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In Vitro Inhibition of Human Immunodeficiency Virus (HIV) Type 1 Replication by C_2 Symmetry-Based HIV Protease Inhibitors as Single Agents or in Combinations

SEIJI KAGEYAMA,[1] JOHN N. WEINSTEIN,[2] TAKUMA SHIRASAKA,[1] DALE J. KEMPF,[3] DANIEL W. NORBECK,[3] JACOB J. PLATTNER,[3] JOHN ERICKSON,[4] AND HIROAKI MITSUYA[1]*

Experimental Retrovirology Section, Medicine Branch,[1] and Theoretical Immunology Section, Laboratory of Mathematical Biology,[2] National Cancer Institute, Bethesda, Maryland 20892; Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, Illinois 60064[3]; and Structural Biochemistry Program, PRI/Dyn Corp., National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201[4]

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C_2 symmetry-based human immunodeficiency virus (HIV) protease inhibitors were examined in vitro as single agents or in combination with 3′-azido-2′,3′-dideoxythymidine (AZT) or 2′,3′-dideoxyinosine for activity against HIV type 1 (HIV-1). Ten C_2 symmetry-based or pseudo-C_2 symmetry-based HIV protease inhibitors were active against a laboratory strain (HIV-1_{IIIB}) in the HIV-1 cytopathic effect inhibition assay. Three inhibitors, A75925, A76928, and A77003, selected to represent a range of aqueous solubility and antiviral activity, were active against four different HIV-1 strains tested. These three inhibitors exhibited a significant inhibition of the cytopathic effect of HIV-1 against the CD4$^+$ ATH8 cell line, with 90% inhibitory concentrations ranging from 0.1 to 4 μM. Cellular toxicity was negligible at up to 20 μM. Furthermore, they completely inhibited the replication of monocytotropic strain HIV-$1_{Ba\text{-}L}$ in purified monocytes and macrophages at 0.75 to 2 μM. Potent inhibitory activity against a primary HIV-1 isolate and an AZT-resistant HIV-1 variant was also observed for all three inhibitors in phytohemagglutinin-activated peripheral blood mononuclear cells. When these three HIV protease inhibitors and AZT or 2′,3′-dideoxyinosine were used in combinations against a primary HIV isolate in phytohemagglutinin-activated peripheral blood mononuclear cells and the results were analyzed with the COMBO program package, their antiviral activities were identified to be synergistic in some cases and additive in others. The present data warrant further investigations of these compounds as potential antiviral agents for the therapy of HIV infections.

In the past 7 years, a number of potentially useful approaches for the therapy of AIDS and its related diseases have emerged (21, 22). The use of the family of synthetic 2′,3′-dideoxynucleoside analogs, including 3′-azido-2′,3′-dideoxythymidine (AZT, or zidovudine) (7, 20, 30), 2′,3′-dideoxycytidine (18, 33), and 2′,3′-dideoxyinosine (ddI, or didanosine) (4, 13, 19, 32), as antiretroviral agents is one such approach. These 2′,3′-dideoxynucleoside analogs are believed to exert an antiretroviral effect by inhibiting human immunodeficiency virus (HIV) reverse transcriptase activity. A late stage in the replication of HIV involves crucial proteolytic processing of viral precursor proteins by HIV protease. This process is required for the maturation of newly assembled virus particles into infectious virions. Thus, HIV protease also represents a virus-specific target for the therapy of HIV infections (11, 12). On the basis of these premises, a number of substrate-based inhibitors have recently been shown to be active against HIV (1, 16, 17, 24, 28). Furthermore, the C_2 symmetrical homodimeric structure of HIV protease has inspired the design of unique, symmetry-based inhibitors (6, 10). However, the lower aqueous solubility of some HIV protease inhibitors may pose difficulties for their application to the experimental therapy of HIV infections (1, 6, 10, 16, 17, 24, 28).

In the current study, we assessed the in vitro antiretroviral activity of 10 C_2 symmetry-based or pseudo-C_2 symmetry-based HIV protease inhibitors and tested three selected compounds, including A77003, with considerable aqueous solubility against four different HIV type 1 (HIV-1) strains in various target cells. We also investigated the effect of combinations of the HIV protease inhibitors with AZT or ddI in vitro.

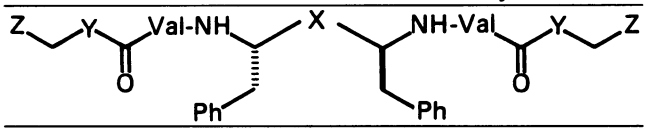
MATERIALS AND METHODS

Reagents. The 10 C_2 symmetry-based and pseudo-C_2 symmetry-based HIV protease inhibitors tested in this study were synthesized as previously described (9, 10). The structures of these 10 compounds are illustrated in Table 1. ddI was provided by the Developmental Therapeutic Program, Division of Cancer Treatment, National Cancer Institute, and AZT was purchased from Sigma Chemical Co., St. Louis, Mo.

