Cyclosporine A, but Not Tacrolimus, Shows Relevant Inhibition of Organic Anion-Transporting Protein 1B1-Mediated Transport of Atorvastatin

Rune Amundsen, Hege Christensen, Behnaz Zabihyan, and Anders Åsberg

Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Oslo, Norway

Received January 15, 2010; accepted June 1, 2010

ABSTRACT:

The aim of this study was to investigate the potential of calcineurin inhibitors [cyclosporine A (CsA) and tacrolimus (Tac)] to inhibit cellular uptake of atorvastatin mediated by the liver-specific organic anion-transporting polypeptide 1B1 (OATP1B1) in vitro. Patients with solid organ transplants are frequently treated with HMG-CoA reductase inhibitors (statins). CsA increases atorvastatin systemic exposure severalfold, an effect not observed with Tac. The effect of CsA and Tac on atorvastatin transport via OATP1B1 was investigated in transfected human embryonic kidney 293 cells. 

An in vitro-in vivo extrapolation (IVIVE) was performed to estimate the clinical potential for CsA and Tac to inhibit OATP1B1-mediated transport. CsA inhibited OATP1B1-mediated uptake of atorvastatin approximately 90-fold more efficiently than Tac, with half-maximal inhibitory concentration (IC50) values of 0.021 ± 0.004 and 1.99 ± 0.42 μM, respectively. Coincubation compared with preincubation with CsA showed a 20-fold lower inhibitory capacity, with an IC50 value of 0.47 ± 0.34 μM. The IVIVE showed that clinically obtainable concentrations of CsA, but not Tac, inhibit OATP1B1 transport of atorvastatin. CsA inhibition ranged from 28 to 77% within a dosing interval, whereas it was less than 1% for Tac, considering free concentrations and assuming competitive inhibition. This does not fully explain the clinically observed interaction with CsA, suggesting that a more complex inhibitory mechanism may be present. This is also supported by the decreased IC50 value of CsA after preincubation. This study provides evidence that OATP1B1 inhibition is a relevant mechanism for the interaction observed between CsA and atorvastatin.

Introduction

The incidence of cardiovascular disease is high in patients receiving solid organ transplants, partly because of a high incidence of dyslipidemia (Aakhus et al., 1999). To reduce the overall cardiovascular risk, transplant recipients are now frequently treated with lipid-lowering drugs, especially HMG-CoA reductase inhibitors (statins). A challenge with statin treatment in this population is the potential for pharmacokinetic interaction with immunosuppressive drugs, especially cyclosporine A (CsA) (Åsberg, 2003). Interactions with CsA are described for all statins, and up to 20-fold increases in systemic exposure are reported (Kallikoski and Niemi, 2009).

Pravastatin and fluvastatin are the preferred lipid-lowering therapies in solid organ transplant recipients, because of the relatively low risk of interaction with CsA (Holdaas et al., 2006; Skalicka et al., 2009). However, in patients with severe hyperlipidemia the efficacy of these two statins may become inadequate. Atorvastatin has been shown to have greater lipid-lowering efficacy than the other statins (Jones et al., 1998), but its use in CsA-treated patients is discouraged because of the severalfold increased systemic exposure with coadministration (Her mann et al., 2004; Lemahieu et al., 2005). Increased systemic exposure of statins is a risk factor for statin-induced adverse events, such as myopathy and in rare cases even rhabdomyolysis (Ballantyne et al., 2003).

Inhibition of CYP3A4-mediated metabolism and transport by P-glycoprotein (P-gp) as well as the organic anion-transporting polypeptide 1B1 (OATP1B1) have been suggested as possible mechanisms for this interaction (Åsberg, 2003; Skalicka et al., 2009). OATP1B1, a member of the solute carrier family SLC, is a liver-specific transporter exclusively expressed on the sinusoidal membrane of the hepatocytes. OATP1B1 facilitates uptake of statins into hepatocytes, thereby increasing the amount of statins available for metabolism by liver enzymes and excretion into the bile (Smith et al., 2005).

