As in Humans, Pregnancy Increases the Clearance of the Protease Inhibitor Nelfinavir in the Nonhuman Primate Macaca nemestrina

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Received February 4, 2009; accepted March 9, 2009

ABSTRACT

The apparent oral clearance of protease inhibitors (PIs) is increased in pregnant women. Although this phenomenon is reproduced in the mouse, because of the multiplicity of mouse cytochrome P450 isoforms, lack of information on their substrate and inhibitor selectivity, and lack of reagents (e.g., antibodies, purified protein), it is difficult to study the mechanistic basis of this phenomenon in this animal model. To investigate the mechanistic basis of this phenomenon in a more representative model, the nonhuman primate, we first determined whether this phenomenon could be reproduced in Macaca nemestrina, using nelfinavir as a model PI. Consistent with the human and mouse studies, we found that the apparent oral clearance of nelfinavir (NFV) in the macaques was significantly increased (3.14-fold) antepartum (n = 3) versus postpartum (n = 4). This increased apparent oral clearance was a result of an increased systemic clearance (1.9-fold) and a decreased bioavailability (~45%) during pregnancy. In vitro, pregnancy significantly enhanced the rate of NFV depletion in hepatic, but not intestinal S-9 fractions. Human CYP3A inhibitors erythromycin (0.5 mM), ketoconazole (0.5 μM), and troleandomycin (0.01–1 mM), but not the CYP2C inhibitor, sulfaphenazole (3 μM), significantly inhibited the depletion of NFV in hepatic S-9 fractions and expressed rhesus CYP3A64 enzyme. Based on these data, we conclude that increased hepatic activity of NFV-metabolizing enzymes (perhaps CYP3A enzymes) results in increased clearance of PIs during pregnancy in the macaques. The M. nemestrina should be further investigated as a model to study the mechanisms by which the clearance of PIs is increased during pregnancy.

Protease inhibitors (PIs) are a class of antiretroviral agents that inhibit protease activity of HIV, thus preventing the postintegration step in HIV replication. For the treatment of the HIV-infected pregnant women and to prevent mother-to-infant HIV transmission, current antiretroviral guidelines recommend triple combination highly active antiretroviral therapy (Centers for Disease Control and Prevention, 1998). Because of its safety, tolerability, and potency, nelfinavir (NFV), together with nucleoside/non-nucleoside reverse transcriptase inhibitors, is the recommended treatment for pregnant women infected with HIV (Mofenson, 2003; Thorne and Newell, 2004).

HIV PIs, among several other classes of drugs, have altered pharmacokinetics during pregnancy (Anderson 2005). Several studies have shown that given the standard dosing regimen, pregnant women achieve lower PI plasma concentrations than nonpregnant women. For example, we have observed that the exposure of antepartum women to indinavir is 68% lower than during the postpartum period (Unadkat et al., 2007). In addition, pregnant women taking saquinavir have approximately 77% lower exposure [area

ABBREVIATIONS: PI, protease inhibitor; NFV, nelfinavir; AUC, area under the curve; P-gp, P-glycoprotein; P450, cytochrome P450; AP, antepartum; PP, postpartum; LC, liquid chromatography; MS, mass spectrometry; ERY, erythromycin; KTZ, ketoconazole; TAO, troleandomycin; SUl, sulfaphenazole; CI, confidence interval.
under the curve (AUC)) to the drug than nonpregnant women (Acosta et al., 2001). Likewise, the antepartum exposure to NFV in pregnancy is lower in pregnant versus postpartum women (Angel et al., 2001; Nellen et al., 2004; van Heeswijk et al., 2004). Likewise, steady-state plasma concentrations and exposure to ritonavir are 40% lower in pregnant versus postpartum women (Acosta et al., 2004). These low plasma concentrations of PIs are of significant clinical concern because they are strongly associated with increased risk of virological failure and may result in transmission of resistant virus to the offspring (Angel et al., 2001; Burger et al., 2003).

Because NFV and other protease inhibitors are cleared predominantly by CYP3A metabolism and the drug efflux transporter P-glycoprotein (P-gp) (Kim et al., 1998; Unadkat and Wang, 2000; Zhang et al., 2001), we have speculated that this decreased exposure to PIs during pregnancy is due to increased CYP3A or P-gp activity or both. To test this hypothesis, we replicated this phenomenon in the pregnant mouse. We found that this decreased exposure to PI during pregnancy could be attributed to a significant decrease in the bioavailability of NFV (Mathias et al., 2006). In addition, we found that the hepatic expression and activity of Cyp3a enzymes are significantly higher in pregnant mice than that in nonpregnant mice, whereas hepatic and intestinal P-glycoprotein expression are unaffected (Mathias et al., 2006; Zhang et al., 2008). However, determining the mechanistic basis for this change is difficult because there are at least six Cyp3a isoforms and two P-gp isoforms present in the mouse, and these isoforms have been shown to be differentially regulated (Anakk et al., 2004; Zhang et al., 2008). In addition, specific antibody or purified protein of each of these P450 isoforms are not available.

