Changes in Pharmacokinetics of Anti-HIV Protease Inhibitors during Pregnancy: The Role of CYP3A and P-glycoprotein

Anita A. Mathias, Lillian Maggio-Price, Yurong Lai, Anshul Gupta, and Jashvant D. Unadkat

Departments of Pharmaceutics (A.A.M., Y.L., A.G., J.D.U.) and Comparative Medicine (L.M.-P.), University of Washington, Seattle, Washington

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ABSTRACT

Human immunodeficiency virus (HIV)-infected women have reduced exposure [area under the curve (AUC)] to anti-HIV protease inhibitors [e.g., nelfinavir (NFV)] during pregnancy. To determine the mechanistic basis of this phenomenon, we administered NFV esmyleate orally (2.5 mg) or intravenously (0.625 mg) to timed pregnant (gestational age: 18–19 days) and non-pregnant FVB mice. After oral but not after i.v. administration, the plasma clearance of NFV was higher (by 134%, \( p < 0.05 \)) and bioavailability was lower (by 32%, \( p < 0.05 \)) in pregnant (\( n = 3 \)) versus nonpregnant mice (\( n = 3 \)). These effects of pregnancy were not due to changes in plasma protein binding of NFV. The half-life of NFV depletion in hepatic S-9 fractions of pregnant mice (\( n = 8 \)) was 2.2-fold faster (\( p < 0.05 \)) than that in nonpregnant mice (\( n = 7 \)). Hepatic CYP3A activity (testosterone 6β-hydroxylation, \( n = 4 \)) and expression (\( n = 8 \)) were significantly higher (by 138 and 49%, \( p < 0.05 \)) in pregnant mice than that in nonpregnant mice. In the intestine, no CYP3A activity was detected and CYP3A protein expression (\( n = 6 \), \( p > 0.05 \)) was not significantly different between the two groups. P-glycoprotein expression (\( n = 6 \)) in hepatic and intestinal tissue of pregnant mice was not significantly different from that in nonpregnant mice. These changes in disposition of NFV during pregnancy are predominately due to a change in its bioavailability. An increase in hepatic CYP3A can explain the reduced bioavailability of NFV during pregnancy. If such up-regulation of hepatic CYP3A activity occurs in pregnant women, it has important implications for dose adjustment of a variety of drugs ingested by pregnant women and cleared predominately via CYP3A metabolism.

Antiretroviral treatment of HIV-infected pregnant women is widely used to prevent mother-to-child HIV transmission and to treat maternal HIV infection (Mofenson, 2003; Thorne and Newell, 2004). The standard of care uses a combination of antiretroviral drugs, including nucleoside reverse transcriptase inhibitors and anti-HIV protease inhibitors (PIs). Recently, several clinical studies in small groups of women have indicated that pregnancy alters the disposition of PIs (Acosta et al., 2001; Angel et al., 2001; Bryson et al., 2002; Kosel et al., 2003; van Heeswijk et al., 2004). Acosta et al. (2001) have shown that the area under the plasma concentration-time curve (AUC\(_{0–8 h}\)) of saquinavir was approximately 77% lower antepartum (gestational age: 27–34 weeks, \( n = 4 \)) than that in nonpregnant adults. Likewise, Bryson et al. (2002) and van Heeswijk et al. (2004) have reported that the antepartum AUC\(_{0–12 h}\) of nelfinavir (NFV) is lower (by approximately 22 and 25%) than that observed postpartum. In another perinatal study, we have observed that indinavir AUC\(_{0–8 h}\) antepartum (gestational age 30 weeks, \( n = 11 \)) is 68% lower than that observed postpartum (6 weeks after delivery) (D. W. Wara and R. E. Tuomala, unpublished results; PACTG 358, co-chairs Drs. Diane Wara and Ruth Tuomala).

To date, the mechanisms by which pregnancy reduces maternal exposure to PIs have not been elucidated. The systemic clearance or bioavailability of the PIs is primarily determined by drug-metabolizing cytochrome P450 enzymes 3A4/5 (CYP3A4/5) and the drug efflux transporter P-glycoprotein (P-gp) (Kim et al., 1998; Sandoval et al., 1998; Unadkat and Wang, 2000a; Zhang et al., 2001). In addition, PIs are predominantly bound to plasma \( \alpha1 \)-acid glycoprotein, with

ABBREVIATIONS: HIV, human immunodeficiency virus; PIs, protease inhibitors; AUC, area under the plasma concentration-time curve; NFV, nelfinavir; CYP3A4/5, cytochrome P450 3A4/5; P-gp, P-glycoprotein; HPLC, high-performance liquid chromatography; SPA, sulfaphenazole; KTZ, ketoconazole; PBS, phosphate-buffered saline; MS, mass spectrometry.
the percentage bound ranging from approximately 60% for indinavir to approximately 98 to 99% for amprenavir, ritonavir, NFV, and saquinavir (Invirase package insert; Roche Pharmaceuticals, Nutley, NJ; Viracept package insert; Agouron Pharmaceuticals, La Jolla, CA) (Anderson et al., 2000; Sadler et al., 2001). Therefore, alterations in metabolism or transport of the PIs or changes in their degree of plasma protein binding could play an important role in influencing the pharmacokinetics of the PIs (Unadkat and Wang, 2000b; Huang et al., 2001; Acosta, 2002).

