Human Organic Anion Transporting Polypeptide-C (SLC21A6) Is a Major Determinant of Rifampin-Mediated Pregnan X Receptor Activation

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ABSTRACT
Rifampin, a member of the rifamycin class of antibiotics, is well known for its ability to induce drug-metabolizing enzymes and transporters, through activation of the pregnane X receptor. Available data suggest rifampin entry into hepatocytes may be transporter-mediated. Accordingly, it is therefore plausible that modulation of the achievable intracellular concentration of rifampin by drug uptake transporters would influence the degree of induction. In this study, we expressed an array of known hepatic uptake transporters to show the key hepatic rifampin uptake transporters are liver-specific members of the organic anion transporting polypeptide family (OATP). Indeed, both OATP-C and OATP8 seemed capable of mediating rifampin uptake into HeLa cells. OATP-C, however, seemed to have far greater affinity and capacity for rifampin transport. In addition, several allelic variants of OATP-C known to be present among European and African Americans were found to have markedly decreased rifampin transport activity. In cell-based, transactivation assays, OATP-C expression was associated with increased cellular rifampin retention as well as potentiation of PXR reporter gene activity. This is the first demonstration of an uptake transporter such as OATP-C, in modulating PXR function, and sheds important new insight into our understanding of the molecular determinants of PXR-mediated inductive processes.

Rifampin belongs to the rifamycin class of antibiotics commonly used in the treatment or prophylaxis of mycobacterial infections. In addition, rifampin is broadly used in clinical studies as a prototypical inducer of drug-metabolizing enzymes and transporters. Recent studies have shown that rifampin-mediated induction of cytochrome P450 (P450) enzymes and drug transporters is mediated by the activation of the nuclear receptor pregnane X receptor (PXR) (Kliwer et al., 1998; Lehmann et al., 1998). Although it is clear that intersubject variability in the attained plasma levels of rifampin could have a major role in modulating the extent of PXR activation, little attention has been paid to the processes involved in rifampin disposition as a potential determinant of PXR activation.

Studies in humans have long indicated that drug transporters are importantly involved in rifampin hepatic uptake. For example, administration of rifamycin SV decreased the elimination rates of bromosulfophthalein (BSP) and indocyanine green, compounds that are also cleared predominantly by hepatic elimination (Acocella et al., 1965). Furthermore, unconjugated bilirubin levels increased during rifamycin SV treatment (Acocella et al., 1965). Indeed, impairment in the hepatic uptake of these organic anions by rifamycins is believed to be the mechanism involved in such drug-drug interactions (Kenwright and Levi, 1973, 1974). Moreover, rifampin treatment is known to increase serum bile acid concentrations (Galeazzi et al., 1980; Berg et al., 1984), suggesting an interaction with hepatic bile acid transporters. Taken together, these findings indicate that the efficient hepatic clearance of rifamycins by human liver is facilitated by sinusoidal membrane uptake system(s) capable of endogenous and exogenous anion transport.

Isolated rat hepatocyte studies have shown evidence for carrier-mediated mechanisms in the hepatic uptake of rifampin, including a high-affinity ($K_m$ value of ~130 $\mu$M), low-capacity system and a low-affinity ($K_m$ value of ~1 mM), high-capacity system (Laperche et al., 1979). These systems are shared by other compounds because rifampin uptake into
hepatocytes could be inhibited by BSP (Kenwright and Levi, 1973; Laperche et al., 1979), cyclosporin (Ziegler and Frimmer, 1986), and renin-inhibiting peptides (Bertram and Ziegler, 1991). Conversely, BSP (Kenwright and Levi, 1974) and bile acid (Anwer et al., 1978) uptake by rat hepatocytes was inhibited by rifamycins.

