CHARACTERIZATION OF THE SELECTIVITY AND MECHANISM OF HUMAN CYTOCHROME P450 INHIBITION BY THE HUMAN IMMUNODEFICIENCY VIRUS-PROTEASE INHIBITOR NELFINAVIR MESYLATE

JAMES H. LILLIBRIDGE, BAI HONG LIANG, BRADLEY M. KERR, STEPHANIE WEBBER, BARRY QUART, BHASKER V. SHETTY, AND CAROLINE A. LEE

Agouron Pharmaceuticals Inc.

(Received December 3, 1997; accepted March 6, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

In vitro studies with human liver microsomes and P450 probe substrates were performed to characterize selectivity and mechanism of cytochrome P450 inhibition by nelfinavir mesylate. At therapeutic concentrations (steady-state plasma concentrations × 4 μM), nelfinavir was found to be a competitive inhibitor of only testosterone 6β-hydroxylase (CYP3A4) with a Kᵢ concentration of 4.8 μM. At supratherapeutic concentrations, nelfinavir competitively inhibited dextromethorphan O-demethylation (CYP2D6), S-mephenytoin 4-hydroxylation (CYP2C19), and phenacetin O-de-ethylase (CYP1A2) with Kᵢ concentrations of 68, 126, and 190 μM, respectively. Nelfinavir did not appreciably inhibit tolbutamide 4-hydroxylation (CYP2C9), paclitaxel 6α-hydroxylation (CYP2C8), or chlorzoxazone 6β-hydroxylation (CYP2E1) activities. The inhibitory potency of nelfinavir toward CYP3A4 suggested the possibility of in vivo inhibition of this isoform, whereas in vivo inhibition of other P450s was considered unlikely. In a one-sequence crossover study in 12 healthy volunteers, nelfinavir inhibited the elimination of the CYP3A substrate terfenadine and the carboxylic metabolite of terfenadine. The 24-hr urinary recoveries of 6β-hydroxycortisol were reduced by an average of 27% during nelfinavir treatment, consistent with CYP3A inhibition by nelfinavir. Inhibition of CYP3A4 by nelfinavir in vitro was NADPH-dependent requiring the catalytic formation of a metabolite or a metabolic intermediate. The catechol metabolite of nelfinavir (M3) was considered unlikely to be responsible for inhibition as the addition of catechol O-methyl transferase, S-adenosyl methionine, and ascorbic acid to the preincubation mixture did not protect against the loss of testosterone 6β-hydroxylase activity. Also, the addition of M3 to human liver microsomes did not inhibit CYP3A4. Although incubations with nelfinavir showed a time- and concentration-dependent loss of CYP3A4 activity, the partial or complete recovery of enzyme activity upon dialysis indicated that inhibition was reversible. Microsomal incubations with nelfinavir and NADPH did not result in a loss of spectral P450 content compared with the NADPH control. Glutathione, N-acetylcysteine, and catalase did not attenuate CYP3A4 inhibition by nelfinavir. Collectively, these results suggest that the probable mechanism for CYP3A4 inhibition by nelfinavir is a transient metabolic intermediate or stable metabolite that coordinates tightly but reversibly to the heme moiety of the P450.

Nelfinavir mesylate is a potent, orally active HIV protease inhibitor (PI) approved for the treatment of HIV infection. Optimal drug therapy for suppression of HIV viral replication is currently considered to be chronic drug treatment involving the combination of two reverse transcriptase inhibitors and a potent HIV-PI (Fauci et al., 1993). Reverse drug reactions and potentially life-threatening drug-drug interactions. Among HIV-PIs, ritonavir is recognized clinically as a broad spectrum P450 inhibitor and a very potent CYP3A4 inhibitor (Kumar et al., 1996), whereas saquinavir (Fitzsimmons and Collins, 1997) and indinavir (Chiba et al., 1996) are generally considered selective and moderately potent inhibitors of CYP3A4. Human liver microsomal studies have demonstrated that P450s are the primary enzymes responsible for the metabolism of nelfinavir (Wu et al., 1996). The objectives of this study were: 1) to investigate the potential for inhibition of human cytochrome P450 by nelfinavir mesylate followed by the determination of the inhibition constants (Kᵢ) in human liver microsomes; 2) to examine the effect of nelfinavir mesylate on the clinical pharmacokinetics of a substrate for the P450 isoform most involved in the metabolism of nelfinavir.

Send reprint requests to: Caroline A. Lee, Ph.D., Agouron Pharmaceuticals Inc., 4245 Sorrento Valley Blvd., San Diego, CA 92121.

1 Abbreviations used are: PI, protease inhibitor; P450, human cytochrome P450; CYP3A4, human cytochrome P450 3A4; HIV, human immunodeficiency virus; COMT, catechol O-methyl transferase; SAM, S-adenosyl methionine; GSH, glutathione; ACN, acetonitrile; ABT, 1-aminobenzotriazole.

2 A coefficient of correlation of r2 = 0.95 was obtained with the logarithm of drug concentrations in plasma. This correlation was calculated by performing a linear regression analysis of the data. The slope of the regression line was then used to calculate the average of 27% during nelfinavir treatment.
potently inhibited by nelfinavir; and 3) to gain insight into the mechanism of inhibition for the P450 most potently inhibited by nelfinavir mesylate.