To determine the mechanisms by which the disposition of PIs is affected by pregnancy, we have conducted studies in the pregnant mouse. Using NFV as our model PI, we first determined whether the pregnancy-related changes in exposure (AUC) to oral NFV, observed in pregnant women, could be replicated in the mouse. Then we determined whether systemic clearance, bioavailability, or plasma protein binding of NFV was altered during pregnancy. Finally, we sought to determine whether enhanced expression and/or activity of mouse CYP3A or P-gp or both was the underlying mechanism for reduced exposure of NFV observed during pregnancy.

Materials and Methods

Chemicals

NFV mesylate and [14C]NFV (specific activity: 60 mCi/mmol) were gifts from Pfizer, Inc. (La Jolla, CA), saquinavir mesylate was a gift from Roche Ltd. (Nutley, NJ), and sulfobutylether-β-cyclodextrin (CAPTISOL) was a gift from CyDex, Inc (Lenexa, KS). Methyl tert-butyl ether (HPLC grade) was purchased from Sigma Chemical Company (St. Louis, MO). Testosterone, phenylmethylsulfonyl fluoride, 6β-hydroxytestosterone, NADPH, 11β-hydroxyprogesterone, sucrose, and sulfaphenazole (SPA) were purchased from Sigma. Ketocnazole (KTZ) was purchased from U.S.P.C. Inc. (Rockville, MD), potassium phosphate monobasic was purchased from J. T. Baker (Phillipsburg, NJ), and ethyl acetate (HPLC grade) was purchased from Mallinkrodt ChromAR (Paris, KY). Protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim, Germany). The BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL). All other reagents used in experiments were purchased from Fisher Scientific (Fairlawn, NJ).

Animal Studies

Pregnant (gestational age: 18–19 days) and nonpregnant FVB wild-type mice (Taconic Farms, Germantown, NY) were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication 85-23, 1985. The experimental studies were approved by the Institutional Animal Care and Use Committee at the University of Washington. The mice had free access to food and water and were maintained on a 12:12-h automatically timed light/dark cycle. Male mice, 7 to 9 weeks of age and weighing 20 to 30 g, were mated with female mice, 7 to 9 weeks of age and weighing 20 to 30 g. Female mice demonstrating sperm plugs were weighed and housed in new cages. Gestational age was calculated based on the estimated time of insemination (presence of the sperm plug). Progression of pregnancy in these female mice was regularly monitored by visual inspection and by measuring the increase in body weight. All experiments were performed on gestational day 18 to 19 (term in mice is approximately 20–21 days). Age-matched nonpregnant female mice, whose weights were comparable with those of pregnant mice on gestation day 0, were used as controls.

NFV mesylate was dissolved in 10% sulfobutylether-β-cyclodextrin and administered orally by gavage (2.5 mg) or intravenously by retro-orbital injection (0.625 mg) to timed pregnant (gestational age: 18–19 days) and nonpregnant mice (Price et al., 1984). After dosing, animals were sacrificed under anesthesia (ketamine-xylazine) by cardiac puncture at one of several time points (0, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min). Blood was collected in heparinized Microtainer tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged (Sigma 3K12; B Braun, Melsungen, Germany), and the harvested plasma was stored at −80°C until analysis. Following organ perfusion (with ice-cold PBS), target tissues, intestine (jejunum), and liver were removed from timed pregnant and nonpregnant mice, rinsed a second time with ice-cold PBS, cut into several smaller pieces, snap-frozen in liquid nitrogen, and stored at −80°C for further analysis. The oral and i.v. doses of NFV were designed such that the maximal NFV plasma concentrations achieved in the mice were comparable with the steady-state plasma concentrations (~3–4 μg/ml) achieved in people with HIV infection (Viracept package insert). Plasma NFV concentrations were determined by a validated HPLC/MS/MS method.

Plasma Protein Binding of NFV

The unbound fraction of NFV in pregnant and nonpregnant mouse plasma was determined by equilibrium dialysis. The dialysis cells (Spectrum Laboratories, Rancho Dominguez, CA) consisted of two Teflon half-cells, each of 1 ml capacity, separated by a semipermeable cellulose membrane (Spectra/Per 4 dialysis membrane, molecular mass cutoff 12,000–14,000 Da; Spectrum). The membranes were prepared by first soaking in deionized water (0.5–1 h) and then overnight in modified Krebs’ mammalian Ringer phosphate buffer (pH 7.4). One compartment of the half-cell contained the buffer solution (200 μl), and the other contained 0.2 ng/ml [14C]NFV in plasma (200 μl) containing 0.2 ng/ml [14C]NFV. The cells were then immersed in a water bath at 37°C and rotated gently for 24 h. A fixed volume of sample (150 μl) was withdrawn from both the buffer and plasma compartments, and total radioactivity in these compartments was measured by liquid scintillation counting. The unbound fraction in the plasma was calculated as the ratio of radioactivity in the buffer compartment after dialysis and the radioactivity in the plasma compartment after dialysis. Preliminary experiments showed that nonspecific binding of NFV to the cellulose membrane was negligible, the time to reach equilibrium was 8 h, and the plasma protein binding of NFV in mouse plasma was linear over the range of 0.1 to 12.5 μg/ml. Plasma protein binding of NFV was determined in pooled samples, because the volume of a single sample was insufficient to do so. For plasma obtained after oral administration, samples containing the maximal concentration of NFV were pooled and tested. Plasma samples after i.v. administration were pooled from samples drawn at earlier time points (e.g., 3 or 15 or 30 min), when the highest concentrations of NFV were achieved. All determinations were conducted in duplicate.

