In Vitro-In Vivo Model for Evaluating the Antiviral Activity of Amprenavir in Combination with Ritonavir Administered at 600 and 100 Milligrams, Respectively, Every 12 Hours

Sandra L. Preston, Peter J. Piliéro, John A. Bilello, Daniel S. Stein, William T. Symonds, and George L. Drusano

Division of Clinical Pharmacology, Clinical Research Initiative, Albany Medical College, Albany, New York, and Clinical Pharmacology and Discovery Medicine, GlaxoSmithKline, Research Triangle Park, North Carolina

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The study objective was to evaluate the pharmacodynamics of amprenavir in an in vitro system, develop an exposure target for maximal viral suppression, and determine the likelihood of target attainment based on the pharmacokinetics of amprenavir and ritonavir in human immunodeficiency virus (HIV)-infected patients. Population pharmacokinetic data were obtained from 13 HIV-infected patients receiving amprenavir and ritonavir in doses of 600 and 100 mg, respectively, every 12 h. A 2,500-subject Monte Carlo simulation was performed. Target attainment was also estimated for a target derived from clinical data. Maximal viral suppression (in vitro) was achieved when amprenavir free-drug concentrations remained greater than four times the 50% effective concentration (EC50) for 80% of the dosing interval. At an amprenavir EC50 of 0.03 μM, the likelihood of target attainment is 97.4%. For reduced-susceptibility isolates for which the EC50s are 0.05 and 0.08 μM, target attainment is 91.0 and 75.8%, respectively. For the clinical target of a trough concentration/EC50 ratio of 5, the target attainment rates were similar. Treatment with amprenavir and ritonavir at doses of 600 and 100 mg, respectively, twice a day provides excellent suppression of wild-type isolates and reduced-susceptibility isolates up to an EC50 of 0.05 μM. Even at 0.12 μM, target attainment likelihood exceeds 50%, making this an option for patients with extensive exposure to protease inhibitors when this treatment is used with additional active antiretroviral agents.

The goal of antiretroviral therapy in the treatment of human immunodeficiency virus (HIV) infection is to reduce the viral burden in the blood to below the limit of assay quantitation, e.g., less than 50 copies/ml. Despite the availability of potent highly active antiretroviral therapy regimens, not all patients achieve this goal. There are several reasons for treatment failure, including suboptimal drug exposure. It is estimated that approximately one-third of patients may have suboptimal serum protease inhibitor concentrations which may result in virologic failure in nearly 50% of these patients (1). While adherence to the drug regimen is clearly a factor in successful therapy (11), some patients who fully adhere to the regimen may still fail therapy because their serum drug concentrations are suboptimal for the drug-resistant viruses they harbor. This situation can be problematic, particularly in patients who have already experienced antiretroviral treatment.

Another challenge is defining an optimal protease inhibitor concentration-time profile that will maximize viral suppression while allowing for a reasonable dosing schedule. An optimal level of drug exposure should be linked to a measure of viral susceptibility (e.g., peak concentration/the 95% effective concentration [EC95], area under the concentration-time curve [AUC]/EC95, time for which the concentrations are above the EC95 [time > EC95]).

One approach to treating patients who have experienced antiretroviral treatment is to provide a protease inhibitor drug exposure that is more likely to be effective against more resistant viral strains. Amprenavir concentrations in serum can be pharmacokinetically enhanced when the drug is dosed with the protease inhibitor ritonavir due to the ability of ritonavir to inhibit the cytochrome P-450-mediated metabolism of amprenavir (13, 14).

A measure of viral susceptibility relative to the EC50 for a wild-type virus is readily obtained by using phenotypic-resistance assays. These data are useful in combination with the known pharmacokinetics of amprenavir to maximize the likelihood of virological suppression. The purposes of the present study were to develop a target for antiviral concentration attainment for amprenavir in a hollow-fiber system (in vitro) and then to determine the likelihood of attainment of the target EC50 for a given virus when a dose of amprenavir-ritonavir is administered to patients (in vivo).

