Cyclosporine A and tacrolimus mediated inhibition of cytochrome P450 3A4 and 3A5 \textit{in vitro}

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Abbreviations

AUC: area under the concentration versus time curve
CL: clearance
$C_{av}$: average concentration
$C_{gut}$: intestinal concentration
$C_{inlet}$: hepatic inlet concentration
$C_{max}$: maximum concentration observed
$C_{u}$: unbound concentration
$C_{wb}$: whole blood concentration
CNI.: calcineurin inhibitors
CsA: cyclosporine A
CYP: cytochrome P-450
$D$: dose
$F$: bioavailability
$F_{a}$: fraction absorbed
$F_{g}$: fraction escaping intestinal metabolism
$f_{m(CYP3A)}$: fraction of drug substrate metabolized by CYP3A
$f_{u}$: free fraction
HPLC: high performance liquid chromatography
$[I]_{av}$: average inhibitor concentration
$[I]_{gut}$: maximal intestinal inhibitor concentration
$[I]_{inlet}$: hepatic inlet concentration
$[I]_{max}$: maximum inhibitor concentration
$IC_{50}$: half maximal inhibitory concentration
IVIVE: in vitro-in vivo extrapolation
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\( k_a \): absorption constant

\( k_{\text{deg,CYP3A}} \): first order degradation rate constants of CYP3A

\( K_i \): inhibition constant

\( K_I \): concentration required for half-maximal inactivation

\( k_{\text{inact}} \): maximal rate of inactivation at saturation

\( k_{\text{obs}} \): initially observed inactivation rate constant

MBI: mechanism-based inhibition

MDZ: midazolam

MS: mass spectrometry

OATP1B1: organic anion transporting polypeptide 1B1

P-gp: P-glycoprotein

\( Q_g \): intestinal blood flow

\( Q_H \): hepatic blood flow

SOT: solid organ transplant

\( T_{1/2} \): terminal half-life

Tac: tacrolimus

TDI: time-dependent inhibition
Abstract

Cyclosporine A (CsA) and tacrolimus (Tac) are immunosuppressive drugs used in the majority of solid organ transplanted patients, generally in combination with a wide range of drugs. CsA and Tac seem not only to be substrates of CYP3A, but have also been described as inhibitors of CYP3A. Especially for CsA, inhibition of CYP3A has been suggested as the main mechanism of interactions 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 probe substrate in co- and preincubation investigations utilizing human liver microsomes (HLMs) as well as specific CYP3A4 and CYP3A5 expressing insect microsomes (Supersomes™). In vitro-in vivo extrapolations (IVIVE) were performed to evaluate the clinical relevance of the inhibition. Both CsA and Tac competitively inhibited CYP3A in HLMs, showing inhibition constants (K_i) of 0.98 µM and 0.61 µM, respectively. Experiments in Supersomes™ revealed that Tac inhibited both CYP3A4 and CYP3A5, while 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 IC_{50} following preincubation, supporting a time-dependent inhibition mechanism in recombinant microsomes. Applying HLM data IVIVE estimated the AUC of MDZ to increase by 73% and 27% with CsA and Tac, respectively. The inhibitory effect was predominantly on the intestinal level, while 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 solid organ transplanted (SOT) patients use either CsA or Tac as backbone of their immunosuppressive regime. Treatment with CNIs is however limited by adverse effects, such as nephrotoxicity, dyslipidemia, hypertension and neurotoxicity. SOT patients 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, though the literature is not unanimous. Especially 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 (de Jonge et al., 2011; Kajosaari et al., 2005; Paine et al., 2000; Åsberg, 2003; Åsberg et al., 1999), but there are also several studies that do not show any CYP3A inhibitory potential, exemplified by studies with atorvastatin and diltiazem as victim drugs (Lemahieu et al., 2005; Åsberg et al., 1999). 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 mainly is mediated via the hepatic uptake transporter organic anion transporting polypeptide 1B1 (OATP1B1), for which CsA, contrary to Tac, is a potent inhibitor. It can however not be ruled out that inhibition of CYP3A mediated metabolism in part plays a role in this interaction (Amundsen et al., 2010; Kajosaari et al., 2005; Neuvonen et al., 2006).