NFV Metabolism in Hepatic S-9 Fractions

To determine whether hepatic metabolism of NFV was elevated during pregnancy, we determined cytochrome P450-mediated depletion of NFV in hepatic S-9 fractions. S-9 fractions of hepatic tissue from pregnant and nonpregnant mice were isolated using previously described standard protocols, with certain 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 harvesting the supernatant. Aliquots of the supernatant were frozen at −80°C until analysis. All subsequent experiments were conducted on the same set or subset of S-9 fractions. In studies examining NFV metabolism, S-9 fractions were preincubated for 5 min at 37°C with 100 mM potassium phosphate; pH 7.4, and 0.11 to 1.51 μM NFV substrate (dissolved in methanol; final methanol concentration 1%). Incubation reactions were initiated by adding 1 mM NADPH (freshly prepared; final incubation volume 1 ml). Controls included incubations without NADPH or substrate but with 1% methanol. Reactions...
were terminated at 0, 5, 10, 20, and 30 min by the addition of 5 ml of methyl-tert-butyl ether and cooling on ice. Fifty microliters of saquinavir (210 ng/ml stock in methanol) was added to the mix as an internal standard. The pH of the incubation mix was adjusted to 9.5 to 10 by the addition of 100 μl of 50 mM NaOH. Samples were centrifuged at 400g for 20 min at 4°C. The supernatant was dried, and the reconstituted residue was injected onto the HPLC/MS/MS system for analysis (as described below). All incubations were conducted in duplicate. Using log-linear regression, a first-order decay model was fit to the percentage of NFV remaining in the incubation to estimate the half-life of NFV depletion. In preliminary experiments, the optimal protein concentration for incubations in which NFV depletion followed first-order decay kinetics was found to be 2 mg/ml. The half-life of NFV depletion was unchanged over the concentration range 0.11 to 0.30 μM; therefore, all further substrate depletion experiments (as described below) were carried out at 0.11 μM NFV using 2 mg/ml protein.

To determine whether selective human CYP3A or CYP2C inhibitors could inhibit NFV depletion in mouse hepatic S-9 fractions, S-9 fractions were incubated as described above but with the inclusion of SPA (2.5 μl of 10 or 50 μM SPA in methanol) or KTZ (2 μl of 1 μM KTZ in methanol). Reactions were terminated at 5 min, and NFV concentrations were determined by the HPLC/MS/MS method as described below.

Mouse Hepatic and Intestinal CYP3A Activity

Intestinal S-9 fractions were prepared using the methodology described for hepatic S-9 fractions, except that an additional centrifugation step at 600g for 5 min was included followed by 11,000g for 15 min at 4°C. Hepatic and intestinal CYP3A activities in the S-9 fractions of both pregnant and nonpregnant mice were examined as described above, except that the selective murine CYP3A substrate testosterone was used as a substrate (Emoto et al., 2000; Mankowski et al., 2000; Nallani et al., 2003). Reactions were terminated at 15, 30, and 45 min by the addition of 5 ml of ethyl acetate and cooling on ice. Fifty microliters of 11-hydroxyprogesterone (internal standard) was added to the incubation mix. The samples were centrifuged (200g for 20 min). The supernatant was dried under nitrogen on an evaporator. The residue was reconstituted in 100 μl of methanol-water (50:50) and assayed by HPLC/UV (as described below). For all further experiments, the time of incubation (t = 15 min) and concentrations of S-9 fraction protein (1 mg/ml for hepatic tissue) and testosterone (250 μM) were chosen as optimal because under these conditions the rate of metabolite formation (6β-hydroxytestosterone) was maximal. We were unable to measure mouse CYP3A activity in pregnant and nonpregnant intestinal samples, containing up to 4 mg of protein and incubated for 45 min, possibly because of the low CYP3A activity observed there. In addition, to confirm that KTZ was an inhibitor of mouse CYP3A activity, we conducted the above hepatic S-9 fraction incubations in the presence of KTZ (1 μM concentration added to each preincubation).

Mouse Hepatic and Intestinal CYP3A and P-gp Expression

Mouse CYP3A and P-gp (gene products: mdr1a and mdr1b) expression in hepatic and intestinal S-9 fractions (pregnant and nonpregnant) was quantified by Western blot analysis according to the method of Towbin et al. (1979). In brief, for mouse CYP3A detection, hepatic S-9 fraction proteins (1 μg of protein) and intestinal S-9 fraction proteins (10 μg of protein) were separated on a 10% SDS-polyacrylamide gel (Criterion Blotter apparatus; Bio-Rad, Hercules, CA) and transferred electrophoretically to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). After transfer, the membranes were incubated with a 1:1000 dilution of the primary antibody, polyclonal goat anti-rat 3A2 anti-asea (Daichi Pure Chemicals, Tokyo, Japan), followed by incubation with polyclonal anti-rabbit IgG with peroxidase (Bio-Rad) as secondary antibody (1:2000 dilution for intestinal samples and a 1:4000 dilution for liver samples). To quantify P-gp expression, hepatic (40 μg of protein) or intestinal S-9 fractions (80 μg of protein) were separated on a 4 to 15% gradient SDS-polyacrylamide gel (Criterion Blotter apparatus) and transferred electrophoretically to a nitrocellulose membrane. After transfer, the membranes were incubated with a 1:200 dilution of the primary antibody, monoclonal antibody C219 (ID Labs Inc., London, ON, Canada), followed by incubation with the polyclonal antibody, mouse anti-IgG with peroxidase (Bio-Rad), as the secondary antibody (1:2000 dilution for intestinal samples and liver samples). CYP3A and P-gp bands were visualized by enhanced chemiluminescence (Amersham Biosciences), imaged (X-Omat Blue XB-1; PerkinElmer Life Sciences, Rochester, NY), and quantified by scanning densitometry. The relative intensity of each protein band was determined using the Bio-Rad Chemi-Doc and Quantity One Program. Data were expressed as optical density units per microgram of protein.