MATERIALS AND METHODS

The hollow-fiber system was utilized for our in vitro experiments. This technique allows for growth and maintenance of cells at densities near those in tissues, provides access for sampling for drug exposure, and allows for drug exposure of cells to a concentration-time profile that is identical to that achievable in humans (2, 3).

Cell culture. The human T-lymphoblastoid cell line CEM and this line chronically infected with the IIIB strain of HIV type 1 (HIV-1) was originally obtained from the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Cells were cultured in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum, 25
mM HEPES, and gentamicin (50 μg/ml; Paragon Biotech, Baltimore, Md.). The medium for hollow-fiber bioreactor culture was modified for growth in the absence of CO2 by the omission of Na2HCO3 during formulation. Hollow-fiber bioreactors with a 10,000-molecular-weight cutoff (Spectrum Laboratories, Inc., Rancho Dominguez, Calif.) were used for this study. Experimental hollow-fiber units (previously perfused for 16 to 24 h with test media) were initiated by introduction of 7 ml of a 1:100 mixture of HIV-infected and uninfected CEM cells at a density of 3 × 10^5 cells per ml into the extracapillary space. An initial sample was taken for determining the cell number, the percentage of infected cells, viability by trypan blue exclusion, and p24 level. In continuous-infusion experiments, the drug-containing medium was recirculated, and the replenishment of drug-containing medium was done daily to maintain adequate stability of the agent.

**HIV antigen assay.** Samples for the p24 antigen assays were taken at the time points indicated in Fig. 1 after the initiation of experiments. Aliquots of cells and tissue culture media were removed through the extracapillary space access ports on the bioreactors at the time points indicated in Fig. 1 and centrifuged for 5 min at 1,500 × g. The levels of HIV gag p24 protein in cell-free supernatants were measured by using the Coulter Immunology (Hialeah, Fla.) p24 enzyme-linked immunosorbent assay according to the manufacturer’s guidelines. Some of the HIV p24 assays performed at SRA Life Sciences (Rockville, Md.) used p24 kits from Organon-Tecknika. Absorbance was measured and data were analyzed with a computer-supported microplate reader (Molecular Devices, Menlo Park, Calif.). Levels of the p24 protein were calculated by the SoftMax program provided by the manufacturer of the microplate reader. The levels of p24 at baseline (day 0) were subtracted from values obtained on later days.

**MTT virus infectivity assays.** The cytotoxicity of HIV-1 in the presence and absence of an antiviral agent was correlated to the formation of formazan in an assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described by Drusano et al. (4). Cultures from days 5 to 7 of MT-2 cells treated with or without antiviral agent and/or other additives and actively infected with HIV-1 in 96-well microtiter plates were pulsed for 4 h with 1 mg of MTT per ml in RPMI1640 plus 10% fetal calf serum. The supernatant was removed from the cells, and the formazan was solubilized with 85 μl of acid isopropanol per well. Absorbance was read at 540 nm with a Molecular Dynamics plate reader. The choice of the wavelength was determined by analysis of the spectrum of MTT absorbance as described previously (8). The means of determinations for quadruplicate wells ± standard deviations were used for calculations of cytotoxicity or inhibition of HIV-1-mediated apoptosis.

**Sensitivities of viral isolates to amprenavir.** The sensitivities of the viral isolates to amprenavir were determined by dose-response determinations in an MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (8). The means of determinations for quadruplicate wells ± standard deviations were used for calculations of cytotoxicity or inhibition of HIV-1-mediated apoptosis.

**Final drug concentrations.** The EC50 or EC90-95 (4). It is important to recognize that it would be inappropriate to attempt to determine EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half. Obviously, the EC50s turn down viral replication only by half.
for which concentrations were above the EC₉₀ (time > EC₅₀), including time before the peak concentration.