To determine whether the phenomenon of decreased exposure to PIs during pregnancy could be replicated in an alternative animal model that is genetically and physiologically closer to humans, we studied the pregnant nonhuman primate, Macaca nemestrina. To be specific, we used NFV as a model PI to determine whether 1) pregnancy-related changes in exposure to oral NFV, observed in pregnant women, could be replicated in the macaque; 2) the systemic clearance and bioavailability of NFV are altered during pregnancy; and 3) pregnancy accelerates in vitro metabolism of NFV in hepatic and proximal small intestine S-9 fractions.

Materials and Methods

Materials. Nelfinavir mesylate powder was a kind gift from Pfizer (La Jolla, CA). -Cyclohexidrin was purchased from CyDex Pharmaceuticals, Inc. (Lenexa, KS). H-Labeled nelfinavir mesylate was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Saquinavir mesylate was a kind gift from Roche Ltd. (Nutley, NJ). Lactated ringer was purchased from Cardinal Health (Dublin, OH). Erythromycin, ketocazole, and troleandomycin were purchased from Sigma Chemie (Deisenhofen, Germany). High-performance liquid chromatography-grade methanol, acetonitrile, water, methylene chloride, and methyl tert-butyl ether were purchased from Thermo Fisher Scientific (Waltham, MA). CYP3A64 was a kind gift from the Drug Metabolism Department, Merck Research Labs (West Point, PA). All other reagents and materials were purchased from standard vendors and were of the highest available purity.

Animals. Three pregnant [antepartum (AP), 125–144 days; term is 167 days] and four nonpregnant [postpartum (PP) 102–125 days] macaques (M. nemestrina, 7.0–12 years, 6.5–10 kg) were studied. The animals were housed in individual cages in unidirectional airflow rooms with controlled temperature (22 ± 2°C), relative humidity (50 ± 10%), and 12-h light/dark cycles according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Animals were fed a standard animal diet (LabDiet, Richmond, IL); food and tap water were available ad libitum. Under anesthesia, the animals were chronically catheterized (gastric catheter, femoral artery, and femoral vein) and placed in a tether system described previously (Morton et al., 1987). All surgical procedures were conducted using aseptic techniques in a special-purpose operating suite. Venous and arterial catheters were constantly flushed with lactated ringer containing 3000 U/liter heparin at a rate of 18 ml/h. Weight of each animal, standard hematology, and blood chemistry panel were obtained on each animal the day before each study. Studies were not conducted unless the hematocrit and chemistry values were within normal ranges. During oral study, animals were fasted overnight, and food was not allowed until the 4-h blood sample was collected. Before initiating the study, the protocol was approved by the Institutional Animal Care and Use Committee.

Dose Administration and Blood Sampling. After at least 10 days of recovery from surgery, the animals were sequentially administered NFV orally (NFV mesylate, 100 mg/kg in 10–12% -cyclodextrin solution in water) and intra-arterially (NFV mesylate, 7.5 mg/kg in 10–12% -cyclodextrin), separated by at least 5 days. For safety reasons, the dose was administered intra-arterially, and blood samples were collected from the venous catheter. For the oral study, blood samples were collected from the femoral vein predose and at 30 min and 1, 2, 3, 4, 6, 8, 10, 12, and 24 h after dosing. For the intra-arterial study, blood samples were collected from the femoral vein predose and at 3, 5, 15, and 30 min and at 1, 2, 4, 6, 8, 10, 12, and 24 h after dosing. Blood samples were immediately centrifuged, and plasma samples were stored at −20°C until analysis. All dosing solutions were analyzed by LC-MS to confirm the concentrations of solutions administered.