Analytical Methods

NFV HPLC/MS/MS Assay. Plasma concentrations of NFV (as free base) were determined by a validated HPLC/MS/MS assay. In brief, to 50 μl of blank plasma (pooled from donor mice) or unknown sample, 20 μl of the NFV calibrator solution (final concentration 12.5–800 ng/ml) or methanol (for unknown sample) was added. Twenty microliters of saquinavir (21 ng/ml) as an internal standard was added to the plasma, followed by 50 μl of 50 mM sodium hydroxide (pH adjusted to 9.5–10). The solution was mixed on a vortex-type mixer, and 2 ml of methyl-tert-butyl ether was added. The samples were mixed on a vortex-type mixer for 30 s and agitated (Eberbach Corp., Ann Arbor, MI) for 25 min. The samples were then centrifuged (Dyna Centrifuge, Clay Adams division of Becton Dickinson, Parsippany, NJ) at 2500g for 5 min. The methyl-tert-butyl ether layer was aspirated into borosilicate glass centrifuge tubes (VWR, West Chester, PA). The organic layer was dried under nitrogen (Reacti-Therm III Heating Module; Pierce). The residue was reconstituted in 50 μl of mobile phase B, methanol-acetonitrile (1:1), and 5 to 10 μl of the reconstituted extract was injected onto the HPLC/MS/MS system for analysis. Quality control samples were prepared at the low (25 ng/ml), mid (100 ng/ml), and high ends (400 ng/ml) of the calibration range. The stock solutions and the quality control samples were stored in 100-μl aliquots at −80°C.

The HPLC/MS/MS assay for analysis of NFV in plasma was developed using a Shimadzu LC-10 AD liquid chromatograph with a Shimadzu SIL-10 Advp auto-injector interfaced with a VG Quattro II Micromass instrument) triple quadrupole mass spectrometer. Chromatography was performed at room temperature on a C18 reverse phase column (Zorbax Eclipse XDB-C18, 2.1 × 50 mm, 5 μm; Agilent Technologies, Palo Alto, CA) equipped with a guard column (Zorbax Extend-C18, Narrow Bore guard column, 2.1 × 12.5 mm, 5 μm; Agilent). The mobile phase contained A 0.1% acetic acid, pH 3.0, and B = methanol-acetonitrile (1:1). Analytes were eluted at a flow rate of 250 μl/min with a linear gradient of 40% B to 90% B over 3 min, followed by an additional 1 min at 90% B and then a column wash with 100% B. Mass spectrometer conditions were set as follows: cone voltage for all m/z, 35 V; source temperature, 150°C; desolvation temperature, 400°C; collision gas pressure (argon), 1.1 × 10⁻³ mBarr; and positive ion mode. NFV was detected in multiple reaction monitoring mode as follows: m/z 568.4→330.2 (collision energy, 30 eV) and 568.4→135.0 (collision energy: 50 eV). The internal standard saquinavir was detected in selective ion recording mode m/z 671.3. The assay was validated by assaying calibrators and quality control plasma samples in triplicate on 5 different days. Quality control samples were assayed in triplicate on the same day to estimate the intraday coefficient of variation and the accuracy of the assay. Calibration lines were constructed by linear regression of the peak area ratios (NFV/saquinavir) plotted versus the NFV concentration. The concentration of NFV in unknown samples was determined by inverse regression.
Testosterone and 6β-Hydroxytestosterone Assay. The HPLC assay (kindly provided by Yang Xu, Department of Pharmaceutics, University of Washington, Seattle, WA) for analysis of testosterone and its primary CYP3A metabolite, 6β-hydroxytestosterone, was performed on a HPLC system consisting of a Shimadzu LC-600 liquid chromatograph, interfaced with a Shimadzu SPD-6A UV detector and a Waters 717 autosampler. The chromatography was performed on an Econosil C18 reverse phase column (250 × 4.6 mm, 5 μm; Alltech Associates, Deerfield, IL) with a guard column (150 × 4.6 mm, 5 μm; Alltech) at room temperature. The mobile phase consisted of solvent A (30% methanol-2% acetonitrile-68% water) and solvent B (80% methanol-5% acetonitrile-15% water). Gradient elution at a flow rate of 1 ml/min was programmed from 30 to 100% B over 31 min. All analytes and the internal standard were detected at λ = 244 nm. Calibrators ranged from 0.05 to 0.8 μg of 6β-hydroxytestosterone spiked in the incubation matrix. 11α-Hydroxyprogesterone (0.5 μg) was used as the internal standard.