Materials and Methods

**Chemicals.** Testosterone, troleandomycin, diethylthiobarbituric acid, retinoic acid, 6β-hydroxytestosterone, 11α-hydroxyprogesterone, chloropropamide, pentoxifylline, glutathione, N-acetylcysteine, acetylmethionine, phenacetin, quinidine, sulfaphenazole, 7,8-benzoflavone, catalase, catechol O-methyltransferase, S-adenosyl methionine, NADPH, ascorbic acid, EDTA, and diazolam were purchased from the Sigma Chemical Company (St. Louis, MO). 1-Aminobenzotriazole and 4-hydroxy-3-(α-aminobenzyl)-1-methyl-6-phenypryridin-2(1H)-one (which is used as an internal standard and is referred to in this paper as ALD25033-3) were purchased from Aldrich (Milwaukee, WI). Paclitaxel, 4-hydroxy-S-mephenytoin, tolbutamide, 4-hydroxybutyramid, dextromorphan D-tartrate, dextromethorphan hydrobromide, chloroxazone, 6β-hydroxychloroxazone, and ketoclonazole were purchased from Research Biochemicals International (Natick, MA). S-Mephenytoin was obtained from Cedra Corp. (Austin, TX). Magnesium chloride was obtained from GibCO BRL (Gaithersburg, MD). Seldane™ was purchased from Marion Merrell Dow (Kansas City, MO). Human liver tissue and pooled human liver microsomes were purchased from the Pennsylvania Regional Skin Bank (Exton, PA). Nelfinavir mesylate, 3-methoxy-4-hydroxy (N1), 3,4-dihydroxy nelfinavir (N3), and nelfinavir hydroxy-r-butylamidine (M8) were synthesized and indinavir, ritonavir, and saquinavir were isolated at Agouron Pharmaceuticals Inc. (La Jolla, CA). All reagents used in the extraction and analysis were HPLC grade (Fisher Scientific).

**Microsomal Incubations.** The concentrations of nelfinavir selected for the *in vitro* studies were based on steady-state total (free plus bound) C_mic plasma concentrations of nelfinavir that averaged approximately 5.3 to 7.0 μM after a multiple oral dosing regimen of 750 mg t.i.d. (Agouron Pharmaceuticals Inc., 1997). For incubation studies designed to determine the inhibition constant (K_i) of nelfinavir inhibition toward various P450s, the preincubation mixture contained 1.0 mg/ml microsomal protein (except for chloroxazone 6β-hydroxylation, a CYP2E1 probe substrate, where 1 nmol/ml P450 was used), and various P450 isoform-specific probe substrates (for specific concentrations, see P450 probe substrate assays) in 100 mM phosphate buffer, pH 7.4, in a final volume ranging from 0.25 to 0.5 ml at 37°C in a shaking water bath. CYP3A4-related probe substrates (for specific concentrations, see P450 probe substrate assays) were 0.5 ml at 37°C in a shaking water bath. CYP3A4-related incubations were based on steady-state total (free plus bound) C_mic plasma concentrations of nelfinavir after a 30-min incubation; 5 ml of methylene chloride for chloroxazone and testosterone after a 15-min incubation; and 3 ml of ethyl acetate for S-mephenytoin after a 30-min incubation. Preliminary experiments were performed to optimize conditions so that metabolite formation was linear with respect to time and protein content (data not shown).

Quenched incubation samples for phenacetin, paclitaxel, and dextromethorphan were vortexed for 10 min on an SP Multitube Vortexer (Baxter, McGaw Park, IL) and centrifuged at 2,500g for 15 min on an IEC Centra-DR (Damon, Needham Heights, MA). The organic layer was removed and evaporated on a Dri-Block sample concentrator (Techne, Princeton, NJ) under nitrogen at 40°C. Quenched incubation samples for tolbutamide, S-mephenytoin, chloroxazone, and testosterone were spiked with internal standards of chloropropamide (400 ng), ALD25033-3 (200 ng), pentoxifylline (1 mM), 11β-hydroxyprogesterone (250 ng), respectively, and then vortexed and centrifuged as described above. Similarly, the organic layer was removed and evaporated under nitrogen at 40°C.