Tissue Collection. After the in vivo studies were completed, the macaques were euthanized (100 mg/kg phenobarbital) to harvest tissues. Although the animals were breathing under anesthesia, the small intestine was removed and perfused with ice-cold phosphate-buffered saline on ice. Then, the intestine was cut open longitudinally on a cooled plate and sliced into several pieces (proximal, middle, and distal). Next, the mucosa was removed by gently scraping the luminal surface using a fine-tissue culture scraper. The liver was perfused in situ with ice-cold phosphate-buffered saline and then was excised from the body. In additional animals, instead of the above procedure, liver biopsies were obtained from antepartum (n = 3) and postpartum (n = 2) macaques using a percutaneous Tru-Cut needle. All the tissues were segmented into smaller pieces, snap-frozen in liquid nitrogen, and stored at −80°C until analysis.

S-9 Fraction Preparation. To determine whether hepatic or intestinal rate of NFV metabolism was elevated during pregnancy, we determined the depletion of NFV in hepatic and intestinal S-9 fractions. S-9 fractions of hepatic tissue from pregnant and nonpregnant macaques were isolated using previously described standard protocols, with minor modifications (Pang et al., 1985). In brief, hepatic S-9 fractions were prepared by centrifuging the hepatic tissue homogenate at 11,000g for 15 min at 4°C and then collecting the supernatant. Aliquots of the supernatant were frozen at −80°C until further analysis. Intestinal mucosal S-9 fractions were similarly prepared, with an additional centrifugation step at 600g for 5 min before centrifuging at 11,000g for 15 min. Protein concentration of the S-9 fractions was determined by the BCA protein assay, following manufacturer’s protocol (Thermo Fisher Scientific).

Nelfinavir Depletion in S-9 Fractions. NFV depletion was measured using a method published previously (Mathias et al., 2006). In brief, 0.2 mg/ml hepatic or 0.5 mg/ml intestinal S-9 frac-
tions were preincubated for 5 min at 37°C with 100 mM potassium phosphate, pH 7.4, containing 0.1 mM EDTA and 0.2 μM NFV (dissolved in methanol; final methanol concentration, 1%) in a shaking water bath. Incubation reactions were initiated by adding 1 mM NADPH (freshly prepared; final incubation volume, 100 μl). Controls included incubations without NADPH or substrate but with 1% methanol. Reactions were terminated by the addition of 100 μl of ice-cold acetonitrile containing the internal standard saquinavir. Samples were vortexed and kept on ice for 30 min before centrifuging at 14,000g for 10 min at room temperature. Supernatant was then collected and injected directly onto a LC-MS system for analysis, following the method described below. All incubations were conducted in duplicate. Preliminary experiments were carried out to determine the protein concentrations and incubation time under which the depletion of NFV was linear/log-linear.

To determine whether NFV depletion was mediated by CYP3A or CYP2C, selective human CYP3A inhibitors, erythromycin (ERY; 0.5 mM), ketoconazole (KTZ; 0.5 μM), and a selective CYP2C inhibitor sulfaphenazole (SUL; 3 μM) were included in the assay. All inhibition incubations were carried out for 5 min for hepatic S-9 fractions and 10 min for intestinal S-9 fractions before termination with ice-cold acetonitrile. Except for TAO, all inhibitors were coincubated with substrate and S-9 fractions for 5 min before adding NADPH. TAO was coincubated with S-9 fractions and NADPH for 30 min before adding the substrate. NFV concentrations were determined by the LC-MS method as described below. As a positive control, depletion of NFV was also carried out in macaque CYP3A46 superosomes (2 pmol/100 μl) for 10 min, using conditions described above. The NFV concentration in each incubation was expressed as a percentage of that in the corresponding 0-min control.

**NFV Analysis by LC-MS.** Plasma concentrations of NFV (as free base) were determined using LC-MS. In brief, 0.5 ml of plasma samples and 50 ng of saquinavir (internal standard) were alkalized with 100 μl of concentrated ammonium hydroxide and extracted with 5 ml of methyl-tert-butyl ether. Detection of NFV (m/z 568.4) and saquinavir (m/z 671.3) was performed on a Waters 2690 separations module (Waters, Milford, MA) coupled to a Micromass platform LCZ mass spectrometer (Waters) using positive electrospray ionization. Chromatographic separation was achieved using a gradient method on an Agilent XDB-C18 analytical column (2.1 × 50 mm, 5 μm; Agilent Technologies, Santa Clara, CA) equipped with an Agilent XDB-C18 guard column (2.1 × 12.5 mm, 5 μm) with a mobile phase consisting of solvent A (0.1% acetic acid in H2O) and solvent C (0.1% acetic acid in methanol). Samples were eluted at room temperature over 7.5 min using a gradient of 60 to 100% A from 0.5 to 2.5 min, remaining at 100% A for 1 min, then returning to 60% A from 3.5 to 3.75 min at a flow rate of 0.25 ml/min. A calibration curve and quality control samples ranging from 2.5 to 2500 ng/ml were analyzed in duplicate with all plasma samples. The accuracy of the assay was 108 ± 9.6, 101 ± 8.2, and 99 ± 7.1% and the CV of the precision was 4.8 ± 4.6, 9.4 ± 5.2, and 6.5 ± 3.7% at the low (30 ng/ml), mid (750 ng/ml), and high ends (1500 ng/ml) of the calibration range, respectively.