Some *in vitro* studies investigating the inhibitory activities of CsA and Tac on CYP3A metabolism show large differences in inhibitory potency. The majority of these studies have been carried out using pooled human liver microsomes (HLM), but also rat liver microsomes and recombinant systems have been used, in combination with more or less specific probe substrates (Jacobsen et al., 1999; Lecointre et al., 2002; Niwa et al., 2007; Paine et al., 2000; Picard et al., 2007; Wandel et al., 1999). Most of these studies indicate that both CsA and Tac have too low inhibitory capacity to be clinically relevant. Interestingly however, previous studies have shown that differences in *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, utilizing 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 (Cat. 452161) and baculovirus-infected insect cell microsomes (Supersomes™) expressing either human CYP3A4 (Cat. 456202) or CYP3A5 (Cat. 456256) enzymes were purchased from BD Gentest (Woburn, MA). The CYP3A4 activity of the pooled HLMs was measured to 4900 pmol/(mg protein x min) (testosterone 6β-hydroxylase assay). The HLMs did not contain CYP3A5. The Supersomes™ also expressed human cytochrome P450 reductase and cytochrome b5. Midazolam (MDZ) was a gift from F. Hoffmann-La Roche AB, Switzerland. The metabolite 1'-OH MDZ was purchased from Sigma-Aldrich (St.Louis, MO, USA). CsA was obtained from Sigma (St.Louis, MO). Tac was a gift from Fujisawa GmbH (Munich, Germany). HPLC grade acetonitrile was purchased from Fisher Scientific UK Limited (Leicestershire, UK). All other chemicals were reagent grade and purchased from Sigma (St.Louis, MO).

Study design

MDZ was used as CYP3A probe substrate and both MDZ and its CYP3A-mediated metabolite 1'-OH MDZ were analyzed by a validated LC-MS method (Christensen et al., 2009). Both coincubation and preincubation investigations with CsA and Tac were performed in HLMs as well as in CYP3A4 and CYP3A5 Supersomes™, in order to reveal possible time-dependent inhibition. NADPH-dependency was also investigated. In case of no effect of preincubation, reversible inhibition investigations were performed. A two-step method was used to characterize the kinetic details in case of time dependent inhibition (Polasek & Miners, 2007; Yang et al., 2005).
**Coincubation.** HLMs (total protein concentration 0.25 mg/mL) and Supersomes™ expressing human CYP3A4 or CYP3A5 (3.5 pmol/mL) were incubated for 15 minutes 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. Following this, a mixture of probe substrate (MDZ) and increasing concentrations of inhibitor (0, 0.25, 0.5, 1, 1.5, 2.5, 5, (10) µM for CsA and 0, 0.025, 0.05, 0.1, 0.15, 0.25, 0.5, 1, 2, 4 µM for Tac) dissolved in incubation buffer and methanol was added. The concentration of MDZ was set to 2 µM, the approximate $K_m$ value in the system (Christensen et al., 2009). Total methanol concentration in the incubations was 1 % and the final incubation volume was 500 µL. The samples were incubated for 7.5 minutes in silicone-coated glass containers in a 37°C shaking water bath. The incubation was terminated by adding 300 µL ice-cold acetonitrile including 0.044 µM diazepam as internal standard. After 30 minutes on ice, the samples were centrifuged for 5 min at 1600g (3500 rpm) at 4°C and the supernatant was decanted and analysed for MDZ and 1'-OH MDZ by LC-MS.

**Preincubation.** Inhibitor (CsA or Tac, as described above) 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 minutes at 37°C in the absence of MDZ. Following the preincubation time 2 µM MDZ was added to a final incubation volume of 500 µL and the samples were further incubated for 7.5 minutes. Total methanol concentration in the incubations was 1 %. The samples were incubated, terminated and 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, 5 µM CsA or 0, 0.25, 1 µM Tac) were used to assign a
mechanism of inhibition and estimate an inhibition constant ($K_i$). Incubation reactions were performed as described for coincubations above.

Inactivation kinetics. 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. Second step; aliquots of 20 µL were removed from the preincubation tubes at selected preincubation times (0, 2.5, 5, 10 and 30 minutes), diluted 20-fold in incubation buffer containing a saturating amount of MDZ (20 µM) and incubated for 3 minutes at 37°C, to determine remaining enzyme activity. Total methanol concentration in the incubations was 1% in both incubation steps.