**HPLC Analysis.** Chromatography was performed using a Hewlett Packard 1050 system and monitored using either a Hewlett Packard multichannel UV or fluorescence detector. The standard curves were linear over their respective ranges, and interday and intraday coefficients of variation for the slopes of the standard curves were less than 10%. The probe substrate metabolites were analyzed as described in the literature (Kumar et al., 1994; Miners and Birkett, 1996; Nagata et al., 1986; Shimada et al., 1986; Thummel et al., 1993) with some modifications. Chromatographic separation of the metabolites of interest for phenacetin O-deethylation, paclitaxel 6α-hydroxylation, and tolbutamide 4-hydroxylation were achieved using a Phenomenex prismsphere column (C18, 5 μm; 4.6 × 25 mm, Phenomenex, Torrance, CA); dextromethorphan O-demethylation and S-mephenytoin 4-hydroxylation were quantified using a YMC-AG ODS column (5 μm, 4.6 × 150 mm, YMC Inc., Wilmington, NC); and chloroxazone 6β-hydroxylation and testosterone 6α-hydroxylation were achieved with a Microsorb-MV (C18, 5 μm, 4.6 × 150 mm, YMC Inc., Wilmington, NC). The mobile phase for phenacetin O-deethylation was 90/10% water/ACN (v/v) at a flow rate of 1.0 ml/min. Acetaminophen was monitored by UV absorption at 254 nm. Retention times for acetaminophen and phenacetin were 4.8 and 12.5 min, respectively. 6α-Hydroxypaclitaxel was separated using a two-step isocratic increase in organic of 80/20% ACN/10 mM ammonium phosphate buffer, pH 3.0 (v/v) for 5 min followed by a 25-min gradient to 60/40% ACN/buffer at a flow rate of 1.0 ml/min. 6α-Hydroxypaclitaxel was monitored by UV absorption at 229 nm. Retention times for 6α-hydroxypaclitaxel and paclitaxel were 24.6 and 28.3 min, respectively. An isocratic mobile phase of 65/35% 25 mM ammonium phosphate, pH 4.2/ACN (v/v) was used to separate 4-hydroxytolbutamide at a flow rate of 1.0 ml/min. 4-Hydroxytolbutamide was monitored by UV absorption at 230 nm. Retention times for 4-hydroxytolbutamide, tolbutamide, and chloropropamide were 3.8, 10.5, and 14.5 min, respectively. A two-step isocratic increase in organic of 30/70% ACN/water (v/v) for 15 min followed by a 2-min gradient to 60/40% ACN/water (v/v) at a flow rate of 1.0 ml/min was used to measure 4-hydroxy S-mephenytoin. This metabolite was monitored by UV absorption at 225 nm. The retention times for 4-hydroxy S-mephenytoin, and the internal standard ALD25033-3 were 4.0, 9.5, and 12.5 min, respectively. For dextromethorphan O-demethylation, compounds were eluted by an isocratic mobile phase of
75/25% 25 mM ammonium phosphate, pH 4.5/ACN (v/v) at a flow rate of 1.0 ml/min. Dextrorphan was monitored by fluorescence detection (excitation 230, emission 315 nm). Retention times for dextrorphan and dextromethorphan were 4.4 and 18 min, respectively. The mobile phase for chlorozoxazine 6β-hydroxylation was 80/20% 0.15% (v/v) glacial acetic acid, pH 4.7/ACN (v/v) at a constant flow rate of 1.2 ml/min. 6β-Hydroxychlorozoxazine was monitored by UV absorption at 282 nm. Retention times for 6β-hydroxychlorozoxine, pentoxifylline, and chlorozoxazine were 5.4, 6.6, and 17.2 min, respectively. 6β-Hydroxytestosterone was eluted by a gradient mobile phase consisting of methanol/ACN/water under the following time course of: 0 min, 58/0/42%; 0–15 min, 62/5/33%; 15–24 min, 62/25/13%; 24–30 min, 58/0/42% delivered at a constant flow rate of 1.0 ml/min. The 6β-hydroxytestosterone metabolite was monitored by UV absorption at 254 nm. The retention times for 6β-hydroxytestosterone, 11α-hydroxyprogesterone, and testosterone were 4.4, 9.4, and 13.5 min, respectively.

**Measurement of Cytochrome P450 Content.** Pooled human liver microsomes (1.0 nmol of P450/ml at a final volume of 1.5 ml in 100 mM phosphate buffer, pH 7.4) were incubated in triplicate at 37°C in a shaking water bath to examine the effects of nelfinavir on P450 content. P450 content was determined in samples containing human liver microsomes only, human liver microsomes plus NADPH (1.0 mM) or nelfinavir (10 μM), and human liver microsomes plus NADPH (1.0 mM) and nelfinavir (10 μM) or ABT (100 μM). At 0, 0.5, 5, 10, and 20 min, a 300-μl aliquot of each incubation mixture was transferred to a test tube containing 300 μl of ice-cold phosphate buffer and kept on ice until analysis (≤30 min). The zero time point was the baseline condition prior to the addition of components to microsomes. P450 content was determined by the method of Estabrook et al. (1972) with an extinction coefficient of 100 mM−1 cm−1 using a Shimadzu UV160U-single beam spectrophotometer.

**Dialysis Experiment.** Pooled human liver microsomes were incubated with NADPH in the presence or absence of nelfinavir (1.0, 3.0, 5.0, and 10.0 μM) for 20 min. Samples were immediately placed in 6,000–8,000 molecular weight cutoff Spectra/Per dialysis tubing (Spectrum Medical Industries Inc., Houston, TX) and dialyzed for 18 hr at 4°C against 1000 ml of 100 mM potassium phosphate buffer, pH 7.4, containing 5 mM EDTA. Dialysis buffer was changed once after 6 hr. Protein content was subsequently measured, and 20-min incubation studies were conducted to assess testosterone 6β-hydroxylase activity as described above.