**NFV Plasma Protein Binding.** Plasma protein binding of NFV was determined by ultrafiltration (CentriF YM-30; Millipore Corporation, Billerica, MA). H-Labeled (66.5 ng) and unlabeled NFV mesylate (varying amount) were dried in a cell culture tube. Then, 700 μl of NFV-free plasma collected from pregnant or nonpregnant macaques (predose sample) was added to each tube. After mixing at room temperature for 10 min, duplicate aliquots of 300 μl of the plasma were transferred to the filtration devices and incubated for 30 min at 37°C. The cartridges were then centrifuged in a swinging bucket centrifuge (preset to 37°C) at 1000g for 2.5 min (approximately 35 μl of filtrate was collected). Thirty microliters of plasma (before filtration) and ultrafiltrate were analyzed for total radioactivity on a Packard Tri-Carb 1600RP liquid scintillation counter (PerkinElmer Life and Analytical Sciences). The percentage unbound (fu) was calculated as the percentage of radioactive counts in the filtrate to that in the plasma sample. The percentage of protein binding of NFV was determined to be constant over the range of 0.06 to 10 μg/ml using blank macaque plasma spiked with NFV. The plasma protein binding was determined at an average plasma concentration (150 ng/ml i.a., 350 ng/ml p.o.) observed in the study. Nonspecific binding of [3H]NFV to the filtration cartridge was shown to be insignificant by filtering 300 μl of a 12% β-cyclodextrin solution in water containing 66.5 ng/ml [3H]NFV.

**[3H]NFV Blood/Plasma Partition Coefficient.** The in vitro blood/plasma partition coefficient of [3H]NFV mesylate was determined by incubating 200 μl of fresh female macaque blood with [3H]NFV mesylate (2.66 ng) and varying concentration of unlabeled NFV mesylate (in 12% β-cyclodextrin; less than 2% of blood volume). After gentle agitation and incubation at 37°C for 30 min, duplicate aliquots of 20 μl of blood were removed before centrifuging the remaining sample (1 min at 13,000g) to separate plasma from the erythrocytes. Blood and plasma samples (20 μl of each) were then incubated with 1 ml of Biosol (National Diagnostics, Atlanta, GA) at 50°C for 30 min in a 20-ml scintillation vial. Next, the samples were mixed overnight with 300 μl of hydrogen peroxide (30%). Then, 10 ml of scintillation fluid was added to the vial and stored at room temperature for 1 week before scintillation counting (Packard Tri-Carb 1600RP liquid scintillation counter). The blood/plasma partition ratio was calculated as the percentage of radioactive counts in the blood to that in the corresponding plasma sample. The ratio was found to be independent of NFV concentration in the range 0.1 to 5 μg/ml.

**Pharmacokinetic Data Analysis.** Plasma concentration versus time profiles for NFV were obtained for each individual animal and were analyzed noncompartmentally using WinNonlin 5.0.1 (Pharsight, Mountain View, CA) to recover AUC0–∞ and other parameters. Bioavailability was estimated by dividing each animal’s NFV systemic clearance by the corresponding apparent oral clearance. Data for each pharmacokinetic parameter were reported as arithmetic mean ± S.D. Because pharmacokinetic parameters are typically log-normally distributed, statistical analysis was conducted on log-transformed pharmacokinetic parameters. The geometric mean ratio of each pharmacokinetic parameter, antepartum versus postpartum, was computed, as well as its 95% confidence intervals (CIs). If the 95% CI encompassed the value of unity, then the pharmacokinetic parameter of the drug was considered to be not significantly affected by pregnancy.

