Effects of Cyclosporine A and Itraconazole on Permeability, Biliary Excretion and Pharmacokinetics of Amlodipine

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Abstract: Cyclosporin A (CsA) is a P-glycoprotein (P-gp) inhibitor clinically used as an immunosuppressant. Itraconazole (ITZ) functions as an inhibitor of both the P-gp and CYP3A and is used as a fungistatic/fungicidal agent in human and veterinary medicine. The present studies were designed to investigate the effects of CsA and ITZ on 1) intestinal permeability of amlodipine (a calcium channel blocker used as a cardiovascular agent) in isolated rat everted gut sac model, and 2) biliary excretion and pharmacokinetics of amlodipine in rats. The concentrations of amlodipine in biosamples were measured by the liquid chromatograph mass spectrometer (LC/MS). Both CsA and ITZ significantly increased permeability of amlodipine in the ileum and jejunum of the rat everted gut sac model, and ITZ showed more potent than CsA in this model. Pretreatment of rats with ITZ increased plasma levels and biliary excretion of amlodipine in a dose-dependent manner. In contrast, pretreatment with CsA slightly decreased biliary excretion of amlodipine and made no changes in its plasma levels. In conclusion, ITZ increased in vitro permeability of amlodipine and its levels in plasma and bile in vivo. Whereas, CsA showed no significant effects on the levels of amlodipine in rat plasma and bile probably due to the potent inhibition of ITZ against both CYP3A and P-gp.

Keywords: Amlodipine, cyclosporin A, itraconazole, P-glycoprotein, CYP3A, permeability, biliary excretion, pharmacokinetics.

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INTRODUCTION

P-glycoprotein (P-gp) is an energy-dependent efflux pump associated with the multidrug resistance in tumor cells. It is also expressed in a variety of normal human tissues including liver, brain, kidney and the gastrointestinal tract and plays an important role in the barrier function of these tissues against xenobiotics [1,2]. Substrates transported by P-gp include a variety of structurally and pharmacologically unrelated, hydrophobic compounds such as some anticancer agents, steroid hormones, calcium channel blockers, immunosuppressant agents, β-blockers and others [3]. P-gp modulators seem to inhibit P-gp activity by competing with the binding site and/or through inhibition of ATP hydrolysis [4]. Cyclosporin A (CsA) is a P-gp inhibitor clinically used as an immunosuppressant in transplantation patients and patients suffering from chronic inflammatory diseases [5]. Itraconazole (ITZ) is a fungistatic/fungicidal agent widely used in human and veterinary medicine, and has been described as a potent P-gp and CYP3A inhibitor [6].

Amlodipine, a relatively new potent long-acting calcium channel blocker, is widely used for treatment of hypertension as well as stable and variant angina [7], and is one of the most commonly prescribed cardiovascular agents worldwide [8]. Amlodipine is slowly cleared with a relatively long elimination half-time of 40-50 h, which distinguishes it from other calcium channel-blocking agents. Although structurally related to other dihydropyridine derivatives, amlodipine displays significantly different pharmacokinetic characteristics and is suitable for administration in a single daily dose. Previous studies showed that amlodipine is extensively metabolized in the liver [9]. Moreover, amlodipine was proposed as a potential substrate of P-gp [10]. Interestingly, it was also reported that amlodipine could exhibit an inhibitory effect on P-gp-mediated transport of daunorubicin and digoxin [11]. Enormous efforts have been made to investigate the P-gp-mediated drug transport, which led to the development of pharmacologically active inhibitors [12]. However, until now, there have been only few reports on the interactions between amlodipine and P-gp.

The objectives of the current work were: 1) to assess the involvement of P-gp in the intestinal absorption of amlodipine using the in vitro rat everted gut sac model, and 2) to evaluate the effects of the two different P-gp inhibitors CsA and ITZ at equimolar levels on the pharmacokinetic parameters and biliary excretion of amlodipine in rats.
EXPERIMENTAL

Chemicals

Amlodipine powder (purity> 99 %) was supplied by New Saike Medicine company (Zhejiang Province, China). Internal standard nicardipine (10 μg/ml) was obtained from Center of Drug Metabolism and Pharmacokinetics of China Pharmaceutical University. Methanol (HPLC grade) was purchased from Tedia Company and water was from RO-BUST. All other chemicals were purchased commercially and were of analytical grade.