**Binding Spectra.** The P450 substrate binding spectra were obtained using a Varian Cary 3E dual beam spectrophotometer equipped with a temperature controller, which maintained the samples at 37°C. Both reference and sample cuvettes contained 2 nmol/ml human P450 and 100 mM phosphate buffer, pH 7.4, with or without 1 mM NADPH. Nelfinavir (100 μM) was added to the test cuvette, and scans were recorded every 5 min for 35 min.

**Pharmacokinetic Studies.** Twelve healthy male volunteers, 18 to 36 years of age, body weight within 15% of ideal, gave informed consent to participate in a one sequence (1 × 2) crossover study. On the morning of day 1, a 60-mg dose of terfenadine (60-mg SeldaneTM tablet) was administered 10 min after completion of a standard breakfast in the absence of nelfinavir. Serial plasma samples were collected at predose (0 hr) and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36, 48, and 72 hr post-dose for assay of terfenadine and unchanged terfenadine. On days 6 through 12, 750 mg of nelfinavir (3 × 250-mg Viracept tablets) was administered every 8 hr (the standard clinical dose of Viracept is 750 mg t.i.d.). Each dose of nelfinavir was ingested within 10 min after eating a meal or light snack. On the morning of day 10 (5th day of nelfinavir treatment), a predose plasma sample was drawn from each subject for assay of nelfinavir trough concentration, and a 60-mg dose of terfenadine was administered concomitantly with the morning dose of nelfinavir at 10 min following the standard breakfast. Serial plasma samples were collected on day 10 from predose through 72 hr post-dose (same times as listed above) for assay of terfenadine and unchanged terfenadine. Total 24-hr urine collections were performed on day 5 (before nelfinavir treatment) and day 12 (7th day of nelfinavir) for measurement of 6β-hydroxycortisol. In two other healthy volunteer studies not described here in detail, 24-hr urine collections for measurement of 6β-hydroxycortisol were performed before and on the 5th day of treatment with 600 mg rifampin daily (in combination with 750 mg nelfinavir every 8 hr) or 400 mg ketoconazole daily (in combination with 500 mg nelfinavir every 8 hr).

**Bioanalytical Methods for Pharmacokinetic Studies.** Plasma concentrations of nelfinavir were measured by a validated HPLC method with ultraviolet detection (Wu et al., 1997). The calibration curve for nelfinavir (0.25 ml plasma volume) over the range of 0.05 to 10.0 μg/ml yielded a correlation coefficient (r) >0.999 with precision based on quality control samples within 2.9% and accuracy expressed as per cent of nominal within 96.4–100.2%. Plasma concentrations of terfenadine and terfenadine carboxylate and urinary concentrations of 6β-hydroxycortisol were measured by validated HPLC methods with fluorescence detection (Wilkinson et al., 1996; Wisconsin Analytical and Research Services, 1996). For terfenadine and terfenadine carboxylate (1 ml plasma volume), calibration curves over the range of 5 to 100 ng/ml yielded correlation coefficients (r) of ≥0.999 with precision within 7.6% and accuracy within 95.2–100.4%. For 6β-hydroxycortisol (1 ml urine volume), calibration curves over the range of 10 to 300 ng/ml yielded correlation coefficients (r) of ≥0.997 with precision within 7.6% and accuracy within 95.3–104.3%.

**Pharmacokinetic and Statistical Analysis.** The maximal plasma concentration (Cmax) and time of maximal concentration (tmax) for terfenadine were estimated by inspection of individual subject plasma concentration-time profiles. The elimination rate constant (Kd) for terfenadine carboxylate was estimated by least-squares regression of the terminal log-linear portion of the plasma concentration-time profile. Terminal half-life for the carboxylate was estimated as the ratio of the natural logarithm of 2 divided by Kd. The area under the plasma concentration-time curve for the carboxylate metabolite from time of terfenadine dosing to infinity (AUC∞) was estimated by the trapezoidal method to the time of last measurable concentration with extrapolation to infinity by addition of the quantity Clast/Kd, where Clast represents the last measurable concentration of the carboxylate.

Twenty-four hour urinary recoveries of 6β-hydroxycortisol were estimated as the product of urine volume and concentration of 6β-hydroxycortisol for a 24-hr pooled urine collection. When the concentration of 6β-hydroxycortisol was below the previous limit of quantitation (which occurred in 4 of 12 subjects treated with ketoconazole), the concentration was assumed to be equal to the lower limit of quantitation for the purpose of estimating 24-hr recovery (in which case the 24-hr recovery represents an upper limit, potentially resulting in an underestimate of ketoconazole inhibitory effect).

