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Antiviral Activity of the Dihydropyrone PNU-140690, a New Nonpeptidic Human Immunodeficiency Virus Protease Inhibitor

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PNU-140690 is a member of a new class of nonpeptidic human immunodeficiency virus (HIV) protease inhibitors (sulfonamide-containing 5,6-dihydro-4-hydroxy-2-pyrones) discovered by structure-based design. PNU-140690 has excellent potency against a variety of HIV type 1 (HIV-1) laboratory strains and clinical isolates, including those resistant to the reverse transcriptase inhibitors zidovudine or delavirdine. When combined with either zidovudine or delavirdine, PNU-140690 contributes to synergistic antiviral activity. PNU-140690 is also highly active against HIV-1 variants resistant to peptidomimetic protease inhibitors, underscoring the structural distinctions between PNU-140690 and substrate analog protease inhibitors. PNU-140690 retains good antiviral activity in vitro in the presence of human plasma proteins, and preclinical pharmacokinetic studies revealed good oral bioavailability. Accordingly, PNU-140690 is a candidate for clinical evaluation.

Current antiretroviral therapy for human immunodeficiency virus type 1 (HIV-1) infection is based on inhibition of viral enzymes that play essential roles in replication. For two such targets, HIV protease and reverse transcriptase (RT), there are several inhibitors that are either approved for use or in late-stage clinical trials. While monotherapy with these agents reduces viral replication initially, their failure to achieve complete suppression allows resistant variants to emerge from the large, diverse viral population in infected patients (5, 9, 24, 26). Currently, therapy employing combinations of antiviral agents provides the best chance for achieving significant and durable reductions in viral replication (23). Because the comprehensive clinical evaluation of more than a few combinations is unlikely, in vitro data is valuable for identifying combinations that are likely to be advantageous. Ideal component antiviral agents will be potent, bioavailable, and well tolerated and also will combine with synergy and will fail to select for cross-resistant HIV-1 variants (15). These criteria can be met by combining an RT inhibitor with a protease inhibitor, because each has a distinct molecular target. The combination of two drugs that act similarly on the same target enzyme is less ideal, since mutations that confer cross-resistance to both agents may emerge (6, 20, 24). Still, similar RT inhibitors have been shown to combine advantageously, probably by virtue of the distinct modes of resistance that each typically selects for (1, 27). For example, relatively slow emergence of virus coresistant to both zidovudine (AZT) and lamivudine (3TC) could result from 3TC selective pressure for the RT substitution M to V at position 184, a change that restores a sensitive phenotype to AZT-resistant strains (16). Protease inhibitors that select for distinct patterns of resistance mutations might also achieve more durable antiviral effects when combined. However, significant cross-resistance has been observed in in vitro studies with the currently approved set of peptidomimetic protease inhibitors (29), and indinavir-resistant HIV-1 variants isolated in vivo exhibited significant cross-resistance to saquinavir and the ritonavir analog A-80987 (6). Novel protease inhibitors may be required to realize benefits from combination protease inhibitor therapy.

This work describes the antiviral activity of PNU-140690, a nonpeptidic protease inhibitor that resulted from structure-based design efforts at Pharmacia & Upjohn. In addition to having excellent potency versus a variety of HIV-1 laboratory strains and clinical isolates, PNU-140690 is highly active against HIV-1 isolates that are resistant to the peptidomimetic protease inhibitor ritonavir. PNU-140690 retains good antiviral activity in vitro in the presence of human plasma proteins and is able to act synergistically with RT inhibitors such as AZT or delavirdine (DLV), a nonnucleoside RT inhibitor in phase III trials (7, 25). Preclinical oral bioavailability studies indicate that concentrations of PNU-140690 in blood adequate for these activities can be readily achieved (28).

MATERIALS AND METHODS

Compounds. PNU-140690 (Fig. 1), ritonavir (Abbott Laboratories), indinavir (Merck), nelfinavir (Agouron), AZT (Glaxo-Wellcome), and DLV (Pharmacia & Upjohn) were dissolved in dimethyl sulfoxide (DMSO) at 10 to 30 mM, aliquoted, and frozen. Saquinavir (Roche) was dissolved in sterile deionized water. Fresh vials were thawed just prior to use for each assay. Drug dilutions were prepared in supplemented RPMI 1640 medium (see below).

