DISPOSITION OF TACROLIMUS IN ISOLATED PERFUSED RAT LIVER: INFLUENCE OF TROLEANDOMYCIN, CYCLOSPORINE, AND GG918

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ABSTRACT:

The disposition of tacrolimus and the influence of cyclosporine, troleandomycin, and GF120918 (GG918, or N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)-ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamine) on its hepatic disposition were examined in the isolated perfused rat liver. Livers from groups of rats were perfused in a recirculatory manner following a bolus dose of tacrolimus (100 μg), a substrate for P-glycoprotein (P-gp) and CYP3A, or with felodipine (200 μg), a substrate only for CYP3A. Perusions of each substrate were also examined in groups of rats in the presence of the inhibitors: troleandomycin only for CYP3A. The area under the curve (AUC) from 0 to 30 min was determined. For the dual CYP3A and P-gp substrate, tacrolimus, AUC was increased by GG918 (1,730 ± 270 ng · min/ml, P < 0.05) and was increased by troleandomycin (5,200 ± 2,470 ng · min/ml, P < 0.05) and cyclosporine (4,390 ± 2,080 ng · min/ml, P < 0.05). For the exclusive CYP3A substrate, felodipine, AUC was unchanged from control by GG918 but increased by troleandomycin and cyclosporine. It is concluded that GG918 increased the hepatic exposure of tacrolimus by inhibiting the canalicular P-gp transport, whereas GG918 has no effect on hepatic disposition of felodipine. These results support our hypothesis that the hepatic metabolic clearance of a dual substrate will be increased by inhibiting the efflux transporter.

The interplay between P-glycoprotein (P-gp1) and cytochrome P450 3A4 (CYP3A) is a major factor contributing to the variable bioavailability of many therapeutically available drugs (Wacher et al., 1995; Zhang and Benet, 2001). The considerable overlap in substrate selectivity makes it difficult to differentiate the interactive roles of P-gp and CYP3A in xenobiotic disposition.

In enterocytes, P-gp is located on the apical plasma membrane and CYP3A4 is on the endoplasmic reticulum inside the cells. Absorbed drug molecules may be actively extruded back into the intestinal lumen by P-gp. As we have previously reported (Benet and Cummins, 2001; Cummins et al., 2002a, 2003), through the repeated process of diffusion and active efflux, drug molecules may come in contact with CYP3A intracellularly, increasing the potential for metabolism by the enzyme. Prolonging the intracellular residence time of the parent drug decreases the rate of absorption and results in increased drug clearance by CYP3A4 relative to the parent drug traversing the intestine. We believe that P-gp, in fact, controls the access of drugs to intracellular metabolism by CYP3A4 in the intestine (Benet and Cummins, 2001), which is well supported by bidirectional transport studies using CYP3A-transfected Caco-2 cells (Cummins et al., 2002a) and mdrl gene knockout mice (Lan et al., 2000). Our recent in vivo perfused rat intestinal studies further confirm our hypothesis (Cummins et al., 2003).

In the liver, the absorbed compounds enter the hepatocytes from the sinusoidal blood, then the drugs are either biotransformed, transported back into the blood, or eliminated via biliary secretion. Since P-gp is located on the canalicular membrane of hepatocytes, drug molecules confront CYP3A before P-gp efflux. Hence, P-gp influences the access of drug molecules to CYP3A in an opposite manner to the enterocyte, where the transporter precedes the enzyme. We hypothesize that if P-gp is decreased by chemical inhibitors or via gene disruption (mdrl knockout), the hepatic metabolic clearance of the substrates will be enhanced due to their increased availability to CYP3A. This phenomenon was implied in gene disruption studies by Lan et al. (2000) and was well illustrated for basolateral doses in our recent cellular transport studies (Cummins et al., 2002a). We further calculated that the hepatic extraction ratios of vinblastine, paclitaxel, and doxorubicin were increased in mdrl knockout mice versus wild-type controls (Cummins et al., 2002b). The goal of the studies described here was to directly test our hypothesis of the effect of P-gp inhibition on hepatic metabolism by identifying the contribution of P-gp to CYP3A4 metabolism using an ex situ model (isolated perfused rat liver) with appropriately selected CYP3A4/P-gp substrates and inhibitors.