Paired t test analyses were used to statistically compare terfenadine carboxylate terminal half-life and AUC∞ in the absence vs. presence of nelfinavir and 24-hr urinary recoveries of 6β-hydroxycortisol in the absence vs. presence of drug treatment (nelfinavir, ketoconazole, rifampin). Kd values were determined with PCNONLIN software (SCI Software, Lexington, KY). Raw data were fitted to a Michaelis-Menten competitive inhibition model described by the equation:

\[ \text{Velocity} = \frac{V_{max} \times \text{Concentration} / K_{m}}{1 + (I / K_I) + \text{Concentration}} \]

**Results**

**Selectivity of Nelfinavir Mesylate on the Inhibition of P450 Isoforms.** The inhibition of specific P450 isoforms by nelfinavir mesylate was investigated using various P450 isorm-specific probe substrates. Among the various positive control inhibitors, extent of inhibition was at least 60% at the lowest probe substrate concentration. Kd values for the inhibition of various P450s were determined when the criterion of ≥10% decrease in probe substrate activity was observed with up to 100 μM nelfinavir in preliminary studies. This criterion was met for CYP3A4, CYP2C19, CYP2D6, and CYP1A2 (data not shown), and further studies were conducted to evaluate the Kd of nelfinavir for these specific P450 isoforms. The results are summarized in table 1. Nelfinavir did not significantly inhibit the CYP2E1-, CYP2C8-, or CYP2C9-mediated reactions, and consequently Kd values were not determined. The HIV-PIs ritonavir, indinavir, and saquinavir have been shown to inhibit CYP3A4 (Abbott Laboratories, 1996; Chiba et al., 1996; Fitzsimmons and Collins, 1997; Kumor et al., 1996). To compare the inhibitory potency of these HIV-PIs to that of nelfinavir, testosterone was selected as the common
CYP3A4 activity was nearly restored in the absence of preincubation with NADPH. By comparison, midazolam (10 μM), a known mechanism-based irreversible inactivator of CYP3A4 (Podoll et al., 1996) caused a 90% loss of activity after 20 min. Dialysis experiments were performed to evaluate whether CYP3A4 activity could be restored to that of control (minus nelfinavir) after a 20-min preincubation with nelfinavir and NADPH. Concentrations of 1, 3, 5, and 10 μM nelfinavir were chosen. After an 18-hr dialysis against one change of buffer, microsomes were incubated with testosterone for 20 min, and testosterone 6β-hydroxylase activity was measured (table 3). CYP3A4 activity was nearly restored to that of the control (zero nelfinavir concentration) at all concentrations of nelfinavir tested except at the highest concentration of 10 μM, where only partial activity was regained in comparison with undialyzed samples, suggesting the possibility of multiple inhibition mechanisms with differing rates or extents of reversibility.

**Effect of Modifiers on the Inhibition of CYP3A4.** Several modifiers were chosen to investigate the potential for a reactive metabolite to leave the active site and to inhibit CYP3A4. A supratherapeutic concentration of nelfinavir (10 μM) was selected to increase the metabolism of metabolite levels to assess the effects of the modifiers. The addition of highly reactive nucleophiles such as glutathione (1 and 5 mM) and N-acetylcycteine (1 mM) did not alter the time-dependent loss of CYP3A4 activity (fig. 2). Catalase (100 units) did not affect the extent of inhibition either (data not shown). Because catechols can give rise to reactive ortho-quinones, it was of interest to investigate whether the catechol metabolite of nelfinavir (M3) might be the inhibitory species. To examine potential inhibition by M3, nelfinavir and human microsomal incubations plus COMT (200 units/ml) and SAM (2.0 mM) or the free radical scavenger ascorbic acid (500 μM) were performed (fig. 3). These incubations did not alter the inhibition profile even though LC-MS analysis (ion signal m/z = 598) confirmed the methoxy-catechol metabolite of nelfinavir (M1), for which M3 is the precursor, had been formed (data not shown).

**TABLE 3**

<table>
<thead>
<tr>
<th>Nelfinavir</th>
<th>% of Control Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>Dialyzed (n = 3) Mean ± SD</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 2.3</td>
</tr>
<tr>
<td>1.0</td>
<td>103 ± 2.3</td>
</tr>
<tr>
<td>3.0</td>
<td>82 ± 1.6</td>
</tr>
<tr>
<td>5.0</td>
<td>83 ± 0.8</td>
</tr>
<tr>
<td>10.0</td>
<td>44 ± 15.5</td>
</tr>
</tbody>
</table>

Values for dialyzed samples were calculated from three separate experiments, and for undialyzed samples the values were calculated from two separate experiments.

**Effect of Nelfinavir on P450 Content in Human Liver Microsomes.** The results of spectral studies that assessed the effect of over, human liver microsomal incubation studies demonstrated that 0.1 and 1.0 μM M1 were not inhibitory toward CYP3A4 (data not shown), whereas M3 at 0.5 μM was not inhibitory against CYP3A4 and was only marginally inhibitory (10%) at a high concentration of 5 μM (table 4).

**Effect of Nelfinavir on P450 Content in Human Liver Microsomes.** The results of spectral studies that assessed the effect of...
nelfinavir and/or its metabolite(s) on P450 content over time are presented in fig. 4. The addition of nelfinavir without NADPH to microsomes did not result in a loss of P450 content. P450 content decreased 35% from control by the addition of 1 mM NADPH, as previously observed (Chiba et al., 1995). The combination of nelfinavir and NADPH did not further decrease the loss in P450 content relative to NADPH control after a 20-min incubation period. In contrast, the addition of 100 μM ABT, a known mechanism-based inactivator of CYP450 (Ortiz de Montellano and Matthews, 1981), resulted in a 70% loss of P450 content after 20 min. The lack of P450 destruction by nelfinavir and/or metabolite(s) indicates that binding to the heme moiety of P450 is reversible, which is consistent with the restoration of P450 activity by dialysis (as described above).