Cells and virus strains. The following cells and virus strains were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: H9 and MT-4 cells and HIV-1_{IIIB}, HIV-1_{NL4-3}, and HIV-1_{JR-CSF} strains. Clinical isolates were received from either SRA Technologies, Inc., or Aaron Diamond AIDS Research Center. Human peripheral blood mononuclear cells (PBMC) were obtained from fresh plasmapheresis preparations taken from HIV-1-seronegative donors and prepared by density gradient centrifugation with Ficoll-Hypaque (Organon Teknika, Durham, N.C.). PBMC were collected, washed, and incubated for 3 days in culture medium containing 4 μg of phytohemagglutinin (PHA) per ml. PHA-treated cells were then cultured in assay medium containing recombinant human interleukin-2 (80 U/ml; Gibco). Under our experimental conditions and without change of culture media, uninfected PBMC reached a peak cell number (1.85×10^6 cells/ml) and showed >95% survival on day 5 after seeding.

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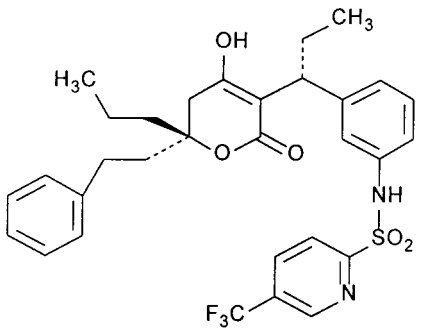

FIG. 1. PNU-140690.

Genotypic evaluation of virus stocks. The protease-coding region of each virus stock used in the cross-resistance studies was sequenced as follows. Virion RNA was isolated from 250 μl of cell-free supernatant virus with TriReagent LS (Molecular Research Center, Cincinnati, Ohio) and was resuspended in 50 μl of RNase-free water. One microliter of the purified RNA was used as template for cDNA synthesis by Superscript II RT (Gibco/BRL). Amplification of the protease-coding region and flanking regions was accomplished via typical PCR protocols with the following primer pairs: ORP+350B, 5′-biotin-CTGTCAAT GGCCATTGTTTAACTTTTGGG-3′ (nucleotides 2602 to 2630 of HXB2); and PRO-106, 5′-CAGAGCCAACAGCCCCACCAGAAGAG-3′ (nucleotides 2146 to 2171 of HXB2). The resulting PCR product was bound to strepavidin-coated beads, denatured with NaOH, and sequenced on the solid phase with Sequenase V2.

Antiviral assays. The PNU-140690 susceptibility of HIV-1_{IIIB} in H9 cells was determined following infection at a multiplicity of infection of 0.005. Infected H9 cells were washed and seeded at 1.6×10^5 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml (RPMI complete) containing PNU-140690 or DMSO and allowed to grow at 37°C for 5 days, at which time culture supernatants were withdrawn for quantitative HIV p24 antigen determinations (Coulter). The PNU-140690 susceptibility of HIV-$1_{JR\text{-}CSF}$ in PBMC was determined following infection of PHA-stimulated normal donor PBMC (11) at a multiplicity of infection of 0.01. Infected PBMC were seeded at 3×10^5 cells/ml in RPMI complete containing 80 U of interleukin-2 per ml and PNU-140690 or DMSO control. After growth for 5 days at 37°C, culture supernatants were assayed for p24 antigen levels. PNU-140690 susceptibility assays of clinical isolates not known to be resistant to RT inhibitors (RTIs) were performed according to the AIDS Clinical Trials Group-U.S. Department of Defense (ACTG-DoD) consensus protocol (11). The AZT and DLV susceptibilities of RTI-resistant clinical isolates were also determined according to the ACTG-DoD protocol, but the PNU-140690 susceptibilities of these isolates were determined by infecting PHA-stimulated PBMC with inocula sufficient to achieve p24 antigen levels between 10^4 and 10^5 picograms/ml by day 4 postinfection. Culture supernatant p24 levels observed at day 4 of growth in the presence of PNU-140690 or DMSO were used to determine 90% inhibitory concentrations (IC_{90}). To determine selectivity indices in either H9 or PBMC culture, IC_{50} of PNU-140690 were determined and divided by 50% cell culture toxicity doses ($CCTD_{50}$) determined in the corresponding culture system. The cytotoxicities of test compounds in uninfected H9 or PBMC cultures were evaluated by measuring the formation of formazan, a tetrazolium dye, in a 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay as described previously (3, 19). The effects of human plasma proteins on PNU-140690 antiviral activity were assessed in H9 and PBMC assays as described for HIV-1_{IIIB} and HIV-$1_{JR\text{-}CSF}$ above but with culture medium supplemented with one of the following: 33, 50, or 75% (vol/vol) heat-inactivated (56°C, 30 min) human plasma from HIV-seronegative donors; 12.8 mg of human plasma albumin (Albutein, lot NS4010A; Alpha Therapeutic Corp.) per ml, which is equivalent to 33% of normal human albumin levels; or 2.0 mg of α1-acid glycoprotein (lot 113H9308; Sigma) per ml. The protease inhibitor susceptibilities of HIV-$1_{NL4\text{-}3}$ and HIV-$1_{NL4\text{-}3}$(p37) replicating in MT-4 cells were determined after infection with inocula sufficient to yield approximately 10^6 of p24 antigen per ml on day 4. The protease inhibitor susceptibilities of ritonavir-resistant clinical isolates replicating in PBMC were determined after infection with inocula sufficient to yield approximately 10^5 pg of p24 antigen per ml by day 5 postinfection.