NADPH-dependency. Single point inactivation experiments were carried out to investigate possible NADPH dependent inhibition by Tac. Supersomes™ expressing human CYP3A4 or CYP3A5 (100 pmol/mL) in incubation buffer was 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 minutes, diluted 20-fold in incubation buffer containing a saturating amount of MDZ (20 µM) and incubated for 3 more minutes at 37°C. Decrease in activity was calculated by the following equation (Walsky & Obach, 2004):

\[
\text{% decrease in activity} = 100 \times \left( \frac{\text{activity with inactivator}}{\text{activity in control}} \right)_{-NADPH} - \left( \frac{\text{activity with inactivator}}{\text{activity in control}} \right)_{+NADPH} \right) \quad (\text{Eq. 1})
\]
Analysis of MDZ and 1'-OH MDZ

MDZ and 1'-OH MDZ was analyzed by a liquid chromatographic method with mass spectrometry detection (Shimadzu, Kyoto, Japan) as described earlier (Christensen et al., 2009). Briefly, separation was carried out on a reverse phase C8 column (50 × 3 mm, 5 μm; Inertsil, GL Sciences Inc. Tokyo, Japan), using gradient elution with two mobile phases; A: 0.05 M ammonium formate buffer (pH 4.4) and B: acetonitrile 95 %/methanol 5 %. The relative contribution of mobile phase B increased linearly from 25 % to 37.5 % during the first five minutes after sample injection. This mobile phase solution was held constant for 2.5 minutes. During the following 2.5 minutes, mobile phase B content was increased to 46.7% and then quickly increased to 80 %. From the 11th to 17th minute, the column was reequilibrated by reversing the mobile phase B to the start concentration of 25 %. The flow rate of the mobile phase was 0.5 mL/min, and the analysis run time per sample was 17 minutes. MDZ was not identified in the present analysis. The retention time was 5.7 min for 1'-OH MDZ and 4.7 min for 4-OH MDZ. Diazepam was applied as internal standard (0.044 μM) and showed a retention time of 9.8 min.

Kinetic analyses

In the time dependent inhibition studies half maximal inhibitory concentration (IC₅₀) values of CsA and Tac on CYP3A mediated 1'-hydroxylation of MDZ were estimated by the Hill equation. The effect of preincubation with inhibitor was assessed by comparing IC₅₀ values of pre- and coincubations. A decrease of at least 1.5- to 2-fold for IC₅₀ following preincubation were used as cut-off for categorizing the drugs as time dependent inhibitors (Grimm et al., 2009).
In the reversible inhibition studies simultaneous non-linear regressions were performed applying both competitive, non-competitive, uncompetitive and mixed inhibition models. The model best fitting the data was determined together with the matching $K_i$ value, using the method of Marquardt and Levenberg, which blends the method of linear descent and the method of Gauss-Newton.

Constants describing the inactivation of CYP3A metabolism, $K_1$ (concentration required for half-maximal inactivation) and $k_{\text{inact}}$ (maximal rate of inactivation at saturation) were determined using the initially observed inactivation rate constant ($k_{\text{obs}}$) at each Tac concentration in the inactivation studies. The values of $k_{\text{obs}}$ were plotted against the Tac concentrations, and the inactivation parameters were obtained by non-linear regression using equation 2,

$$k_{\text{obs}} = \frac{k_{\text{inact}}[I]}{K_1 + [I]}$$

(Eq. 2)

where $k_{\text{inact}}$ is the maximum rate of inactivation, $[I]$ the concentration of Tac, and $K_1$ the concentration of Tac that produces half-maximal rate of inactivation. Unbound $K_i$ ($K_{i,u}$) and $K_1$ ($K_{1,u}$) values were calculated by multiplying $K_i$ and $K_1$ values with the free fraction ($f_u$).

All curve-fitting analyses were performed using GraphPad Prism® (Version 5.01, GraphPad Software, San Diego, CA, US). Pharmacokinetic parameters are expressed as means ± S.D. Studies on time-dependent inhibition and reversible inhibition of Supersomes™ consisted of three individual experiments with single or duplicate determinations. All studies of HLMs and the inactivation studies were carried out as single experiments with three replicates.
In vitro-in vivo extrapolation

