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In Vitro Combination of PNU-140690, a Human Immunodeficiency Virus Type 1 Protease Inhibitor, with Ritonavir against Ritonavir-Sensitive and -Resistant Clinical Isolates

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PNU-140690 (sulfonamide-containing 5,6-dihydro-4-hydroxy-2-pyrone) is a potent, nonpeptidic inhibitor of the human immunodeficiency virus type 1 (HIV-1) protease currently under clinical evaluation. PNU-140690 and ritonavir were studied in two-drug combinations against the replication of HIV-1 clinical isolates in peripheral blood mononuclear cells. A ritonavir-sensitive (301-1x) and -resistant (301-6x) isolate pair derived from an individual before and after monotherapy with ritonavir were used. These isolates showed no significant difference in sensitivity to PNU-140690, but isolate 301-6x was more than 50-fold less sensitive to ritonavir than isolate 301-1x. Mathematical analysis showed that the combination of various concentrations of PNU-140690 with ritonavir yielded additive to moderately synergistic antiviral effects against the ritonavir-sensitive isolate and stronger synergy against the ritonavir-resistant isolate. The mechanism of synergy was not investigated, but the results suggested that both the virological and the observed in vitro pharmacological effects may have contributed to the observed synergy. Importantly, no significant antagonism was observed with the drug combinations studied. These data suggest that PNU-140690 may be useful in combination regimens with a structurally unrelated protease inhibitor such as ritonavir.

The protease of human immunodeficiency virus type 1 (HIV-1) is an enzyme essential for viral replication. Several peptidomimetic, competitive inhibitors of this enzyme (saquinavir, ritonavir, and indinavir) are currently being marketed for the treatment of HIV-1 infection (16). Several newer-generation nonpeptidic inhibitors (PNU-140690, 141W94 or VX-478, and nelfinavir) are still in clinical evaluation, and one of these (nelfinavir) has been recently approved for therapeutic use. Monotherapy with the marketed agents for patients with AIDS resulted in a marked decrease in circulating viral RNA levels with a concomitant increase in CD4 cells (13–15, 26, 34). However, the therapeutic benefit of a single protease inhibitor is often not sustained due to the emergence of resistant viral variants from the large, diverse viral population in infected patients (10, 11, 21, 34, 36). In contrast, when a protease inhibitor is used in combination with one or more inhibitors of viral reverse transcriptase, the emergence of viral resistance to protease inhibitors is greatly reduced (11, 12, 20, 27, 41). Although these drug combinations have been shown to be highly effective, not all patients have benefited from the treatments (11, 20). Furthermore, some patients have experienced severe side effects from these drug combinations (11). Therefore, it is important to explore other treatment regimens.

An alternate approach that may also offer the benefit of reduced resistance to any single inhibitor is the combined use of two or more protease inhibitors with distinct, nonoverlapping resistance patterns. Early studies showed that HIV-1 variants selected with one protease inhibitor were cross-resistant to other protease inhibitors both in vitro (40) and in vivo (14). This led to the belief that protease inhibitors would not be useful as convergent combination therapy. However, recent studies have revealed a more complex picture of cross-resistance among different protease inhibitors (4, 13, 28, 33, 37). An important finding was that unlike the multiply resistant variants selected during prolonged therapy, resistant variants that appeared early in therapy with one inhibitor were still sensitive to other protease inhibitors (13, 28). In addition, a few compounds in development were shown to select for resistance genotypes that differ from those for the existing drugs (29–31, 36). Furthermore, the combined use of certain protease inhibitors such as ritonavir and saquinavir may also result in enhanced efficacy due to improved bioavailability (2, 5). This is supported by a recent report that showed greatly increased concentrations of saquinavir in plasma when saquinavir was coadministered with ritonavir because of the latter's inhibitory effect on cytochrome P-450 3A (25).

Despite the promising prospect for the use of protease inhibitors in combination therapy, few in vitro studies have been reported. The availability of several new drugs now provides the opportunity for in vitro analyses to identify the combinations that may yield enhanced antiviral effects and to exclude others that may be antagonistic. PNU-140690 (sulfonamide-containing 5,6-dihydro-4-hydroxy-2-pyrone) is a new nonpeptidic inhibitor of HIV-1 protease currently under clinical development (39). It is structurally unrelated to other protease inhibitors and has been reported to be highly active against viral variants resistant to other protease inhibitors (31). Here we describe the antiviral effect of PNU-140690 in combination with ritonavir, a combination that imparts a nonoverlapping resistance pattern and potential pharmacologic synergy in vitro.