Antiviral activity of PNU-140690 in combination with AZT or DLV. The inhibition of HIV-$1_{JR\text{-}CSF}$ replication in PBMC by combinations of PNU-140690 with AZT or DLV was evaluated in experiments involving several molar ratios of the drugs. Four concentrations of each drug (PNU-140690 at 0.01, 0.03, 0.1, and 0.3 μM; AZT at 0.0003, 0.001, 0.003, and 0.01 μM; DLV at 0.001, 0.003, 0.01, and 0.03 μM) and two-drug combinations were assayed in duplicate wells. For each molar ratio, three to four datum points were analyzed to yield combination indices (CI) by the multiple drug effect equation of Chou and Talalay (4), assuming mutually nonexclusive drug interactions as previously described (3). The CI values at various fractional inhibitions (50, 75, 90, and 95%) were used to determine whether the combinations were synergistic ($CI < 1$), additive ($CI = 1$), or antagonistic ($CI > 1$).

RESULTS

The availability of three-dimensional structural data for HIV-1 protease–ligand complexes facilitated iterative cycles of rational inhibitor design that ultimately yielded PNU-140690 (Fig. 1). PNU-140690 is neither a transition state analog of peptide substrates of HIV protease nor a C_2 symmetry-based inhibitor. Crystal structure examinations revealed that PNU-140690 bound to the active site of HIV-1 protease, supporting enzymatic data showing high potency and selectivity ($K_i < 0.01$ nM versus HIV-1 protease, $K_i < 1$ nM versus HIV-2 protease, and K_is of 2, 15, and 9 μM versus human pepsin, human cathepsin D, and human cathepsin E, respectively) (28).

PNU-140690 potently suppressed viral replication of laboratory and clinical HIV-1 strains, as summarized in Table 1. In H9 cell cultures, acute infections with laboratory strain HIV-1_{IIIB} were inhibited by an average PNU-140690 IC_{90} of 0.16 μM with a selectivity index ($IC_{50}/CCTD_{50}$) of 502. Acute infection of PBMC with HIV-$1_{JR\text{-}CSF}$, a monocytotropic isolate, was inhibited by a PNU-140690 IC_{90} of 0.18 μM with a selectivity index of 350. When 10 diverse HIV-1 isolates from patients were used to infect PBMC, PNU-140690 also demonstrated consistently potent activity (average IC_{90} of $0.16 \pm .07$ μM). AZT-resistant isolates also tested were fully sensitive to PNU-140690, demonstrating the expected lack of cross-resistance to protease inhibitors. Similarly, PNU-140690 potently inhibited isolates resistant to DLV, a nonnucleoside RT inhibitor in phase III trials.