The extent of drug-drug interactions can be described by the ratio of AUC$_{po}$ in the presence and absence of inhibitor. For drugs being CYP3A substrates, significant first pass metabolism may occur in the intestinal wall, thus affecting the bioavailability. The AUC$_{po}$ 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):

$$\frac{AUC_{po}}{AUC_{po}} = \frac{CL_{int}/F_g}{CL_{int}/F_g} = \frac{F'_g}{F_g} \times \frac{CL_{int}}{CL_{int}'}$$  
(Eq. 3)

where $CL_{int}'$ and $CL_{int}$ are the hepatic intrinsic clearance and $F'_g$ and $F_g$ the intestinal wall availability, in the presence and absence of inhibitor, respectively. Assuming competitive inhibition, the terms $CL_{int}/CL_{int}'$ and $F'_g/F_g$ can be described as follows (Obach et al., 2006):

$$\frac{CL_{int}}{CL_{int}'} = \frac{1}{\left( \frac{f_m(CYP3A)}{1+\left( \frac{\text{ILm}_{\text{m}}}{K_1} \right)} \right)^{1-f_m(CYP3A)}}$$  
(Eq. 4)

$$\frac{F'_g}{F_g} = \frac{1}{F_g+(1-F_g)\times \left( \frac{1}{\text{ILm}_{\text{int}}} \right)}$$  
(Eq. 5)

For time-dependent inhibition the corresponding terms are (Wang et al., 2004):

$$\frac{CL_{int}}{CL_{int}'} = \frac{1}{\left( \frac{f_m(CYP3A)}{1+\left( \frac{K_{\text{inact}} \times \text{ILm}_{\text{m}}}{K_1^* \times K_{\text{deg.CYP3A.}\text{hep}}} \right)} \right)^{1-f_m(CYP3A)}}$$  
(Eq. 6)
\[ \frac{F'_G}{F_G} = \frac{1}{F_G + (1-F_G) \times \left( \frac{1}{k_{inact} \times [I]_{gut}} + \frac{k_{deg,CYP3A,hep} \times [I]_{gut} + k_t}{k_{deg,CYP3A,gut} \times (k_{gut} + k_t)} \right)} \]  

(Eq. 7)

\( [I]_{in\,vivo} \) is the concentration of inhibitor \( \textit{in vivo} \), \( f_{m(CYP3A)} \) is the fraction of drug substrate metabolized by CYP3A and \( k_{deg,CYP3A,hep} \) and \( k_{deg,CYP3A,gut} \) 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 \( [I]_{in\,vivo} \), total and unbound maximum concentrations (\( [I]_{\text{max}} \)), total and unbound average concentrations (\( [I]_{\text{av}} \)), and total and unbound hepatic inlet concentration (\( [I]_{\text{inlet}} \)), calculated according to Kanamitsu et al. (Kanamitsu et al., 2000).

\[ [I]_{\text{inlet}} = [I]_{\text{max}} + \frac{D \times k_a \times F_a \times F_G}{Q_H} \]  

(Eq. 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

\[ C_u = C_{wb} \times \frac{f_u}{R_{b/p}} \]  

(Eq. 9)

where \( C_{wb} \), \( f_u \) and \( R_{b/p} \) are whole blood concentration, free fraction in plasma and blood-plasma partition ratio, respectively. The maximal intestinal inhibitor concentration (\( [I]_{gut} \)) was estimated by the following equation (Rostami-Hodjegan & Tucker, 2004):
where $f_{u,gut}$ is the free fraction in the enterocytes and $Q_g$ is the intestinal blood flow (248 mL/min, (Rostami-Hodjegan & Tucker, 2004)). When estimating the effect of dose staggering an $[I]_{gut}$ equal to $[I]_{max,u}$ was applied (Rostami-Hodjegan & 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 $C_{max}$ (± SD) of 1880 ± 407 ng/mL, $C_{av}$ of 567 ± 158 ng/mL and the mean dose of Sandimmun Neoral® was 163 ± 44 mg administered twice daily. The Tac treated patients showed a mean $C_{max}$ of 23.3 ± 7.2 ng/mL, $C_{av}$ of 11.2 ± 3.6 ng/mL and the mean dose of Prograf® was 8.3 ± 3.9 mg, given twice daily. The assumption of inhibitor $F_a \times F_g$ of 0.28 and 0.26 (Kato et al., 2008), $F_a$ of 0.52 and 0.97 (calculated from a number of reported values of $(F_a \times F_g)$ and $F_g$), $k_a$ of 1.28 h⁻¹ (mean of four published values) and 1.95 h⁻¹ (Kato et al., 2008), $f_u$ of 0.038 and 0.012, and an $R_{b/p}$ 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 (Galetin et al., 2006; Galetin et al., 2010; Gertz et al., 2010; Gorski et al., 1998) and $f_m(CYP3A)$ for MDZ was set to 0.94 (Galetin et al., 2006; Obach et al., 2006) and for $k_{deg,CYP3A,hep}$ and $k_{deg,CYP3A,gut}$ values of 0.000321 min⁻¹ 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 $IC_{50}$ of $2.06 \pm 0.59$ and $1.45 \pm 0.16$ µM for co- and preincubation experiments, respectively. Since no relevant $IC_{50}$ shift was found between these experiments the inhibition was considered not to be time-dependent (Figure 1A). Also in HLMs the inhibitory effect of CsA on CYP3A metabolism was concentration-dependent with $IC_{50}$ of $1.24 \pm 0.27$ and $1.47 \pm 0.22$ µM for co- and preincubation experiments, supporting that the inhibitory effect of CsA was not time-dependent (Figure 1C). Non-linear regression of CYP3A4 activity as a function of MDZ concentration indicated that CsA was a reversible competitive inhibitor of CYP3A4 (Figure 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 (Figure 1B).