MATERIALS AND METHODS

Compounds. Stocks of PNU-140690 (Pharmacia & Upjohn) and Ritonavir (Abbott Laboratories) were prepared as 10 or 30 mM solutions in dimethyl sulfoxide (DMSO), aliquoted, and stored at −80°C. Fresh vials of stock solutions were thawed and diluted in culture medium on the day of assay.

Cells and virus stocks. Human peripheral blood mononuclear cells (PBMC) were obtained from fresh plasmapheresis preparations taken from HIV-1-seronegative donors and prepared by density gradient centrifugation as previously described (6). Paired HIV-1 clinical isolates were obtained from the Aaron Diamond AIDS Research Center, New York University School of Medicine,

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New York, N.Y. These were derived from an HIV-1-seropositive patient before and after ritonavir monotherapy. These isolates were designated 301-1x (pretherapy) and 301-6x (posttherapy). To prepare viral stocks for drug susceptibility testing, aliquots were used to infect phytohemagglutinin p-stimulated PBMC in 25-cm^2 tissue culture flasks containing 10 ml of medium. Cell-free supernatant fluids were harvested 10 days after culture, and titers were determined by the end point dilution method, by using PBMC in 96-well plates as previously described (7).

Antiviral assay. PBMC antiviral assays were set up as described previously (6). The cells were infected by incubation for 2 h with diluted stock virus at multiplicity of infection of 0.01. After 7 days of incubation, culture supernatant showed a mean p24 concentration of 700 ng/ml as measured by an enzyme-linked immunosorbent assay (Coulter Diagnostics, Hialeah, Fla.). The percentages of inhibition of HIV replication on days 5 and 7 of treatment were determined by comparing HIV p24 antigen levels in the supernatant of infected cells treated with inhibitor versus those in supernatant from the control cultures without the compound. The levels of DMSO in the culture medium containing drugs were less than 0.01% for all drug concentrations tested. In previous studies we have shown that up to 0.03% DMSO had no effect on viable cell number or viral p24 production.

Combination studies. The inhibition assays of HIV-1 replication using a two-drug combination of PNU-140690 and ritonavir were set up in duplicate wells in a checkerboard manner, with four concentrations of each drug (4 wells by 4 wells). To assess the antiviral effects of different combination drug treatments, combination indices (CIs) were calculated according to the method described by Chou and Talalay (9) and volumes of synergy or antagonism were assessed according to the method described by Prichard et al. (32). For each molar ratio, three or four data points were analyzed for CIs by the multiple drug effect equation by using the more conservative mutually nonexclusive drug interaction condition. The CI values at various fractional inhibitions (90, 95, and 99%) were used to determine whether the combinations were synergistic (CI $<$ 1), additive (CI = 1), or antagonistic (CI $>$ 1). For these studies, we considered a CI range of 0.90 to 1.10 as nearly additive and 1.10 to 1.20 as weak antagonism in accordance with the suggested guideline of the computer program used. Estimates of CI accuracy were calculated with Monte Carlo simulations (1) and were computed with a new Windows-based software for dose effect analysis called Calcusyn (Biosoft, Cambridge, United Kingdom). The 95% confidence interval at each level of fractional inhibition was calculated according to formula the CI $\pm$ (1.96 $\times$ SD), where SD is the standard deviation and the values CI + (1.96 $\times$ SD) and CI $-$ (1.96 $\times$ SD) correspond to the upper and lower boundaries of the confidence interval, respectively. With the MacSynergy program, the theoretical additive interactions from single-drug treatment groups were plotted as a plane in a three-dimensional graph. The data obtained from combination assays were then compared with predicted values for the additive interaction. Points above the additive plane represent synergistic interactions, while points below the plane represent antagonism. The extent of drug synergy is determined from the volume of the area above the additive plane. According to Prichard et al. (32), values of synergy between 25 and 50 μM^2% are considered minor but significant, whereas values between 50 and 100 μM^2% and $>$100 μM_2% indicate moderate and strong synergy, respectively. The data shown were obtained at the 95% confidence level and were plotted with DeltaGraph software. Separately, a two-sample *t* test at the 95% confidence level was used to compare drug effects between selected treatment groups.

Assay of cytotoxicity. Cytotoxicities of the compounds in uninfected 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 (7). Cytotoxicity assays were performed by incubating uninfected cells in the presence of various concentrations of the drugs tested individually and in combination for 5 days under conditions similar to those for the antiviral assays.