Pharmacokinetic Data Analysis. Mice were randomly assigned within the oral or i.v. NFV-dosing groups. Blood samples (one sample per mouse) obtained at various time points (0–360 min) were used to generate a composite plasma NFV concentration-time profile for each pregnant and nonpregnant mouse study. In total, three plasma concentration-time profiles in pregnant and nonpregnant mice, after oral or i.v. NFV administration, were generated. Pharmacokinetic parameter estimates were calculated using the noncompartmental approach (WinNonLin 3.2). Area under the plasma concentration-time curve from time 0 to infinity (AUC0–∞) was calculated as the ratio of the total plasma clearance (oral or i.v.) and the fraction unbound in plasma. Bioavailability after oral administration of NFV was estimated as the ratio of (AUC0–∞oral/doseoral) to (AUC0–∞i.v./dosei.v.).

For pregnant mice, certain pharmacokinetic parameters (oral and i.v.) were calculated as the ratio of the total plasma clearance (oral or i.v.) and the fraction unbound in plasma. Bioavailability after oral administration of NFV was estimated as the ratio of (AUC0–∞oral/doseoral) to (mean AUC0–∞oral). For pregnant mice, certain pharmacokinetic parameters (oral and i.v.) were calculated as the ratio of the total plasma clearance (oral or i.v.) and the fraction unbound in plasma. Bioavailability after oral administration of NFV was estimated as the ratio of (AUC0–∞oral/doseoral) to (mean AUC0–∞oral).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pregnant Mice (n = 3)</th>
<th>Nonpregnant Mice (n = 3)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC0–∞ (μg/ml/min)</td>
<td>176.5 ± 43.2</td>
<td>396.8 ± 31.7</td>
<td>0.002</td>
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<tr>
<td>CL/F (ml/min)</td>
<td>14.8 ± 4.0</td>
<td>6.3 ± 0.5</td>
<td>0.022</td>
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<tr>
<td>CL/F (ml/min/g)</td>
<td>0.6 ± 0.2</td>
<td>0.3 ± 0.02</td>
<td>0.020</td>
</tr>
<tr>
<td>CL/F unbound (ml/min)</td>
<td>1107.1 ± 297.2</td>
<td>209.5 ± 17.4</td>
<td>0.006</td>
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<tr>
<td>CL/F unbound (ml/min/g)</td>
<td>44.2 ± 11.5</td>
<td>8.4 ± 0.7</td>
<td>0.006</td>
</tr>
<tr>
<td>Cmax (μg/ml)</td>
<td>2.6 ± 0.3</td>
<td>6.2 ± 1.4</td>
<td>0.011</td>
</tr>
<tr>
<td>t1/2 (min)</td>
<td>7.7 ± 4.5</td>
<td>70.4 ± 43.4</td>
<td>0.782</td>
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<tr>
<td>fp (% unbound)</td>
<td>10.8 ± 2.6</td>
<td>15.7 ± 1.3</td>
<td>0.043</td>
</tr>
<tr>
<td>fp (% unbound)</td>
<td>1.3 ± 0.1</td>
<td>3.0 ± 1.4</td>
<td>0.049</td>
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* Parameters were compared using the two-sample t test (equal variance, statistical significance p < 0.05).

Results

The NFV HPLC/MS/MS assay was linear (r = 0.99) over the calibration range 12.5 to 800 ng/ml NFV (free base) in mouse plasma. The accuracy of the assay was 0.6, 5, and 2%, and the precision was 16, 9, and 12% at the low (25 ng/ml), mid (100 ng/ml) and high ends (400 ng/ml) of the calibration range. NFV eluted at approximately 4.4 min, and the internal standard saquinavir eluted at approximately 4.5 min.

After oral administration, AUC0–∞ in pregnant mice (n = 3) was 55% lower than that in the nonpregnant mice (n = 3) (Table 1; Fig. 1A). As a result, the mean NFV total oral plasma clearance in pregnant mice was significantly greater (134%, p < 0.05) than that in nonpregnant mice, irrespective of whether it was normalized to body weight or not. The percentage of NFV unbound in the plasma was significantly (p < 0.05) higher in nonpregnant versus pregnant mice. Consequently, the mean NFV unbound oral plasma clearance in pregnant mice was approximately 5-fold (p < 0.05) greater than that in nonpregnant mice. The maximum plasma concentration achieved in nonpregnant mice was 2.4-fold (p < 0.05) greater than that in pregnant mice. The terminal plasma half-life of NFV after oral administration was not significantly different between pregnant and nonpregnant mice (p > 0.05). Bioavailability of NFV was significantly lower (by 32%, p < 0.05) in pregnant versus nonpregnant mice.

After i.v. administration, AUC0–∞ in pregnant mice (n = 3) was 36% lower than that in nonpregnant mice (n = 3), but the fetus is not a major site of clearance of the drug. In the case of the PIs, this is likely to be true as Smit et al. (1999) have shown that the PIs are excluded from the fetal compartment by placental P-gp. Because the true maternal weight at gestation age 18 to 19 days (without the fetuses) cannot be readily obtained, we used the weight on gestation day 0 to express the values of certain pharmacokinetic parameters.
this difference was not significant ($p > 0.05$) (Table 2; Fig. 1B). The remaining pharmacokinetic parameters in pregnant mice were not significantly different from those observed in nonpregnant mice ($p > 0.05$).

