Cyclosporine A- and Tacrolimus-Mediated Inhibition of CYP3A4 and CYP3A5 In Vitro

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ABSTRACT:

Cyclosporine A (CsA) and tacrolimus (Tac) are immunosuppressive drugs used in the majority of patients with solid organ transplants, generally in combination with a wide range of drugs. CsA and Tac appear not only to be substrates of CYP3A but also described as inhibitors of CYP3A. For CsA, in particular, inhibition of CYP3A has been suggested as the main mechanism of interaction seen clinically with various drugs. The aim of this study was to investigate the inhibitory effect and inhibition characteristics of CsA and Tac on CYP3A4 and CYP3A5 in vitro and to evaluate its clinical relevance. Inhibition by CsA and Tac was studied using midazolam as the probe substrate in coincubation and preincubation investigations using human liver microsomes (HLMs) as well as specific CYP3A4- and CYP3A5-expressing insect microsomes (Supersomes). In vitro-in vivo extrapolations (IVIVEs) were performed to evaluate the clinical relevance of the inhibition. Both CsA and Tac competitively inhibited CYP3A in HLMs, showing inhibition constants (K) of 0.98 and 0.61 μM, respectively. Experiments in Supersomes revealed that Tac inhibited both CYP3A4 and CYP3A5, whereas CsA only inhibited CYP3A4. In contrast to the HLM experiments, studies in Supersomes showed inhibition by Tac to be NADPH- and time-dependent, with a 5-fold reduction in IC₅₀ after preincubation, supporting a time-dependent inhibition mechanism in recombinant microsomes. By application of HLM data, IVIVE estimated the area under the concentration versus time curve of midazolam to increase by 73 and 27% with CsA and Tac, respectively. The inhibitory effect was predominantly on the intestinal level, whereas hepatic intrinsic clearance seemed unaffected.

Introduction

The calcineurin inhibitors (CNI), cyclosporine A (CsA) and tacrolimus (Tac), are immunosuppressive drugs widely used in many therapeutic areas. For instance, the majority of patients with solid organ transplants use either CsA or Tac as the backbone of their immunosuppressive regimen. However, treatment with CNIs is limited by adverse effects, such as nephrotoxicity, dyslipidemia, hypertension, and neurotoxicity. Patients with solid organ transplants generally use a wide range of other drugs, both for other underlying diseases and to deal with the side effects of CNI treatment.

Both CsA and Tac are substantially metabolized by CYP3A enzymes and hence affected significantly by concomitant treatment with potent CYP3A inhibitors such as diltiazem, erythromycin, and itraconazole (Campana et al., 1996; Mignat, 1997; Shiraga, 1999). The general clinical perception has been that both drugs also inhibit CYP3A and might influence CYP3A-mediated metabolism of other drugs, although literature reports are not unanimous. In particular, CsA has been described as a CYP3A inhibitor, and it has even been suggested that CsA is a mechanism-based inhibitor of CYP3A (Neuvonen et al., 2006; Zhou et al., 2007). Inhibition of CYP3A has, for example, been discussed as the major mechanism for the interactions seen with diltiazem, statins, repaglinide, midazolam, and felodipine (Åsberg et al., 1999; Paine et al., 2000; Åsberg, 2003; Kajosaari et al., 2005; de Jonge et al., 2011), but there are also several studies that do not show any CYP3A inhibitory potential, exemplified by studies with atorvastatin and diltiazem as victim drugs (Åsberg et al., 1999; Lemahieu et al., 2005). Tac, on the other hand, which commonly is considered a more potent “CsA-like” drug, has only been shown to affect CsA pharmacokinetics to a clinically relevant degree, probably due to inhibition of presystemic CYP3A inhibition (Venkataramanan et al., 1991). However, other mechanisms of interaction may also play a role, as exemplified by the effect on atorvastatin pharmacokinetics (Lemahieu et al., 2005). In retrospect, it has been shown that this interaction is mainly mediated via the hepatic uptake transporter organic anion-transporting polypeptide 1B1, for which CsA, contrary to Tac, is a potent inhibitor. However, the possibility that inhibition of CYP3A-mediated metabolism may play a role in this interaction cannot be ruled out (Kajosaari et al., 2005; Neuvonen et al., 2006; Amundsen et al., 2010).