Viruses and cells. HIV-1 was pelleted by ultracentrifugation from the culture supernatants of HIV-1_{IIIB} (also known as HIV_{LAI})-producing H9 cells and was prepared to contain 7.39×10^{10} virus particles per ml (23). HIV-$1_{Ba\text{-}L}$, a monocytotropic HIV-1 strain which was originally isolated from a sample of lung tissue from a patient with an HIV-1 infection (8), was propagated in purified normal fresh peripheral monocytes and macrophages (M/M). The supernatant of M/M cultures was collected and used as a source of infectious monocytotropic virus as previously described by Gartner et al. (8). Two clinical HIV-1 strains, HIV-1_{ERS103} and HIV-1_{ERS205}, were isolated by coculturing peripheral blood

* Corresponding author.

TABLE 1. Structures of C_2 symmetry-based HIV protease inhibitors examined in the current study[a]



HIV protease inhibitor	X	Y	Z
A75925	(*R*,*R*)-CH(OH)-CH(OH)	O	Phenyl
A77212	(*R*,*S*)-CH(OH)-CH(OH)	O	2-Pyridyl
A76890	(*S*,*S*)-CH(OH)-CH(OH)	O	2-Pyridyl
A76928	(*S*,*S*)-CH(OH)-CH(OH)	NCH_3	2-Pyridyl
A77003	(*R*,*S*)-CH(OH)-CH(OH)	NCH_3	2-Pyridyl
A76264	(*R*,*R*)-CH(OH)-CH(OH)	O	3-Pyridyl
A77002	(*R*,*R*)-CH(OH)-CH(OH)	O	4-Pyridyl
A76792	(*R*,*R*)-CH(OH)-CH(OH)	O	2-Pyridyl
A76889	(*R*,*R*)-CH(OH)-CH(OH)	NCH_3	2-Pyridyl
A75912	CH(OH)	O	2-Pyridyl

[a] Ph, phenyl.

mononuclear cells (PBM) from two different patients with AIDS with unfractionated phytohemagglutinin-activated PBM (PHA-PBM) from normal seronegative individuals. In brief, 10^6 PBM obtained from patients with AIDS were cultured with an equal number of PHA-PBM in the presence of exogenous interleukin-2 (50 U/ml; Amgen, Thousand Oaks, Calif.), and the culture supernatants were collected on day 7 of culturing.

The 50% tissue culture infective dose ($TCID_{50}$) per milliliter of cell-free HIV-1 preparations was determined by an endpoint titration method as previously described by Leland and French (15). A cloned $CD4^+$ T-cell line (ATH8) (19, 20) was used as a target cell line for infection by HIV-1, whereas M/M and PHA-PBM served as target cells for HIV-$1_{Ba\text{-}L}$ and clinical HIV-1 isolates, respectively.

HIV cytopathic effect inhibition assay. The HIV cytopathic effect inhibition assay was performed as previously described (19, 20). In brief, target $CD4^+$ T cells (ATH8) (2×10^5) were exposed to 3,200 or 1,600 $TCID_{50}$s of HIV-1 for 1 h, resuspended in 2 ml of fresh complete medium (RPMI 1640 supplemented with 4 mM L-glutamine, 15% undialyzed and heat-inactivated fetal calf serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml) containing 15% (vol/vol) interleukin-2 (lectin depleted; Advanced Biotechnologies Inc., Silver Spring, Md.) and 50 U of recombinant interleukin-2 (Amgen) per ml, and incubated at 37°C in 5% CO_2-containing humidified air. Control cells were treated similarly but were not exposed to the virus. At various times, viable cells were counted in a hemacytometer under a microscope by the trypan blue dye exclusion method. Data were subjected to further analysis only when the number of viable ATH8 cells exposed to the virus and cultured in the absence of drugs was less than 10% the number of control, non-virus-exposed, non-drug-exposed cells.

Determination of HIV-1 Gag protein production by PHA-PBM or M/M. HIV-1 Gag protein production by PHA-PBM or M/M was determined as previously described (26). In brief, target PHA-PBM (2×10^6) were preincubated with drugs for 2 h and exposed to 50 and 100 $TCID_{50}$s of clinical isolates HIV-1_{ERS103} and HIV-1_{ERS205}, respectively. PHA-PBM were cultured in 2 ml of culture medium in the presence or absence of drugs. M/M (10^6) were exposed to drugs for 24 h, exposed to 20 $TCID_{50}$s of HIV-$1_{Ba\text{-}L}$, and resuspended in 1 ml of culture medium in the presence or absence of drugs. Every 4 to 5 days, 50% of the culture medium for PHA-PBM or 90% of the culture medium for M/M was replaced with an equal amount of fresh medium. Cells were continuously exposed to the same concentrations of each drug. The amounts of Gag protein in M/M and PHA-PBM cultures were determined by a radioimmunoassay (RIA; Du Pont, NEN Research Products, Boston, Mass.).