Although a large body of compelling evidence indicates an important role of carrier-mediated mechanisms for rifampin uptake into hepatocytes, the identity of the responsible proteins has not been determined. Recently, Fattinger et al. (2000) demonstrated that the transport activity of members of the rat organic anion transporting polypeptide family (OATP), including rOatp1 (Jacquemin et al., 1994) and rOatp2 (Noé et al., 1997), was differentially inhibited by rifampin and rifamycin SV. Although these proteins are localized to the basolateral hepatocyte membrane and are capable of transporting compounds that are known to interact with the hepatic uptake of rifampin, the ability of rat Oatps to directly transport rifampin was not demonstrated (Fattinger et al., 2000).

In this study, a comprehensive screening of a variety of rat and human uptake transporters, including members of the OATP, organic anion transporter (OAT), organic cation transporter (OCT), and sodium-dependent taurocholate transporter (NTCP) families, has identified the liver-specific transporter OATP-C as the major rifampin transporter. Moreover, we directly demonstrate that OATP-C expression enhances cellular accumulation of rifampin and potentiates PXR activation.

Materials and Methods

Chemicals. [3H]Rifampin (18.5 Ci/mmol, radiochemical purity >97%) was obtained from Moravek Biochemicals (Brea, CA). [3H]Estradiol-17β-β-glucuronide (E2G) (44 Ci/mmol, radiochemical purity >97%) was obtained from PerkinElmer Life Sciences (Boston, MA). BSP, caffeine, cyclosporin A (CyA), and rifampin were obtained from Sigma-Aldrich (St. Louis, MO). Hyperforin was obtained from Apap Chemicals (Oxfordshire, UK). Indinavir, nelfinavir, ritonavir, and saquinavir were generously supplied by Merck Research Laboratories (West Point, PA), Agouron Pharmaceuticals (La Jolla, CA), Abbott Diagnostics (Abbott Park, IL), and Roche Discovery (Rahway, NJ), respectively.

Expression Plasmids. Preparation of expression plasmids containing cDNAs for human NTCP (Kim et al., 1999), OATP-A (Cvetkovic et al., 1999), OATP-C and its naturally occurring variants (Tirona et al., 2001), rat oatp1, oatp2, and oct1 (Cvetkovic et al., 1999) has been described previously. Human OATP-A and rat oatp3 cDNAs were kindly provided Drs. Peter Meier (University Hospital, Zurich, Switzerland) and Paul Dawson (Wake Forest University, Winston-Salem, NC). All other expression plasmids were obtained by reverse transcription-polymerase chain reaction followed by isolation of cDNAs into pEF6/V5-His vector (Invitrogen, Carlsbad, CA). Ziegler, 1991). Conversely, BSP (Kenwright and Levi, 1974) and bile acid (Anwer et al., 1978) uptake by rat hepatocytes was inhibited by rifamycins. The following primers were used for polymerase chain reaction: hOCT1, 5'-ATG GGA CCC AGG ATA GGG CCA GCG G-3' and 5'-GTC TCT CTC TGG CAG ACG TCT CGC TGC TCC TCC AGC GAA-3'; hOCT2, 5'-AGT GGC ATG GCC ATC TTC TGG GAG ATC TTC G-3'; rOAT1, 5'-GAG GTC CTC AGT GAT CGA CCA CTC AGC-3' and 5'-TCA AAG TCC ATT CCT CTC TGG TG-3'; and rOAT3, 5'-GTT GTC CTC AGC TGG TCC GCA CCA GGC CGG G-3' and 5'-AGT GCC ATG GCC TTC TTC GAG ATC TTC G-3'; rOAT1, 5'-GAG GTC CTC AGT GAT CGA CCA CTC AGC-3' and 5'-TCA AAG TCC ATT CCT CTC TGG TG-3'; and rOAT3, 5'-GTT GTC CTC AGC TGG TCC GCA CCA GGC CGG G-3' and 5'-AGT GCC ATG GCC TTC TTC GAG ATC TTC G-3'; rOAT1, 5'-GAG GTC CTC AGT GAT CGA CCA CTC AGC-3' and 5'-TCA AAG TCC ATT CCT CTC TGG TG-3'; and rOAT3, 5'-AGT GCC ATG GCC TTC TTC GAG ATC TTC G-3'.