Some in vitro studies investigating the inhibitory activities of CsA and Tac on CYP3A3 metabolism show large differences in inhibitory potency. The majority of these studies have been performed using pooled human liver microsomes (HLMs), but rat liver microsomes and recombinant systems have also been used, in combination with more or less specific probe substrates (Jacobsen et al., 1999; Wandel et al., 1999; Paine et al., 2000; Lecointre et al., 2002; Niwa et al.,

ABBREVIATIONS: CNI, calcineurin inhibitors; CsA, cyclosporine A; Tac, tacrolimus; HLM, human liver microsomes; MDZ, midazolam; LC-MS, liquid chromatography-mass spectrometry; AUC, area under the concentration versus time curve; P450, cytochrome P450.
2007; Picard et al., 2007). Most of these studies indicate that the inhibitory capacity of both CsA and Tac is too low to be clinically relevant. Previous studies have shown that differences in the in vitro test systems applied may influence the evaluation of both mechanism and the degree of inhibition by various drugs (Galetin et al., 2006; Polasek et al., 2010).

The aim of the present study was hence to investigate the inhibitory effect and inhibition characteristics of both CsA and Tac on CYP3A in HLMs as well as in recombinant CYP3A4 and CYP3A5 microsomes, using midazolam as a probe drug. The clinical relevance of this CYP3A inhibition was also evaluated by in vitro-in vivo extrapolation.

Materials and Methods

Materials. Pooled HLMs and baculovirus-infected insect cell microsomes (Supersomes) expressing either human CYP3A4 or CYP3A5 enzymes were purchased from BD Gentest (Woburn, MA). The CYP3A4 activity of the pooled HLMs was measured to 4900 pmol/mg protein × min (testosterone 6β-hydroxylase assay). The HLMs did not contain CYP3A5. The Supersomes expressed human cytochrome P450 reductase and cytochrome b₅, Mida-zolam (MDZ) was a gift from F. Hoffmann-La Roche AB (Basel, Switzerland). The metabolite 1'-OH MDZ was purchased from Sigma-Aldrich (St. Louis, MO). CsA was obtained from Sigma-Aldrich. Tac was a gift from Fujisawa GmbH (Munich, Germany). High-performance liquid chromatography-grade acetonitrile was purchased from Fisher Scientific UK Limited (Leicestershire, UK). All other chemicals were reagent grade and were purchased from Sigma-Aldrich.

Study Design. MDZ was used as the CYP3A probe substrate, and both MDZ and its CYP3A3-mediated metabolite 1'-OH MDZ were analyzed by a validated LC-MS method (Christensen et al., 2009). Both coinubation and preincubation investigations with CsA and Tac were performed in HLMs and in CYP3A4 and CYP3A5 Supersomes to reveal possible time-dependent inhibition. NADPH dependence was also investigated. If there was no effect of preincubation, reversible inhibition investigations were performed. A two-step method was used to characterize the kinetic details for time-dependent inhibition (Yang et al., 2005; Polasek and Miners, 2007).

Coinubcation. HLMs (total protein concentration 0.25 mg/ml) and Supersomes expressing human CYP3A4 or CYP3A5 (3.5 pmol/ml) were incubated for 15 min in an incubation buffer consisting of Tris-H₂SO₄ (pH 7.5, 150 mM (final concentration)), MgSO₄ (0.5 mM), and NADPH (1.6 mM) at 37°C. Then, a mixture of probe substrate (MDZ) and increasing concentrations of inhibitor (0, 0.25, 0.5, 1, 1.5, 2.5, 5, and 10 μM for CsA and 0, 0.025, 0.05, 0.1, 0.15, 0.25, 0.5, 1, 2, and 4 μM for Tac) dissolved in incubation buffer and methanol were added. The concentration of MDZ was set to 2 μM, the approximate Kₘ value in the system (Christensen et al., 2009). The total methanol concentration in the incubations was 1%, and the final incubation volume was 500 μl. The samples were incubated for 7.5 min in silicone-coated glass containers in a 37°C shaking water bath. The incubation was terminated by addition of 300 μl of ice-cold acetonitrile including 0.044 μM diazepam as internal standard. After 30 min on ice, the samples were centrifuged for 5 min at 16000g (3500 rpm) at 4°C, and the supernatant was decanted and analyzed for MDZ and 1'-OH MDZ by LC-MS.