Tac inhibited both CYP3A4 and CYP3A5 mediated 1'-OH MDZ formation in insect microsomes (Figure 3). Preincubation with Tac resulted in $IC_{50}$ values 5-fold lower than coincubation with Tac. The $IC_{50}$ values for Tac on CYP3A4 and CYP3A5 were $0.62 \pm 0.12$ and $0.63 \pm 0.18$ µM for coincubation and $0.12 \pm 0.02$ and $0.14 \pm 0.06$ µM for preincubation experiments, respectively (Figure 3A and 3B). This indicated that Tac is a time-dependent inhibitor of CYP3A4 and CYP3A5 in insect microsomes. The inactivation kinetic parameters characterizing the time-dependent inhibition, $k_{\text{inact}}$ and $K_i$, obtained for Tac were $0.30 \text{ min}^{-1}$ and $2.66$ µM for CYP3A4 and $0.21 \text{ min}^{-1}$ and $2.69$ µM for CYP3A5, respectively, as calculated from the inactivation plot in Figure 4. The NADPH dependency studies showed that preincubating with NADPH in the Tac experiments resulted in a 42% and 52% decreased
metabolic activity for CYP3A4 and 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, $IC_{50}$ values for coincubation and preincubation were $0.94 \pm 0.14 \, \mu M$ and $0.74 \pm 0.08 \, \mu M$, respectively (Figure 3C). Non-linear regression of CYP3A4 activity as a function of MDZ concentration indicated that Tac was a reversible competitive inhibitor with a $K_i$ of $0.61 \, \mu M$ when studied in HLMs (Figure 5).

**In vitro-in vivo extrapolation**

CsA was assumed to be a competitive inhibitor of CYP3A4, based on the findings in the present study. AUC of MDZ was estimated to increase by 73 % and 76 % 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 based on CYP3A4 inhibition affecting both intestinal wall availability ($F_g$) and hepatic intrinsic clearance ($CL_{\text{int}}$), and the inhibitory effect of CsA was shown to be predominantly on $F_g$, while 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 using HLM data (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 147 % increase in midazolam AUC was estimated based on 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 µM and 0.61 µM, respectively. This 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.

Previous studies have however shown CsA to induce clinically detectable interactions with a variety of drugs. These interactions are most probably mainly due to other mechanisms, however, such as inhibition of OATP1B1 or the efflux transporter P-glycoprotein (Amundsen et al., 2010; Nooter et al., 1990). 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). It cannot be ruled out that inhibition of CYP3A in part may play a role in these clinically observed interactions.
A closer look at the in vitro-in vivo extrapolations show that while hepatic $CL_{int}$ is unaffected by CsA or Tac coadministration, there might be an effect on intestinal CYP3A metabolism. By application of the HLM data, the estimated increase in $F_G$ was 64 % and 27 % for CsA and Tac respectively. This is due to the assumption of much higher unbound inhibitor concentrations present in the enterocytes, compared to that in the systemic circulation. In line with these findings, Paine et al. reported an oral clearance ($CL_{int}/F$) of MDZ in kidney transplant patients on CsA of 31.2 L/h, about half what was reported in healthy volunteers (Paine et al., 2000). The systemic clearance was however within the range reported for normal subjects, suggesting a potential effect of CsA on oral bioavailability. Also Kajosaari et al. and de Jonge et al. have shown similar results in CsA-treated patients on repaglinid and MDZ, respectively (de Jonge et al., 2011; Kajosaari et al., 2005). The present analyses support these in vivo studies, indicating a potential effect on the intestinal level. The lower predicted interaction potential for Tac as compared with CsA, is reflected in the low number of clinical reports describing potential CYP3A inhibition by Tac. Actually, the recent study by de Jonge et al. showed no effect of Tac on MDZ metabolism in vivo (de Jonge et al., 2011). The choice of MDZ as probe substrate is however not optimal for investigating interactions on bioavailability. Since MDZ has a relatively high initial $F_G$, a doubling of bioavailability is the maximal achievable effect.