RESULTS

Effects of PNU-140690 or ritonavir alone. We first determined the antiviral effect of PNU-140690 in comparison to that of ritonavir in acutely infected PBMC. Shown in Fig. 1 are the dose responses for PNU-140690 and ritonavir in cultures infected with either a pre-ritonavir therapy isolate (301-1x) or a posttherapy isolate (301-6x). Ritonavir was highly active versus HIV-1 isolate 301-1x (50% inhibitory concentrations [IC_{50}] and IC_{90} [$\pm$standard error {SD}]: 0.035 $\pm$ 0.007 and 0.11 $\pm$ 0.01 μM, respectively) but was about 50-fold less active versus isolate 301-6x (IC_{50} and IC_{90}: 2.13 $\pm$ 0.27 and 5.05 $\pm$ 0.60 μM, respectively). However, both isolates displayed similar sensitivities to PNU-140690 (IC_{50} = 0.075 $\pm$ 0.007 μM and IC_{90} = 0.20 $\pm$ 0.01 μM for isolate 301-1x; IC_{50} = 0.12 $\pm$ 0.03 μM and IC_{90} = 0.33 $\pm$ 0.08 μM for isolate 301-6x). In similar assays, we also evaluated the antiviral activities of two other marketed protease inhibitors, saquinavir and indinavir. Compared to the pretherapy isolate (301-1x), isolate 301-6x was 4.2- and 9.0-fold less sensitive to saquinavir and indinavir, respectively. PNU-140690 showed little toxicity to uninfected PBMC, with 50% cytotoxic concentrations of greater than 30 μM (data not shown) and a selectivity index of $>$400. From these data, concentration ranges of PNU-140690 and ritonavir that would best demonstrate drug interactions in the subsequent combined therapy experiments under similar assay conditions were selected.

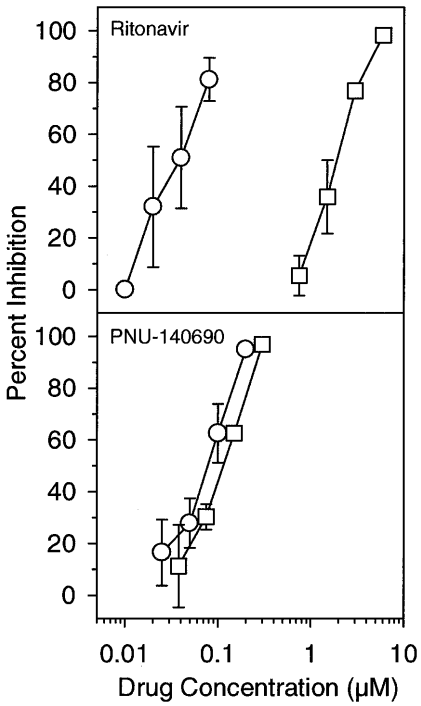

FIG. 1. Dose response of PNU-140690 and ritonavir against ritonavir-sensitive (○) and ritonavir-resistant (□) clinical isolates. Data presented are the means $\pm$ SEs from four experiments.

Effects of PNU-140690 and ritonavir combinations. We studied the effects of combinations of PNU-140690 and ritonavir on HIV-1 replication while monitoring the potential cytotoxicity of these agents to PBMC. Figure 2 shows that over the time course of an acute infection with the ritonavir-sensitive HIV-1 isolate, combination treatment with PNU-140690 and ritonavir at a molar ratio of 2.5:1 was significantly ($P < 0.01$) more effective than treatment with either agent alone. This drug combination ratio reflected the fact that PNU-140690 is about 2.5 times less active than ritonavir on a molar basis, and so the compounds were combined at equivalent activities. Similar time course infection assays using the ritonavir-resistant viral isolate (301-6x) also showed that the combination was significantly more active than either agent alone (Fig. 3). Much higher concentrations of ritonavir (0.75, 1.5, 3.0, and 6.0 μM) were needed in order to show an inhibitory effect against the resistant virus. Consequently, PNU-140690 was used in combination with ritonavir at a 1:20 ratio so that both compounds would be used at equivalent activities.