Preincubation. Inhibitor (CsA or Tac, as described under Coinubcation) was preincubated with HLMs (total protein concentration 0.25 mg/ml) or Supersomes expressing human CYP3A4 or CYP3A5 (3.5 pmol/ml) in the incubation buffer for 15 min at 37°C in the absence of MDZ. After the preincubuation time, 2 μM MDZ was added to a final incubation volume of 500 μl, and the samples were further incubated for 7.5 min. The total methanol concentration in the incubations was 1%. The samples were incubated, the incubation was terminated, and the samples were prepared for LC-MS analysis as described above.

Reversible inhibition. A matrix of substrate (0.5, 1, 2, 5, 10, 20, and 40 μM MDZ) and inhibitor concentrations (0, 1, and 5 μM CsA or 0.025, and 1 μM Tac) was used to assign a mechanism of inhibition and estimate an inhibition constant (Kᵢ). Inhibition reactions were performed as described for coionubcations above.

Inactivation kinetics. In the first step (the inactivation step), a concentrated mixture of Supersomes expressing human CYP3A4 or CYP3A5 (100 pmol/ml) in incubation buffer was preincubated at 37°C with various concentrations of Tac. In the second step, aliquots of 20 μl were removed from the preincubation tubes at selected preincubation times (0, 2.5, 5, 10, and 30 min), diluted 20-fold in incubation buffer containing a saturating amount of MDZ (20 μM), and incubated for 3 min at 37°C to determine remaining enzyme activity. The total methanol concentration in the incubations was 1% in both incubation steps. NADPH dependence. Single-point inactivation experiments were performed to investigate possible NADPH-dependent inhibition by Tac. Supersomes expressing human CYP3A4 or CYP3A5 (100 pmol/ml) in incubation buffer were preincubated at 37°C with Tac (2 μM + control) in the absence and presence of NADPH. Aliquots of 20 μl were removed from the preincubation tubes at 0 and 30 min, diluted 20-fold in incubation buffer containing a saturating amount of MDZ (20 μM), and incubated for 3 more min at 37°C. The decrease in activity was calculated by eq. 1 (Walsky and Obach, 2004):
In Vitro-In Vivo Extrapolation. The extent of drug-drug interactions can be described by the ratio of AUC_{p.o.} in the presence and absence of inhibitor. For drugs that are CYP3A substrates, significant first-pass metabolism may occur in the intestinal wall, thus affecting the bioavailability. The AUC_{p.o.} ratio is therefore affected by both hepatic intrinsic clearance (CL_{int}) and intestinal wall availability (F_{g}), and the effects on intestinal and hepatic CYP3A-mediated metabolism are combined as a product (Wang et al., 2004) as in eq. 3:

\[
\frac{\text{AUC}_{p,o}}{\text{AUC}_{p,o}^{\text{abs}}} = \frac{\text{CL}_{\text{int}}/F_{g}}{\text{CL}_{\text{int}}/F_{g}'} \times \frac{F_{g}'}{F_{g}}
\]

(3)

where CL_{int} and CL_{abs} are the hepatic intrinsic clearance and F_{g} and F_{g}' are the intestinal wall availability in the presence and absence of inhibitor, respectively. If competitive inhibition is assumed, the terms CL_{int}/CL_{abs} and F_{g}/F_{g}' can be described as in eqs. 4 and 5 (Obach et al., 2006):

\[
\frac{\text{CL}_{\text{int}}}{\text{CL}_{\text{abs}}} = \frac{1}{1 + \left(\frac{I_{\text{abs}}}{K_{I}}\right)} + \left(1 - f_{\text{int}}\right)
\]