Realistic estimates of the drug concentration at the site of metabolism in both liver ($C_{in\text{vivo}}$) and gut wall ($C_{\text{gut}}$) are crucial for in vitro-in vivo extrapolations to be reliable. The unbound concentration of the inhibitor is usually applied in extrapolations and maximum unbound concentration at the inlet to the liver ($C_{\text{inl, u}}$) has been reported to give the most accurate predictions (Obach et al., 2006). In the present study, not even $C_{\text{inl, u}}$, the highest unbound $C_{\text{in\text{vivo}}}$, affected hepatic $CL_{int}$ in a clinical relevant magnitude (less than 10 % reduction in $CL_{int}$).
There are however reports suggesting that application of total concentrations of inhibitor give more reliable predictions (Bachmann & Lewis, 2005; Ito et al., 2004). In that case, though unlikely, the inhibitory capacity of CsA would increase severalfold, and a potentially relevant clinical effect on hepatic $CL_{int}$ is obtained from the extrapolations performed. Interestingly, de Jonge et al. recently reported a 45% increase in systemic exposure of MDZ in CsA treated renal transplant patients compared to 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 ($f_{u,gut}$) of 1 in the present analyses provides the worst case scenario, as some degree of drug binding in the gut is also plausible. However, a report by Yang et al. indicated that this assumption ($f_{u,gut} = 1$) results in the most accurate predictions (Yang et al., 2007). Further research in respect to estimation of a reliable concentration of inhibitors in the gut wall would be 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), as $C_{gut}$ 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 $K_i$ values ranging from 0.30 μM to 7.6 μM (Jacobsen et al., 1999; Wandel et al., 1999; Paine et al., 2000). CsA did on the other hand not inhibit CYP3A5 in recombinant insect microsomes. Even though CsA hepatic $CL_{int}$ is about 2.3-fold higher for CYP3A4 than for CYP3A5 (Dai et
al., 2006), it is likely to believe 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 is in accordance with previous in vitro studies, mainly in HLMs, showing competitive inhibition and $K_i$ values in the range 0.36 µM to 3.7 µM (Lecointre et al., 2002; Niwa et al., 2007; Wandel et al., 1999). It is however worth mentioning that Lecointre et al. who reported a six-fold higher $K_i$ value than in the present study (3.7 µM), applied a substrate concentration of MDZ (15 µM) about 10 times higher than the $k_m$ value, i.e. not optimal conditions for inhibition experiments. In insect microsomes Tac was showing time-dependent and NADPH-dependent inhibition, which may suggest a mechanism-based inhibitory effect. Interestingly, in contrast to CsA, Tac inhibited CYP3A4 and CYP3A5 to a similar extent with comparable inactivation coefficients in this test system. This may be explained by Tac showing similar binding affinities for the two enzymes, as reported by Dai et al. (Dai et al., 2006). Prediction of the in vivo interaction potential based on these data resulted in a four- to five-fold larger change in AUC-ratio, compared with the HLM data. In this respect it is interesting to note that recombinant enzyme models, such as recombinant E.coli and insect microsomes also previously have been reported to overestimate CYP mediated interactions when used in in vitro-in vivo extrapolations (Polasek & Miners, 2007). This has been discussed to be partly due to the relative high amount of NADPH-cytochrome P450 reductase in recombinant enzyme models, increasing the risk of formation of reactive intermediates inactivating the CYP-enzymes (Polasek & Miners, 2007). HLMs have thus recently been suggested as the
optimal in vitro test system for evaluation of CYP-mediated interactions (Polasek & 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 were almost completely caused by an effect on intestinal CYP3A metabolism.
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Authorship Contributions

Participated in research design: Amundsen, Åsberg, Ohm and Christensen.