The results of other drug combination ratios obtained by checkerboard titrations of the two compounds at equivalent activity ranges were plotted with the MacSynergy program (Fig. 4). The results of these experiments using both ritonavir-sensitive (301-1x) and -resistant (301-6x) isolates are summa-

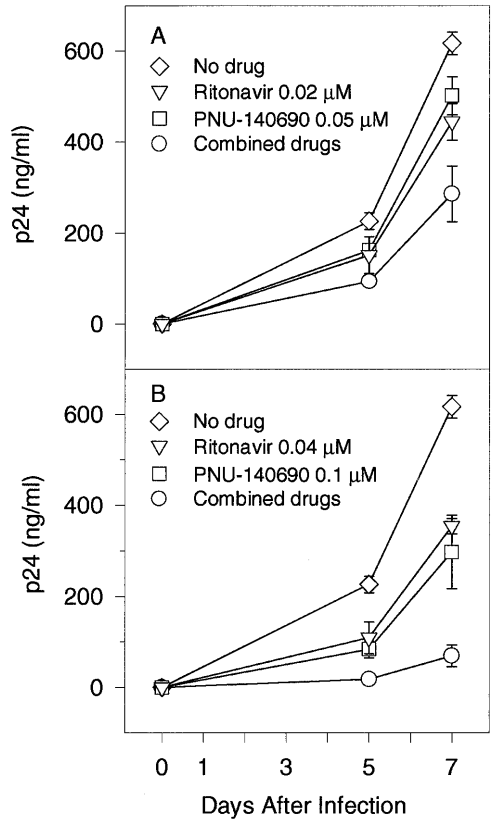

FIG. 2. Inhibition of HIV-1 p24 antigen production in acutely infected PBMC treated with PNU-140690 or ritonavir alone or in two-drug combinations against the ritonavir-sensitive isolate (301-1x). HIV-1 p24 antigen concentrations in culture supernatant were measured on days 5 and 7 postinfection from drug-treated wells and non-drug-treated control wells. Data presented are the means (±SEs) of the results from two experiments (experiments 2 and 3), each with two replicate wells separately infected, treated, and assayed for p24 antigen. Symbols with no error bars indicate a small standard error.

rized in Table 1. In cultures infected with isolate 301-1x, combinations of PNU-140690 and ritonavir in the dose range tested showed synergy volumes suggestive of minor to moderate synergistic effects at the 95% confidence level (Fig. 4; Table 1, experiments 2 and 3). However, little synergistic drug effect was observed when lower concentrations of ritonavir were used (Table 1, experiment 1). In contrast, much greater synergistic drug effects were observed in all experiments using isolate 301-6x-infected cultures (Fig. 4; Table 1, experiments 4, 5, and 6). Importantly, no significant drug antagonism was observed in cultures infected either with the ritonavir-sensitive or -resistant isolates (Table 1).

The differing levels of drug synergy between ritonavir-sensitive and -resistant isolates were unexpected. To confirm this, we also analyzed the data from each of these experiments by the methods of Chou and Talalay (9). For the computation of CIs, the dose effects for each single agent and the combinations were used to make a series of median-effect plots. From these plots, CI values at various combination ratios that allowed the two compounds to be used together in a wide activity range were computed. CIs were calculated at the 90% effective concentration (EC_{90}), EC_{95}, and EC_{99} levels since these are most representative of in vivo therapeutic objectives. Against the ritonavir-sensitive isolate, the drug interactions observed ranged from additive to moderate synergy, depending on the ratios of the two compounds used (Table 2). With ritonavir and PNU-140690 in combination at a 1:5 ratio, CI values suggestive of slight antagonism were seen for the day 5 assays only. Table 3 shows that moderate synergy was observed for all ratios tested in cultures infected with the ritonavir-resistant isolate. Thus, both methods of analysis showed that the combination of PNU-140690 and ritonavir yielded greater synergy versus the ritonavir-resistant than against the ritonavir-sensitive isolate.

With either of these single agents or their combinations, we did not detect any cytotoxicity or antiproliferative activity as measured by MTT reduction assay (data not shown). The data suggested that there was no significant toxicity with the combinations and that the enhanced effects of the combinations are due to specific antiviral activities.

DISCUSSION

To provide a rationale for combination therapy, we investigated the antiviral effect of combining PNU-140690 with ritonavir against ritonavir-sensitive and -resistant clinical isolates. We showed that the combination of PNU-140690 with ritonavir provides an overall moderately synergistic interaction with no significant antagonism. Deminie et al. also reported additive-to-weakly synergistic interactions between an experimental peptidomimetic protease inhibitor, BMS-PI, and saquinavir or indinavir (17). Another study reported that protease inhibitor 141W94 was synergistic when combined with saquinavir and additive when combined with ritonavir or indinavir, but data were not shown (38). Unlike the authors of