(4)

\[
\frac{F_{g}'}{F_{g}} = \frac{1}{F_{g} + (1 - F_{g}) \times \left(\frac{1}{1 + \left(\frac{I_{\text{abs}}}{K_{I}}\right)}\right)}
\]

(5)

For time-dependent inhibition, the corresponding terms are shown in eqs. 6 and 7 (Wang et al., 2004):

\[
\frac{\text{CL}_{\text{int}}}{\text{CL}_{\text{abs}}} = \frac{1}{1 + \left(\frac{I_{\text{abs}}}{K_{I}}\right)} + \left(1 - f_{\text{int}}\right)
\]

(6)

\[
\frac{F_{g}'}{F_{g}} = \frac{1}{F_{g} + (1 - F_{g}) \times \left(\frac{1}{1 + \left(\frac{I_{\text{abs}}}{K_{I}}\right)}\right)}
\]

(7)

where \[I_{\text{abs}}\] is the concentration of inhibitor in vivo, \(f_{\text{int}}\) is the fraction of drug substrate metabolized by CYP3A, and \(k_{\text{deg,CYP3A,hep}}\) and \(k_{\text{deg,CYP3A,gut}}\) are the first-order degradation rate constants of the CYP3A enzyme in the liver and intestine, respectively.

Predictions were made with six different values for \[I_{\text{abs}}\] total and unbound maximum concentrations (\([I_{\text{abs}}]\), total and unbound average concentrations (\([I_{\text{ave}}]\), and total and unbound hepatic inlet concentration (\([I_{\text{inlet}}]\), calculated according to eq. 8 (Kanamitsu et al., 2000):

\[
[I_{\text{inlet}}] = [I_{\text{max}}] + \frac{D \times k_{a} \times F_{a} \times F_{g}}{Q_{h}}
\]

(8)

where D is the dose, \(k_{a}\) is the absorption rate constant, \(F_{a}\) is fraction absorbed, \(F_{g}\) is fraction escaping intestinal metabolism, and \(Q_{h}\) is hepatic blood flow (1610 ml/min). The unbound concentration (\(C_{u}\)) was estimated by eq. 9:

\[
C_{u} = C_{w} \times \frac{f_{u}}{R_{wz}}
\]

(9)

where \(C_{w}, f_{w}\) and \(R_{wz}\) are whole blood concentration, free fraction in plasma, and blood-plasma partition ratio, respectively. The maximal intestinal inhibitor concentration ([I_{gut}]) was estimated by eq. 10 (Rostami-Hodjegan and Tucker, 2004):

\[
[I_{\text{gut}}] = f_{u,gut} \times \left(\frac{D \times k_{a} \times F_{a}}{Q_{h}}\right)
\]

(10)

where \(f_{u,gut}\) is the free fraction in the enterocytes and \(Q_{h}\) is the intestinal blood flow (248 ml/min) (Rostami-Hodjegan and Tucker, 2004). When the effect of dose staggering was estimated, an [I_{gut}] equal to [I_{\text{max},a}] was applied (Rostami-Hodjegan and Tucker, 2004).

In these calculations, clinical data from a study in renal transplant patients were applied (Falck et al., 2008). The CsA-treated patients showed a mean ± S.D. maximum concentration (\(C_{\text{max}}\)) of 1880 ± 407 nm/gl and average concentration (\(C_{\text{ave}}\)) of 567 ± 158 nm/gl, and the mean dose of cyclosporine (Sandimmune Neoral, Basel, Switzerland) was 163 ± 44 mg administered twice daily. The Tac-treated patients showed a mean \(C_{\text{max}}\) of 23.3 ± 7.2 nm/gl and \(C_{\text{ave}}\) of 11.2 ± 3.6 nm/gl, and the mean dose of Tac (Prograf; Astellas Pharma, Tokyo, Japan) was 8.3 ± 3.9 mg, given twice daily. The assumptions of inhibitor \(F_{g} \times F_{g}'\) of 0.28 and 0.26 (Kato et al., 2008), \(F_{g}\) of 0.52 and 0.97 [calculated from a number of reported values of \((F_{g} \times F_{g}')\) and \(k_{a}\), \(k_{a}\) of 1.28 h^{-1} (mean of four published values) and 1.95 h^{-1} (Kato et al., 2008), \(f_{u}\) of