Transient Transfection and Uptake Transport Assays. Transient transfection assays were performed using the recombinant vaccinia virus (VTF-7) expression method detailed previously (Cvetkovic et al., 1999). Briefly, human cervical carcinoma cells (HeLa) (American Type Culture Collection, Manassas, VA) were seeded into 12-well plates, infected with vaccinia virus, and then transfected with expression plasmid or vector control using Lipofectin reagent (Invitrogen). Sixteen hours thereafter, cells were washed with transport media (OptiMEM; Invitrogen) and treated with radiolabeled drug in the presence or absence of transport inhibitors. At various time intervals, cells were washed three times with ice-cold medium then lysed with 1% SDS. Retained cellular radioactivity was quantified by liquid scintillation spectrometry.

PXR Transactivation Assay. HeLa cells with stably inducible expression of OATP-C were prepared by hygromycin selection after transfection with the pMEP4 expression vector containing OATP-C. rC1a DNA by a procedure described previously (Shi et al., 1995). Expression of OATP-C, which is controlled by the metallothionein IIa promoter present in pMEP4, is activated by zinc exposure. In brief, HeLa-OATP-C cells were plated in 12-well plates (0.5 × 10⁶ cells/well) in growth medium (Dulbecco's modified Eagle's medium with 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) in the presence of 100 μM zinc sulfate (100 μM) was added to the growth media when desired to induce OATP-C expression. Eight hours later, cells were transfected with a cytchrome P450 3A4 luciferase reporter plasmid (CYP3A4-XREM-Luc, 250 ng/well) (Zhang et al., 2001), pRL-TK (10 ng/well; Invitrogen), human PXR expression plasmid (pEP-HPXR, 250 ng/well), and human HNF4α expression plasmid (pEP-HNF4a, 250 ng/well) in OptiMEM using Lipofectin reagent. The preparation of the PXR and HNF4α plasmids, as well as details on the requirement of HNF4α for CYP3A4 promoter transactivation in HeLa cells will be described elsewhere. Again, zinc sulfate (100 μM) was added to the media when desired for OATP-C expression. After 16 h, the media were replaced with media (OptiMEM) containing rifampin (0–50 μM) with or without zinc sulfate (150 μM). Twenty-four hours thereafter, cells were lysed and luciferase activities (Dual Luciferase Assay System; Promega, Madison, WI) were determined.

Data Fitting and Statistical Analysis. Parameters for saturation kinetics (Vmax and Km) as well as EC50 values for inhibition of uptake transport and PXR activation were estimated by nonlinear curve fitting using Prism (GraphPad Software, Inc., San Diego, CA) or Scientist (MicroMath Inc., Salt Lake City, UT). Pairwise analysis was performed using Student’s t test. A P < 0.05 was considered as a statistically significant difference.

Results

Rifampin Uptake Is Mediated by OATP-C, OATP8, and rOAT4. To identify the proteins responsible for the hepatic uptake of rifampin, transport experiments were performed in HeLa cells transiently transfected with a variety of cDNAs coding for members of human and rat OAT, OAT, OCT, and NTCP families. At a concentration of 0.5 μM, statistically significant higher uptake rates for [3H]rifampin were found in cells transfected with the human hepatocyte-specific transporters OATP-C and OATP8 (Fig. 1). In addition, rOAT4, the rat ortholog of OATP-C, was capable of transporting rifampin (Fig. 1). Other members of human or
rat OATP, OAT, OCT, and NTCP were incapable of facilitating rifampin transport under these conditions, although they could transport their prototypical substrates (estrone sulfate, p-aminohippurate, tetraethylammonium, and taurocholate, respectively) (data not shown). OATP-C-specific uptake of rifampin in transfected cells was time-dependent, reaching a plateau over 30 min (Fig. 2A). Concentration-response experiments indicated that OATP-C-specific rifampin uptake was saturable with a $K_m$ of approximately 1.5 $\mu$M (Fig. 2B).

