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In vitro anti-HIV-1 activity of HIV protease inhibitor KNI-272 in resting and activated cells: implications for its combined use with AZT or ddI

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Abstract

KNI-272, a conformationally constrained human immunodeficiency virus (HIV) protease inhibitor containing a P1 allophenylnorstatine (Apns) ((2*S*,3*S*)- 3-amino-2-hydroxy-4-phenylbutyric acid), has been shown to be a selective and potent inhibitor of the replication of a wide spectrum of HIV strains in vitro. When KNI-272 was tested in combination with 3′-azido-2′,3′-dideoxythymidine (AZT) or 2′,3′-dideoxyinosine (ddI) against a primary HIV-1 isolate in phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBM), its activity was identified to be additive, but not synergistic or antagonistic, as analyzed with the COMBO program package. When tested alone for anti-HIV-1 activity in resting PBM (R-PBM) and PHA-PBM, KNI-272 was found to be comparably potent against the virus in both target cell populations, whereas AZT was more potent in PHA-PBM than in R-PBM and ddI was more potent in R-PBM. These data suggest a potential clinical application of KNI-272 and its analogs.

Keywords: Acquired immunodeficiency syndrome (AIDS); Antiviral therapy; Azidothymidine; Dideoxyinosine; Protease inhibitor; Combination therapy

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Abbreviations: AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-2′,3′-dideoxythymidine; ddI, 2′,3′-dideoxyinosine; ddNs, 2′,3′-dideoxynucleosides; FCS, fetal calf serum; HIV, human immunodeficiency virus; IC_{50}, drug concentration yielding 50% inhibition; IC_{90}, drug concentration yielding 90% inhibition; PHA-PBM, phytohemagglutinin-activated peripheral blood mononuclear cells; R-PBM, resting peripheral blood mononuclear cells; $TCID_{50}$, 50% tissue culture infectious dose.

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1. Introduction

Since the clinical usefulness of 3′-azido-2′,3′-dideoxythymidine (AZT, zidovudine) was established in patients with advanced human immunodeficiency virus (HIV) infection in 1987 (Mitsuya et al., 1985; Yarchoan et al., 1986; Fischl et al., 1987), a number of potentially useful drugs for antiviral therapy of acquired immunodeficiency syndrome (AIDS) and related diseases have emerged. Among them are a variety of HIV-1 protease inhibitors (Ashorn et al., 1990a; DesJarlais et al., 1990; Erickson et al., 1990; Kageyama et al., 1992, 1993; Kort et al., 1993; Lambert et al., 1993; Craig et al., 1994), which are currently in various stages of clinical or preclinical trials in the United States and European countries. A conformationally constrained HIV protease inhibitor containing a P1 allophenylnorstatine (Apns) ((2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid), KNI-272 (Fig. 1), has been shown to be a selective and potent inhibitor of the replication of a wide spectrum of HIV strains in vitro (Kageyama et al., 1993) and is currently undergoing clinical trials at the National Cancer Institute.

It should be noted that long-term antiviral therapy has been complicated by the emergence of HIV-1 variants less susceptible to a variability of antiviral agents (Larder and Kemp, 1989; Larder et al., 1989; Shirasaka et al., 1993; Kojima et al., 1995). With the relatively low therapeutic index of most anti-HIV drugs and the propensity of HIV to develop resistance by mutation, it is likely that the optimal therapy for HIV-1 infection should involve a combination of drugs and approaches. There are several potential advantages to the combination therapy of AIDS. One is the potential for reducing overall toxicity by combining drugs with different toxicity profiles. Another reason for combining various drugs is the potential for drug synergy. In addition, different drugs may complement each other and may achieve more effective antiviral activity when properly combined. For example, different dideoxynucleoside analogs appear to be differently phosphorylated intracellularly depending on the activation state of target cells (Perno et al., 1989; Gao et al., 1993, 1994), thereby making it possible to combine such nucleosides for potentially more effective antiretroviral therapy (Gao et al., 1993, 1994).

In this study, we asked whether KNI-272 combined with two therapeutic dideoxynucleoside analogs, AZT and ddI, had synergistic or antagonistic antiviral activity in vitro. To our knowledge, there has been no report studying the antiviral activity of HIV

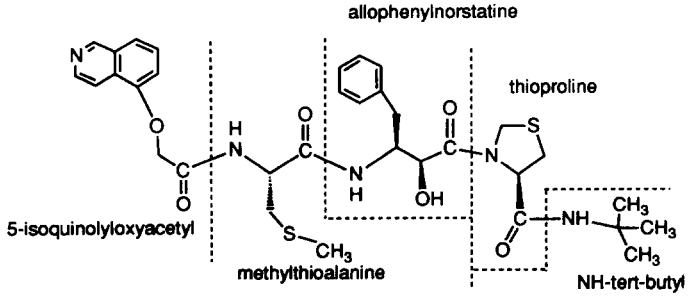

Fig. 1. Structure of KNI-272.

protease inhibitors in resting cells. Therefore, we also asked whether KNI-272 exerted its antiviral activity differently in resting and activated target cells.

2. Materials and methods

2.1. Compounds

3′-Azido-2′,3′-dideoxythymidine (AZT, zidovudine) and 2′,3′-dideoxyinosine (ddI, didanosine) were purchased from Sigma (St. Louis, MO) and Calbiochem-Novabiochem Co. (San Diego, CA), respectively. KNI-272, an HIV protease inhibitor, was provided by Japan Energy, Co., Ltd., Tokyo. Properties of KNI-272 have been reported elsewhere (Mimoto et al., 1991; Kageyama et al., 1993, 1994).