Atorvastatin is administered in its active form, as the hydroxy acid, but equilibrates with the inactive lactone form in vivo. Atorvastatin is almost completely metabolized before excretion, primarily by CYP3A4 in the liver. The acid form of atorvastatin has been reported to be both a substrate and an inhibitor of OATP1B1 (Chen et al., 2005; Kameyama et al., 2005). Inhibition of OATP1B1-mediated hepatic uptake will not only reduce the elimination of atorvastatin but also directly limit its availability at the site of action within hepatocytes. The lack of a proportional lipid-lowering effect despite a severalfold increased systemic atorvastatin exposure in CsA-treated patients, reported by Åsberg et al. (2001), suggests that inhibition of OATP1B1-mediated uptake into hepatocytes is a plausible mechanism for the interaction observed with CsA.
Lemahieu et al. (2005) investigated the effect of tacrolimus (Tac) as well as CsA coadministration on atorvastatin pharmacokinetics. In contrast to the significant increase in systemic exposure of atorvastatin experienced after concomitant treatment with CsA, treatment with Tac did not affect atorvastatin pharmacokinetics.

The primary aim of this study was to investigate OATP1B1-mediated transport of atorvastatin and compare the efficacy of CsA and Tac to inhibit this transport in vitro to provide an explanation for the clinically observed interaction. In vitro-in vivo extrapolation of the inhibitory efficacy was also performed to elucidate the contribution of OATP1B1 inhibition to the overall clinical interaction.

Materials and Methods

Materials. Acid and lactone forms of atorvastatin and d5-atorvastatin acid and lactone were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Tacrolimus was a gift from Fujisawa GmbH (Munich, Germany). Human embryonic kidney (HEK293) cells transfected with OATP1B1*1a was kindly donated by Professor Ikumi Tamai, Tokyo University of Science, Chiba, Japan (Nozawa et al., 2005). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Invitrogen (Paisley, UK). Hanks’ buffered salt solution, penicillin-streptomycin, and sodium pyruvate were purchased from PAA Laboratories GmbH (Pasching, Austria). High-performance liquid chromatography-grade acetonitrile was purchased from Fisher Scientific UK Limited (Leicestershire, UK). All other chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO).

Uptake Experiments in HEK293 Cells. OATP1B1 and mock-transfected HEK293 cells were grown in high-glucose (4.5 g/l) DMEM containing 10% fetal bovine serum, sodium pyruvate, penicillin, and streptomycin. The selection antibiotic G418 was added to the medium every other passage to maintain the transfection. The cells were incubated at 37°C in a humidified 5% CO2 atmosphere.

Two days before the uptake experiments, OATP1B1-transfected cells and mock cells were seeded onto six-well plates coated with poly-d-lysine, at a density of 4.5 × 10⁵ cells/well. To enhance the expression of OATP1B1, the medium was replaced with medium containing 5 mM sodium butyrate 24 h before assays.

Uptake of atorvastatin acid and lactone by OATP1B1 was assessed both as a function of time and of concentration, in the ranges 0.5 to 15 min and 0 to 10 µM, respectively. The inhibitory potency of CsA and Tac on OATP1B1-mediated uptake of atorvastatin acid was investigated in a 0 to 5 µM concentration range of the respective calciumein inhibitor. The inhibition experiments with CsA were performed by both coinubation and preincubation with the inhibitor. For Tac, only preincubation experiments were performed. It was also investigated whether CsA and Tac are substrates of OATP1B1-mediated transport.

The uptake studies were initiated by addition of serum- and antibiotic-free DMEM containing substrate (atorvastatin acid or lactone), and incubation was performed at 37°C. The incubation time was fixed at 1 min in the concentration studies, and the substrate concentration was fixed at 0.5 µM in the time studies. In the studies of the inhibitory potency of CsA and Tac, the experiments were initiated by incubating 1 h with medium with or without inhibitor added, for the pre- and coinubation experiments, respectively. This was followed by coinubation of inhibitor and 0.5 µM substrate for 1 min. The medium was removed, and the cell layers were washed with 2 × 2 ml of phosphate-buffered saline before addition of 1 ml of lysis solution (acetonitrile-water, 90:10). At this point the internal standard, d5-marked substrate was added to the wells. Cells were then detached from the surface by scraping, and the resulting suspension containing cells were transferred to Eppendorf tubes and frozen at −70°C for 1 h to obtain complete cell lysis. After centrifugation at 20,000g for 10 min at 4°C, the supernatant was transferred to new Eppendorf tubes and evaporated to dryness in a SpeedVac system. Atorvastatin was analyzed by liquid chromatography (LC)-tandem mass spectrometry (MS/MS). The cell pellet was solubilized in 750 µl of 0.1 M NaOH, and an aliquot of 10 µl was used to determine the total protein concentration with a Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA) by the method of Bradford (1976), with bovine serum albumin as standard.