**Binding Spectra of Nelfinavir Mesylate with Human Liver Microsomes.** The addition of 100 μM nelfinavir resulted in type I binding spectra characterized by λmax and λmin of 379 and 419 nm, respectively (data not shown). With the addition of NADPH to the test cuvette, the binding spectra changed to type II binding, characterized by λmax and λmin of 426 and 410 nm (fig. 5). The trough of 410 nm observed when NADPH was added to the cuvette containing nelfinavir and microsomes is consistent with a type II spectral change that is characterized by a broad trough ranging between 390 and 410 nm (Jefcoate, 1978). The type II spectral change suggests the formation of a metabolic intermediate or stable metabolite that binds tightly but reversibly to the heme of CYP450 (Ortiz de Montellano, 1995; Vickery, 1992).

**Pharmacokinetics.** Plasma concentrations of unchanged terfenadine were below the lower limit of quantitation (<5.00 ng/ml) in all 12 subjects at all sampling time points after treatment with terfenadine in the absence of nelfinavir. When terfenadine was administered during nelfinavir treatment, plasma concentrations of terfenadine were transiently measurable in all 12 subjects (fig. 6) with Cmax ranging from 5.5 to 15.3 ng/ml, tmax ranging from 2 to 6 hr, and last occurrence of measurable plasma terfenadine at 12 hr. The plasma AUC0-6 hr and half-life for terfenadine carboxylate (1.15 ± 0.38 mg*hr/liter and 4.4 ± 1.2 hr in the absence of nelfinavir) were significantly greater (p < 0.001 for each parameter) when terfenadine was administered during treatment with nelfinavir (1.62 ± 0.33 mg*hr/liter and 27.0 ± 7.2 hr). Steady-state trough plasma concentrations of nelfinavir on day 10 just prior to administration of terfenadine averaged 2.62 ± 1.09

**FIG. 2. Effect of nucleophiles on the time-dependent decrease of testosterone 6β-hydroxylase activity by nelfinavir mesylate in human liver microsomes.**

The human liver microsomal incubations consisted of 10 μM nelfinavir, 1 mM NADPH, and 1 mg/ml microsomal protein. Each point is the average of three determinations, and the values are normalized to control at time zero. □, nelfinavir control; △, 1 mM GSH; ●, 5 mM GSH; ■, 1 mM NAC.

**FIG. 3. Effect of the modifiers COMT/SAM and ascorbate on the time-dependent decrease of CYP3A4 activity by nelfinavir mesylate.**

Microsomal incubation conditions were similar to that described in fig. 2. Each point is the average of three determinations, and the values are normalized to control at time zero. □, nelfinavir control; △, 200 units of COMT and 2 mM SAM; ○, 500 M

---

**TABLE 4**

<table>
<thead>
<tr>
<th>M3 Concentration (μM)</th>
<th>Testosterone 6β-hydroxylase activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td>0.5</td>
<td>0.59 ± 0.05 (7%)</td>
</tr>
<tr>
<td>5.0</td>
<td>0.56 ± 0.01 (11%)</td>
</tr>
</tbody>
</table>

Activity is expressed as mean ± SD. Values in parentheses represent the percentage of inhibition of CYP3A4 by M3 in comparison with control samples that do not contain M3.
mg/liter (4.6 ± 1.9 μM) with a range of 0.55–4.42 mg/liter (1.0–7.8 μM).

Twenty-four hour urinary recoveries of 6β-hydroxycortisol, an in vivo marker of CYP3A activity, were reduced by an average of 27% (p < 0.03) on the 7th day of nelfinavir treatment (77 ± 40 μg) as compared with baseline (117 ± 45 μg) for the 12 subjects participating in the terfenadine study. Collective results from subjects participating in interaction studies with terfenadine, ketoconazole, and rifampin indicated that treatment with 750 mg nelfinavir thrice daily for 5–7 days in the absence of other drugs reduced the 24-hr urinary recoveries of 6β-hydroxycortisol by an average of 40% vs. baseline (table 5). In comparison, 6β-hydroxycortisol recoveries were reduced by 74% on the 5th day of treatment with the potent CYP3A inhibitor ketoconazole (in combination with nelfinavir) and were increased by 117% on the 5th day of treatment with the potent CYP3A inducer rifampin (in combination with nelfinavir) (table 5).

Discussion

The human liver microsomal studies described herein showed that only CYP3A4 was inhibited at clinically relevant concentrations of nelfinavir. The Ki values of nelfinavir for CYP2D6, CYP2C19, and CYP1A2 were 13- to 36-fold greater than the typical Cmax (5.3 μM) of nelfinavir achieved in humans at therapeutic doses (Agouron Pharmaceuticals, 1997). These findings suggest that nelfinavir drug interactions involving the CYP3A family are possible, whereas clinical inhibition of other isoforms is not expected. These in vitro inhibitory data were used to prioritize clinical drug interaction studies that focused on CYP3A4.

