the gene and functional expression results strongly implicated the involvement of OATP-A, *X. laevis* oocytes into which OATP-A cRNA had been injected were used to confirm the involvement of OATP-A and to characterize the kinetics of SQV interactions with OATP-A. Current results show that the rate of SQV uptake by oocytes into which OATP-A cRNA had been injected was significantly ($p < 0.05$) higher than that of control oocytes and was concentration-dependent and saturable with a Michaelis constant of 36.4 $\pm$ 21.8 μM. The K_m values determined in oocyte studies are comparable to SQV plasma concentrations (4−10 μM),[15] suggesting that OATP-A expressed in the liver or brain may be clinically relevant to SQV's disposition. Due to the relatively low K_m, these results also suggest that the impact on intestinal transport after oral delivery is questionable since, at typical luminal concentrations, OATP-A would be saturated for much of the drug's residence time in the intestine. However, the abundance of OATP-A at the apical domain of enterocytes was not measured because of the lack of a specific competitive inhibitor of OATP-A for SQV, but this could significantly affect SQV uptake and, ultimately, absorption. The K_m values determined in oocytes and Hep G2 cells are significantly different since the apparent K_m of Hep G2 cells also accounts for SQV interactions with apical efflux membrane transporters such as P-gp and MRP2 or possibly with other yet-to-be-identified basolaterally located absorptive transporters.

It is known thus far that OATP-A, OATP-C, OATP8, and OATP-B (SLC21A9) are the major members of the organic anion-transporting polypeptide family whose expression has been identified in human liver.[28−31] Unlike OATP-C and OATP8, whose expression is restricted to the liver, OATP-A is also found in the small intestine, kidney, and blood−brain barrier at the mRNA level.[32] The current Western blot results further demonstrate that OATP-A is expressed in human liver, small intestine, and the parietal lobe of the brain at the protein level. Although OATP-A has been suggested by other research groups to be an apically located absorptive transporter in the small intestine,[7] it may not play a significant role in facilitating SQV absorption due to the low K_m and possible saturation effects. However, the interplay between absorptive and secretory transporters on the apical membrane deserves further study, as does the role of the brain since typical SQV plasma concentrations are in the range of the OATP-A K_m. In this report, we have not investigated the potential roles of OATP-C, OATP8, and OATP-B.

It has been well documented that SQV is a substrate of P-gp and MRP2.[4,5] The role of P-gp and/or MRP2 in hepatic SQV transport was confirmed by using GF120918 and MK571, specific inhibitors of P-gp and the MRP family of transporters, respectively. The fact that significantly greater intracellular accumulation of SQV was observed in Hep G2 cells when these two inhibitors were used strongly suggests that P-gp and MRP2 are involved with SQV efflux from these cells. GF120918 has also been reported to inhibit breast cancer resistant protein (BCRP)-mediated cancer drug transport,[18] so the potential contribution from BCRP to the efflux of SQV from Hep G2 cells cannot be ruled out.

In conclusion, we show for the first time that OATP-A is capable of transporting SQV in vitro. The current results also suggest that basolaterally located OATP-A (influx transporter) together with apically located P-gp and/or MRP2 (efflux transporters) in human hepatocytes may act in a concerted manner for the vectorial transport and excretion of SQV into bile. Although the in vivo importance of OATP-A in SQV's disposition requires further investigation, these results provide a molecular biopharmaceutical basis for further preclinical and clinical studies.

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