**Results**

**Pharmacokinetics of NFV after Oral Administration.** After oral administration, NFV was absorbed rapidly (Tmax ~2–3 h), with a large interindividual variability observed antepartum. The mean maximum plasma concentration achieved antepartum was significantly lower than that observed postpartum (~32%). Antepartum dose-normalized AUC0–∞ (n = 3) was only approximately 32% of the postpartum value (n = 4) (Table 1; Fig. 1A). The percentage of NFV unbound in the plasma was approximately 1.2% and was not affected by pregnancy. As a result, the antepartum mean apparent oral NFV plasma clearance or the corresponding unbound clearance was significantly greater (~200%) than that observed postpartum, irrespective of whether it was normalized to body weight or not. The bioavailability of NFV was significantly decreased antepartum (10.5%) and was approximately half of that postpartum (19%). The mean plasma terminal half-life of NFV was approximately ~3 h after oral administration and was not significantly different antepartum versus postpartum.

**Pharmacokinetics of NFV after Intra-Arterial Administration.** After intra-arterial administration, the es-
Antepartum and postpartum pharmacokinetic parameters (arithmetic mean ± S.D.) of NFV in *M. nemestrina* after oral administration of the drug (NFV mesylate, 100 mg/kg)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>AP (n = 3)</th>
<th>PP (n = 4)</th>
<th>Geometric mean ratio (AP/PP, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{\text{max}}/\text{dose} ) (ng/ml/mg)</td>
<td></td>
<td>1.32 ± 0.39</td>
<td>4.36 ± 2.23</td>
<td>0.32 (0.17–0.61)*</td>
</tr>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td></td>
<td>2.95 ± 2.55</td>
<td>1.77 ± 0.51</td>
<td>1.34 (0.46–3.90)</td>
</tr>
<tr>
<td>( \text{AUC}_{0-\infty}/\text{dose} ) (h·ng/ml/mg)</td>
<td></td>
<td>5.90 ± 0.75</td>
<td>21.0 ± 12.4</td>
<td>0.32 (0.18–0.60)*</td>
</tr>
<tr>
<td>( \text{CL/F} ) (liter/h)</td>
<td></td>
<td>171 ± 24.0</td>
<td>61.5 ± 33.5</td>
<td>3.14 (1.73–5.70)*</td>
</tr>
<tr>
<td>( \text{CL/F}_{\text{unbound}} ) (liter/h)</td>
<td></td>
<td>21.4 ± 0.57</td>
<td>9.04 ± 5.22</td>
<td>2.73 (1.47–5.10)*</td>
</tr>
<tr>
<td>( \text{CL/F}_{\text{unbound}} ) (liter/h/kg)</td>
<td></td>
<td>14,589 ± 3004</td>
<td>5,383 ± 3,325</td>
<td>3.04 (1.67–5.61)*</td>
</tr>
<tr>
<td>( \text{t}_{1/2} ) (h)</td>
<td></td>
<td>1,817 ± 190</td>
<td>791 ± 503</td>
<td>2.65 (1.43–4.88)</td>
</tr>
<tr>
<td>( f_u ) (%)</td>
<td></td>
<td>10.5 ± 0.89</td>
<td>19.0 ± 8.51</td>
<td>0.60 (0.38–0.94)*</td>
</tr>
<tr>
<td>( f_u^* ) (%)</td>
<td></td>
<td>1.19 ± 0.09</td>
<td>1.18 ± 0.30</td>
<td>1.03 (0.79–1.35)</td>
</tr>
</tbody>
</table>

\( C_{\text{max}}/\text{dose} \), dose-normalized maximum plasma concentration; \( T_{\text{max}} \), time to reach maximum plasma concentration; \( \text{CL/F} \), total oral plasma clearance; \( \text{CL/F}_{\text{unbound}} \), unbound oral plasma clearance; \( \text{t}_{1/2} \), terminal plasma half-life; \( F \), bioavailability; \( f_u \), unbound in plasma.

* Significantly different antepartum versus postpartum.

As a result, the mean NFV total plasma clearance or the unbound plasma clearance was significantly higher antepartum (by ~80%) versus postpartum, irrespective of whether it was normalized to body weight or not. The steady-state volume of distribution of NFV was ~2.8-fold greater antepartum versus postpartum. The terminal plasma half-life of NFV after intra-arterial administration was not significantly different between the two groups.

**NFV Depletion in Hepatic S-9 Fractions.** The depletion of NFV in hepatic S-9 fractions occurred only in the presence of NADPH. The depletion rate in hepatic S-9 fractions obtained antepartum (Fig. 2A) was more rapid than in S-9 fractions obtained postpartum. To gain insight into the enzymes responsible for the increased depletion rate of NFV in hepatic S-9 fractions during pregnancy, we determined whether the human prototypic CYP3A and CYP2C inhibitors could inhibit NFV depletion. KTZ (CYP3A inhibitor; 0.5 \( \mu \)M) almost completely inhibited NFV depletion in hepatic S-9 fractions from both antepartum and postpartum animals. ERY (0.5 mM) inhibited the depletion to a lesser degree. SUL (CYP2C inhibitor; 3 \( \mu \)M) did not significantly inhibit NFV depletion (Fig. 2A). Another human CYP3A inhibitor, TAO, inhibited NFV depletion in hepatic S-9 fractions in a concentration-dependent manner (Fig. 2B).