The results of experiments assessing the antiviral activity of PNU-140690 combined with AZT or DLV are shown in Table

TABLE 1. Antiviral activity of PNU-140690 in cell culture

Virus/cell culture	IC_{90} (μM)[a] of:		
	AZT	DLV	PNU-140690[b]
HIV-1_{IIIB}/H9			0.16 ± 0.07[c]
HIV-$1_{JR\text{-}CSF}$/PBMC			0.18
HIV-1 clinical isolates/PBMC			
UJ00004			0.8
UJ00007			0.15
UJ00009			0.32
N. Amer1			0.17
N. Amer2			0.17
N. Amer3			0.07
N. Amer4			0.15
N. Amer5			0.17
N. Amer6			0.12
N. Amer7			0.16
RTI-resistant clinical isolates/PBMC			
458	0.3	7.7	0.04
477	<0.3	6.7	0.04
6	1.6	0.1	0.04
410	6.6	>10	0.04
472	1.4	>10	0.03

[a] Concentration required to inhibit HIV-1 p24 antigen production by 90% relative to that in no-drug controls. Values were determined according to the ACTG-DoD consensus protocol (see Materials and Methods).

[b] Values for HIV-1_{IIIB}, HIV-$1_{JR\text{-}CSF}$, and RTI-resistant clinical isolates were determined as described in Materials and Methods. Values for other clinical isolates were determined according to the ACTG-DoD consensus protocol.

[c] Mean of three assays.

TABLE 2. CI values for two-drug combination regimens of U-140690 with AZT or DLV assayed against acute HIV-1_{JR-CSF} infection of PBMC

Drug combination	Ratio[a]	CI at % inhibition[b]			
		50	75	90	95
PNU-140690 + AZT	10:1	0.85	0.78	0.72	0.69
	30:1	0.87	0.95	1.02	1.09
PNU-140690 + DLV	3:1	0.85	0.74	0.63	0.59
	10:1	0.56	0.65	0.76	0.86

[a] Molar ratio of drug combinations.
[b] For each combination ratio, CI values were computed based on the mean percent inhibition of two experiments (4). Values <1, =1, and >1 indicate synergism, additive effect, and antagonism, respectively.

2. Combinations of PNU-140690 with either AZT or DLV in the dose ranges tested were consistently more effective than the individual agents. The dose-effect plots for each single agent and the PNU-140690 combinations were used to make a series of median effect plots and to estimate the CI as described above. The drugs were combined in two molar ratios likely to equalize their individual antiviral effects. Additive-to-synergistic interactions were observed at all levels of inhibition for all ratios tested except the 30:1 ratio of PNU-140690 to AZT. At this somewhat extreme ratio, CI reflected additive antiviral effects. No cytotoxicity was evident by MTT assay with any of these drugs alone or combined at the highest two concentrations tested.

Some peptidomimetic HIV protease inhibitors have reduced in vitro activity as a result of high-affinity binding to plasma proteins, in particular to α1-acid glycoprotein (2, 12, 17). Since the in vivo antiviral activity of protease inhibitors is likely to be influenced by serum protein binding, the effects of human plasma, human plasma albumin, and α1-acid glycoprotein on the antiviral activity of PNU-140690 were assessed with HIV-1_{IIIB} in H9 cell culture and HIV-1_{JR-CSF} in PBMC culture. The PNU-140690 IC_{90} under these conditions are shown in Table 3. The fold increase in the IC_{90} resulting from the presence of the additional protein is also tabulated and ranges from 1.7 to 6.2, with the 6.2-fold increase being caused by the presence of 2 mg of α1-acid glycoprotein per ml in the HIV-1_{IIIB}/H9 acute infection assay. The presence of high percentages of human plasma in culture medium containing 10% fetal bovine serum had less effect on PNU-140690 potency, and in no case did the IC_{90} exceed 2.1 μM.

TABLE 3. Effect of human plasma proteins of the antiviral activity of PNU-140690

Virus/cells	Additional plasma protein present[a]	IC_{90} (μM)[b]	Fold change[c]
HIV-1_{IIIB}/H9	0% human plasma	0.34 ± 0.13[d]	
	33% human plasma	0.57 ± 0.10[d]	1.7
	2 mg of α1-acid glycoprotein/ml	2.1	6.2
HIV-1_{JR-CSF}/PBMC	0% human plasma	0.41	
	33% human plasma	0.95	2.8
	12.8 mg of human plasma albumin/ml	0.25	1

[a] In medium containing 10% fetal bovine serum.
[b] Concentration of PNU-140690 required to inhibit HIV-1 p24 antigen production by 90% relative to that in no-drug controls.
[c] Fold difference compared to control with no addition.
[d] Mean of three experiments ± standard deviation.