A second target was determined from the minimum or trough concentration (Cₘᵢₙ)/EC₅₀ ratio based on a previously published relationship among exposure, viral load decrease, and the probability of maintaining a viral log decrease through 8 weeks in a population highly experienced with retrovirals (D. S. Stein, Y. Lou, J. Johnson, and S. Randall, Abstr. 2nd Int. Workshop Clin. Pharmacol. HIV Ther., poster 5.6, 2001). The previously described relationship determined the link between the Cₘᵢₙ/EC₅₀ and the probability of maintaining at least a 1.0-log₁₀ time-weighted average change in viral load from baseline (in copies per milliliter) for combination therapy that included amprenavir. In the previous study, the protein binding correction was 10-fold rather than the 7.4-fold used in the in vitro model based on 90% protein binding. A protein binding-adjusted Cₘᵢₙ/EC₅₀ ratio of 5 was associated with approximately a 90% probability of maintaining at least a 1-log time-weighted change in viral load from baseline to week 8.

**RESULTS**

**Hollow-fiber experiments and determination of a target.** The data from the hollow-fiber experiment are shown in Fig. 1 for no-drug, continuous-infusion, BID, and TID regimens. An inhibitory sigmoid Eₘₐₓ model was fit to the data (Fig. 2), which provided a relationship between effect (p24 suppression) and the time > EC₉₅ variable: p24 = 2,580,000 – [2,466,000 × (time > EC₉₅)¹⁰.¹⁹/(time > EC₉₅)¹⁰.¹⁹ + 0.6³¹⁰.¹⁹)]. Based upon this relationship, amprenavir free-drug concentrations should remain above the EC₉₀₉₅ for 80% of the dosing interval to achieve near-maximal viral suppression.

**Population pharmacokinetic analysis.** Of 13 patients, 12 were included in the population pharmacokinetic analysis. The patient demographics are shown in Table 1. One subject had predose and 12-h plasma drug concentrations determined at purported steady state that differed by a value that would be explained by greater than five half-lives. As the predose concentration was much lower than the 12-h concentration, this strongly suggested that the patient had missed taking the previous dose of amprenavir. Because of this, we chose to exclude this patient’s data from the analysis.

<table>
<thead>
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<th>Characteristic</th>
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<td>Trial participants (n = 13)</td>
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<tr>
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<tr>
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<tr>
<td>Range</td>
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<td>42–618</td>
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<td>Baseline viral load (no. of log₁₀ copies/ml)</td>
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<tr>
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<td>4.25</td>
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<tr>
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</table>

*a* One patient was excluded from the pharmacokinetic analysis.
The parameter estimates from the population pharmacokinetic analysis are shown in Table 2. The scatterplot for observed versus predicted concentrations based on parameter means is shown in Fig. 3. The $r^2$ was 0.925, and the least-squares line of best fit was calculated as follows: observed concentration = 0.176 + (0.993 × predicted concentration). The bias as a mean error was −0.149 μg/ml, and the precision as bias-adjusted precision was 0.323 (μg/ml)$^2$.

### Determination of fractional target attainment

The fractional target attainment for the first target derived from our hollow-fiber system for a range of EC50s is shown in Fig. 4A for 2,500 simulated patients. At an amprenavir EC50 of 0.03 μM, the likelihood of target attainment (a virologically active amprenavir concentration greater than four times the EC50 for ≥80% of the dosing interval) is 97.4%. For an isolate with reduced susceptibility to amprenavir, i.e., for which the amprenavir EC50 is 0.050 or 0.080 μM, the likelihood of target attainment falls to 91.0 or 75.8%, respectively.

For the second target of a $C_{\text{min}}$/EC50 ratio of 5 based on a human study that showed this to be associated with a 90% probability of attaining at least a 1.0-log10 time-weighted average decline in viral load from baseline (copies per milliliter) over 8 weeks, the distribution of the target attainment rates for the range of EC50s is displayed in Fig. 4B. As can be seen, the distribution of target attainment rates is quite similar to that in Fig. 4A, irrespective of the target chosen, especially in the range of EC50s that were ≤0.03 μM.

### DISCUSSION

Achieving an optimal outcome for patients taking highly active antiretroviral therapy requires a therapeutic regimen that will generate a high probability of driving the viral load below the limit of assay quantitation. This fact has been shown repeatedly in multiple investigations (5, 7, 12). There are a number of conditions that need to be met in order for a patient to achieve this goal. First and foremost, the regimen prescribed needs to be virologically potent. Adhering well to a virologically inadequate regimen will still result in failure to achieve the goal of reducing viral load to less than the limit of assay quantitation (11). Therefore, one must generate a properly potent regimen and do so with a schedule of administration and pill burden to which a patient can adhere.