Two compounds were tested: one a dual P-gp and CYP3A4 substrate (tacrolimus) and the other an exclusive CYP3A4 substrate (felodipine). Tacrolimus was metabolized by CYP3A4 (Lampen et al., 1995) and is a good substrate for P-gp. Felodipine is metabolized by
CYP3A4 but is not a substrate for P-gp efflux (Cummins et al., 2002a). The substrates were administered with and without the inhibitors troleandomycin (inhibitor of CYP3A, but not P-gp), N-[4-[(2,1,2,3,4-tetrahydro-6,7-dimethoxy-2-isouquinolinyl)-ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamine (GG918, an inhibitor of P-gp, but not CYP3A), or cyclosporine (an inhibitor of both P-gp and CYP3A). With these relatively selective substrate/inhibitor studies, it is revealed that when P-gp alone was inhibited, the extent of metabolism of the dual CYP3A4 and P-gp substrate was increased, but there was no change in the extent of metabolism for the exclusive CYP3A4 substrate. These data indicate that hepatic P-gp, when inhibited, can increase the CYP3A4 metabolism without disturbing the metabolic activity of the enzymes.

Materials and Methods

Chemicals. Cyclosporine (Novartis; Basel, Switzerland), felodipine (Interchem Corp., Paramus, NJ), and GG918 (GF120918; GlaxoSmithKline, Research Triangle Park, NC) were kind gifts of the manufacturers. Tacrolimus was obtained as Prograf (5 mg/ml; Fujisawa Pharmaceutical, Osaka, Japan); troleandomycin, taurocholate, ketamine/xylazine mixture solution, and Krebs-Ringer buffer were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents for the analysis were of analytical grade and used as supplied commercially.

Surgery and Perfusion of Livers. Male Sprague-Dawley rats (300–400 g; Bantin and Kingman, Fremont, CA) were housed in the University of California San Francisco (UCSF) animal care facility with a 12 h light/dark cycle, and allowed free access to water and food. Approval for their use in experiments was obtained from the Committee on Animal Research, UCSF. Anesthesia was conducted by intraperitoneal injection with a 1-ml/kg dose of ketamine/xylazine (80 mg, 12 mg/kg) before surgery. Livers were isolated for perfusion ex situ, as described previously from our laboratory (Prueksaritanont et al., 1992). Krebs-Ringer buffer (pH 7.4), supplemented with 1% bovine serum albumin, glucose (10 mM), and sodium taurocholate (220 nmol/min), was pumped through the liver at a flow rate of 40 ml/min via a catheter inserted in the portal vein. Perfusion was performed at 37°C in a recirculatory manner from a reservoir containing 110 ml of medium, through a 10-μm in-line filter, oxygenator, and bubble trap placed before the liver. Liver viability was judged on macroscopic appearance (uniformly pink to brown), the flow of bile (>5 μl/min), portal vein pressure (8–10 mm Hg), and the pH of perfusate (range, 7.35–7.45).

After a 20-min stabilization period, the recirculatory perfusion was started with addition of the compound of interest to the reservoir. Samples of perfusate (0.5 ml) were taken from the reservoir immediately (0 min) and at 2, 5, 10, 15, 20, 30, 45, and 60 min after the addition of tacrolimus or felodipine. All bile was collected at intervals of 15 min.

Experimental Design. To examine the influence of troleandomycin, GG918, and cyclosporine on the hepatic disposition of tacrolimus and felodipine, 24 rats for tacrolimus and 12 rats for felodipine were divided equally into four groups, and each group was perfused with tacrolimus or felodipine at an initial concentration of 1,000 ng/ml and 2,000 ng/ml, respectively. While one group served as the control, the other three served as treatment groups. The inhibitors (final concentration), troleandomycin (20 μM), cyclosporine (10 μM), and GG918 (1 μM), were added 5 min before tacrolimus or felodipine was added.

Analysis of Perfusate. Samples of perfusate and bile (diluted with drug-free perfusate to 200 μl) were extracted with methyl tertiary butyl ether. The methyl tertiary butyl ether layer was separated and evaporated to dryness under nitrogen, and the residue was reconstituted with mobile phase (300 μl). Aliquots (20–μl) were injected on to a Microsorb-MV C8 column (Rainin Instruments, Woburn, MA). 4.6 × 15 mm (tacrolimus and GG918), silica column, 4.6 × 15 mm (felodipine) in a Quattro liquid chromatography/electrospray ionization (Micromass Inc., Beverly, MA). Tacrolimus and GG918 were eluted with a mobile phase of methanol/formic acid/2.5 μM sodium acetate (80:0.1:19.9%); felodipine was eluted with acetoni- ter/difluoroacetic acid/water (80:0.1:19.9%); all mobile phases were pumped at 0.8 ml/min (LC-10AD; Shimadzu, Kyoto, Japan). As metabolite standards were not available for O-demethyl-tacrolimus and dehydrofelodipine, an MS response factor for O-demethyl-tacrolimus was calculated using the ratio of peak areas for metabolite and parent peaks obtained by UV detection (215 nm) and comparing these with the ratio of peak areas of metabolite and parent obtained for the same sample when measured by MS. The MS response factor was obtained by dividing the UV metabolite/parent ratio by the MS metabolite/parent ratio. After adjusting the metabolite peak areas obtained by MS by their respective response factors, quantitation was performed using the tacrolimus MS calibration curve. The UV ratios were not obtained for dehydrofelodipine. Therefore, the response area reading from the mass spectrum was used as an arbitrary unit for the comparison.