![Fig. 1. IC_{50} curves showing the effect of coinubation (○) and preincubation (■) with cyclosporine in recombinant CYP3A4 (A), recombinant CYP3A5 (B), and human liver microsomes (C). Each value represents the mean ± S.E.M. of three experiments/paralelons.](downloaded_from_dnd.aspetjournals.org_at_ASPE)
0.012, and $R_b/p$ of 1.58 (mean of three published values) and 3.4 were made for CsA and Tac, respectively (Amundsen et al., 2010). $F_g$ of MDZ was estimated as a mean of four published values to be 0.51 (Gorski et al., 1998; Galetin et al., 2006, 2010; Gertz et al., 2010) and $f_{m(CYP3A)}$ for MDZ was set to 0.94 (Galetin et al., 2006; Obach et al., 2006), and for $k_{deg, CYP3A, hep}$ values of 0.000321 and 0.000481 min$^{-1}$ were applied (Obach et al., 2007). All data sources are available from the authors on request.

Results

**Inhibition Studies.** CsA inhibited CYP3A4-mediated 1'-OH MDZ formation in insect microsomes in a concentration-dependent manner with IC$_{50}$ values of 2.06 ± 0.59 and 1.45 ± 0.16 μM for coincubation and preincubation experiments, respectively. Because no relevant IC$_{50}$ shift was found between these experiments, the inhibition was considered not to be time-dependent (Fig. 1A). In addition, in HLMs the inhibitory effect of CsA on CYP3A metabolism was concentration-dependent with IC$_{50}$ values of 1.24 ± 0.27 and 1.47 ± 0.22 μM for coincubation and preincubation experiments, supporting the fact that the inhibitory effect of CsA was not time-dependent (Fig. 1C). Nonlinear regression of CYP3A4 activity as a function of MDZ concentration indicated that CsA was a reversible competitive inhibitor (Fig. 2), and the $K_i$ values were estimated to 0.89 μM in insect microsomes and 0.98 μM in HLMs. CsA did not inhibit CYP3A5 to any relevant degree (Fig. 1B).

Tac inhibited both CYP3A4- and CYP3A5-mediated 1'-OH MDZ formation in insect microsomes (Fig. 3). Preincubation with Tac resulted in IC$_{50}$ values 5-fold lower than those for coincubation with Tac. The IC$_{50}$ values for Tac on CYP3A4 and CYP3A5 were 0.62 ± 0.12 and 0.63 ± 0.18 μM for coincubation and 0.12 ± 0.02 and 0.14 ± 0.06 μM for preincubation experiments, respectively (Fig. 3, A and B). This result indicated that Tac is a time-dependent inhibitor of CYP3A4 and CYP3A5 in insect microsomes. The inactivation kinetic parameters characterizing the time-dependent inhibition, $k_{inact}$ and $K_a$, obtained for Tac were 0.30 min$^{-1}$ and 2.66 μM for CYP3A4 and 0.21 min$^{-1}$ and 2.69 μM for CYP3A5, respectively, as calculated from the inactivation plot in Fig. 4. The NADPH dependence studies showed that preincubation with NADPH in the Tac experiments resulted in 42 and 52% decreased metabolic activity for CYP3A4 and

![Fig. 2. Nonlinear regression curves as a function of midazolam concentration with varying cyclosporine concentration: 0 μM (○), 1 μM (■), and 5 μM (▲), in recombinant CYP3A4 (A) and human liver microsomes (B).](image)

![Fig. 3. IC$_{50}$ curves showing the effect of coincubation (○) and preincubation (■) with tacrolimus in recombinant CYP3A4 (A), recombinant CYP3A5 (B), and human liver microsomes (C). Each value represents the mean ± S.E.M. of three experiments/parallels.](image)
CYP3A5, respectively, suggesting that Tac behaves as a time-dependent inhibitor of the enzymes in the recombinant microsomes. However, in the HLM studies the inhibitory effect of Tac was less pronounced, and the inhibitory effect was not time-dependent, IC\text{50} values for coincubation and preincubation were 0.94 ± 0.14 and 0.74 ± 0.08 μM, respectively (Fig. 3C). Nonlinear regression of CYP3A4 activity as a function of MDZ concentration indicated that Tac was a reversible competitive inhibitor with a \( K_i \) of 0.61 μM when studied in HLMs (Fig. 5).