**NFV Depletion in Proximal Small Intestinal S-9 Fractions.** The depletion of NFV in proximal small intestine S-9 fractions was unaffected by pregnancy and was less and more variable than that with hepatic S-9 fractions (Fig. 3, A and B). KTZ (0.5 \( \mu \)M) and TAO (10 \( \mu \)M), but not SUL (0.3 \( \mu \)M), significantly inhibited the depletion in the S-9 fractions in the postpartum group (Fig. 3).

**NFV Depletion in Expressed CYP3A64 Enzyme.** To confirm that macaque CYP3A can catalyze the metabolism of NFV, we measured the depletion of NFV by rhesus macaque CYP3A64 cDNA-expressed enzyme. NFV was rapidly depleted by 2 pmol CYP3A64 (Fig. 4A). To confirm that the inhibitors used in the tissue S-9 fraction NFV depletion studies demonstrated specificity for CYP3A, we measured their ability to inhibit CYP3A64. ERY (0.5 mM) or KTZ (0.5 \( \mu \)M) significantly inhibited NFV depletion mediated by CYP3A64 (Fig. 4A). TAO showed a concentration-dependent inhibition of NFV depletion by CYP3A64 (Fig. 4B). In contrast, SUL (3 \( \mu \)M) did not inhibit CYP3A64-mediated NFV depletion (Fig. 4A).
Student's t test.

**Discussion**

The macaque reproduced the pregnancy-associated changes in the disposition of oral NFV observed in pregnant women (Nellen et al., 2004; van Heeswijk et al., 2004). In the macaque, the maximum plasma concentration and the dose-normalized AUC after oral NFV were significantly decreased ~68% by pregnancy (Table 1). As a consequence, the apparent oral clearance of the drug was increased by 3.1-fold during pregnancy, irrespective of whether it was normalized to body weight or not. This increase could be caused by an increase in the systemic clearance of NFV or a decrease in bioavailability or both.

To determine whether pregnancy changed the systemic clearance of NFV, the drug was administered intra-arterially to the macaques. The dose-normalized AUC_{0-\infty} was significantly lower antepartum versus postpartum (Table 2). As a result, the systemic clearance of NFV was increased 1.9-fold during macaque pregnancy, irrespective of whether it was normalized to body weight or not (Table 2). However, the terminal plasma half-life of NFV (~2–3 h), similar to that observed in humans and cynomolgus monkeys (Shetty et al., 1996; Markowitz et al., 1998; van Heeswijk et al., 2004), was not significantly affected by pregnancy. Because the terminal half-life of a drug is dependent on both systemic clearance and volume of distribution (Vss), this unchanged half-life and increased systemic clearance indicates that volume of distribution (Vss) was increased during pregnancy. A 2.8-fold increase in Vss was observed (Table 2). The Vss value (1.9 liter/kg) obtained in M. nemestrina (Table 2) was similar to that observed in cynomolgus macaques (Shetty et al., 1996).

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**TABLE 2**

Antepartum and postpartum pharmacokinetic parameters (arithmetic mean ± S.D.) of NFV in *M. nemestrina* after intra-arterial administration of the drug (NFV mesylate, 7.5 mg/kg)

<table>
<thead>
<tr>
<th>Parameter Unit</th>
<th>AP (n = 3)</th>
<th>PP (n = 4)</th>
<th>Geometric Mean Ratio (AP/PP, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{0}/dose (µg/mL/mg)</td>
<td>0.16 ± 0.05</td>
<td>0.70 ± 0.39</td>
<td>0.29 (0.10–0.82)*</td>
</tr>
<tr>
<td>AUC_{0-\infty} (h · ng/mL/mg)</td>
<td>55.9 ± 2.71</td>
<td>107 ± 24.6</td>
<td>0.54 (0.42–0.69)*</td>
</tr>
<tr>
<td>CL (liter/h)</td>
<td>17.9 ± 0.87</td>
<td>9.82 ± 2.58</td>
<td>1.87 (1.46–2.40)*</td>
</tr>
<tr>
<td>CL (liter/kg)</td>
<td>2.25 ± 0.14</td>
<td>1.41 ± 0.38</td>
<td>1.65 (1.27–2.10)*</td>
</tr>
<tr>
<td>Vss (liter)</td>
<td>34.2 ± 2.7</td>
<td>13.5 ± 7.3</td>
<td>2.80 (1.69–4.62)*</td>
</tr>
<tr>
<td>Vss (liter/kg)</td>
<td>4.33 ± 0.74</td>
<td>1.93 ± 1.07</td>
<td>2.45 (1.46–4.10)*</td>
</tr>
<tr>
<td>t_{1/2} (h)</td>
<td>2.27 ± 1.01</td>
<td>2.12 ± 0.33</td>
<td>1.02 (0.62–1.67)</td>
</tr>
<tr>
<td>f_{u} (%)</td>
<td>1.69 ± 0.10</td>
<td>1.62 ± 0.34</td>
<td>1.06 (0.85–1.32)</td>
</tr>
</tbody>
</table>