Conducted experiments: Amundsen.

Performed data analysis: Amundsen.

Wrote or contributed to the writing of the manuscript: Amundsen, Åsberg, Ohm and Christensen.
DMD #43018

References


Legends for figures

**Figure 1** $IC_{50}$ curves showing the effect of coincubation (●) and preincubation (■) with cyclosporine in (A) recombinant CYP3A4, (B) recombinant CYP3A5 and (C) human liver microsomes. Each value represents mean ± SEM of three experiments/parallels.

**Figure 2** Non-linear regression curves as a function of midazolam concentration with varying cyclosporine concentration; 0 µM (●), 1 µM (■) and 5 µM (▲), in (A) recombinant CYP3A4 and (B) human liver microsomes.

**Figure 3** $IC_{50}$ curves showing the effect of coincubation (●) and preincubation (■) with tacrolimus in (A) recombinant CYP3A4, (B) recombinant CYP3A5 and (C) human liver microsomes. Each value represents mean ± SEM of three experiments/parallels.

**Figure 4** Time-dependent inhibition of CYP3A4 and CYP3A5 by Tacrolimus. (A) Time- and concentration-dependent inhibition of CYP3A4. (B) Plot of $k_{obs}$ of CYP3A4 versus tacrolimus concentration. (C) Time- and concentration-dependent inhibition of CYP3A5. (D) Plot of $k_{obs}$ of CYP3A5 versus tacrolimus concentration.

**Figure 5** Non-linear regression curves as a function of midazolam concentration with varying tacrolimus concentration; 0 µM (●), 0.25 µM (■) and 1 µM (▲), in human liver microsomes.
Table 1 *In vitro-in vivo* extrapolation showing estimated change in exposure of MDZ (%) following administration of CsA or Tac (increase in $F_g$/reduction of $CL_{int}$ in parantheses (%))

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Test system</th>
<th>Administration</th>
<th>Mechanism</th>
<th>Estimated change in exposure of MDZ (%) using different inhibitor concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$[l]_{av,u}$</td>
</tr>
<tr>
<td>CsA</td>
<td>Recomb CYP3A4</td>
<td>Coadministration</td>
<td>Comp.</td>
<td>68 (67/1)</td>
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<tr>
<td></td>
<td></td>
<td>Dose staggering</td>
<td>Comp.</td>
<td>3 (2/1)</td>
</tr>
<tr>
<td></td>
<td>HLM</td>
<td>Coadministration</td>
<td>Comp.</td>
<td>66 (64/1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose staggering</td>
<td>Comp.</td>
<td>3 (2/1)</td>
</tr>
<tr>
<td>Tac</td>
<td>Recomb CYP3A4/5</td>
<td>Coadministration</td>
<td>TDI</td>
<td>102 (94/4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose staggering</td>
<td>TDI</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>HLM</td>
<td>Coadministration</td>
<td>Comp.</td>
<td>27 (27/0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose staggering</td>
<td>Comp.</td>
<td>0 (0/0)</td>
</tr>
</tbody>
</table>
CsA: cyclosporine A; Tac: tacrolimus; HLM: pooled human liver microsomes; Comp.: competitive inhibition; TDI: time-dependent inhibition; $F_g$: fraction escaping intestinal metabolism; $CL_{int}$: hepatic intrinsic clearance; $[I]$: inhibitor concentration; av: average; max: maximum; inlet: hepatic inlet; u: unbound
Figure 4

(A) and (C) Show the decline in enzymatic activity over time for different concentrations of Tac.

(B) and (D) Show the relationship between Tac concentration and observed rate constant (k_{obs}).

Parameters:
- $K_i = 2.66 \mu M$
- $k_{inact} = 0.30 \text{ min}^{-1}$
- $K_i = 2.69 \mu M$
- $k_{inact} = 0.21 \text{ min}^{-1}$
Figure 5

Activity (pmol/min/mg protein) vs. Midazolam concentration (µM)

- Solid circles: Control
- Solid squares: Treatment A
- Solid triangles: Treatment B

Error bars indicate variability in the data.