**In Vitro-In Vivo Extrapolation.** CsA was assumed to be a competitive inhibitor of CYP3A4, based on the findings in the present study. The AUC of MDZ was estimated to increase by 71 and 74% in vivo if CsA was coadministered with MDZ, using HLM and insect microsome data, respectively, and estimated unbound CsA concentration at the inlet to the liver (\( C_{\text{inlet}} \)) and concentrations within enterocytes (\( C_{\text{gut}} \)) (Table 1). Thus, for CsA data, the test system did not show any relevant effect on the in vitro-in vivo extrapolation. This increase in AUC for midazolam was estimated on the basis of CYP3A4 inhibition affecting both intestinal wall \( F_e \) and hepatic CL\text{int}, and the inhibitory effect of CsA was shown to be predominantly on \( F_e \), whereas the effect on hepatic CL\text{int} was minimal (Table 1).

For Tac, on the other hand, the results of the extrapolations varied several fold, depending on the test system applied. The predicted increase in MDZ AUC was minimal when HLM data were used (27%) (Table 1). However, when the inactivation kinetic parameters obtained from the recombinant CYP3A4 microsomes, which revealed time-dependent inhibition by Tac, were applied in the simulations, a 120% increase in midazolam AUC was estimated on the basis of unbound \( C_{\text{inlet}} \) of Tac (Table 1).

**Discussion**

The main finding of the present study is that both CsA and Tac inhibit CYP3A-mediated metabolism of MDZ in vitro, and in vitro-in vivo extrapolations indicate that this inhibition may induce clinically relevant interactions. At least in the case of CsA, adjustment of concomitant MDZ doses could be necessary. Data from the HLM experiments suggested that both CsA and Tac inhibited CYP3A in a competitive manner, with \( K_i \) values of 0.98 and 0.61 μM, respectively. This finding indicates that Tac is a slightly more potent inhibitor than CsA but considering that Tac is administered in 10-fold lower molar doses than CsA, the latter still seems to be the drug with highest potential for clinical interactions. However, clinical systemic unbound concentrations of both drugs are low, and unbound peak concentrations of both drugs are well below the \( K_i \) values determined in the present study. The hepatic drug metabolism will hence not be affected to any relevant degree.
However, previous studies have shown CsA to induce clinically detectable interactions with a variety of drugs. These interactions are probably mainly due to other mechanisms, however, such as inhibition of organic anion-transporting polypeptide B1 or the efflux transporter P-glycoprotein (Nooter et al., 1990; Amundsen et al., 2010). Reports on Tac as a perpetrator drug are few, but increased exposure of CsA has been reported in combination with Tac (Venkataramanan et al., 1991). The possibility that inhibition of CYP3A may play a role in these clinically observed interactions cannot be ruled out.

A closer look at the in vitro--in vivo extrapolations show that although hepatic CLint is unaffected by CsA or Tac coadministration, there might be an effect on intestinal CYP3A metabolism. By application of the HLM data, the estimated increases in Fg were 63 and 27% for CsA and Tac, respectively. This result is due to the assumption of much higher unbound inhibitor concentrations present in the enterocytes compared with that in the systemic circulation. In line with these findings, Paine et al. (2000) reported an oral clearance (CLint/F) of MDZ in renal transplant patients receiving CsA of 31.2 l/h, approximately half of what was reported in healthy volunteers. However, the systemic clearance was within the range reported for normal subjects, suggesting a potential effect of CsA on oral bioavailability. In addition, Kajosaari et al. (2005) and de Jonge et al. (2011) have shown similar results in CsA-treated patients receiving repaglinide and MDZ, respectively. The present analyses support these in vivo studies, indicating a potential effect on the intestinal level. The lower predicted interaction potential for Tac compared with that for CsA is reflected in the low number of clinical reports describing potential CYP3A inhibition by Tac. In fact, the recent study by de Jonge et al. (2011) showed no effect of Tac on MDZ metabolism in vivo. However, the choice of MDZ as a probe substrate is not optimal for investigating interactions on bioavailability. Because MDZ has a relatively high initial Fg, a doubling of bioavailability is the maximal achievable effect.