Animals

Male Sprague-Dawley rats (weight 200 to 240g) were purchased from Southeast University, Jiangsu, China. All rats were maintained in a clean room at a temperature of 23±2°C and relative humidity of 50%±10%, and had free access to water and standard animal diet. All animal handling procedures were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People’s Republic of China.

Intestinal Absorption of Amlodipine in Rat Everted Gut Sac Model

Before the experiment, the rats were starved for 12 hours but allowed for tap water ad libitum. After anesthesia, the abdomen was opened by a midline incision. The entire intestine was removed by cutting across the upper end of the duodenum and the lower end of the colon and stripping the mesentery manually. The intestine was washed out carefully with the normal saline (0.9% NaCl) using a syringe equipped with blunt end. Intestinal segments (6.0±1.5 cm) were gently everted over a glass rod (200 mm long and 1.5 mm in diameter). The everted intestine was then slipped off the glass rod and placed in a flat dish containing the Krebs-Henseleit bicarbonate buffer (25 mM NaHCO₃, 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 1.2 mM CaCl₂, 10 mM Glucose) oxygenated with O₂/CO₂ (95%/ 5%) at 37°C. One end was clamped and tied with a silk suture before filling it with the buffer at 37°C using a 1-ml plastic syringe. The filled intestinal sac was then slipped off the needle carefully and the loose ligature at the proximal end was tightened. The sacs were placed in the individual incubation chambers containing 20 ml of the oxygenated buffer at 37°C.

To study the effects of the P-gp inhibitors on tissue uptake or serosal transfer of amlodipine, amlodipine (4 μg/ml) was added to the chambers in the presence and absence of CsA (10 μM) or ITZ (10 μM). The external incubation buffer was bubbled with a gas mixture of 95% O₂ and 5% CO₂ throughout the experiment. At the end of the incubation period (60 min), the sac was removed from the organ bath and blotted dry. The serosal fluid was drained through a small incision into a test tube. Gentle pressure was applied to make the sac empty. The volume of the serosal fluid was accurately measured using a 100 μl blunted-ended syringe. The segments were blotted again, cut lengthwise, flattened, and measured as accurately as possible to calculate the crude surface area. All samples were rapidly cooled and stored at -20°C until analysis.

Biliary Excretion and Pharmacokinetic Profiles After Intravenous Administration of Amlodipine

Twenty-eight male Sprague Dawley rats were used in this trial. Rats that had fasted for at least 12 h with free access to water were anesthetized by 20% ethylurethane (1 ml/kg, i.p.). The rats were placed in a supine position on a heating pad under a surgical lamp to maintain normal body temperature. After midline longitudinal abdominal incision, a polyethylene cannula (0.4 mm i.d., 0.8 mm o.d., Natsume, Tokyo, Japan) was inserted into the bile duct. CsA (5, 10 and 20 mg/kg ) or ITZ (5, 10 and 20 mg/kg) was intravenously administered by bolus injection from tail vein to pretreat the rats. 5 min after the pretreatment, amlodipine (400 μg/kg, dissolved in sterile saline with 20% ethanol) was intravenously administered by bolus injection from tail vein. Bile was then collected at designated intervals over 120 min. In parallel, 250-μl aliquots of blood samples were collected into heparinized centrifuge tubes at the midpoint of each bile collection. Plasma samples were obtained by centrifuging blood samples at 12000×g for 10 min. A control experiment was performed in parallel using the vehicle for the pretreatment.

Analytical Method and Procedures

The extraction of amlodipine from all samples were prepared following the liquid-liquid extraction method. To samples of sac fluids (500 μl), plasma (100 μl), and bile (100 μl), 50, 10 and 10 μl of nicardipine (internal standard) were added, respectively. Then 3 ml of tert-butyl methyl ether was added to each preparation, which was vortex-mixed for 3 min, and centrifuged at 4000×g for 5 min. 1.5 ml organic supernatant was transformed to a clean test tube and evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 200 μl of the mobile phase, vortex-mixed, and centrifuged at 12000×g for 10 min. Aliquots of 10 μl of sample were injected into the following LC–MS system for analysis of amlodipine by the autosampler.