The half-life of NFV depletion in hepatic S-9 fractions (Fig. 2A) from pregnant mice ($8.1 \pm 1.0 \text{ min, } n = 8$) was 2.2-fold faster ($p < 0.05$) than that in S-9 fractions from nonpregnant mice ($17.2 \pm 7.1 \text{ min, } n = 7$). To determine the enzymes responsible for this enhanced depletion of NFV in hepatic S-9 fractions from pregnant mice, we determined the effect of CYP3A and CYP2C inhibitors on this depletion. NFV depletion in hepatic S-9 fractions ($n = 3$ and 4 for pregnant and nonpregnant mice, respectively) was almost completely inhibited by $1 \mu M$ KTZ (a CYP3A inhibitor) (Fig. 2B) but not by $10$ or $50 \mu M$ SPA (a CYP2C inhibitor, $n = 3$). Higher concentrations of KTZ (up to $50 \mu M$) did not produce any further inhibition of NFV depletion. These data suggest that NFV depletion in S-9 fractions is mediated by mouse CYP3A and that CYP3A activity is enhanced in pregnancy.

To confirm that mouse hepatic and intestinal CYP3A ac-

### Table 2
Pharmacokinetic parameters (means ± S.D.) of NFV after i.v. administration of NFV (0.625 mg) to pregnant (gestational age: 18–19 days) and nonpregnant FVB mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pregnant Mice (n = 3)</th>
<th>Nonpregnant Mice (n = 3)</th>
<th>$p$ Value $^a$</th>
</tr>
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<tbody>
<tr>
<td>AUC$_{0-\infty}$ (µg/ml/min)</td>
<td>411.2 ± 88.5</td>
<td>645.8 ± 166.3</td>
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<tr>
<td>$\text{CL}(\text{ml/min})$ $^b$</td>
<td>1.6 ± 0.3</td>
<td>1.0 ± 0.3</td>
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<td>$\text{CL}(\text{ml/min/g})$</td>
<td>0.1 ± 0.01</td>
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<tr>
<td>$\text{CL}_{\text{unbound}} (\text{ml/min})$ $^c$</td>
<td>60.7 ± 12.5</td>
<td>40.4 ± 10.0</td>
<td>0.092</td>
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<tr>
<td>$\text{CL}_{\text{unbound}} (\text{ml/min/g})$ $^d$</td>
<td>2.5 ± 0.5</td>
<td>1.7 ± 0.5</td>
<td>0.092</td>
</tr>
<tr>
<td>$V_{ss}$ (ml) $^e$</td>
<td>8.6 ± 2.9</td>
<td>4.2 ± 1.9</td>
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<tr>
<td>$V_{ss}$ (ml/g) $^f$</td>
<td>94.4 ± 37.9</td>
<td>71.2 ± 32.2</td>
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<tr>
<td>$t_{1/2}$ (min) $^e$</td>
<td>2.6 ± 0.7</td>
<td>2.5 ± 0.3</td>
<td>0.801</td>
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</table>

$^a$ Parameters were compared using the two-sample $t$ test (equal variance, statistical significance $p < 0.05$).

$^b$ Clearance.

$^c$ Unbound clearance.

$^d$ Volume of distribution at steady state.

$^e$ Terminal plasma half-life.

$^f$ Percent unbound in plasma.
tivity is indeed enhanced during pregnancy, we measured the hydroxylation of testosterone, a classic mouse CYP3A substrate, to 6β-hydroxytestosterone in hepatic and intestinal S-9 fractions. The accuracy of the assay was 11, 12, and 4%, and the precision was 9, 6, and 4% at the low (0.05 μg), mid (0.2 μg), and high ends (0.8 μg) of the calibration range, respectively. 6β-Hydroxytestosterone eluted at approximately 18 min, the internal standard 11α-hydroxyprogesterone eluted at approximately 22 min, and the parent drug testosterone eluted at approximately 30 min. Hepatic CYP3A activity in pregnant mice was significantly greater (138%, 2.4-fold) than that in nonpregnant mice (n = 4, p < 0.05) (Table 3). We were unable to measure CYP3A activity in pregnant and nonpregnant intestinal sample S-9 fractions, containing up to 4 mg of protein and incubated for 45 min, possibly because of the low CYP3A activity observed there. Therefore, further experiments to measure intestinal CYP3A activity using testosterone or NFV as substrate were discontinued.

On Western blotting for mouse CYP3A in hepatic and intestinal tissue, we observed that multiple isoforms of CYP3A were recognized by the anti-rat CYP3A2 antibody. All bands migrated closely and were within the expected size of 50 to 60 kDa. In hepatic S-9 fractions from nonpregnant mice, we observed three bands (2, 3, and 4 in Fig. 3A) within 50- to 60-kDa molecular mass, whereas we observed four distinct bands, (1, 2, 3, and 4 in Fig. 3A) within 50 to 60 kDa molecular mass in hepatic S-9 fractions from pregnant mice. Because purified standards or specific antibodies to discriminate between mouse CYP3A isoforms are not available to us, we were unable to identify the different isoforms. Interestingly, we observed that band 1 was present in all hepatic S-9 fractions from pregnant mice but was present in trace amounts in only a few hepatic S-9 fractions from nonpregnant mice. In intestinal samples (Fig. 3B), we observed three distinct bands (1, 2, and 3) in both pregnant and nonpregnant mice. Because the bands migrated very closely and the identity of each band could not be determined due to the lack of standards, we quantified all bands between 50 and 60 kDa molecular mass and represented the data as the sum of the total CYP3A isoforms. Using this approach, hepatic CYP3A expression by Western blot was significantly higher during pregnancy (49%, n = 4, p < 0.05) than that in nonpregnant mice. Expression of intestinal CYP3A was low compared with hepatic CYP3A and not significantly different (n = 6, p > 0.05) between tissue derived from pregnant and nonpregnant mice (Fig. 4). Hepatic and intestinal P-gp protein expression in pregnant mice was not significantly different (n = 6, p > 0.05) from that in nonpregnant mice.