* C_{0}/dose, dose-normalized initial plasma concentration; CL, clearance; CL_{unbound}, unbound clearance; Vss, volume of distribution at steady state; t_{1/2}, terminal plasma half-life; f_{u}, unbound in plasma.

* Significantly different antepartum versus postpartum.
and larger than the total body water volume, indicating extensive tissue binding of NFV. An increase in the unbound fraction of NFV in the plasma also could contribute to the observed increase in the systemic clearance of the drug. Consistent with reported human data, we found that NFV was bound extensively to macaque plasma proteins (>98%). However, pregnancy did not affect NFV plasma protein binding (Table 1). For a moderate extraction ratio drug, such as NFV, an increase in hepatic blood flow could increase its systemic clearance. Assuming that, as in humans (Zhang et al., 2001), NFV is eliminated primarily by metabolism, the reported 50% increase in hepatic blood flow in pregnant women (Nakai et al., 2002) would not completely explain the doubling of NFV systemic clearance observed in the pregnant macaque. Thus, we conclude that the increased systemic clearance of NFV must be due, at least in part, to increased hepatic metabolism of NFV (see below).

To determine whether hepatic or intestinal bioavailability of NFV was affected by pregnancy, we first computed the oral bioavailability ($F_b$) of NFV. The oral bioavailability of NFV is determined as a product of the fraction of drug absorbed ($F_a$), fraction that escapes metabolism by the small intestine ($F_g$), and fraction that escapes metabolism by the liver ($F_h$). A decrease in one or more of these parameters will reduce the total oral bioavailability ($F = F_a \cdot F_g \cdot F_h$). Based on the intra-arterial data, we first computed $F_h$ of NFV. To do so, we assumed that NFV is cleared solely by the liver, hepatic blood flow in the macaque is 3.1 liter/h/kg (Boxenbaum 1980) and, as in humans, is increased by 50% during pregnancy (Nakai et al., 2002). In addition, we used the observed NFV blood/plasma partition ratio of 0.67. With these values, the average hepatic bioavailability $F_h$ was computed to be 0.32 ± 0.18 postpartum and 0.27 ± 0.05 antepartum. However, there is no consensus regarding change in hepatic blood flow during pregnancy. Another study showed that it does not change during human pregnancy (Robson et al., 1990). If we assume no change in hepatic blood flow during macaque pregnancy, $F_h$ cannot be calculated because the computed blood clearance of NFV antepartum is larger than the reported hepatic blood flow (Boxenbaum 1980). Assuming that $F_g$ is not affected by pregnancy, we computed $F_a$ of NFV to determine whether $F_a$ and $F_h$ are both affected by pregnancy. The computed $F_a$ was found to be 1.00 ± 1.12 postpartum compared with 0.39 ± 0.09 antepartum. Although neither $F_a$ nor $F_h$ of NFV was significantly lower during pregnancy, the net bioavailability ($F_a \cdot F_g \cdot F_h$) was. Because nelfinavir is a highly permeable drug (log $P$ value of 2.9; Ford et al., 2004), it is unlikely that the fraction of drug absorbed ($F_a$) would have been affected by the decreased GI tract motility observed in human pregnancy (Parry et al., 1970; Hytten and Leitch, 1971). Thus, we conclude that a combined reduction in $F_a$ and $F_h$ resulted in reduced bioavailability of NFV in the pregnant macaque.