Atorvastatin Analysis. Atorvastatin acid and lactone were analyzed with a cross-validated, slightly modified LC-MS/MS method as described previously (Hermann et al., 2005). Both the mobile phase composition and gradient were somewhat changed to reduce the run time. In short, the evaporated supernatants were resolved in mobile phase A (acetonitrile-10 mM ammonium formate, 30:70, v/v) and centrifuged at 20,000g for 10 min at 4°C, and 100 µl of the supernatant was injected in the high-performance liquid chromatographic system. The analytes were separated on an Omnisphere C18 (3 µm, 30 × 2 mm) analytical column with a ChromSep (3 µm, 10 × 2 mm) guard column (both obtained from Varian, Harbor City, CA). The mobile phase system consisted of mobile phase A and mobile phase B (acetonitrile-10 mM ammonium formate, 60:40, v/v), and the gradient was started at 100% mobile phase A and changed linearly to 60% A and 40% B during the first 3 min, a combination held for 5 min before reequilibration with mobile phase A. The method had a linear range of 0.1 to 15 ng/ml, and the lower limit of quantification was 0.1 ng/ml for both the acid and lactone form of atorvastatin. CsA and Tac were analyzed by validated LC-MS/MS methods (Falk et al., 2007; Taylor et al., 2007).

Data Analysis. The OATP1B1-mediated uptake is presented as mock cell uptake subtracted from the uptake in OATP1B1-transfected cells. Michaels-Menten nonlinear curve fitting was applied to estimate values for the maximal uptake rate \( V_{\text{max}} \) and the Michaelis-Menten constant \( K_m \) of atorvastatin acid and lactone. Half-maximal inhibitory concentration \( IC_{50} \) values of the CsA and Tac inhibitory effect on OATP1B1-mediated atorvastatin acid uptake were estimated by the Hill equation. Curve-fitting analyses were performed using GraphPad Prism (version 5.01; GraphPad Software Inc., San Diego, CA). Pharmacokinetic parameters are expressed as means ± S.D. of three individual experiments.

In Vivo-In Vivo Extrapolation. An in vitro-in vivo extrapolation was performed based on the \( IC_{50} \) values obtained in the cellular uptake experiments to get an estimate of the potential for CsA and Tac to inhibit OATP1B1-mediated transport in an in vivo setting. \( IC_{50} \) values of both pre- and coenucubation were used in the extrapolations. With the assumption of competitive inhibition, the inhibition constant, \( K_i \), was estimated by eq. 1 (Macarron and Hertzberg, 2002):

\[
K_i = \frac{IC_{50}}{1 + S/K_m}
\]

where \( S \) is the substrate concentration.

Because the atorvastatin concentration in patients is much lower than \( K_m \), the intrinsic membrane transport clearance by OATP1B1 \( (PS_{\text{int}}) \) can be approximated by eq. 2:

\[
PS_{\text{int}} = \frac{V_{\text{max}}}{K_m}
\]

Assuming competitive interaction, the intrinsic membrane transport clearance in the presence of an inhibitor can be described by eq. 3 (Kusuhara and Sugiyama, 2002):

\[
PS_{\text{int}}(\text{inhibitor}) = \frac{V_{\text{max}}}{K_m(1 + I/K_i)}
\]

where \( I \) represents the concentration of the inhibitor at the site of the transporter. The ratio, \( R \), of the OATP1B1 membrane transport clearance values with and without inhibitor, respectively, will be a measure of the degree of inhibition of OATP1B1 \( PS_{\text{int}} \) (Kusuhara and Sugiyama, 2002) and is described by eq. 4:

\[
R = \frac{PS_{\text{int}}(\text{inhibitor})}{PS_{\text{int}}(\text{control})} = \frac{1}{1 + I/K_i}
\]

and the fraction inhibited is expressed as eq. 5:

\[
\text{Fraction inhibited} = 1 - \frac{K_i}{K_i + I}
\]