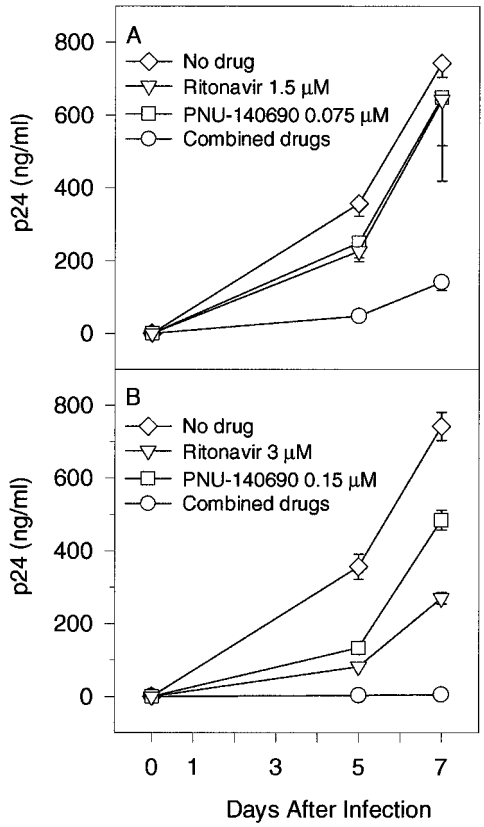

FIG. 3. Inhibition of HIV-1 p24 antigen production in acutely infected PBMC treated with PNU-140690 or ritonavir alone or in two-drug combinations against the ritonavir-resistant isolate (301-6x). HIV-1 p24 antigen concentrations in culture supernatant were measured on days 5 and 7 postinfection from drug-treated and non-drug-treated control wells. Data are as described for Fig. 2.

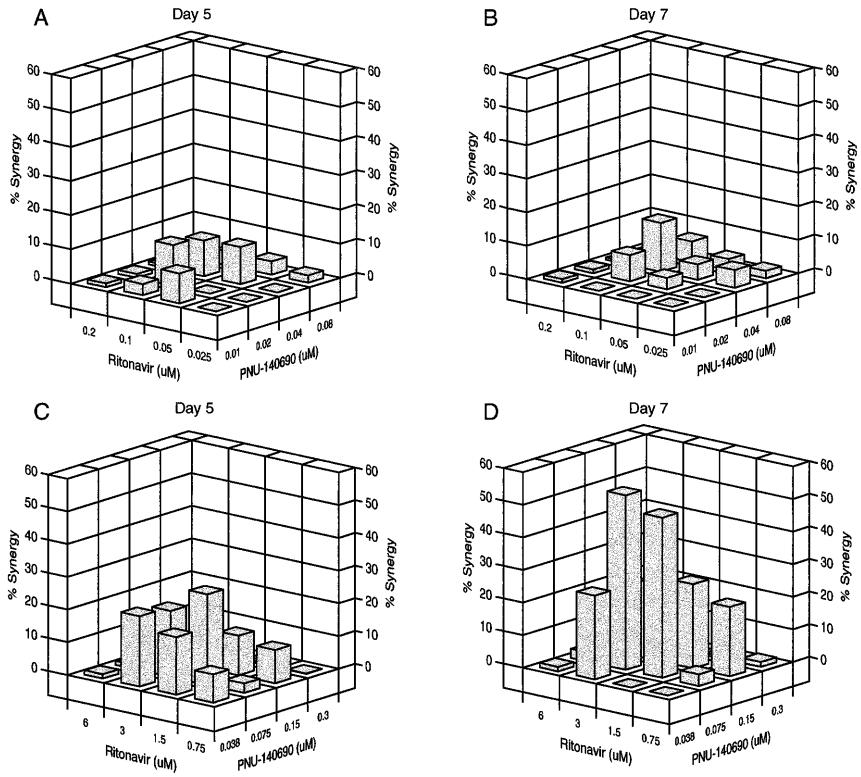

FIG. 4. Drug interaction analysis (MacSynergy) of the combination of PNU-140690 and ritonavir against ritonavir-sensitive (A and B; HIV-1 isolate 301-1x; experiments 2 and 3) or -resistant (C and D; HIV-1 isolate 301-6x; experiments 5 and 6) isolates, 5 or 7 days after acute infection of PBMC. The drug concentrations are labeled on the *x* and *y* axes, and the *z*-axis values are percent synergy values. Statistically significant synergy at the 95 percent confidence level is shown. The height of bars indicates the amount of synergy.

these studies, we have used HIV-1 clinical isolates and have further showed that the combination of two protease inhibitors with nonoverlapping resistance patterns may yield a synergistic effect versus a resistant posttherapy viral isolate.