TABLE 4. PNU-140690 potently blocks replication of HIV-1 broadly cross-resistant to peptidomimetic protease inhibitors

Protease inhibitor	IC_{90} (μM)[a] vs:		Fold increase in IC_{90} vs HIV-1_{NL4-3}(p37)
	Parental HIV-1_{NL4-3}	HIV-1_{NL4-3}(p37)	
PNU-140690	0.07	0.45	6
Ritonavir	0.08	6.4	80
Indinavir	0.03	1.4	47
Nelfinavir	0.025	>3.1	>125
Saquinavir	0.03	3.75	125

[a] Compound concentration required to inhibit HIV-1 p24 antigen production in MT-4 cell culture by 90% relative to that in no-drug controls.

HIV-1 variants that exhibit cross-resistance to several protease inhibitors have been described, so the antiviral activity of PNU-140690 against a broadly resistant HIV-1_{NL4-3} variant was evaluated. The resistant variant, HIV-1_{NL4-3}(p37), resulted from 37 passages of HIV-1_{NL4-3} in the presence of ritonavir. The character of the viral stock through passage 22 has been previously described (18), and the 22-passage variant exhibited a 10- to 25-fold increase in resistance to ritonavir compared to the parent strain. Further passage in the presence of ritonavir yielded HIV-1_{NL4-3}(p37), which was 80-fold more resistant to ritonavir, 47-fold more resistant to indinavir, and at least 125-fold more resistant to nelfinavir and saquinavir than the parent strain (Table 4). As expected, genotypic analysis of HIV-1_{NL4-3}(p37) revealed several coding changes associated with broad resistance to protease inhibitors (Table 5). Nevertheless, PNU-140690 retained good activity against HIV-1_{NL4-3}(p37), inhibiting it with an IC_{90} of 0.45 μM, while parental HIV-1_{NL4-3} was inhibited by an IC_{90} of 0.07 μM. The mutations in HIV-1_{NL4-3}(p37) that confer profound resistance to several peptidomimetic protease inhibitors thus caused only a 6.5-fold increase in resistance to PNU-140690.

TABLE 5. Protease substitutions predicted from genotypic analysis[a] of passaged HIV-1_{NL4-3} and HIV isolates from patients treated with ritonavir

Viral RNA source	Amino acid differences from consensus protease sequence[b]
HIV-1_{NL4-3}	L10I
HIV-1_{NL4-3}(p37)	L10I, M46I, L63P, I84V/A[c]
Isolate 301, pretherapy	M36M/I, I62V, L63A
Isolate 301, posttherapy	L10I, K20R, M36I, I54V, I62V, L63A, A71V, V82A, L90M
Isolate 304, pretherapy	K70R
Isolate 304, posttherapy	M46L, I54V, K70K/R, V77V/N, V82A
Isolate 306, pretherapy	K14R, M36I
Isolate 306, posttherapy	K14R, K20R, M36I, I54V, I62V, A71V, V82A, L90M
Isolate 313, pretherapy	I13V, G16E, L63A, V77I
Isolate 313, posttherapy	I13V, G16E, L33F, M36V, P39P/S, L63A, V77I, V82F

[a] The protease region of isolated viral RNA was amplified by RT-PCR and sequenced directly.
[b] Relative to the North American/European clade B consensus sequence. Entries show the amino acids and position, e.g., L10I means a change from L to I at position 10.
[c] Coding changes for both indicated amino acids were observed.