To predict the degree of CsA-mediated inhibition of OATP1B1 \( PS_{\text{int}} \), we used 12-h pharmacokinetic profiles of CsA from nine renal transplant patients (Falk et al., 2008). Inhibition of uptake was presented as a function of time over the dosing interval for total whole blood and unbound plasma concent-
OATP1B1-Mediated Uptake of Atorvastatin Acid and Lactone.

The concentration-dependent uptake of atorvastatin acid and lactone into OATP1B1-transfected HEK293 cells is shown in Fig. 1. Uptake of atorvastatin acid showed Michaelis-Menten kinetics and a high affinity for OATP1B1, with a $K_m$ value of 0.93 ± 0.23 $\mu$M. $V_{\text{max}}$ for the uptake of atorvastatin acid was 3.84 ± 0.27 pmol/min/mg total protein. There was no apparent OATP1B1-mediated uptake of atorvastatin lactone (Fig. 1).

The time dependence of OATP1B1-mediated uptake of atorvastatin lactone was observed (Fig. 2). The maximal inhibition of OATP1B1-mediated uptake of atorvastatin ranged from 94 to 99% using the total concentration of CsA in the estimations, inhibition of OATP1B1-mediated uptake of atorvastatin acid was described as a function of time for a dosing interval. The maximal inhibition of OATP1B1-mediated uptake by Tac was estimated to be 0.77% when we considered the maximum total concentration at the inlet to the liver, averaged from 28 to 77% with preincubation and 2 to 13% with coincubation. By using the total concentration of CsA in the estimations, inhibition of OATP1B1-mediated uptake of atorvastatin ranged from 94 to 99% with preincubation and from 42 to 82% with coincubation in the dose interval. The maximal inhibition of OATP1B1-mediated uptake by Tac was estimated to be 0.77% when we considered the maximum total concentration at the inlet to the liver, estimated on the basis of a $C_{\text{max}}$ value of 0.097 $\mu$M (78 ng/ml) (Niwa et al., 2007).
but not of Tac, on atorvastatin systemic exposure in patients receiving transplants to a large extent may be due to an interaction on OATP1B1-mediated uptake (Hermann et al., 2004; Lemahieu et al., 2005). Inhibition of OATP1B1 also provides a plausible explanation for the relative lack of an increased lipid-lowering effect that would be expected from the severalfold increase in plasma concentrations of atorvastatin when it is used in combination with CsA (Åsberg et al., 2001) because inhibition of OATP1B1 will also affect the delivery of atorvastatin to its site of action.

The in vitro-in vivo extrapolation of uptake inhibition by CsA was performed to elucidate the importance of OATP1B1 inhibition on the severalfold increase in systemic exposure of atorvastatin observed when it is coadministered with CsA (Hermann et al., 2004; Lemahieu et al., 2005). Inhibition of OATP1B1 also provides a plausible explanation for the relative lack of an increased lipid-lowering effect that would be expected from the severalfold increase in plasma concentrations of atorvastatin when it is used in combination with CsA (Åsberg et al., 2001) because inhibition of OATP1B1 will also affect the delivery of atorvastatin to its site of action.

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In the in vitro-in vivo extrapolation studies based on preincubation data, the OATP1B1 $PS_{\text{int}}$ was only reduced by 77% when estimated maximal unbound concentrations of CsA at the inlet to the liver were applied (Fig. 4), indicating that this mechanism only partly explains the interaction observed clinically. In addition, the effect of dose staggering in this case would probably be limited, as the degree of inhibition is substantial throughout the dosing interval (Fig. 4).

Theoretically, an increased unbound concentration of CsA could explain the extensive interaction observed. CsA is highly bound to

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**Fig. 3.** Inhibition of OATP1B1-mediated uptake of atorvastatin acid (0.5 μM) into transfected HEK293 cells by cyclosporine A preincubation (○) and coincubation (■) (A) and tacrolimus (B). Uptake velocity is corrected for mock cell uptake. Each value represents the mean ± S.E.M. of at least three experiments.