The measurement of amlodipine concentrations in all samples was carried out by LC-MS (Shimadzu 2010EV; Shimazu Corp., Kyoto, Japan) system consisted of a Shimadzu LC/MS-2010EV liquid chromatograph-mass spectrometer, SIL-20AC autosampler, two LC-10AVP pumps and an electrospray-ionization interface (Kyoto, Japan). The analytical column was a reverse phase Hypersil ODS2 column (3 μm, 2.1×100 mm I.D., Sepax Technologies, Inc.) kept in the CTO-20A column oven (Shimadzu, Japan) at 40°C. The mobile phase was composed of methanol and ammonium acetate (5 mM in H₂O containing 0.1% methanoic acid at a ratio of 65:35 (v/v)). The flow-rate was 0.3 ml/min.

Mass spectrometry was performed in the selected ion monitoring (SIM) mode using positive target ions at m/z 431.10 for amlodipine and m/z 480.30 for the internal standard, respectively. The detector voltage was 1.60 kv. The heat block temperature was 200°C and the CDL temperature was 250°C. The nebulizer gas flow was 1.5 l/min.

The analytical procedures, including chemical extraction and HPLC analysis of amlodipine in all samples were validated. Calibration curves were prepared in a range between...
Effects of Cyclosporine A and Itraconazole on Permeability

Drug Metabolism Letters, 2008, Vol. 2, No. 3 165

Pharmacokinetic and Statistical Analyses of the Data

Pharmacokinetic parameters of amlodipine after intravenous administration were obtained by applying a non-compartmental pharmacokinetic analysis to the plasma concentration–time courses using the computer program WinNonlin 5.0.1 (Pharsight corporation, Mountain View, CA, USA). The terminal elimination rate constant (\(k_t\)) was determined by a linear regression of at least three data points from the terminal portion of the plasma concentration–time courses. The area under the concentration versus time curve up to the last measured time (\(AUC_{last}\)) was calculated and extrapolated to the infinity (\(AUC_{inf}\)) using a correction term, namely \(C_{last}/k_t\). The total body clearance (\(Cl_{tot}\)) was calculated by dividing the intravenous dose by \(AUC_{inf}\). The biliary clearances (\(Cl_{bile}\)) of amlodipine was estimated by dividing the total amount of the drug excreted into the bile during the study period by the \(AUC_{last}\), and normalized by the body weight. The area under the first-moment curve to the last measured concentration (\(AUMC_{last}\)) was also calculated using the linear trapezoidal rule and the addition of a correction term after the last measured point (\(t_{last}\)) until the infinity (\(AUMC_{inf}\)). The distribution volume at steady state (\(Vd_{ss}\)) was then calculated using the formula \(Cl_{tot} \times AUMC_{inf} / AUC_{inf}\). A \(P\) value of less than 0.05 was considered statistically significant following a \(t\) test between the two means for unpaired data using the Social Package of Statistical Sciences (SPSS, version 11.5). All results were expressed as means±standard deviations.

RESULTS

Analytical Method Validation

The linear regression lines showed correlation coefficients ranging between 0.997 and 0.999. The mean recoveries of amlodipine were in a range between 70 and 80% for the different samples analyzed. The mean recoveries of amlodipine were in a range between 70 and 80% for the different samples analyzed. The limit of quantification was established as the lowest concentration measured with a recovery higher than 70%.

Effects of CsA and ITZ on Amlodipine’s Permeability Across Rat Everted Gut Sac Model

Figure 1 shows the transepithelial apparent permeability (\(P_{app}\)) values of amlodipine in the duodenum, jejunum, ileum and colon after incubation of the segments with amlodipine alone or in the presence of either CsA or ITZ. The \(P_{app}\) was expressed in cm/ sec as described previously [15,16] using the following equation for calculation:

\[
P_{app} = \frac{dQ/dt}{A \cdot C_0}
\]

where \(dQ/dt\) is the rate of appearance of compound in the receiver chamber, \(C_0\) is the substrate concentration in the donor chamber, and \(A\) is the cross-sectional area of the tissue.