**Discussion**

Our results indicate that the mouse replicates the pregnancy-related changes in the disposition of oral NFV observed in pregnant women (Acosta et al., 2001; Angel et al., 2001; Bryson et al., 2002; Kosel et al., 2003; van Heeswijk et al., 2004). The maximum plasma concentration and AUC were found to be significantly decreased by pregnancy. Consequently, the oral plasma clearance of the drug was increased during pregnancy, irrespective of whether it was normalized to body weight or not. This increase may be due to an increase in systemic clearance of the drug or a decrease in NFV bioavailability or both. The former could occur because of an increase in the percentage of NFV unbound in the plasma. Consistent with reported data in humans, we found that NFV was extensively bound to mouse plasma proteins, but contrary to our expectations, the percentage of NFV unbound in plasma from pregnant mice was lower than that in plasma from nonpregnant mice. Therefore, a decrease in plasma protein binding of NFV cannot explain the increase in oral plasma clearance of NFV observed during pregnancy.

The observed increase in unbound oral plasma clearance in

**TABLE 3**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pregnant Mice (n = 4)</th>
<th>Nonpregnant Mice (n = 4)</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6β-Hydroxytestosterone (mol/min/mg protein)</td>
<td>0.12 ± 0.04</td>
<td>0.05 ± 0.02</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

* Parameters were compared using the two-sample t test (equal variance, statistical significance p < 0.05).
pregnant mice suggests that intestinal and/or hepatic activity of CYP3A, P-gp, or both, which collectively determines the bioavailability and systemic clearance of NFV, may be increased during pregnancy. However, the terminal plasma half-life was not significantly different between the two groups. This suggests that the systemic clearance is unchanged during pregnancy (although a change of equal magnitude in NFV volume of distribution and systemic clearance cannot be discounted). Indeed, all i.v. NFV pharmacokinetic parameters, including AUC and clearance (total and unbound), were unaffected by pregnancy. Consistent with the oral data, the terminal plasma half-life of the drug was unaffected by pregnancy. Based on these i.v. data, we computed the bioavailability of NFV and found that it was significantly reduced by pregnancy. Thus, pregnancy increases the oral plasma clearance of NFV by reducing its bioavailability and not its systemic clearance.

Because the bioavailability of NFV and the other PIs in humans is determined by CYP3A and P-gp (Sandoval et al., 1998; Unadkat and Wang, 2000a; Zhang et al., 2001), reduced bioavailability of NFV in pregnant mice could be due to increased intestinal/hepatic first-pass metabolism of NFV (presumably via mouse CYP3A metabolism) or reduced absorption via either enhanced P-gp activity or some other mechanism not related to an efflux transporter. Interestingly, we observed that the half-life of NFV depletion in hepatic S-9 fractions from pregnant mice was 2.2-fold shorter than that in S-9 fractions from nonpregnant mice and was NADPH-dependent. This NADPH-dependent NFV depletion was almost completely inhibited (\(>87\%\)) by 1 \(\mu M\) KTZ, a CYP3A inhibitor (Emoto et al., 2000; Warrington et al., 2000), but not by 10 or 50 \(\mu M\) SPA, a CYP2C inhibitor (Hickman et al., 1998; Kobayashi et al., 2003). These data suggest that the increased total oral plasma clearance of NFV is a result of increased first-pass hepatic CYP3A metabolism of NFV. Although human adults express two major isoforms of CYP3A, CYP3A4 and CYP3A5, the mouse expresses eight different CYP3A isoforms (Itoh et al., 1994; Dai et al., 2001; Sakuma et al., 2002).Six of the mouse CYP3A isoforms exhibit 69 to 92% homology in the amino acid sequence. Some of these CYP3A isoforms are gender-specific (CYP3A41 and CYP3A44) or developmentally regulated (CYP3A16). Thus, based on the above data, we conclude that the activity of one or more of these mouse hepatic CYP3A isoforms is increased during pregnancy. Consistent with the NFV depletion results, the catalytic activity of hepatic CYP3A, as measured by 6\(\beta\)-hydroxylation of testosterone (Emoto et al., 2000; Mankowski et al., 2000; Nallani et al., 2003) in hepatic S-9 fractions, was 2.4-fold higher in pregnant mice than that in nonpregnant mice. Moreover, this activity was completely inhibited by 1 \(\mu M\) KTZ.