TABLE 6. PNU-140690 exhibits submicromolar IC_{90} against ritonavir-resistant clinical isolates

Clinical isolates[a]	IC_{90} (μM)[b] of:		
	PNU-140690	Ritonavir	Indinavir
301, pretherapy	0.21 ± 0.08[c]	0.05 ± 0.00	0.03 ± 0.01
301, posttherapy	0.60 ± 0.32 (3)[d]	3.36 ± 0.33 (67)	0.60 ± 0.16 (20)
304, pretherapy	0.22 ± 0.05	0.05	0.02 ± 0.01
304, posttherapy	0.36 ± 0.07 (2)	1.59 (32)	0.41 (20)
306, pretherapy	0.17	0.06	0.02 ± 0.02
306, posttherapy	0.29 (2)	3.84 (64)	0.66 ± 0.21 (33)
313, pretherapy	0.22	0.05	0.03 ± 0.02
313, posttherapy	0.38 (2)	3.03 (61)	0.09 ± 0.01 (3)

[a] Clinical isolates from patients treated with ritonavir used to infect PBMC.
[b] Compound concentration required to inhibit HIV-1 p24 antigen production by 90% compared to that of drug-free controls.
[c] Results represent the means ± standard deviations for three or fewer replicate assays.
[d] Fold increase compared to the value for the pretherapy isolate.

To determine whether PNU-140690 also blocked clinical isolates of HIV-1 with phenotypic resistance to peptidomimetic protease inhibitors, four paired clinical isolates from patients treated with ritonavir (pre- and posttreatment) were used to infect PBMC to determine susceptibilities to PNU-140690, ritonavir, and indinavir. A genotypic analysis of these isolates revealed a variety of coding changes previously observed in protease inhibitor-resistant variants (26) (Table 5). As shown in Table 6, PNU-140690 IC_{90} values were between 0.17 and 0.22 μM for the pretherapy isolates and between 0.29 and 0.60 μM for the post-ritonavir-therapy isolates. Thus, increases in IC_{90} between pre- and posttherapy isolates were only 2- to 3-fold for PNU-140690 compared to the 32- to 67-fold increases observed for ritonavir and the 3- to 33-fold increases observed for indinavir. In addition, it is evident from the inhibition curves for three of these resistant isolates (Fig. 2) that PNU-140690 achieves much greater reductions in culture p24 levels than ritonavir or indinavir. Because these isolates reflect the diverse population of virus present after ritonavir therapy, inability to inhibit the most resistant quasispecies might cause the descending inhibition curve to reach a plateau. If so, the absence of such a plateau with PNU-140690 suggests inhibition of those viral variants most resistant to ritonavir and indinavir.

DISCUSSION

Recently gained insight into HIV pathogenesis makes clear the importance of profound suppression of viral replication as a means of delaying the emergence of drug-resistant viral populations. It is clear that with as many as 10^{10} virions produced per day in an infected patient, error-prone replicative machinery will give rise to many viral variants able to evade drug surveillance. The ability of targeted HIV enzymes to accommodate amino acid substitutions is impressive, and there is a large and growing list of changes that emerge in response to various drugs (26). With the current emphasis on the use of drug combinations to achieve lasting reductions in viral burden, the patterns of resistance elicited by individual drugs have become important factors in the design of ideal combinations. Similarities in the resistance patterns for current protease inhibitors argue against their combination or sequential use and underscore the importance of continued efforts to design novel compounds. While future generations of protease inhibitors are not expected to avoid resistance development, they may select for distinct patterns of resistance and thus retain activity against HIV isolates resistant to previous groups of inhibitors.

PNU-140690 is a novel HIV protease inhibitor that resulted from structure-based drug design efforts at Pharmacia and Upjohn. As a candidate for treatment of HIV infection, PNU-140690 has several attractive attributes. Foremost, it has high potency against a variety of HIV strains and isolates in several cell culture systems. As expected, its activity does not decline against isolates that are resistant to AZT or DLV. To better approximate conditions likely to be encountered in vivo, PNU-140690 activity was assessed in the presence of high concentrations of human plasma proteins. Peptidomimetic HIV protease inhibitors like ritonavir may incur 10-fold reductions in activity due to high-affinity plasma protein binding (13), and α1-acid glycoprotein in particular has been shown to bind a variety of basic and neutral drugs and to reduce in vitro activity of HIV protease inhibitors (2, 17). The inclusion of 2 mg of α1-acid glycoprotein per ml in culture media caused a 6.2-fold reduction in the potency of PNU-140690, increasing the IC_{90} to 2.1 μM. The inclusion of human plasma or human plasma albumin caused less-than-threefold reductions in the antiviral activity of PNU-140690. After 10-mg/kg of body weight oral dosing in rats, absolute bioavailability was 30% and levels of PNU-140690 in blood exceeded 1 μM for 8 to 12 h (28).