**Fig. 4.** Degree of inhibition of OATP1B1-mediated uptake of atorvastatin acid as a function of time after administration of cyclosporine A, estimated from unbound (○) and total (■) concentration at the inlet to the liver, based on preincubation (A) and coincubation data (B). Each value represents the mean ± S.D. of estimates from nine renal transplant patients.
lipoproteins in plasma and the lipid-lowering effect of statins will possibly alter its free fraction, as described with simvastatin (Akhlaghi et al., 1997). During the absorption phase one could also hypothesize the possibility of delayed binding to and saturation of erythrocyte binding sites, temporarily increasing the unbound concentration of CsA.

A limitation of the in vitro-in vivo extrapolation performed is the assumption of pure competitive inhibition. Pharmacokinetic interactions involving cytochrome P450 enzymes have been thoroughly investigated and during the last years an increasing focus has been put on mechanism-based inhibition (Kato et al., 2008). Until now, transport prediction models have generally been based on the assumption of reversible competitive inhibition (Hinton et al., 2008). The effect of preincubation shown in this study could suggest that a more complex mechanism is present. A possible irreversible inhibition of OATP1B1 by CsA would potently inhibit the transporter even at low concentrations, offering an alternate explanation for the interaction observed clinically. In this respect, it is interesting to note that Shitara et al. (2009) recently reported that CsA exhibited a long-lasting inhibitory effect on transporters in rat and rat hepatocytes, with a reduced IC50 value on preincubation, in line with our findings. It is not likely that the mechanism can be explained by models from the enzyme kinetics field in which the inhibitors are required to be processed catalytically by the enzyme for inhibitory effect. Given a more complex mechanism of inhibition, eq. 3 will probably not describe the extent of inhibition precisely, and the assumption of reversible competitive inhibition will underpredict the interaction. Thus, future research should be focused on elucidation of transporter inhibition mechanisms to develop better prediction models for transport interactions that may provide more informative data.

Previous clinical studies have also reported an increase in the exposure of atorvastatin lactone after treatment with known inhibitors of OATP1B1, for instance, CsA and rifampin (Hermann et al., 2004; Lemahieu et al., 2005; Lau et al, 2006). Lau et al. (2006) suggested that this observation could be explained by the lactone form being a substrate for OATP1B1 as well. A wide range of compounds have been reported as substrates for OATP1B1, including cationic and neutral compounds. However, results of the atorvastatin lactone up-take experiments in this study suggest that the lactone form is not a substrate for OATP1B1 (Figs. 1 and 2).

In addition to the interaction with atorvastatin, CsA has also been shown to increase the plasma concentrations of all other marketed statins. Inhibition of OATP1B1 can probably to a large extent explain the majority of these interactions. Even though other mechanisms may also apply, including inhibition of CYP3A4, P-gp, OATP1B3, and OATP2B1, there is reason to believe that the findings in this study may be extrapolated to other interactions between CsA and statins (Kalliokoski and Niemi, 2009).

In conclusion, the present study provides evidence that OATP1B1 inhibition is a relevant mechanism for the interaction observed between CsA and atorvastatin. Tac, on the other hand, does not have the properties to induce a similar interaction in vivo. However, the Ki value obtained for inhibition of OATP1B1 by CsA, assuming competitive inhibition by the free concentration, cannot fully explain the severe interaction observed, indicating that a more complex inhibitor mechanism may be present. This finding is also supported by a decreased IC50 value of CsA after preincubation.

Acknowledgments. We thank Siri Johannesen at the School of Pharmacy for performing the protein analyses and for always being there when needed. We thank Dr. Nils Tore Vethe at the Department of Medical Biochemistry, Rikshospitalet University Hospital, for analyzing the Tac samples. We also thank Professor Ikumi Tamai for the supply of OATP1B1-transfected HEK293 cells.

References


**Address correspondence to:** Rune Amundsen, University of Oslo, School of Pharmacy, P.O. Box 1068 Blindern, 0316 Oslo, Norway. E-mail: rune.amundsen@farmasi.uio.no