In humans, PIs are primarily cleared by CYP3A and P-gp (Kim et al., 1998; Unadkat and Wang, 2000; Zhang et al., 2001). Both are present in the liver and the enterocytes of the small intestine. In the latter, they work synergistically to prevent the drug from entering the systemic circulation (Wacher et al., 1995; Zhang et al., 2001). Therefore, if the activity of one or both of these proteins is increased during pregnancy, the first-pass extraction of NFV would be greater, resulting in a decreased bioavailability of NFV. To gain insight into whether hepatic or intestinal enzymes responsible for the metabolism of NFV have increased activity, we determined the rate of NFV depletion by NADPH-dependent oxidases in hepatic and intestinal S-9 fractions. As a positive control, we used CYP3A64, a recently identified rhesus macaque CYP3A enzyme. CYP3A64 catalyzes testosterone 6β-hydroxylation, midazolam 1'-hydroxylation, nifedipine oxidation, and 7-benzoxycarbonylmethylcoumarin dealkylation with higher intrinsic clearance compared with human CYP3A4 enzyme (Carr et al., 2006). Based on the in vitro and in vivo characterization, CYP3A64 is considered to be the CYP3A4 ortholog in rhesus macaques (Carr et al., 2006; Pruksaritnanont et al., 2006). *M. nems.strina, Macaca fascicularis* (cynomolgus macaque), and *Macaca mulatta* (rhesus macaque) all belong to the Old-World macaques, and they share high sequence similarity to the human genome, and between each other (Manness et al., 2005; Gibbs et al., 2007). For example, the rhesus genome shares ~93% identity to the human genome (Gibbs et al., 2007), and at the amino acid level, rhesus CYP3A64 sequence is 100% identical to the cynomolgus CYP3A8 (Carr et al., 2006). Therefore, it is very likely that CYP3A4s in *M. nemestrina* will have similar enzyme characteristics as CYP3A64 of the rhesus macaque. The depletion of NFV in hepatic S-9 fractions, but not in small intestinal S-9 fractions, was significantly greater antepartum versus postpartum (Fig. 2A). The depletion in hepatic S-9 fractions was almost completely inhibited by 0.5 μM KTZ, a prototypic inhibitor of human CYP3A4, but not by 3 μM SUL, a prototypic inhibitor of human CYP2C9, or 20 μM omeprazole, a prototypic inhibitor of human CYP2C19 (data not shown). In addition, KTZ has been shown to inhibit midazolam metabolism in cynomolgus macaques (Kanazu et al., 2004) and testosterone 6β-hydroxylase activity in rhesus macaque liver microsomes and in cDNA-expressed CYP3A64 enzymes (Carr et al., 2006). Another prototypic human...
CYP3A inhibitor, TAO, was also included in our depletion study. However, at a concentration of 10 μM, only marginal inhibition of NFV-depletion by hepatic S-9 fractions and CYP3A4 was observed (Figs. 2B and 3B), whereas in human liver microsomes, 10 μM TAO almost completely abolished testosterone 6b-hydroxylase activity (Polasek and Miners 2006). These results indicate that although TAO inhibits macaque CYP3A4-mediated NFV metabolism, it is not as potent an inhibitor of macaque CYP3A4 as it is of human CYP3A4. Our data suggest collectively that hepatic, but not intestinal, activity of NFV-metabolizing oxidative enzyme(s) is enhanced during pregnancy. Although it is possible that one of these enzymes is the macaque CYP3A4, the significant inhibition of both hepatic and intestinal NFV-metabolizing enzymes by KTZ suggests that this inhibitor may not be a selective inhibitor of the macaque enzymes.

In summary, consistent with previous reports, the pregnant macaque reproduced the increased apparent oral clearance of PIs observed in pregnant women. This increased apparent oral clearance of NFV was a synergistic effect of increased bioavailability and increased systemic clearance of the drug. The pregnancy induced increased systemic clearance, and the first-pass extraction by the liver was due (at least in part) to the increased hepatic NFV-metabolizing enzyme(s) activity. In addition, because these enzymes are NADPH-dependent, they are likely to be P450 enzymes. This P450 enzyme(s) may be potentially the macaque hepatic CYP3A4 enzyme(s). To identify this enzyme(s) and to investigate the mechanistic basis of this phenomenon, additional studies are required. These studies could be similar to those that we have conducted in pregnant women where we observed enhanced CYP3A and renal P-gp activity in pregnant women as measured by the apparent oral clearance of midazolam (a prototypic CYP3A substrate) and renal clearance of digoxin, respectively (Hebert et al., 2008). Such studies are in progress in our laboratory.

Acknowledgments

We thank Dr. Keith Vogel, Mike Gough, and Carol Elliot for invaluable technical assistance in performing the animal studies.

References


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