We found that enhanced CYP3A activity in hepatic tissue obtained from pregnant mice was consistent with expression of CYP3A proteins. In both hepatic and intestinal tissues, we observed multiple isoforms of CYP3A. When represented as a sum, we observed that hepatic CYP3A expression in pregnant mice was 49% higher than that in nonpregnant mice. Interestingly, band 1 (Fig. 3A) was present in all hepatic tissue from pregnant mice but was present in trace amounts in only a few hepatic tissues from nonpregnant mice. It is likely that this band may represent female-specific CYP3A41 or CYP3A44. Sakuma et al. (2002) demonstrated that CYP3A41 and CYP3A44 showed 91% homology in their amino acid sequence and that both isoforms (mRNA) are inducible by estradiol. Therefore, it is possible that when plasma concentrations of estradiol are increased during pregnancy, this may lead to an increase in the expression of one or both of these isoforms. Standards and specific antibodies to these CYP3A isoforms are needed to confirm the identity of these proteins in the literature of tissue-specific regulation of human CYP3A (Thummel et al., 2001), and, therefore, an increase in hepatic CYP3A expression could occur independently of that in other tissues.

To address the contribution of P-gp to the decreased AUC (exposure) of NFV, seen after oral administration of the drug, we examined hepatic and intestinal expression of P-gp. No difference in expression of P-gp protein was observed between pregnant and nonpregnant mice in these tissues.

In summary, our data show that the mouse replicates the pregnancy-related changes in the disposition of PIs observed in pregnant women after oral administration. The effect of pregnancy on the disposition of NFV is predominately due to changes in the bioavailability of NFV rather than to changes in plasma protein binding or systemic clearance of the drug. Our observation that NFV clearance is increased after only oral administration but not after i.v. administration is not surprising for medium-to-high extraction ratio drugs such as NFV. For such drugs, based on fundamental pharmacokinetic principles, an increase in the hepatic intrinsic clearance (in this case, increased CYP3A activity) does not result in a proportional increase in total systemic clearance of the compound because the clearance depends on hepatic blood flow and hepatic intrinsic clearance (Gibaldi and Perrier, 1982). If the same compound were administered orally, then an increase in hepatic intrinsic clearance would result in a proportional increase in oral clearance of the compound because the oral clearance (but not the i.v. clearance) is dependent only on the hepatic intrinsic clearance and not on hepatic blood flow. Therefore, an increase in hepatic metabolic activity will translate into increased first-pass metabolism after oral administration but will not appreciably change the systemic clearance of the drug. Numerous studies in the literature have demonstrated this pharmacokinetic principle, such as for metoprolol and propranolol (Herman et al., 1983; Hogstedt et al., 1983, 1985).

Our in vitro data indicate that the increased hepatic first-pass effect of NFV during pregnancy was due to the increased mouse hepatic CYP3A activity and expression and not due to a change in intestinal CYP3A expression or changes in hepatic or intestinal P-gp expression. The magnitude of change observed in mouse hepatic CYP3A activity is remarkably consistent with the change observed in oral plasma clearance of NFV. The mechanisms by which mouse hepatic CYP3A activity is increased during pregnancy cannot be elucidated without first identifying the specific isoform(s) affected. To do so, specific substrates/antibodies that can discriminate among the various mouse hepatic CYP3A isoforms will be necessary.

Although no data are available in the literature to suggest
that P-gp activity is enhanced during pregnancy, studies have suggested that CYP3A activity is increased during pregnancy. For example, Matsumura et al. (2001) have reported that pregnancy elevates mouse hepatic CYP3A11 (mouse isoform of CYP3A4) transcripts by approximately 17- to 20-fold. In pregnant women (gestational age 37–42 weeks, isoform of CYP3A4) transcripts by approximately 17- to 20-fold of mouse cytochrome P450 3A11 (mouse CYP3A11), the clearance of clorazepate (after i.m. administration), a CYP3A substrate, is 109% higher than that observed in non-pregnant (n = 7) women (Rey et al., 1979; Sachs et al., 2001). Tracy et al. (2005) have also found that CYP3A activity, as determined by the urinary ratio of dextromethorphan to its metabolite 3-hydroxydextromethorphan (mediated by CYP3A4/5), is significantly increased (35–38%) during all stages of pregnancy (14–40 weeks).

From a teleological point of view, it makes sense that, during pregnancy, nature would want to up-regulate CYP3A activity in maternal liver and/or intestine as a first line of defense against potentially toxic xenobiotics. If such changes do occur, they have significant implications for a large number of drugs administered to pregnant women as CYP3A and P-gp are involved in the disposition of a significant number of drugs on the market. Significant up-regulation of CYP3A during pregnancy would translate into changes in dosing regimens of drugs administered to pregnant women that are cleared predominately by these proteins.

From a therapeutic point of view, the PIs are essential for effective treatment of the HIV-infected pregnant woman. The reduced exposure (AUC) to PIs during pregnancy is of considerable clinical concern because of possible emergence of resistant viruses. A low plasma concentration of the PIs during pregnancy may result in a breakthrough resistant virus, which could be transmitted to the infant, thus compromising effective therapy of both the mother and her infant. To prevent inadequate exposure of the pregnant woman to the PIs, either the dosing regimens of the PIs will need to be pharmacologically enhanced by coadministration with ritonavir, a potent CYP3A inhibitor.

References


Address correspondence to: Dr. Jashvant (Jash) Unadkat, Department of

Adjunct Professor, University of Washington, Box 357610, Seattle, WA 98195.

E-mail: jaslab@uw.edu

CYP3A and P-gp in Pregnancy

1209

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