Terfenadine is a CYP3A4 substrate that undergoes extensive first pass metabolism following oral administration (Honig et al., 1992; Jurima-Romet et al., 1994). In the absence of a drug interaction, the carboxylate metabolite is the principal circulating entity in plasma, whereas unchanged terfenadine, a drug known to cause torsades de pointes, is normally not present at measurable concentrations (Honig et al., 1992, 1993). Terfenadine cardiotoxicity is potentiated via drug interactions with CYP3A4 inhibitors such as erythromycin or ketoconazole, which increase plasma concentrations of unchanged terfenadine (Eller and Okerholm, 1991; Honig et al., 1992, 1993). In light of in vitro results showing that therapeutic concentrations of nelfinavir inhibited CYP3A4 activity, it was considered important to investigate the nelfinavir interaction with terfenadine. Treatment with a standard multiple dose regimen of nelfinavir was found to impair the metabolism of terfenadine as evidenced by the appearance of measurable unchanged terfenadine in plasma. Trough plasma concentrations of nelfinavir associated with impaired terfenadine metabolism in vivo (4.6 ± 1.9 μM) were consistent with the nelfinavir in vitro Ki concentration toward CYP3A4 (4.8 μM). The marked prolongation of terminal half-life for terfenadine carboxylate was also consistent with inhibition of CYP3A4 by nelfinavir, as other selective CYP3A4 inhibitors are reported to inhibit elimination of the carboxylate (Hoechst Marion Rousell, 1996).

The decreased 6β-hydroxycortisol urinary recoveries during nelfinavir treatment confirmed that nelfinavir is an in vivo inhibitor of CYP3A4. The urinary ratio of 6β-hydroxycortisol to unchanged cor-

![Image](https://example.com/image1.png)

**FIG. 5.** Type II difference binding spectra upon incubating nelfinavir mesylate (100 μM) with human liver microsomes (2 nmol/ml) after a 20-min incubation with 1 mM NADPH.

![Image](https://example.com/image2.png)

**FIG. 6.** Median plasma concentrations of terfenadine and terfenadine carboxylate following administration of terfenadine alone and in combination with nelfinavir.

Terfenadine concentrations after administration of terfenadine alone (●) and in combination with nelfinavir (○) and terfenadine carboxylate concentrations after administration of terfenadine alone (■) and in combination with nelfinavir (□).

![Image](https://example.com/image3.png)

**TABLE 5**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of Patients</th>
<th>Treatment</th>
<th>Percent Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>During</td>
<td></td>
</tr>
<tr>
<td>NFV</td>
<td>24</td>
<td>148 ± 52</td>
<td>77 ± 32</td>
</tr>
<tr>
<td>Rifampin/NFV</td>
<td>12</td>
<td>150 ± 30</td>
<td>315 ± 88</td>
</tr>
<tr>
<td>Ketoconazole NFV</td>
<td>12</td>
<td>134 ± 32</td>
<td>34 ± 18</td>
</tr>
</tbody>
</table>

All treatment values were significantly different before vs. during drug treatment, paired t-test p < 0.0002.
tisol [suggested to be a more robust measure of CYP3A4 activity (Joellenbeck et al., 1992)] also seemed to be decreased by nelfinavir (data not shown), but this ratio was not consistently measurable owing to low cortisol concentrations in some subjects. Clinical data from this study do not rule out the possibility that, like other CYP3A4 inhibitors such as macrolide antibiotics (Amacher et al., 1991; Schuetz et al., 1993), nelfinavir may be an inducer as well as an inhibitor of CYP3A4. However, the terfenadine and 6β-hydroxycortisol results from this study suggest that for most CYP3A4-metabolized drugs, the net effect of nelfinavir is likely to be inhibition rather than induction of metabolic clearance. This expectation is confirmed by abstract reports of nelfinavir inhibiting the \textit{in vivo} clearance of rifabutin (Kravcik et al., 1997) and saquinavir (Kerr et al., 1997), two other recently confirmed substrates of CYP3A4 (Fitzsimmons and Collins, 1997; Iatsimirskaia et al., 1997).

Based on recent \textit{in vitro} Ki concentrations and effects on 6β-hydroxycortisol recoveries, nelfinavir does not seem to be as potent a CYP3A4 inhibitor as ketoconazole. Among the HIV-protease inhibitors tested, nelfinavir was the least potent \textit{in vitro} inhibitor of CYP3A4. These comparative \textit{in vitro} inhibitory potencies of the protease inhibitors do not necessarily translate directly into relative extents of inhibition \textit{in vivo} because clinical inhibition depends upon additional factors that are not easily accounted for \textit{in vitro}, such as concentration time course, plasma protein binding, and partitioning from plasma to liver. Thus, nelfinavir and indinavir seem to have similar inhibitory effects on the \textit{in vivo} clearances of the CYP3A4 substrates rifabutin (Iatsimirskaia et al., 1997; Kerr et al., 1997) and saquinavir (Kravcik et al., 1997; McCrea et al., 1997) despite indinavir being a more potent CYP3A4 inhibitor \textit{in vitro}. This apparent discrepancy may be the result of indinavir having a much greater fluctuation of plasma concentration during a dosing interval, which may result in a transient achievement of inhibitory concentrations. Alternatively, the possible existence of unidentified metabolites that may potently inhibit CYP3A4 could confound \textit{in vitro/in vivo} correlations for inhibitory potencies of parent drugs. Despite the limitations in extrapolating \textit{in vitro} results to the \textit{in vivo} setting, it is notable that the most potent inhibitor \textit{in vitro}, ritonavir, has a greater inhibitory effect than either nelfinavir or indinavir on the \textit{in vivo} clearances of rifabutin and saquinavir (Cato et al., 1996; Kempf et al., 1997; Merry et al., 1997). Although nelfinavir may not be an extremely potent inhibitor of CYP3A4, the clinical interaction with terfenadine nevertheless highlights the need for caution when nelfinavir is coadministered with potentially toxic drugs that are predominantly metabolized by CYP3A4.