Analysis of drug interactions. Drug interactions were analyzed on a personal computer with the COMBO program package, which uses the MLAB environment (Civilized Software, Inc., Bethesda, Md.). The methods are described in detail elsewhere (2, 3, 29). In brief, the data were fitted by iteratively reweighted nonlinear least-squares regression to a "robust potentiation" model defined by the implicit equation:

$$1 = \{(1/z - 1)^{-1/B_1}(C_1/IC_{50_1})[1 + (PI_2C_2)^{BP_2}]^{\pm 1}\} + \{(1/z - 1)^{-1/B_2}(C_2/IC_{50_2})[1 + (PI_1C_1)^{BP_1}]^{\pm 1}\}$$

This equation is equivalent to the equation in reference 2 but parameterized in terms of PIs rather than PC50s. $z = (y - d)/(a - d)$ is the normalized effect, where y, the measured p24 level in natural units, a, the measured p24 level in the absence of drug, and d, the measured p24 level at indefinitely high drug levels, define a surface over C_1 and C_2. C_1 and C_2 are the concentrations of the two drugs. IC_{50_1} and IC_{50_2} are the 50% effective concentrations of the two drugs used separately. B_1 and B_2 are the corresponding 50% effect slopes. PI_1 and PI_2 are the potentiation indices for drug 1 acting on drug 2 and drug 2 acting on drug 1, respectively. PI = 0 indicates additivity; PI > 0 indicates potentiation when the exponent outside the square brackets is +1 and antagonism when it is −1; and PI = +1 indicates that the drug is as effective through its potentiating activity as it is through its intrinsic activity. BP_1 and BP_2 are the corresponding slopes for the potentiation effects. For the present data, it is sufficient to set $BP_1 = BP_2 = 1$. For the potentiation of drug 1 by drug 2, PI_1 was set at zero, and for the potentiation of drug 2 by drug 1, PI_2 was set at zero. Weights for the fitting procedure were determined from the error structure of the data set itself by use of a Gaussian kernel windowing technique based on estimated responses (2, 3, 29). Extensive simulations indicated that all parameter estimates were approximately normally distributed and in agreement with the asymptotic estimates produced by the MLAB curve fitter. Hence, the normal error estimates and confidence limits are presented.

RESULTS

In vitro antiretroviral activity of C_2 symmetry-based HIV protease inhibitors against HIV-1. We first tested 10 C_2 symmetry-based and pseudo-C_2 symmetry-based HIV protease inhibitors for their in vitro antiviral activity against a laboratory HIV-1 strain, HIV-1_{IIIB}, in the HIV-1 cytopathic effect inhibition assay with HIV-1-sensitive $CD4^+$ ATH8 cells as target cells. All inhibitors tested in this system were found to be active against HIV-1_{IIIB} (Table 2). Three compounds, A75925, A77212, and A76890, were most potent on a molar basis. The 90% inhibitory concentration (IC_{90}) of these most potent compounds was 0.1 μM. Other compounds were also active, and their IC_{90}s ranged between 0.8 and 8.5 μM. A76264 was least active, and its IC_{90} was >10 μM.

Three compounds were selected for further studies on the basis of antiviral activity and aqueous solubility: the proto-

TABLE 2. In vitro anti-HIV activity of C_2 symmetry-based HIV protease inhibitors as assessed by the HIV cytopathic effect inhibition assay with ATH8 cells[a]

Protease inhibitor	Concn (μM)[b]	% Protection	IC_{90} (μM)	TC_{50} (μM)	Solubility at pH 7.4 (μg/ml)
A75925	0.01, 0.05, 0.1	0, 45, 94	0.1	34	<0.03
A77212	0.01, 0.05, 0.1	4, 33, 91	0.1	>0.1[c]	2.4
A76890	0.01, 0.05, 0.1	0, 18, 91	0.1	>0.1[c]	0.21
A76792	0.1, 1, 10	48, 100, 100	0.8	>10[c]	5.0
A76928	0.05, 0.1, 1, 10	14, 30, 100, 100	0.9	>100	3.6
A77003	0.05, 0.1, 1, 10	16, 23, 89, 92	4.0	>100	197
A77002	0.1, 1, 10	2, 83, 94	6.7	>10[c]	55
A76889	0.1, 1, 10	8, 41, 100	8.5	>10[c]	225
A75912	0.1, 1, 10	0, 45, 100	8.4	>10[c]	51
A76264	0.1, 1, 10	5, 89, 72	>10	>10[c]	31

[a] The percent protection was determined by the following formula: 100 × [(number of total viable cells exposed to HIV-1_{IIIB} and cultured in the presence of the compound − number of total viable cells exposed to HIV-1_{IIIB} and cultured in the absence of the compound/number of viable cells cultured alone − number of viable cells exposed to HIV-1_{IIIB} and cultured in the absence of the compound)]. TC_{50}, drug concentration that inhibited cellular growth by 50%. TC_{50}s were determined by the trypan blue exclusion method. Solubility in phosphate buffer (pH 7.4) was determined as previously described (9).