In this evaluation, we chose to investigate how to optimize the protease inhibitor component of an antiretroviral regimen. It is important to note that in clinical care, one would also have the antiviral activities of other agents used in the combination therapy to drive antiviral activity. The first thing that needed to be defined was the pharmacodynamically linked variable. To do this, we studied amprenavir in our hollow-fiber pharmacodynamic model. As was demonstrated previously for the protease inhibitor atazanavir (4), the time that free-drug concentrations remain above four times the EC50 (time $> 4 \times \text{EC50}$) is the pharmacodynamically linked variable. This can be clearly seen in Fig. 1, where there is an untreated control hollow-fiber unit and three treated hollow-fiber units. All three treated units have identical 24-h AUC exposures to amprenavir. The difference is that one unit has a continuous infusion at a concentration of 4 times the EC50, which generates a 24-h exposure of 24 times 4 times the EC50. For the other two units, the

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TABLE 2. Pharmacokinetic parameters

<table>
<thead>
<tr>
<th>Determination</th>
<th>$k_{el}$ (h$^{-1}$)</th>
<th>$K_{el}$ (h$^{-1}$)</th>
<th>$V_1$ (liter)</th>
<th>$K_{cp}$ (h$^{-1}$)</th>
<th>$K_{pc}$ (h$^{-1}$)</th>
<th>Time lag (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.179</td>
<td>16.78</td>
<td>100.6</td>
<td>5.049</td>
<td>12.42</td>
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<td>Median</td>
<td>0.180</td>
<td>8.550</td>
<td>92.50</td>
<td>0.720</td>
<td>0.386</td>
<td>0.470</td>
</tr>
<tr>
<td>SD</td>
<td>0.0614</td>
<td>15.48</td>
<td>46.12</td>
<td>8.967</td>
<td>17.55</td>
<td>0.300</td>
</tr>
</tbody>
</table>

*a* $k_{el}$, elimination rate constant; $K_{el}$, absorption rate constant; $V_1$, volume of distribution in the central compartment; $K_{cp}$, intercompartmental rate constant from the central to the peripheral compartment; $K_{pc}$, intercompartmental rate constant from the peripheral to the central compartment; time lag, lag time to absorption.

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**FIG. 3.** The plot of observed versus predicted amprenavir concentrations in plasma after population pharmacokinetic modeling and MAP-Bayes estimation is shown.
24-h AUCs are identical (96 times the EC₅₀), but in one unit, the AUC is broken up into two equal exposures of 48 times the EC₅₀, administered 12 h apart, while in the other, it is broken into three equal parts (32 times the EC₅₀), administered every 8 h. If the ratio of the AUC to four times the EC₅₀ were the pharmacodynamically linked variable, all treated units would give the same degree of viral suppression. If the ratio of the peak concentration to four times the EC₅₀ ratio were linked to viral suppression, then dosing every 12 h (q12h) would be best. Neither of these outcomes occurred; rather, continuous infusion was best and administration q8h had an equivalent outcome, with q12h dosing giving the least viral suppression, clearly demonstrating that time > 4 × EC₅₀ is the appropriate dynamic variable for evaluation.

In order to set a specific goal for regimen evaluation, we fit an inhibitory sigmoid maximal-effect model to the data (Fig. 2). Clearly, the degrees of viral suppression were virtually identical with the continuous-infusion and q8h hollow-fiber units. The latter attained drug concentrations at least four times the EC₅₀ for approximately 80% of the dosing interval. We chose this as the amprenavir exposure target that would indicate adequate therapy for that portion of an individual’s regimen. The necessity to keep drug concentrations in excess of four times the EC₅₀ for approximately 80% of the dosing interval was also seen in previous work with atazanavir in our laboratory (4). It should be recognized that in the case of atazanavir, the dosing interval was 24 h. Therefore, the dosing interval coverage number of 80% is independent of the duration of the dosing interval, at least out to 24 h.