An unexpected finding was that drug synergy was greater against the ritonavir-resistant isolate than against the drug-sensitive isolate. This discrepancy was confirmed by two different methods of mathematical analysis. Both mathematical methods used showed that at some ratios and effective levels, additivity may exist but that a low-to-moderate level of synergy appears to be the general outcome of combining these two inhibitors. The two methods are not expected to give identical

TABLE 1. Volumes of antiviral synergy and antagonism for two-drug combination regimens of PNU-140690 with ritonavir against HIV-1 replication in PBMC

Expt	Day after infection	Virus isolate	Concns (μM) of:		Volume ($\mu M^2\%$) at 95% confidence level[a]	
			Ritonavir	PNU-140690	Synergy	Antagonism
1	5	301-1x	0.008, 0.015, 0.03, 0.06	0.025, 0.05, 0.1, 0.2	18.1	15.9
2	5	301-1x	0.01, 0.02, 0.04, 0.08	0.025, 0.05, 0.1, 0.2	96.6	0
	7		0.01, 0.02, 0.04, 0.08	0.025, 0.05, 0.1, 0.2	66.2	0
3	5	301-1x	0.01, 0.02, 0.04, 0.08	0.025, 0.05, 0.1, 0.2	25.3	13.5
	7		0.01, 0.02, 0.04, 0.08	0.025, 0.05, 0.1, 0.2	34.7	1.6
4	5	301-6x	0.375, 0.75, 1.5, 3.0	0.075, 0.15, 0.3, 0.6	216.4	<0.1
5	5	301-6x	0.75, 1.5, 3.0, 6.0	0.038, 0.075, 0.15, 0.3	164.6	0.1
	7		0.75, 1.5, 3.0, 6.0	0.038, 0.075, 0.15, 0.3	186.3	<0.1
6	5	301-6x	0.75, 1.5, 3.0, 6.0	0.038, 0.075, 0.15, 0.3	99.5	0.1
	7		0.75, 1.5, 3.0, 6.0	0.038, 0.075, 0.15, 0.3	232.6	0

[a] Volumes of synergy and antagonism were computed from a surface area plot of percent drug interaction versus drug concentrations by using the MacSynergy II program. According to the program, values of synergy or antagonism of $<25\ \mu M^2\%$ at the 95% confidence level should be regarded as insignificant. Values of 25 to 50 $\mu M^2\%$, 50 to 100 $\mu M^2\%$, and over 100 $\mu M^2\%$ indicate minor, moderate, and strong synergy, respectively.

TABLE 2. CI values for two-drug combination regimens of U-140690 with ritonavir against the ritonavir-sensitive isolate

Treatment and drug ratio[a]	Expt no. (day)	Median-effect plot parameter values[b]			CI[c] ± SD at fractional inhibition of:		
		m	D_m	*r*	0.90	0.95	0.99
Ritonavir	1 (5)	1.58 ± 0.27	0.020	0.98			
PNU-140690		2.84 ± 0.84	0.066	0.97			
Ritonavir/690							
1:1.7		2.67 ± 0.30	0.045	0.99	0.93 ± 0.08	0.82 ± 0.09	0.63 ± 0.11
1:3.3		2.96 ± 0.21	0.046	0.98	0.99 ± 0.18	0.93 ± 0.20	0.83 ± 0.24
1:6.6		2.87 ± 0.19	0.06	0.99	1.03 ± 0.21	0.95 ± 0.22	0.83 ± 0.24
Ritonavir	2 and 3[d] (5)	2.56 ± 0.88	0.036	0.99			
PNU-140690		2.18 ± 0.63	0.072	0.97			
Ritonavir/690							
1:1.25		3.22 ± 0.39	0.06	0.99	0.71 ± 0.18	0.55 ± 0.17	0.34 ± 0.47
1:2.5		3.32 ± 0.19	0.06	1.00	1.04 ± 0.23	0.95 ± 0.24	0.77 ± 0.30
1:5.0		2.87 ± 0.19	0.06	0.99	1.17 ± 0.27	1.18 ± 0.35	1.2 ± 0.30
Ritanovir	2 and 3[d] (7)	2.07 ± 0.38	0.04	0.97			
PNU-140690		2.50 ± 0.71	0.088	0.98			
Ritonavir/690							
1:1.25		3.03 ± 0.73	0.07	0.97	0.74 ± 0.26	0.64 ± 0.26	0.6 ± 0.19
1:2.5		3.49 ± 0.48	0.06	0.98	0.90 ± 0.20	0.82 ± 0.22	0.66 ± 0.26
1:5.0		3.43 ± 0.19	0.07	0.99	1.04 ± 0.21	0.95 ± 0.26	0.91 ± 0.29