Because anti-HIV drug candidates are likely to be used in combination therapies, interactions with other drugs are a significant concern. Drug combinations including AZT remain most common, and in vitro experiments suggest that PNU-

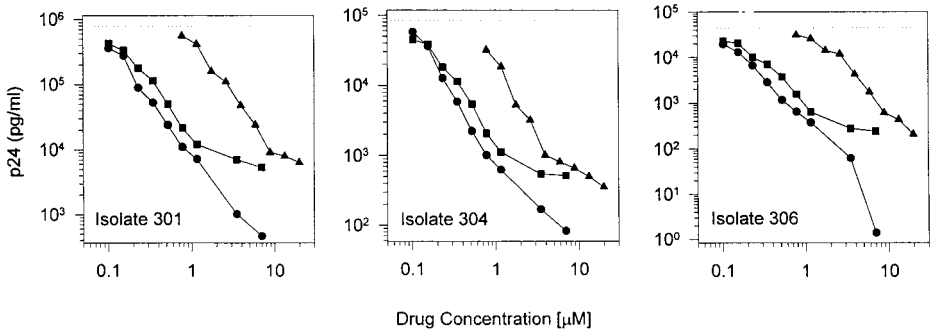

FIG. 2. PNU-140690 can suppress replication of ritonavir-resistant clinical isolates to low levels. PBMC were infected with HIV clinical isolates known to be resistant to inhibition by ritonavir as described in Materials and Methods. Virus replication in the presence of increasing micromolar drug concentrations was quantified by measuring p24 antigen levels (in picograms/milliliter). ●, PNU-140690; ■, indinavir; ▲, ritonavir; ·····, no drug.

140690 is able to interact synergistically with AZT. Similarly, PNU-140690 and DLV combine synergistically to inhibit HIV-1. Complete suppression of viral replication will probably require synergy among the drugs used in combination. Another important element of potential drug interactions is selection for individual resistance patterns that do not overlap. While this work does not describe the resistance pattern that PNU-140690 selects for, it demonstrates the good activity of PNU-140690 against viral variants that are broadly resistant to several other protease inhibitors. Amino acid substitutions that confer broad cross-resistance include those occurring at positions 46, 82, 84, and 90. Changes at these positions are often accompanied by changes at positions 10, 63, and 71 that are thought to be compensatory but whose influence is not fully understood (6, 10, 18, 21, 22). HIV-1_{NL4-3}(p37) and the ritonavir-resistant patient isolates used in this study contain various combinations of these substitutions and thus reflect several stages of broad resistance development. As expected, the multiple substitutions present in HIV-1_{NL4-3}(p37) had the greatest effect on PNU-140690 potency (sixfold increase in resistance). The ritonavir-resistant patient isolates were only two- or threefold less susceptible to PNU-140690. Despite the increased PNU-140690 IC_{90} required for these isolates, high concentrations of PNU-140690 still achieved dramatic reductions in culture p24 antigen levels. Suppression of replication to this extent suggests exertion of selective pressure on even the most resistant quasispecies present in the isolate. In contrast, the antiviral effects of indinavir and ritonavir against these isolates leveled out, apparently allowing replication despite increasing drug concentration. Retention of significant antiviral activity against resistant intermediates is a desirable attribute of protease inhibitors and may explain the observation that high concentrations of protease inhibitors can delay the emergence of resistant viral populations in vivo (14).

Significant efforts have been made to derive PNU-140690-resistant HIV-1 in cell culture. HIV-1_{NL4-3} and HIV-1_{NL4-3} (p37) have been passaged over 30 times in increasing concentrations of PNU-140690, much as described by Markowitz et al. (18). In both cases, less than a threefold increase in resistance has developed through 30 passages. This rate of resistance development is dramatically lower than those found in our previous efforts to derive resistance in vitro to RTIs (8). As with other HIV protease inhibitors, resistance to PNU-140690 may require multiple amino acid substitutions or particular substitutions that reduce proteolytic activity. A full picture of viral resistance development, as well as in vivo data regarding safety, pharmacokinetics, and antiviral activity, should emerge through the course of clinical studies with PNU-140690 planned to begin before the end of 1996.

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