[b] The concentrations listed represent the ones used for the determination of the anti-HIV activity of each compound. The order of the concentrations correspond to the order of the percent protection values.

[c] The highest concentration used for the assay was 0.1 or 10 μM, and the TC_{50} was not further defined in the current study.

typic inhibitor A75925, which had the most potent antiviral activity and very poor solubility; A76928, which had potent antiviral activity and modest solubility; and A77003, which had potent antiviral activity and improved solubility. The toxicity of these three HIV protease inhibitors was negligible or marginal even at 20 μM. The antiviral activity of these compounds was also tested against monocytotropic HIV-1 in the culture system with M/M as target cells. Following exposure to HIV-1_{Ba-L}, in the absence of drugs, M/M began to produce a detectable amount of HIV-1 by day 12 of culturing, and by day 26, they produced up to 25 ng of p24 Gag protein per ml, as assessed by an RIA. However, when M/M were cultured in the presence of 0.75 μM A77003, 0.75 μM A76928, or 2 μM A75925, the production of p24 Gag protein was suppressed to below detectable limits (Fig. 1).

Three compounds, A75925, A76928, and A77003, can suppress the replication of clinical isolates of HIV-1 in vitro. We next asked whether any of the three selected C_2 symmetry-based HIV protease inhibitors could suppress the replication of clinical isolates of HIV-1 in PHA-PBM in vitro. PHA-PBM were preincubated with drugs for 2 h, exposed to HIV-1_{ERS103} or HIV-1_{ERS205}, and cultured in the presence or absence of drugs (Fig. 2). In a representative experiment, 0.05 to 0.1 μM A75925 brought about marginal to moderate levels of suppression of HIV-1_{ERS205}; however, at 0.2 μM, the compound brought about complete suppression of HIV-1 replication. It is noteworthy that the precipitous nature of this dose response was also observed when A77003 and

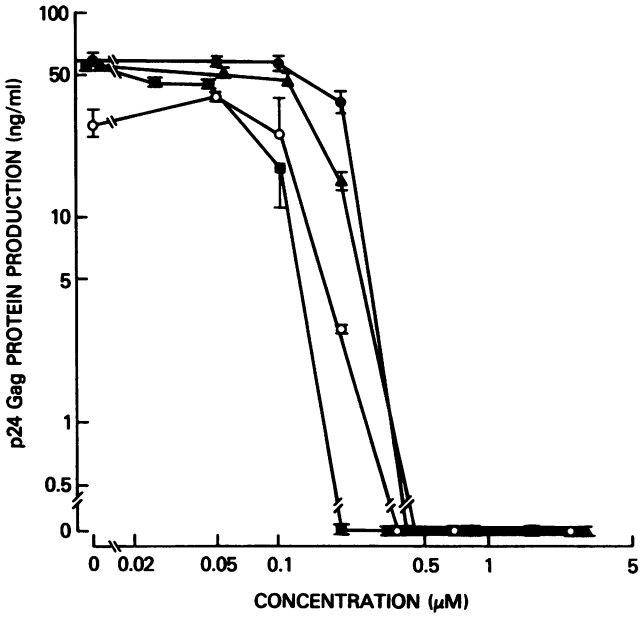


FIG. 2. Suppression of HIV-1 replication in PHA-PBM by HIV protease inhibitors A75925, A76928, and A77003 as single agents. Normal PHA-PBM were exposed to the primary HIV-1 isolates HIV-1_{ERS205} (100 $TCID_{50}$s) and HIV-1_{ERS103} (50 $TCID_{50}$s) and cultured in the presence of various concentrations of each HIV protease inhibitor. The amount of Gag protein released in the culture supernatant was determined by an RIA. Symbols: ■, A75925 against HIV-1_{ERS205}; ▲, A76928 against HIV-1_{ERS205}; ●, A77003 against HIV-1_{ERS205}; ○, A77003 against HIV-1_{ERS103}.