Despite that a high-level expression of transporters could be detected by Western blot analysis (data not shown), the affinity ($K_m$) for rifampin by OATP8 or rOATP4 could not be accurately determined due to low apparent transport efficiency.

**Inhibition of OATP-C-Mediated E2G Uptake by Rifampin and Other Drugs.** To further evaluate the interactions between rifampin and OATP-C, transport inhibition studies were performed. OATP-C-specific transport of the prototypical substrate E2G was inhibited by rifampin in a concentration-dependent manner (Fig. 3B). The concentration for half-maximal inhibition for E2G uptake (0.94 $\mu$M) was similar to the $K_m$ (1.5 $\mu$M) for OATP-C-mediated rifampin uptake. Inhibition of OATP-C-specific E2G uptake by rifampin was comparable with that of other drugs, including BSP, protease inhibitors (indinavir, nelfinavir, saquinavir, and ritonavir), and CyA, and by a constituent of St. John’s Wort, hyperforin (Fig. 3A). Caffeine, at concentrations as high as 100 $\mu$M, did not inhibit OATP-C-mediated E2G uptake. The relative affinities for drug interactions toward OATP-C could be determined from concentration-response studies (Fig. 3B) and indicate the following rank order potencies (BSP > CyA > nelfinavir > ritonavir > hyperforin > indinavir).

**Decreased Rifampin Transport by Naturally Occurring OATP-C Variants.** We have previously reported that several genetic polymorphisms in OATP-C were associated with markedly decreased transport activity of E2G and estrone sulfate in vitro (Tirona et al., 2001). Experiments using HeLa cells transfected with the cDNAs of 16 OATP-C allelic variants indicates that rifampin uptake is markedly de...

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**Fig. 1.** Transport of rifampin in HeLa cells with transient expression of uptake transporters. HeLa cells were transiently transfected with expression plasmids for various members of the human and rat OATP, OAT, OCT, and NTCP families or vector control using a recombinant vaccinia virus method. Cellular uptake of $[^3]$H]rifampin (0.5 $\mu$M) was determined at 5 min and expressed as a percentage of vector control. Data are shown as the mean ± S.E. of experiments performed on at least three separate occasions. **, $P < 0.01$; ***, $P < 0.001$ versus vector control.

**Fig. 2.** Kinetics of rifampin uptake by OATP-C. A, HeLa cells were transiently transfected with an expression plasmids for wild-type OATP-C(*1a) or blank vector control, and the uptake of $[^3]$H]rifampin (0.5 $\mu$M) was determined over 30 min. B, concentration-dependent uptake of $[^3]$H]rifampin (0.5–10 $\mu$M). Uptake rates were determined over the initial 5-min period. Data are shown as the mean ± S.E. of experiments performed on at least three separate occasions. Lines and kinetic parameters were obtained by nonlinear regression analysis.

**Fig. 3.** Inhibition of OATP-C transport activity by rifampin and other drugs. A, HeLa cells were transiently transfected with an expression plasmid for OATP-C and the inhibition of $[^3]$H]E2G (0.5 $\mu$M) uptake at 5 min was determined for various drugs at 10 $\mu$M with the exception of caffeine (100 $\mu$M). B, concentration-dependent inhibition of $[^3]$H]E2G (0.5 $\mu$M) uptake by various drugs in OATP-C transfected HeLa cells. Data are shown as the mean ± S.E. Lines and kinetic parameters were obtained by nonlinear regression analysis.
creased by the *1b, *2, *3, *5, *6, *7, *9, *11, *12, and *13 variants (Fig. 4). For the OATP-C*2, *3, *5, *6, *9, *11, and *12 variants, altered rifampin transport is partially attributable to decreased fractional cell surface expression resulting from either F73L, V82A, E156G, V174A, I353T, or G488A amino acid substitutions (Tirona et al., 2001). A reduction in rifampin transport activity by the OATP-C*1b allele (N130D) is interesting in that this variant was not previously associated with altered transport function toward estrone sulfate nor E2G. Similarly, the OATP-C*7 allele (N432D) showed significant loss of transport toward rifampin despite only a modest reduction in transport activity toward E2G and an unaffected estrone sulfate uptake capacity as shown in our previous work.