\textit{In vitro} studies demonstrated that time-dependent inhibition of CYP3A4 was not observed for nelfinavir alone; however, the addition of NADPH to the preincubation mixture containing nelfinavir and human liver microsomes resulted in a time-dependent loss of CYP3A4 activity, perhaps owing to formation of an inhibitory metabolite. Even though a time-dependent loss of CYP3A4 was observed, the mechanism of inhibition was reversible as evidenced by complete recovery of catalytic activity with 1.0 μM nelfinavir, almost complete recovery at the higher concentrations of 3.0 and 5.0 μM nelfinavir, and partial recovery at a supratherapeutic concentration of 10 μM. According to Silverman (1988), as partial or all enzyme activity was regained after dialysis at 4°C, the enzyme-nelfinavir related inhibitor complex is considered tight and noncovalent in nature. In addition, the lack of an effect of the inhibitory metabolite intermediate on P450 content and the observed type II binding spectrum for nelfinavir in the presence of NADPH instead of a type III spectrum further supports a reversible inhibition mechanism. Similarly, data reported by Kempf et al. (1997) have shown that ritonavir is also a reversible inhibitor as it gives rise to a type II spectral perturbation, which is believed to be the result of a reversible interaction with the oxidized heme iron of CYP3A4.

Nelfinavir can undergo various enzyme-mediated oxidation processes to generate a multitude of metabolites. The steady-state plasma concentrations for hydroxy-β-butylamide metabolite of nelfinavir (M8) are typically one-third of nelfinavir concentrations in humans receiving the standard 750-mg t.i.d. dose (Zhang et al., 1997). M8, which together with nelfinavir accounts for ≈95% of circulating drug-derived material in human plasma, was no more potent an inhibitor than nelfinavir itself. Another metabolite, the catechol metabolite of nelfinavir (M3), was considered a possible inhibitory moiety, as this metabolite could theoretically undergo oxidation to an ortho-quinone. Ortho-Quinones are extremely reactive, especially towards sulfhydryl groups (Joecyn, 1972; McLean et al., 1996). However, the addition of COMT/SAM and ascorbic acid to the incubation mixture containing nelfinavir did not protect against the loss of testosterone 6β-hydroxylase activity. These results suggest that the inhibitory metabolite is probably not the catechol or a catechol derivative such as a reactive ortho-quinone intermediate. It is interesting to note that a modification of nelfinavir with a hydroxyl group to form the catechol moiety (M3) or the methoxy catechol (M1) greatly diminished CYP3A4 inhibition. The addition of catalase, glutathione, and N-acetylcysteine to the incubation mixture did not attenuate the inhibition of testosterone 6β-hydroxylase activity, which suggests that inhibition of CYP3A4 may occur prior to the release of some unidentified metabolite from the active site and that an analogous inhibitory metabolite or metabolic intermediate would be derived from both nelfinavir and M8. Alternatively, the lack of an effect of these modifiers may indicate that the inhibitory metabolite is released from the active site but is stable and not reactive.

In summary, nelfinavir is a moderately potent inhibitor of only CYP3A4 at clinically relevant concentrations. Based on the \textit{Ki} values, drug interactions involving potentially toxic substrates of CYP3A4 are of clinical concern, whereas inhibitory interactions involving other P450s are not anticipated. Nelfinavir’s inhibitory potency in comparison with the other marketed HIV-PIs is similar to that of saquinavir and is less potent than either indinavir or ritonavir when testosterone 6β-hydroxylase activity was used to assess CYP3A4 activity. As anticipated, nelfinavir inhibited the elimination of terfenadine and the carbamazepine metabolite in 12 healthy human volunteers, consistent with inhibition of CYP3A4. Moreover, the decrease in 6β-hydroxy-cortisol urinary recoveries during nelfinavir treatment further supports nelfinavir as an \textit{in vivo} CYP3A4 inhibitor. The mechanism of CYP3A4 inhibition by nelfinavir is mediated by a metabolite that is NADPH dependent. Our studies indicate that the inhibitory metabolite binds tightly based on the low \textit{Ki} value but is reversible owing to complete or partial recovery of CYP3A4 activity after dialysis, type II binding spectrum, and the lack of an effect of nelfinavir on the P450 content when compared with NADPH-treated control microsomes.

Acknowledgment. We thank Dr. Kanyin Zhang for performing the LC-MS analysis of the catechol-metabolite of nelfinavir.

References


Aguonum Pharmaceuticals Inc. (March, 1997) Prescribing information for VIRACEPT (nelfinavir mesylate) tablets and oral powder. La Jolla, CA.