[a] Ritonavir/690, combination of ritonavir and PNU-140690. The drug ratios are the ratios of the concentrations of ritonavir to those of PNU-140690.
[b] *m* is the slope ± standard error, D_m is the median-effect concentration, and *r* is the correlation coefficient.
[c] CI values of <1, 1, and >1 indicate synergy, additivity, and antagonism, respectively. CI values were determined for a mutually nonexclusive interaction. Confidence intervals were calculated by the Monte Carlo technique with $n = 250$ to 500, where n is the number of statistical computations.
[d] CI values were computed from mean percent inhibition values from two experiments.

interpretations, because the MacSynergy plots represented outcomes from all wells of a checkerboard titration and thus included many drug ratios, while CIs were determined at selected drug ratios. We presented CI values for higher levels of antiviral effects (EC_{90}, EC_{95}, and EC_{99}) because we believe that these are more representative of in vivo therapeutic objectives. For antimicrobial agents, synergism at high effect levels is clinically more relevant than synergism at low effect levels. In addition, ineffective or suboptimal levels of HIV-1 protease inhibitors have been shown to result in earlier and higher incidence of resistance (13, 28, 34). Therefore, it is not surprising that current thinking favors high-dose drug regimens that rapidly achieve and maintain highly suppressive plasma concentrations in order to control viral resistance (28, 34).

The mechanism of drug synergy involving two protease in-

TABLE 3. CI values for two-drug combination regimens of U-140690 with ritonavir against the ritonavir-resistant isolate

Treatment and drug ratio[a]	Expt no. (day)	Median-effect plot parameter values[b]			CI[c] ± 1.96 SD at fractional inhibition of:		
		m	D_m	*r*	0.90	0.95	0.99
Ritonavir	4 (5)	3.10 ± 0.67	2.65	0.96			
PNU-140690		3.38 ± 0.11	0.120	1.00			
Ritonavir/690							
2.5:1		3.66 ± 1.06	0.34	0.96	0.90 ± 0.22	0.90 ± 0.21	0.88 ± 0.34
5:1		3.11 ± 0.47	0.41	0.98	0.79 ± 0.14	0.81 ± 0.14	0.83 ± 0.17
10:1		3.30 ± 0.16	0.56	1.00	0.78 ± 0.03	0.80 ± 0.32	0.84 ± 0.04
Ritonavir	5 and 6[d] (5)	3.17 ± 0.22	1.88	0.99			
PNU-149690		2.52 ± 0.60	0.107	0.99			
Ritonavir/690							
10:1		3.93 ± 0.39	0.73	1.00	0.95 ± 0.13	0.90 ± 0.13	0.77 ± 0.18
20:1		3.54 ± 0.42	0.95	0.99	1.01 ± 0.15	0.96 ± 0.06	0.87 ± 0.17
40:1		3.99 ± 0.521	1.36	0.99	0.89 ± 0.10	0.82 ± 0.09	0.70 ± 0.11
Ritonavir	5 and 6[d] (7)	3.21 ± 0.63	2.38	0.97			
PNU-140690		2.31 ± 0.87	0.120	0.95			
Ritonavir/690							
10:1		4.52 ± 0.13	0.83	1.00	0.73 ± 0.13	0.64 ± 0.20	0.53 ± 0.22
20:1		3.94 ± 0.29	1.02	0.99	0.82 ± 0.24	0.75 ± 0.24	0.62 ± 0.21
40:1		4.80 ± 0.413	1.63	1.00	0.64 ± 0.14	0.62 ± 0.07	0.58 ± 0.07

[a] For definitions of ritonavir/690 and the drug ratios, see footnote *a* to Table 2.
[b] Median-effect plot parameters are as defined in footnote *b* to Table 2.
[c] See footnote *c* to Table 2.
[d] See footnote *d* to Table 2.