Pharmacokinetic Analysis. Measured concentrations of tacrolimus and felodipine and their primary metabolites were used to calculate the trapezoidal AUC for 0 to 30 min (since GG918 was only at 10% of its initial concentration at this time point). The ratios of AUC_{0–inf} for metabolite to parent drug in the perfusate were also calculated.

Results and Discussion

The importance of drug transporters has become increasingly appreciated in defining pharmacokinetic properties and understanding drug interactions. Here, we delineate how P-gp can affect drug metabolism by influencing the access of the drug to the intracellular metabolizing enzyme. Figure 1 shows the concentrations of tacrolimus and felodipine determined in perfusate when livers were perfused in the absence and presence of three different inhibitors. Disposition of the specific P-gp inhibitor, GG918, is also shown in Fig. 1C. As GG918 was rapidly eliminated from the system, comparisons up to 30 min only were included in the analysis (Fig. 1 and Table 1). Troleandomycin, a specific CYP3A inhibitor, increased tacrolimus AUC (5,200 versus 2,260 ng · min/ml, P < 0.05) by inhibition of the CYP3A metabolism. GG918, a specific P-gp inhibitor, decreased the AUC (1,730 versus 2,260 ng · min/ml, P < 0.05) of tacrolimus presumably by increasing the residence time of tacrolimus in the hepatocytes by inhibition of P-gp efflux. Cyclosporine, which inhibits both P-gp and CYP3A, showed a profile between that of troleandomycin- and GG918-treated groups, revealing a composite of the two opposite directions of the interaction.

The exclusive CYP3A substrate, felodipine showed a significant increase of AUC (6,150 versus 4,380 ng · min/ml, P < 0.05) from CYP3A inhibition with troleandomycin. Similar results were obtained with cyclosporine, CYP3A, and P-gp inhibitor, but because of the limited number of studies with felodipine, statistical significance was not reached. As would be expected, GG918 has no effect on felodipine AUC (4,000 versus 4,380 ng · min/ml, N.S.). The results support our hypothesis that P-gp may control the access of dual CYP3A/P-gp substrates to the enzyme in the liver. Using a cellular system, Cummins et al. (2002a) showed that during basolateral-to-apical transport of dual CYP3A/P-gp substrates, CYP3A metabolism increased when P-gp was inhibited due to greater access of the substrate to the enzyme. We pointed out further that this cellular basolateral-to-apical transport mimicked the orientation of enzyme and efflux transporter in the liver and that differences in hepatic metabolism could be explained by differences in P-gp activity for dual substrates (Cummins et al., 2002b). Here we confirm this hypothesis using the ex situ isolated perfused rat liver, where significantly lower AUCs of tacrolimus (a dual CYP3A/P-gp substrate) but not felodipine (a CYP3A substrate only) are observed when P-gp is inhibited (Fig. 1 and Table 1). The results from liver perfusion studies reveal that P-gp causes opposite effects to what occurs in the intestine. In the intestine, inhibition of P-gp decreases metabolism (Cummins et al., 2003), whereas in the liver, inhibition of P-gp increases access to the enzyme and increases metabolism.

Although metabolite standards were unavailable, we did obtain a measure of the demethylated tacrolimus metabolites using UV/MS.
response factors, allowing us to report the ratio of this response factor to parent drug (Table 1). The ratios (AUCₘ/AUC) are quite consistent for the control, troleandomycin, and cyclosporine groups, suggesting that metabolite disposition is inhibited to a similar extent as parent tacrolimus. We suspect that the demethylated tacrolimus metabolites are substrates for CYP3A and P-gp (Christians et al., 2002). Although not statistically significant, the ratio of metabolite to parent appears to decrease with GG918, suggesting that the metabolites may be more susceptible than the parent to CYP3A metabolism.