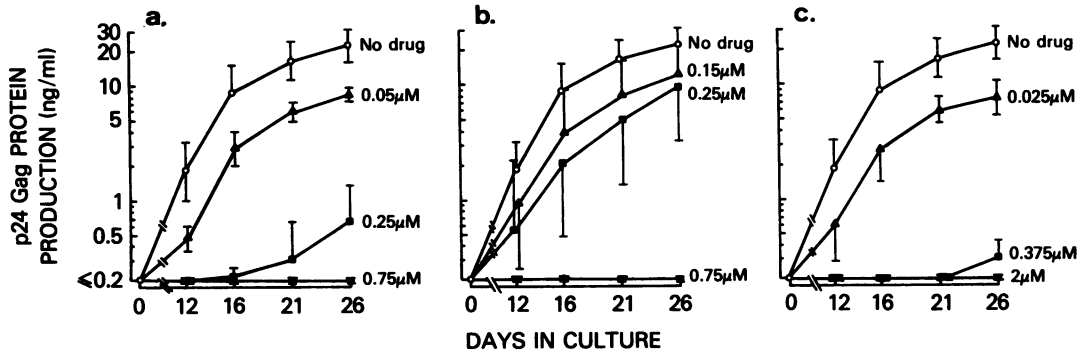


FIG. 1. Suppression of HIV-1 replication in M/M by HIV protease inhibitors A77003 (a), A76928 (b), and A75925 (c). Purified M/M (10^6) were preincubated with various concentrations of each C_2 symmetry-based HIV protease inhibitor, exposed to M/M-tropic HIV-1_{Ba-L} (20 $TCID_{50}$s), and cultured with the same concentrations of each drug for an additional 26 days. The amount of Gag protein released in the culture supernatant was determined by an RIA.

TABLE 3. In vitro antiretroviral activity of the combination of A75925 and AZT against HIV-1_{ERS205} as assayed in PHA-PBM[a]

AZT concn (μM)	p24 Gag protein production in the presence of the following A75925 concn:				
	0 μM	0.025 μM	0.05 μM	0.1 μM	0.2 μM
0	57.4, 55.7, 55.9, 50.0, 55.1, 51.2, 57.9 (0)	45.7, 45.4 (17)	44.6, 44.3 (19)	24.1, 12.8 (68)	0, 0 (100)
0.02	37.8, 46.2 (24)	34.8, 35.5 (36)	20.1, 22.7 (61)	1.4, 1.9 (97)	0, 0 (100)
0.08	28.7, 18.2 (58)	7.6, 2.0 (93)	0, 3.9 (99)	0, 0 (100)	0, 0 (100)
0.32	0, 4.2 (99)	0, 0 (100)	0, 0 (100)	0, 0 (100)	0, 0 (100)
1.28	0, 0 (100)	0, 0 (100)	0, 0 (100)	0, 0 (100)	0, 0 (100)

[a] p24 Gag protein production (in nanograms per milliliter) was determined by an RIA (Materials and Methods). Each combination was tested in duplicate. Values for cultures without drugs were determined in heptaplicate. Values in parentheses represent percent inhibition, relative to the geometric mean p24 Gag protein production (54.7 ng/ml) determined in heptaplicate.

A76928 were tested against HIV-1_{ERS205}. The mechanism of this precipitous dose response is not clear.

A77003 is active against an AZT-resistant HIV-1 clinical isolate in vitro. Larder and coworkers and Rooke and coworkers reported in 1989 that AZT-resistant HIV-1 strains were isolated from patients with AIDS receiving long-term AZT therapy (14, 25). A77003 was tested for its activity against an AZT-resistant clinical HIV-1 isolate, HIV-1_{ERS103}, which was isolated from the PBM of a patient with AIDS who had received AZT therapy for 3 years and 5 months, by coculturing the patient's cells with PHA-PBM from a normal volunteer (27). The IC_{50} and IC_{90} of AZT against HIV-1_{ERS103} were quite high (2.2 and 23.0 μM, respectively), while those against an AZT-susceptible HIV-1 clinical isolate, HIV-1_{ERS205}, were as low as 0.06 and 0.27 μM, respectively. However, A77003 showed virtually equipotent antiviral activity against both AZT-resistant and -susceptible HIV-1 isolates. The IC_{50} and IC_{90} of A77003 against HIV-1_{ERS103} were 0.13 and 0.2 μM, respectively, and those against HIV-1_{ERS205} were 0.21 and 0.36 μM, respectively.