hibitors is not clear. However, it has been reported that the combination of two inhibitors with similar enzyme targets may produce a more thorough blockage resulting in additive-to-synergistic effects (35). The resistant viral isolate used in this study may contain heterogeneous viral populations with differing levels of sensitivity to ritonavir. Thus, we observed a 50-fold increase in the concentration of ritonavir necessary to achieve the same level of viral inhibition. However, in the presence of drug mixtures, the virus variants least sensitive to ritonavir would be readily inhibited by PNU-140690, thus exposing the more sensitive variants to a greater concentration of ritonavir. This is an important point because a greatly elevated concentration of ritonavir was used in cultures infected with the resistant isolate. It is possible that the net effect of these drugs in combination may serve to reduce or abolish the impact of drug resistance in viral cultures. We suggest that such a scenario may partly explain why the combination of PNU-140690 and ritonavir showed greater synergy against the resistant isolate than against the sensitive isolate. A related phenomenon was reported by other investigators who showed that the combination of zidovudine (AZT) with another nucleoside reverse transcriptase inhibitor was synergistic against AZT-resistant virus (18, 22). Their proposed mechanism is that AZT inhibits the sensitive viral population in the mixture of viruses in a clinical isolate.

In addition to resulting from antiviral interactions, synergy may also result from the pharmacologic interaction of drugs in cell cultures. In cultures infected with the drug-sensitive isolate, very low levels of each drug were used and drug concentrations in the mixtures are not likely to significantly affect the extent of drug binding to proteins. However, 50- to 75-fold-higher concentrations of ritonavir were used in cultures infected with the ritonavir-resistant isolate. These high drug concentrations may saturate protein binding leading to greater free-drug levels of both agents in the culture medium. Therefore, reduced protein binding may also explain the finding of greater synergy in cultures infected with the resistant isolate than in those infected with the drug-sensitive isolate. Other published studies have shown that synergy may result from combining antiviral agents with compounds that enhance drug uptake or that increase the level of an active metabolite in cultured cells (3, 19, 23). Ritonavir and PNU-140690 are known to bind to human plasma albumin and alpha-1 acid glycoprotein (24, 31). However, it is not known whether these drugs exhibit competitive binding in mixtures or whether one drug displaces the binding of the other. Clearly, further studies are needed to further dissect the respective roles of protein binding and antiviral interactions in the drug synergy between these agents.

The combination of ritonavir and PNU-140690 showed an overall effect of weak-to-moderate synergy against the ritonavir-sensitive isolate. For day 5 assays, one of the combination ratios (1:5) showed a mean CI value ($\pm$ SD) of 1.19 $\pm$ 0.04 at the 90 to 99% inhibition level, which was suggestive of weak antagonism. However, we believe that this CI level is more reflective of an additive effect because we used the more conservative mutually nonexclusive analysis, which may overestimate the amount of antagonism (8). Mutual exclusivity is used when drugs 1 and 2 yield parallel lines in the median-effect plot and the mixture of the two drugs also yields a parallel line. However, in our studies and in most other complex biological assays, drugs 1 and 2 do not yield parallel lines in the median-effect plots. Consequently, it is difficult to determine exclusivity. Our interpretation is supported by two other observations: (i) the same assays showed synergy for all test ratios (1:1.25, 1:2.5, and 1:5) on day 7 of culture, and (ii) analysis using MacSynergy showed no significant antagonism for all combinations of ritonavir and PNU-140690 studied.

The main conclusion of this study is that combinations of PNU-140690 and ritonavir gave additive-to-synergistic effects and yielded no significant antagonism. By using the pre- and posttherapy HIV-1 isolates, we clearly showed that PNU-140690 retains its activity versus the ritonavir-resistant viral isolate. This is an important property for PNU-140690 and is clinically relevant because several other ritonavir-resistant isolates were also sensitive to PNU-140690 (31). In comparison, both saquinavir and indinavir were more affected by ritonavir resistance, showing four- and ninefold increases in inhibitory concentrations, respectively. For isolate 301-6x, the amino acid substitutions that confer resistance to ritonavir include the following: L10I, K20R, M36I, I54V, I62V, L63A, A71V, V82A, and L90M. Apart from not sharing cross-resistance with ritonavir and indinavir, the activity of PNU-140690 is relatively unaffected by high concentrations of human plasma proteins (31). In a preclinical evaluation, excellent oral bioavailability of PNU-140690 in two animal species was demonstrated (39). Preliminary results from a recent clinical study showed that antivirally effective blood levels of PNU-140690 can be attained in humans (3a). Although much higher levels of ritonavir (up to 6 μM) were used in our assays using the resistant virus, this is still below the level that can be attained in humans (26). Thus, the concentrations of the drugs we employed to show in vitro synergy are lower than the plasma concentration that can be achieved in patients. These findings suggest that PNU-140690 is potentially a good candidate for combination trials with ritonavir or another protease inhibitor that does not share cross-resistance.

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