Realistic estimates of the drug concentration at the site of metabolism in both liver (Cin vivo) and gut wall (C gut) are crucial for in vitro--in vivo extrapolations to be reliable. The unbound concentration of the inhibitor is usually applied in extrapolations, and the maximum unbound concentration at the inlet to the liver (Cint,w) has been reported to give the most accurate predictions (Obach et al., 2006). In the present study, not even Cin,w were the highest unbound Cin,vivo affected hepatic CLint in a clinical relevant magnitude (less than 10% reduction in CLint). However, there are reports suggesting that application of total concentrations of inhibitor give more reliable predictions (Ito et al., 2004; Bachmann and Lewis, 2005). In that case, although it is unlikely, the inhibitory capacity of CsA would increase severalfold, and a potentially relevant clinical effect on hepatic CLint is obtained from the extrapolations performed. Of interest, de Jonge et al. (2011) recently reported a 45% increase in systemic exposure of MDZ in CsA-treated renal transplant patients compared with patients not receiving CsA (de Jonge et al., 2011), and our extrapolations using unbound CsA concentration best fit with these in vivo findings.

The unbound C gut is one of the factors determining the degree of intestinal inhibition. Assumption of a free fraction of inhibitor in the intestine (fu,gut) of 1 in the present analyses provides the worst-case scenario, because some degree of drug binding in the gut is also plausible. However, a report by Yang et al. (2007) indicated that this assumption (fu,gut = 1) results in the most accurate predictions. Further research on estimation of a reliable concentration of inhibitors in the gut wall is essential for in vitro--in vivo extrapolation of metabolism-related drug-drug interactions in the intestinal wall. The estimated increase in exposure of MDZ seen in this study is, in addition, based on coadministration of CsA or Tac. From the in vitro--in vivo extrapolations, it is shown that a separation of dosing times by only a few hours will abolish this effect of CsA/Tac (Table 1), because C gut will be severely reduced, easily limiting the clinical relevance of the interaction.

CsA was shown to inhibit CYP3A4 competitively in both HLMs and recombinant CYP3A4 insect microsomes, in agreement with other reports for a variety of substrates, with Ki values ranging from 0.3 to 7.6 μM (Jacobsen et al., 1999; Wandel et al., 1999; Paine et al., 2000). CsA, on the other hand, did not inhibit CYP3A5 in recombinant insect microsomes. Even though CsA hepatic CLint is approximately 2.3-fold higher for CYP3A4 than for CYP3A5 (Dai et al., 2006), it is likely that individuals expressing functional CYP3A5 will be less affected by the interaction with CsA. Depending on the CYP3A4/CYP3A5 ratio, CYP3A5 may, to a certain degree, replace the inhibited CYP3A4 enzymes as the metabolizing enzyme.

In contrast to CsA, Tac showed different mechanisms of inhibition in the two test systems used. The HLM studies revealed a competitive inhibition mechanism as mentioned above. This result is in accordance with previous in vitro studies, mainly in HLMs, showing competitive inhibition and Ki values in the range of 0.36 to 3.7 μM (Wandel et al., 1999; Lecointre et al., 2002; Niwa et al., 2007). However, it is worth mentioning that Lecointre et al. (2002), who reported a 6-fold higher Ki value than that in the present study (3.7 μM), applied a substrate concentration of MDZ (15 μM) approximately 10 times higher than the Kin value, i.e., not optimal conditions for inhibition experiments.