We also wished to examine the attainment of a target that

![Diagram A](image1.png)

**FIG. 4.** (A) The target attainment rate for a given EC₅₀ for amprenavir and ritonavir at doses of 600 and 100 mg, respectively, is shown with a target of more than four times the EC₅₀. (B) The target attainment rate for a given EC₅₀ for amprenavir and ritonavir at doses of 600 and 100 mg, respectively, is shown with a target Cₘᵢₙ/EC₅₀ ratio of 5.
was based on clinical (in vivo) data. To do this we employed a previously developed relationship for amprenavir relating a protein binding-adjusted \( C_{\text{min}}/EC_{50} \) ratio to the probability of maintaining at least a 1.0-log10 time-weighted decline in HIV RNA concentrations from baseline to week 8 in a highly antiretroviral-experienced population. A \( C_{\text{min}}/EC_{50} \) ratio of 5 was associated with approximately a 90% probability of attaining this goal.

The enhancement of protease inhibitor drug levels by adding a small amount of ritonavir is an important new approach to antiretroviral therapy (10). With this approach, the concentration of amprenavir is increased and the drug can be administered less frequently and with fewer capsules so as to optimize patient adherence. In the clinical research study performed, 600 mg of amprenavir was boosted with 100 mg of ritonavir, with both drugs being given on a q12h dosing schedule. For all of the subjects, the concentration-time profiles obtained during serial pharmacokinetic sampling (178 plasma samples) were population modeled. The model fit the data well, as demonstrated in Fig. 3, where, after MAP-Bayes estimation, the predicted-value-observed-value plot had a regression line with a slope of 0.993 (1.0 is a perfect regression slope) and an intercept of 0.176 (0.0 is a perfect intercept). The overall \( R^2 \) was 0.925, with quite acceptable measures of bias and precision.

In order to obtain an estimate of how well a specific dose of drug will be able to attain its therapeutic target in a population, the full variability of the pharmacokinetic parameter values needs to be taken into account. That is, the relatively small pharmacokinetic trials that are performed do not allow an estimate of the number of patients that will fall in the tails of the distribution to be obtained. Monte Carlo simulation allows the investigator to obtain an idea of the true impact of between-patient variability in the pharmacokinetics of the drug on the ability of that dose of drug to attain a therapeutic goal. It has recently been demonstrated through Monte Carlo simulation (4, 6) that such an estimate can be made, and the predictions have been borne out in a clinical setting.

We performed a 2,500-subject Monte Carlo simulation for the combination of amprenavir and ritonavir, and steady-state amprenavir concentrations were simulated. We then examined the fraction of the simulated subjects that had their free-drug concentrations exceed four times the \( EC_{50} \) (approximately the \( EC_{95} \)) for 80% of the 12-h dosing interval. These results (Fig. 4A) are quite impressive. Target attainment exceeded 90% when subjects received amprenavir up to an \( EC_{50} \) of 0.050 \( \mu \)M. Target attainment exceeded 50% for the population receiving amprenavir up to an \( EC_{50} \) of 0.120 \( \mu \)M. The results imply that this combination can be used without monitoring the concentrations of amprenavir when the \( EC_{50} \) is less than or equal to 0.050 \( \mu \)M (target attainment approximately 90%).

For patients with viral isolates for which the amprenavir \( EC_{50} \) were greater than 0.050 \( \mu \)M, there is still a reasonable expectation of attaining the exposure target, indicating that this can be part of a regimen for salvage patients and that when other active antiretroviral agents are used, a high probability of success can be anticipated; but for these patients, drug concentration monitoring might be utilized as part of the patients’ management programs. It is important to emphasize that the protease inhibitor component of the regimen is only one part of a multidrug therapeutic combination. Even if the protease inhibitor part is not completely optimized, it is still possible to achieve maximal viral suppression due to the additional non-protease antiretroviral drugs.