A very different pattern in the metabolite/parent ratios is noted for felodipine, although the absolute metabolite concentrations may be less accurate since we did not derive the response factor. In all cases the ratio increases versus control. These data would suggest that the felodipine metabolite is a substrate for both P-gp and CYP3A. How-

FIG. 1. Perfusate concentration-time profile.

Perfusate concentration-time profile for tacrolimus (A) and felodipine (B) without (control) and in the presence of three different P-gp/CYP3A inhibitors over 30 min. Perfusate time course of GG918 (C).

TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Troleandomycin</th>
<th>GG918</th>
<th>Cyclosporine</th>
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<tbody>
<tr>
<td>Tacrolimus</td>
<td></td>
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<tr>
<td>AUC (ng/ml·min)</td>
<td>2.260 ± 430</td>
<td>5.200 ± 2.470*</td>
<td>1.730 ± 270*</td>
<td>4.390 ± 2.080*</td>
</tr>
<tr>
<td>AUCₘ (ng/ml·min)</td>
<td>22.900 ± 10.900</td>
<td>72.100 ± 7.900*</td>
<td>12.100 ± 4.500</td>
<td>70.200 ± 27.200*</td>
</tr>
<tr>
<td>AUCₘ/AUC</td>
<td>9.35 ± 1.94</td>
<td>10.3 ± 2.4</td>
<td>6.24 ± 1.74</td>
<td>11.9 ± 2.1</td>
</tr>
<tr>
<td>Felodipine</td>
<td></td>
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</tr>
<tr>
<td>AUC (ng/ml·min)</td>
<td>4.380 ± 320</td>
<td>6.150 ± 900*</td>
<td>4.000 ± 50</td>
<td>5.310 ± 950</td>
</tr>
<tr>
<td>AUCₘ</td>
<td>693 ± 38</td>
<td>3.120 ± 890*</td>
<td>1.190 ± 110*</td>
<td>1.520 ± 670</td>
</tr>
<tr>
<td>AUCₘ/AUC</td>
<td>0.158 ± 0.007</td>
<td>0.504 ± 0.116*</td>
<td>0.297 ± 0.024*</td>
<td>0.285 ± 0.116*</td>
</tr>
</tbody>
</table>

* Significantly different from control, P < 0.05

AUC₀–₃₀ min (noncompartmental model) of parent (AUC) and metabolite (AUCₘ).

Tacrolimus metabolite measured by UV/MS response factor.

Felodipine metabolite peak area measured by MS, arbitrary unit.
ever, this finding needs to be confirmed by studies using isolated or synthesized metabolite.

Figure 2 shows the influence of different treatments on the cumulative amount of tacrolimus and metabolites excreted in bile. Cumulative excretion of tacrolimus to 60 min was decreased significantly (P<0.05) by troleandomycin (79.6% decrease) and cyclosporine (78.5% decrease). The biliary clearance of tacrolimus was statistically unchanged by either of the inhibitors, which may indicate that the inhibitory effect is more dominant within the hepatocytes in terms of metabolism than in terms of biliary excretion, and the biliary metabolite accumulation may be a sensitive indicator for the modulation of enzymatic effects in addition to the perfusate tacrolimus profile. Note that the changes of metabolite in bile are not reflective of what is observed in the perfusate. We should also note that we chose the commonly used red blood cell-free perfusion technique, which requires higher flow rates then observed physiologically to provide sufficient oxygen-carrying capacity. It is possible that our results could be influenced by the higher rates, although our studies always use control perfusions, and the differentiation of the interactive effects with tacrolimus and felodipine correspond to that predicted from our cellular studies (Cummins et al., 2002a).

To summarize, the decreased AUC and increased clearance of tacrolimus after specific inhibition of P-gp showed the importance of the transporter in affecting CYP3A metabolism. The fact that inhibition of P-gp and CYP3A in the intestine is synergistic (Cummins et al., 2003) but opposing in the liver demonstrates the complicated interplay between P-gp and CYP3A in these two organs. The CYP3A4-transfected Caco-2 cells appear to provide a good predictive model of the interactive effects of inhibitors of CYP3A and P-gp. The interplay of P-gp and CYP3A in the liver could be mimicked by a basolateral dose in Caco-2 cells, whereas in this study we further proved that it also held true in the isolated perfused rat liver. Studies are ongoing with the isolated perfused rat liver in our lab to investigate multiple transporters and metabolic enzymes as significant factors affecting the hepatic disposition of drugs and to explore the possible implication that could be of use in clinical situations.

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References