Antiviral activity of A75925, A77003, and A76928 combined with AZT or ddI. We next asked whether the three selected drugs, A75925, A77003, and A76928, could act in synergy against a clinical isolate of HIV-1 when combined with AZT or ddI. When PBM were cultured in the presence of 0.08 μM AZT, the replication of HIV-1_{ERS205} was suppressed by about 50% (Table 3). A75925 at 0.05 μM exerted only a marginal antiviral effect. However, when 0.08 μM AZT and 0.05 μM A75925 were combined, virtually complete suppression was observed. The data in Table 3 were further analyzed for possible synergy by use of the COMBO program package (2, 3, 29). Table 4 shows the parameter estimates resulting from COMBO analyses of six different experiments. Fits were done in two ways: (i) considering the protease inhibitor as the potentiator and (ii) considering the dideoxynucleoside as the potentiator. Synergy corresponds to mutual potentiation. Of most interest in Table 4 is the dimensionless potentiation index, PI_i. PI_1 and PI_2 are the potentiation indices for drug 1 acting on drug 2 and drug 2 acting on drug 1, respectively. PI = 0 indicates additivity; PI > 0 indicates potentiation (or antagonism when the entry in Table 4 is enclosed in parentheses); and PI = +1 indicates that the drug is as effective through its potentiating activity as it is through its intrinsic activity. A useful derived parameter is PC_{50_i}, defined as IC_{50_i}/PI_i, the concentration of drug i required to increase the apparent potency of the other drug (i.e., decrease its apparent IC_{50_i}) by a factor of 2 (beyond what would be expected on the basis of the intrinsic activity of drug i). The lower the value of PC_{50}, the stronger the potentiation; additivity corresponds to PC_{50_1} and PC_{50_2} approaching infinity.

Figure 3a illustrates graphically the relationships for the potentiation of A75925 by AZT. As assessed by COMBO analyses, AZT was found to be synergistic with A75925 ($P < 0.001$ for PI_1 and $P < 0.001$ for PI_2). This was also the case when A76928 was combined with AZT ($P = 0.013$ for PI_1 and $P = 0.025$ for PI_2) (Table 4). There was also a strong trend

TABLE 4. Summary of data on combinations of HIV protease inhibitors and AZT or ddI (COMBO analyses)[a]

Calculation[b]	Drug 1	Drug 2	Dideoxynucleoside IC_{50} (μM)	Protease inhibitor IC_{50} (μM)	Dideoxynucleoside PC_{50} (μM)	Protease inhibitor PC_{50} (μM)	PI	P value[c]	Sum of squared deviations/degree of freedom
A	AZT	A77003	0.060 ± 0.006	0.210 ± 0.005		0.318	0.66 ± 0.46	0.156	0.011
B	AZT	A77003	0.063 ± 0.006	0.225 ± 0.009	0.099		0.63 ± 0.29	0.029	0.011
A	AZT	A75925	0.070 ± 0.005	0.086 ± 0.004		0.040	2.16 ± 0.52	<0.001	0.007
B	AZT	A75925	0.065 ± 0.006	0.085 ± 0.003	0.037		1.76 ± 0.40	<0.001	0.008
A	AZT	A76928	0.072 ± 0.008	0.181 ± 0.016		0.130	1.39 ± 0.62	0.025	0.013
B	AZT	A76928	0.072 ± 0.008	0.164 ± 0.009	0.073		0.98 ± 0.40	0.013	0.014
A	ddI	A77003	6.182 ± 0.482	0.223 ± 0.007		2.20	0.10 ± 0.27	0.705	0.018
B	ddI	A77003	6.383 ± 0.460	0.226 ± 0.008	23.1		0.28 ± 0.22	0.207	0.020
A	ddI	A75925	5.281 ± 0.514	0.086 ± 0.005		(0.405)	(0.21 ± 0.31)	0.498	0.022
B	ddI	A75925	5.552 ± 0.507	0.088 ± 0.005	56.5		0.10 ± 0.23	0.664	0.025
A	ddI	A76928	5.250 ± 0.531	0.178 ± 0.012		(0.578)	(0.31 ± 0.33)	0.353	0.017
B	ddI	A76928	5.806 ± 0.548	0.181 ± 0.010	73.6		0.08 ± 0.21	0.712	0.016

[a] Values in parentheses indicate a trend toward antagonism (rather than potentiation).
[b] A, potentiation of drug 1 by drug 2; B, potentiation of drug 2 by drug 1.
[c] Two-tailed Student's *t* test for the null hypothesis of additivity.