OATP-C Modulates PXR Transactivation of a CYP3A4 Reporter Gene. HeLa cells with stable, zinc-induced expression of OATP-C were found to accumulate rifampin over 15 min to a greater extent than uninduced cells (Fig. 5A). Similar accumulation levels were seen after 30 min (data not shown), indicating that equilibrium in rifampin partitioning between cells and medium occurred somewhat rapidly. When HeLa-OATP-C cells were transfected with a CYP3A4 luciferase reporter construct and an expression plasmid for human PXR, concentration-dependent, rifampin-mediated transcriptional activation differed among cells expressing OATP-C in comparison with cells deficient in this transporter (Fig. 5B). The apparent EC50 values for rifampin activation were 0.9 and 2.5 μM for cells expressing and lacking OATP-C, respectively. Thus, PXR activation occurs at lower rifampin concentrations in OATP-C-expressing cells compared with those that are OATP-C naïve. Maximal reporter activation was similar between OATP-C-expressing and deficient cells, indicating that non-OATP-C-mediated rifampin uptake (Fig. 2A), perhaps by passive diffusion, at high concentrations was enough to elevate intracellular drug concentrations and saturate PXR.

Discussion

It is now increasingly recognized that drug disposition is a complex interplay between the processes involved in drug transport and metabolism. Moreover, transcriptional activation of such processes, mediated by ligand-activated nuclear receptors seems to be a key determinant of intersubject variation in drug disposition profile of rifampin as a variable in the activation of PXR. In this study, we demonstrate that rifampin is primarily transported by the liver-specific uptake transporter human OATP-C (also known as liver-specific transporter-1 and OATP2) (Abe et al., 1999, Hsiang et al., 1999; König et al., 2000b; Tamai et al., 2000) and to a modest extent by OATP8 (also known as LST-2) (König et al., 2000a; Abe et al., 2001). Other transporters localized to the human hepatocyte basolateral membrane such as OATP-B (Tamai et al., 2000; Kullak-Ublick et al., 2001), NTCP (Hagenbuch and Meier, 1994), and hOCT1 (Zhang et al., 1997) were not found to transport this antibiotic. Human transporters that are expressed in other organs such as the kidney, including OATP-A, hOAT1, hOAT3, hOCT1, and hOCT2 did not transport rifampin, consistent with the relatively low renal clearance of the drug (Acocella, 1983). The identification of OATP-C and OATP8 provides the molecular basis for the observed rifampin-organic anion interactions in liver (Acocella et al., 1965). It now seems likely that acute plasma elevations of known or probable OATP-C and OATP8 substrates such as BSP (Cui et al., 2001; Kullak-Ublick et al., 2001), indocyanine green (Cui et al., 2001), and bile acids (Abe et al., 1999, 2001; König et al., 2000b; Kullak-Ublick et al., 2001) by rifampin coadministration are attributable to inhibition of these particular uptake transporters.