Based on our model, we have used 80% of the dosing interval for free-drug concentrations to exceed four times the \( EC_{50} \) as a target. In the clinic, for a 12-h dosing interval, this would translate to \( h \) 9.6 were there no lag time to absorption. As demonstrated in Table 1, there is a modest lag time with a central tendency of 0.616 (mean) to 0.470 (median) \( h \) and a 95% confidence interval from 0 to 1.82 \( h \). To take this lag time into account, and to be conservative with regard to the clinical circumstance, we recommend that if the concentration in serum is monitored, the \( h \) 12 trough concentration would be most appropriate for determining whether or not a target of free drug more than four times the \( EC_{50} \) is attained, as monitoring levels at the time point of 80% of the dosing interval (\( h \) 9.6) is not practical. To be explicit, were one to monitor trough concentrations of amprenavir, we would recommend dividing the total drug concentration by a factor of 7.4 to adjust for the impact of drug binding to serum proteins. The \( EC_{50} \) should be multiplied by 4. The target, then, would be to have the protein binding-adjusted drug concentration at trough exceed four times the \( EC_{50} \).

The second, clinical-data-based target gives a similar target attainment rate distribution (Fig. 4B). It is important to note the relationship between the time > 4 \( \times \) \( EC_{50} \) variable and the \( C_{\text{min}}/EC_{50} \) ratio. Clearly, the goal is to have the number of rounds of viral replication be as low as possible, as this will provide the highest probability of driving the viral load to a level below assay quantitation as well as lowering the probability of the emergence of resistance. A measure of the time above threshold is bounded from above at 100%, while the \( C_{\text{min}}/EC_{50} \) is not bounded. For the in vitro-derived target, adequate suppression of viral turnover is provided by having the protein binding-corrected drug concentrations exceed a target value that provides near-maximal effect (here, four times the \( EC_{50} \) for a large fraction (80%) of the dosing interval. For the \( C_{\text{min}}/EC_{50} \) ratio, one must be careful in interpreting these values. A ratio of 1.0 guarantees only that the drug concentrations will provide half the maximal effect. When drugs are compared with regard to their \( C_{\text{min}}/EC_{50} \) ratios, the meaning of the targets, the methodologies used for the protein binding corrections, the \( EC_{50} \) determinations, and the viral strains compared should be clear, and the ratios should, of necessity, have values of >1.0. It is also important to recognize that the clinical analysis used an average decline in viral load by taking the AUC of the viral load to the point of evaluation and dividing by the time. This will, of necessity, give a smaller viral load decline because of the effect of the viral loads determined early on in therapy. Further, the protein binding adjustment was done differently than in the in vitro evaluation, where the correction factor used was 10.0, not 7.4 (D. S. Stein et al., Abstr. 2nd Int. Workshop Clin. Pharmacol. HIV Ther.). The difference in the correction factor along with the need for a free target \( C_{\text{min}}/EC_{50} \) ratio of 5 rather than 4 accounts for the differences between Fig. 4A and B.

In summary, we have demonstrated that, as for another protease inhibitor (atazanavir), time > 4 \( \times \) \( EC_{50} \) is the pharmacodynamically linked variable for amprenavir. It should be recognized that the target set here for attainment is very con-
servative and that attainment of this target results in shutdown of virtually all rounds of viral replication in an in vitro model. We have also examined the variability of a ritonavir-enhanced protease inhibitor in the population and showed that the regimen studied performs very well up to an EC_{50} of 0.040 to 0.050 μM (95 to 90% target attainment rates). Even with more resistant isolates with EC_{50}s around 0.120 to 0.140 μM, target attainment rates still exceed or approximate 50%. Amprenavir can be used with confidence in patients infected with viruses sensitive to protease inhibitors. For antiretroviral-experienced patients infected with less sensitive isolates, ritonavir-enhanced amprenavir still provides a reasonable probability of attaining an optimal exposure target, especially when combined with other active antiretroviral agents. The monitoring of serum drug concentrations may have a role in the treatment of patients infected with the more resistant, drug-experienced isolates. If the therapeutic monitoring of drug concentrations is considered, it should probably be performed relatively early in the therapeutic course to maximally improve the probability of good, long-term suppression.

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