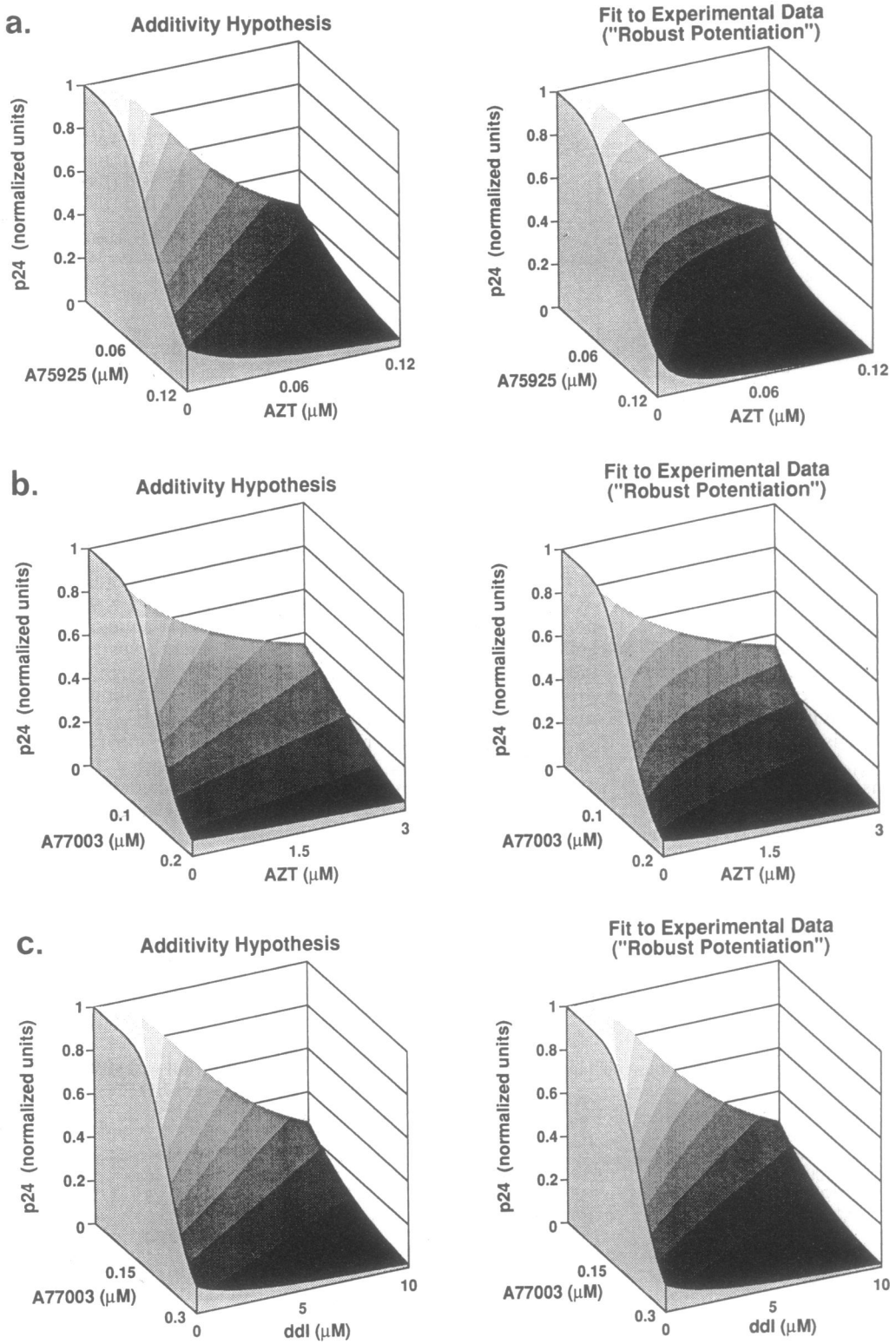


FIG. 3. Synergistic or additive interactions of HIV protease inhibitors when combined with a DNA chain-terminating antiviral nucleoside analog, AZT or ddI. The culture conditions were the same as those described in the legend to Fig. 2. The left panels represent surfaces of response corresponding to the null hypothesis that drug interactions are additive. Hence, the contour lines run linearly from axis to axis in each panel. The right panels represent best-fit data. A bowl-shaped, inward curvature indicates a trend toward synergy, but the statistics must be examined to decide whether the trend is statistically significant. See Table 4 for detailed parameter values and statistics. Calculations were performed for the effect of HIV protease inhibitors on AZT or ddI, but the panels looked almost identical to those showing the effect of AZT or ddI on the HIV protease inhibitors.

toward synergy ($P = 0.029$ for PI_1 and $P = 0.156$ for PI_2) when A77003 was combined with AZT (Table 4 and Fig. 3b). As indicated in Table 4 and Fig. 3c, ddI was found to be essentially additive with all three HIV protease inhibitors under the same conditions.

DISCUSSION

A large array of new anti-HIV drugs are in the developmental phase, and a variety of drugs are now undergoing clinical testing. A logical extension of current approaches for the therapy of HIV infections would be the use of combinations of multiple antiviral agents which have different antiretroviral mechanisms or use different metabolic pathways for conversion to their corresponding active moieties (21, 22, 31). Such combination therapy may enhance the antiretroviral activity and reduce the adverse effects of each drug. In addition, the development of drug-resistant HIV variants may also be delayed with the combined use of multiple drugs versus the use of single drugs.