### Table 1

<table>
<thead>
<tr>
<th>Inhibitor and Test System</th>
<th>Mechanism</th>
<th>Estimated Change in Exposure of MDZ Using Different Inhibitor Conc. (Increase in Fg/Reduction of CLint)</th>
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<tbody>
<tr>
<td><strong>CsA</strong></td>
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</tr>
<tr>
<td>Recombinant CYP3A4</td>
<td>Comp</td>
<td></td>
</tr>
<tr>
<td>Coadministration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLM</td>
<td>Comp</td>
<td>67 (65/1)</td>
</tr>
<tr>
<td>Dose staggering</td>
<td></td>
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</tr>
<tr>
<td><strong>Tac</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recombinant CYP3A4</td>
<td>TDI</td>
<td>102 (94/4)</td>
</tr>
<tr>
<td>Coadministration</td>
<td></td>
<td></td>
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<tr>
<td>HLM</td>
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<td>27 (27/0)</td>
</tr>
<tr>
<td>Dose staggering</td>
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<td>0 (0/0)</td>
</tr>
</tbody>
</table>

Comp, competitive inhibition; TD, time-dependent inhibition; N.A., not applicable.

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**Observed Aspects**

- Reduction in CLint.
- Reports suggesting application.
- Oral clearance (CLint/F).
- In line with these findings, Paine et al. (2000) reported.
- CsA and Tac coadministration.
- CsA reported to give the most accurate predictions.
- Unbound concentration at the inlet to the liver (Cint,w).
- Realistic estimates of the drug concentration at the site of metabolism.
- Both liver (Cin,vivo) and gut wall (Cgut).
- In vitro--in vivo extrapolations.
- Reliable predictions.
- Competitive inhibition.
- Kin values ranging from 0.3 to 7.6 μM.
- In HLMs, showing competitive inhibition.
- Optimal conditions for inhibition experiments.
- Insect microsomes Tac was showing.

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**Additional Insights**

- P-glycoprotein (Nooter et al., 1990).
- Tac as a perpetrator drug.
- Increased exposure of CsA.
- Inhibitory capacity of CsA.
- Unbound CsA concentration.
- Competitive inhibition mechanism.
- Ki values.
- Competitive inhibition mechanism.
- Optimal conditions.
- Insect microsomes Tac was showing.

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**Table 1**

In vitro--in vivo extrapolation showing estimated change in exposure of MDZ after administration of CsA or Tac.
time-dependent and NADPH-dependent inhibition, which may suggest a mechanism-based inhibitory effect. Of interest, in contrast with CsA, Tac inhibited CYP3A4 and CYP3A5 to a similar extent with comparable inactivation coefficients in this test system. This finding may be explained by Tac showing similar binding affinities for the two enzymes, as reported by Dai et al. (2006). Prediction of the in vivo interaction potential based on these data resulted in a 4- to 5-fold larger change in the AUC ratio, compared with the HLM data. In this respect, it is interesting to note that recombinant enzyme models, such as recombinant Escherichia coli and insect microsomes also previously have been reported to overestimate P450-mediated interactions when used in in vitro-in vivo extrapolations (Polakse and Miners, 2007). This has been thought to be partly due to the relatively high amount of NADPH-cytochrome P450 reductase in recombinant enzyme models, increasing the risk of formation of reactive intermediates inactivating the P450 enzymes (Polakse and Miners, 2007). HLMs have thus recently been suggested to be the optimal in vitro test system for evaluation of P450-mediated interactions (Polakse and Miners, 2007), which also is in line with the present data.

In conclusion, both CsA and Tac were shown to inhibit CYP3A4-mediated metabolism of MDZ in vitro, but only Tac inhibited CYP3A5. In vitro-in vivo extrapolations suggested that the interactions, at least for CsA, may be of clinical relevance. The extrapolations also revealed that the potential effect on MDZ exposure by Tac and CsA was almost completely caused by an effect on intestinal CYP3A metabolism.

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Authorship Contributions
Participated in research design: Amundsen, Åsberg, Ohm, andChristensen. Conducted experiments: Amundsen. Performed data analysis: Amundsen. Wrote or contributed to the writing of the manuscript: Amundsen, Åsberg, Ohm, andChristensen.

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