During the 60-min incubation, amlodipine permeated from the donor chamber to the receiver chamber in all segments of the rat intestine with the transepithelial permeability ranked in the following order: ileum> jejunum> duodenum> colon. The \(P_{app}\) values of amlodipine in duodenum, jejunum, ileum and colon were 2.7, 3.29, 3.99 and 1.42 \(10^{-8}\) cm/s, respectively. The \(P_{app}\) values of amlodipine in some intestinal segments were increased after the pre-incubation of

![Fig. (1). The effects of CsA (10 \(\mu\)M) and ITZ (10 \(\mu\)M) on the permeability of amlodipine across rat everted gut sac model. * denotes values that are statistically different \((P < 0.05)\) from those obtained from amlodipine alone. The data represent the mean±S.E. (n= 6).](image-url)
the segments with CsA or ITZ in comparison with those of amlodipine alone (Fig. 1). The permeability of amlodipine significantly increased in jejunum by 26.3% and 53.9%, and in ileum by 71.3% and 114.6% in the presence of CsA and ITZ, respectively. In addition, the \( P_{app} \) value of amlodipine in the colon showed a significant increase by 42.5% in the presence of ITZ in comparison with the amlodipine alone group. However, there were no significant differences in permeability between the duodenum group pretreated with either CsA or ITZ and the amlodipine alone group (Fig. 1).

**Effects of CsA and ITZ on Biliary Excretion and Pharmacokinetics of Amlodipine in Rats**

Figure 2 shows the effects of CsA- or ITZ-pretreatment on the plasma concentration-time profiles and biliary excretion of amlodipine after intravenous administration (400 μg/kg) to rats. The pharmacokinetic parameters of amlodipine are listed in Table 1. Following CsA-pretreatment, there were no significant changes in plasma concentrations of amlodipine and its pharmacokinetic parameters \( AUC_{last} \), clearance and \( Vdss \) as compared to those of control rats (Fig. 2a, Table 1). In contrast, following ITZ-pretreatment, the plasma amlodipine concentrations and the \( AUC_{last} \) or \( AUC_{inf} \) values were increased significantly in a dose-dependent manner from 5-20 mg/kg, while the \( C_{tot} \) values were decreased significantly in a dose-dependent manner from 5-20 mg/kg (Table 1), indicating that ITZ somehow reduces the clearance of amlodipine from the body.

With CsA pretreatment, the cumulative amount of the biliary excretion and clearance (\( C_{ bile } \)) of amlodipine was decreased at doses of 10-20 mg/kg (Fig. 2c, Table 1). The \( C_{ bile } \) of amlodipine after CsA pretreatment decreased markedly (by 26-57%) at doses of 10-20 mg/kg compared to the control. With ITZ pretreatment, the cumulative amount of amlodipine in the bile and its clearance (\( C_{ bile } \)) showed a slight decrease at 5 mg/kg. However, when the doses of ITZ increased to 10 and 20 mg/kg, both clearance and \( Vdss \) of amlodipine were significantly decreased, while biliary excretion of amlodipine were significantly increased (Fig. 2 and Table 1).

**DISCUSSION**

To assess the *in vitro* involvement of P-gp or CYP3A in the intestinal permeability of amlodipine, the rat everted gut sac model was used in the present study. The above results (Fig. 1) show that the permeability of amlodipine in jejunum and ileum was significantly increased in the presence of CsA or ITZ, and the magnitude of the ITZ effect on the amlodipine absorption in rat everted gut sac model was more marked than that of CsA probably due to the dual inhibitory effects of ITZ on CYP3A and P-gp. Moreover, there are various reports showing the regional difference in the expression and activity of P-gp. The expression of MDR1 mRNA is higher in the lower part of the intestine in humans [13] and the expression of *mdr1* mRNA in rat intestine is moderate in the duodenum and the jejunum, maximal in the ileum, and becomes lower through the proximal and distal colon. These reports may form the basis of our observations of the disparity in permeability of amlodipine in the different segments of the isolated tissue (Fig. 1).

Previous studies show that CsA is a P-gp inhibitor and itraconazole is a potent inhibitor of both P-gp and CYP3A. The effect of P-gp or CYP3A on the pharmacokinetics of many drugs has been extensively investigated [17], with CsA and ITZ generally used as inhibitors for the efflux system (mediated by P-gp) and metabolism (mediated by CYP3A), respectively. A number of tools are available to study the role of transporters and drug-metabolizing enzymes, for example, isolated enzyme preparations, specific protein-overexpressing cell lines, specific P-gp inhibitors and mdr1 knockout mice. However, the results obtained using such methods may not provide a general understanding of the effects of the inhibition of CYP3A or P-gp because the results of these methods were obtained under special conditions and can not precisely reflect the normal *in vivo* conditions [14]. Our findings clearly evaluated the effect of CsA or ITZ used at equimolar doses on the *in vivo* plasma and biliary disposition kinetics of amlodipine in rats. Following CsA pretreatment, the biliary excretion of amlodipine was reduced by CsA basically in a dose-dependent manner, as shown in Fig. 2c. However, it was found that the contribution of P-gp to the efflux of amlodipine in the liver was very