![Fig. 4. Rifampin transport by naturally occurring OATP-C variants.](image-url)
In terms of in vivo relevance or contribution of OATP-C versus OATP8, our in vitro data suggest that OATP-C is far more efficient and capable rifampin transporter, with a $K_m$ value (1.5 μM) that is readily attained in vivo (Acocella, 1978). Moreover, given the recent data that suggest that the expressed level of OATP8 in liver is a log order of magnitude lower than OATP-C (Abe et al., 2001), hepatic uptake of rifampin likely is most affected by OATP-C expression and function in vivo. During the course of preparing this manuscript, Cui et al. (2001) demonstrated that OATP8 was capable of transporting rifampin in canine kidney cells double-transfected with OATP8 and multidrug resistance-associated protein 2. Similarly, Vavricka et al. (2002) recently described rifampin transport by OATP-C and OATP-8. It is notable that the $K_m$ value for rifampin transport by OATP-C in Xenopus laevis oocytes (13 μM) (Vavricka et al., 2002) differs from that found in HeLa cells (1.5 μM) (Fig. 2B). Differences in experimental systems (X. laevis oocytes versus mammalian cells) may be an explanation for such disparities in transport characteristics. Similar findings have been described for solute transport by rat Oatp2 in various expression systems (Sugiyama et al., 2001). Therefore, the present report confirms these latest findings and demonstrates that other uptake transporters such as OATs, OCTs, and NTCP do not participate in rifampin disposition. Furthermore, our results indicate that the putative rat ortholog of OATP-C rOatp4rLst1 (Kakyo et al., 1999; Cattori et al., 2000) mediates rifampin transport. Moreover, although rifampin is an inhibitor of rOatp2 with an apparent inhibition constant ($K_i$) of $\sim$1.5 μM (Fattinger et al., 2000), it does not seem to be a substrate, consistent with discrepancies in the $K_m$ values for rifampin uptake into isolated rat hepatocytes ($K_m$ values of 130 and 1000 μM).

The relevance of altered OATP-C activity in rifampin-mediated PXR activation is highlighted by the presence of naturally occurring allelic variants of OATP-C (Tirona et al., 2001) and indicates an important role for amino acids at positions 130 and 432 in drug–transporter interactions.

It is well recognized that the ability of rifampin to induce drug-metabolizing enzymes and transporters such as cytochrome P450s (Goodwin et al., 1999, 2001; Gerbal-Chaloin et al., 2002) and P-glycoprotein (Geick et al., 2001) depends upon activation of the adopted nuclear receptor PXR (Lehmann et al., 1998). Previously, the efflux transporter P-glycoprotein has been shown to affect rifampin-mediated inducibility of CYP3A (Schuetz et al., 1996). Moreover, the magnitude of rifampin-induced intestinal expression of P-glycoprotein is related to MDR1 genotype (Hoffmeyer et al., 2000). Because the hepatocellular concentration of rifampin would be influenced by the presence of uptake transport, we directly assessed the importance of OATP-C expression on drug-metabolizing enzyme inducibility. Expression of OATP-C in HeLa cells increased cellular accumulation of rifampin and significantly potentiated rifampin-mediated PXR activation. Given that the expressed level of OATP-C was much lower than that from a human liver homogenate (data not shown), the potentiation in rifampin-mediated PXR activation is likely to be much greater in vivo. Accordingly, the level of hepatic expression, as well as functional genetic variations in OATP-C is expected to modulate the degree of rifampin-mediated PXR activation, thereby altering the extent of P450 and drug transporter induction. Interestingly, the OATP-C*1b, *5, and *9 alleles, possessing compromised rifampin transport function in vitro, are common nonsynonymous polymorphisms observed in European or African American. Because we now know that functionally relevant polymorphisms in the human PXR are rarely seen in the population (Hustert et al., 2001; Zhang et al., 2001), altered rifampin hepatic uptake due to the presence of variant OATP-C may be a determinant of hepatic PXR activation.

In conclusion, we demonstrated that the human liver-specific transporter OATP-C mediates the hepatocellular uptake of rifampin. Furthermore, several naturally occurring OATP-C variants were found to have markedly reduced rifampin transport activity. Expression of OATP-C enhances rifampin-mediated PXR activation as a result of increased intracellular substrate retention. These findings suggest that uptake transporters such as OATP-C may affect the degree to which hepatic drug-metabolizing enzymes and transporters are induced in vivo by rifampicin and perhaps other drugs. Furthermore, acute elevations in the plasma concentrations of certain drugs and endogenous substances by rifampicin coadministration may be attributed to inhibition of OATP-C. To our knowledge, data presented in this study are the first of this type in directly demonstrating the importance of drug uptake transporters in PXR activation. Considering the frequencies of functionally relevant polymorphisms in OATP-C, variability in OATP-C activity may be a major determinant of the extent of observed intersubject variability in induction of P450 enzymes and transporters.

References


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