In 1990, the Abbott antiviral group developed C_2 symmetry-based inhibitors of HIV protease which were designed to complement the C_2 symmetry of the enzyme active site (6, 10). In the current work, 10 C_2 symmetry-based or pseudo-C_2 symmetry-based HIV protease inhibitors were tested for antiviral activity against HIV-1 in the cytopathic effect inhibition assay with the ATH8 cell line. The structures of A77212, A77003, and A75912 vary from perfect C_2 symmetry at one small part of the molecule (9). Since the stereochemistries of the two central hydroxyl groups of A77212 and A77003 are different from each other, they do not superimpose upon each other when one performs a C_2 rotational operation. However, every other atom does superimpose on the corresponding atom on the other side of the C_2 axis. These molecules thus retain a high degree of symmetry, leading to the term pseudo-C_2 symmetry-based HIV protease inhibitors.

All 10 compounds examined were found to be active against HIV-1 in this system. In assessing the potential clinical activity of a given antiviral compound, testing should be done with diverse strains of HIV-1 as sources of infectious virions for in vitro assays. It is especially important to use freshly isolated clinical HIV-1 strains, since certain laboratory strains have been found to be less susceptible to the inhibitory effects of certain antiviral agents, such as soluble CD4 (5). It has also been shown that HIV-1 strains isolated from patients receiving AZT can exhibit resistance to AZT and that such AZT-resistant HIV-1 strains show cross-resistance to certain dideoxynucleoside analogs (14). In this regard, the three inhibitors selected in the current study exerted potent antiviral activity against several freshly isolated primary HIV-1 strains, including an AZT-resistant strain isolated in PHA-PBM as target cells. These three compounds also exhibited potent antiviral activity against $HIV\text{-}1_{Ba\text{-}L}$ in M/M at concentrations that did not suppress the growth of the M/M target cells.

In the current study, we used an RIA for the determination of p24 Gag protein production for several reasons. Both the RIA and the enzyme-linked immunosorbent assay (ELISA) require a relatively small volume (100 or 200 μl) of supernatants for the assay and are therefore desirable for a microtiter culture study like the current one. Since we use a manifold for harvesting the radioactive DNA synthesized, the determination of the activity of reverse transcriptase is more labor-intensive and more time-consuming. Finally, under the conditions that we used, p24 Gag protein production without antiviral agents ranged between 10 and 100 ng/ml. In this regard, the detection range of the ELISA is about 12 to 200 pg/ml, while that of the RIA is about 0.6 to 20 ng/ml. Therefore, the RIA, which required minimal dilution, was chosen for this study.

Under the conditions used in the present study, C_2 symmetry-based compounds clearly showed potent antiretroviral activity against divergent HIV-1 strains in vitro. However, the prototypic C_2 symmetry-based HIV protease inhibitor, A75925, was poorly soluble in aqueous media (Table 2; 6, 10). This deficiency would very likely hamper the formulation of this class of drugs for the treatment of HIV infections. However, a number of analogs of A75925 and another prototypic C_2 symmetry-based HIV protease inhibitor, A74704, with improved solubility and potent antiviral activity, have recently been synthesized (9) by the introduction of polar, solubilizing functionality into the aromatic rings of the terminal carbobenzyloxy groups of the prototypic inhibitors (9). Among the newly reported compounds, A77003, which has considerable aqueous solubility (~200 μg/ml) and potent antiretroviral activity (Table 2), is currently being considered as an experimental antiviral drug for the therapy of HIV infections.

In this study, we also tested whether C_2 symmetry-based HIV protease inhibitors had synergistic antiviral activities when combined with the DNA chain-terminating antiviral nucleoside analogs AZT and ddI. To this end, we used the recently developed COMBO program package, which provides us with (i) a flexible choice of interaction models, (ii) parametric and nonparametric statistical methods to estimate P values and confidence limits, (iii) flexibility with respect to experimental design (e.g., checkerboard, constant-ratio, etc.), and (iv) statistical techniques to reduce the sizes of experiments by reducing the number of replicates required. Using this program, we found that the HIV protease inhibitors tested here were synergistic or had a strong tendency toward synergism when combined with AZT. The interactions of ddI with HIV protease inhibitors were essentially additive. These data suggest that HIV protease inhibitors may affect differently the anabolism of the two dideoxynucleosides to their corresponding active moieties or influence the sizes of normal deoxynucleotide pools. Conceivably, AZT and ddI may also affect the compartmentalization of HIV protease inhibitors differently inside target cells. Experimental variability may also explain the difference between AZT and ddI combinations with HIV protease inhibitors at least to some extent. More indepth studies must be conducted.

Taken together, in view of their potent antiretroviral activities and synergistic or additive interactions in vitro with two important antiviral nucleoside agents, AZT and ddI, C_2 symmetry-based HIV protease inhibitors such as A77003 appear to have considerable potential as candidate antiviral drugs. Further investigations of the clinical applications are warranted.

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