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**Table 1. Effects of CsA and ITZ on the Pharmacokinetic Profiles of Amlodipine After Intravenous Administration to Rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>( AUC_{last} ) (ng h/ml)</th>
<th>( AUC_{inf} ) (ng h/ml)</th>
<th>( Cl_{tot} ) (1/h/kg)</th>
<th>( Vdss ) (1/kg)</th>
<th>( Q_{bile} ) (ng)</th>
<th>( C_{bile} ) (ml/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56.48±4.69</td>
<td>67.25±4.95</td>
<td>5.97±0.45</td>
<td>37.25±4.56</td>
<td>166.31±18.91</td>
<td>15.07±3.25</td>
</tr>
<tr>
<td>CsA dose (mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>55.20±3.56</td>
<td>68.67±7.23</td>
<td>5.87±0.59</td>
<td>40.99±5.29</td>
<td>183.91±31.01</td>
<td>16.97±3.54</td>
</tr>
<tr>
<td>10</td>
<td>57.63±5.58</td>
<td>68.31±6.85</td>
<td>5.90±0.64</td>
<td>35.79±3.69</td>
<td>121.66±18.15</td>
<td>11.14±2.38</td>
</tr>
<tr>
<td>20</td>
<td>60.83±4.62</td>
<td>72.18±4.54</td>
<td>5.56±0.35</td>
<td>33.45±3.88</td>
<td>79.35±13.20</td>
<td>6.50±1.00*</td>
</tr>
<tr>
<td>ITZ dose (mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>69.72±7.53*</td>
<td>93.11±12.17**</td>
<td>4.36±0.63**</td>
<td>36.31±7.47</td>
<td>118.07±10.72</td>
<td>8.52±1.33</td>
</tr>
<tr>
<td>10</td>
<td>82.97±10.00**</td>
<td>132.19±21.52**</td>
<td>3.10±0.57**</td>
<td>38.08±8.20</td>
<td>330.86±40.15*</td>
<td>20.52±6.07</td>
</tr>
<tr>
<td>20</td>
<td>119.23±11.92**</td>
<td>164.21±15.53**</td>
<td>2.45±0.23**</td>
<td>22.05±3.13**</td>
<td>652.77±230.38**</td>
<td>29.03±12.74**</td>
</tr>
</tbody>
</table>

Values represent mean±S.E.M. (n = 4). * p<0.05, ** p<0.01 compared to the control.

limited because the values of \( C_{\text{bile}} \) of amlodipine with or without pretreatment with CsA were especially small, ranging from 6.50 to 16.98 ml/h/kg (Table 1). In contrast, CYP3A contributed importantly to the metabolism of amlodipine in the liver, since a significant dose-dependent increase in the biliary excretion of amlodipine was found when rats were pretreated with ITZ (Fig. 2d). The increase of about 1.2 to 2.1-times in the value of \( AUC_{\text{inf}} \) together with marked decreases in the values of \( C_{\text{infl}} \) and \( V_{\text{ss}} \) indicated that the metabolism of amlodipine via CYP3A in the liver was inhibited by ITZ. The inhibition may reduce the clearance of amlodipine through kidney and prolong the circulation of amlodipine in blood, and hence contribute, in part, to the increase of the cumulated amount of amlodipine in bile. In addition, the ITZ-pretreatment decreased the volume of distribution of amlodipine in tissues, which may partially contribute to the increase of amlodipine in blood stream, and in turn, of its accumulation in bile.

Moreover, the dose-dependent increase of biliary excretion of amlodipine indicated that the passive efflux of amlodipine from the inside to the outside of cells that is independent of transporters was enhanced due to the inhibition of CYP3A by ITZ. Taking into consideration the fact that ITZ has the ability to inhibit P-gp function, the contribution of CYP3A in the liver is larger than that of P-gp with respect to altering the pharmacokinetics of amlodipine in rats.

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REFERENCES