2.2. Viruses and cells

A primary HIV-1 strain, HIV-$1_{ERS104pre}$, was isolated from an AZT-naive patient (patient ERS104) with advanced HIV-1 infection prior to antiviral therapy (Shirasaka et al., 1993). Briefly, peripheral blood mononuclear cells (PBM, 10^6 cells) obtained from a patient with AIDS (patient ERS104) prior to antiviral therapy were cultured with an equal number of a healthy volunteer's PBM which had been exposed to phytohemagglutinin (PHA; Gibco/BRL) for 2 days (PHA-PBM) in complete culture medium (RPMI 1640 containing 15% FCS, 4 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml)) in the presence of exogenous interleukin-2 (5 ng/ml; Amgen, Thousand Oaks, CA). Culture supernatants collected on day 7 of culture were titrated for the 50% tissue culture infective dose ($TCID_{50}$) by using an endpoint titration method (Leland and French, 1988) and used as the source of infectious virions. This isolate was phenotypically sensitive to AZT, ddC and ddI as tested in PHA-PBM, and is genotypically wild-type in the *pol*-encoding region as determined by nucleotide sequence analysis (Shirasaka et al., 1993).

2.3. Determination of antiviral activity of drugs in combination

The antiviral activity of drug(s) in PHA-PBM was determined by using the inhibition of p24 Gag protein production as an endpoint as previously described (Kageyama et al., 1992; Richman et al., 1993). Briefly, PHA-PBM (5×10^5) were preincubated in the presence of various concentrations of KNI-272, AZT or ddI in 48-well microtiter culture plates in 1 ml of culture medium containing recombinant IL-2 for 2 h, then exposed to HIV-$1_{ERS104pre}$. The cells were continuously exposed to the drug. On day 7 of culture, the amounts of p24 Gag protein in culture medium were determined by radioimmunoassay (DuPont, NEN, Boston, MA). Data are expressed as the concentration that inhibited p24 Gag protein production by 50% (IC_{50}) and 90% (IC_{90}) as previously described (Kageyama et al., 1992; Richman et al., 1993). The results were subjected to an analysis using the COMBO program package (vide infra).

2.4. Analysis of drug interactions

Drug interactions were analyzed with the COMBO program package, which uses the MLAB environment (Civilized Software, Inc., Bethesda, MD). The methods are described in detail elsewhere (Ashorn et al., 1990b; Bunow and Weinstein, 1990; Weinstein et al., 1990; Kageyama et al., 1992). Briefly, the antiviral data were fitted by iteratively reweighted non-linear least-squares regression to a 'robust potentiation' model defined by the implicit equation:

$$1 = \left\{(1/Z-1)^{-1/B_1}(C_1/IC_{50_1})\left[1+(PI_2C_2/IC_{50_2})^{BP_2}\right]^{\pm 1}\right\}$$

$$+\left\{(1/Z-1)^{-1/B_2}(C_2/IC_{50_2})\left[1+(PI_1C_1/IC_{50_1})^{BP_1}\right]^{\pm 1}\right\}$$

This equation is equivalent to the equation in reference (Ashorn et al., 1990b), but parameterized in terms of PIs (in Kageyama et al., 1992, the expressions, PI_2C_2/IC_{50_2} and PI_1C_1/IC_{50_1} were accidentally rendered as PI_2C_2 and PI_2C_2, respectively; but all calculations were done with the correct equation). 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 (or antagonistic) activity as it is through its intrinsic activity. $Z = (y-d)/(a-d)$ 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, 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% effective slopes. 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. These two parameterizations (i.e., in terms of PI_1 or PI_2) represent two ways of looking at the same phenomenon, i.e., as a mutual potentiation (synergy) or a mutual antagonism. One of the parameterizations may happen to fit somewhat better than the other for a given data set, but the two are mathematically indistinguishable if $B_1 = B_2$ and $BP_1 = BP_2 = 1$. Occasional data sets may fit demonstrably better if both PI_1 and PI_2 are included for $B1 \neq B2$, but that is not usually the case. The special case of pure potentiation, in which one agent has no intrinsic activity but potentiates the other, is handled by a different set of equations ('Pure-Pot') in the COMBO package.

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 response. Extensive simulations indicated that all parameter estimates were approximately normally distributed and in agreement with the asymptomatic estimates produced by the MLAB curve fitter. Hence, the normal error estimates and confidence limits are presented.

2.5. Determination of HIV-1 Gag protein production by PHA-PBM

PHA-PBM (5×10^5) were preincubated in the presence of various concentrations of KNI-272, AZT or ddI (0.005, 0.05, 0.5, 5, and 50 μM) in 48-well microtiter culture plates in 1 ml of culture medium containing recombinant IL-2 for 2 h, then exposed to HIV-$1_{ERS104pre}$. The cells were continuously exposed to the drug. On day 7 of culture, the amounts of p24 Gag protein in culture medium were determined by radioimmunoassay (Du Pont). Data are expressed as the concentration that inhibits p24 Gag protein production by 50% (IC_{50}) and 90% (IC_{90}).

2.6. Determination of anti-HIV-1 activity in resting PBM

PBM obtained from each of 3 healthy HIV-1 seronegative donors, A, B, and C, were split in two: one for determination of antiviral activity of a drug in PHA-PBM and the other in resting PBM. The antiviral activity of drug(s) in PHA-PBM was determined as described above. For determination of antiviral activity in R-PBM, immediately after PBM were isolated from donors and split in two, one half of PBM were left unstimulated and incubated with various concentrations of KNI-272, AZT or ddI (0.005, 0.05, 0.5, 5, and 50 μM) in 48-well microtiter culture plates (5×10^5 cells in 1 ml culture medium per well) for 16 h and then exposed to HIV-$1_{ERS104pre}$ for 8 h. The cells (note that the cells were not washed after viral exposure) were subsequently exposed to a mixture of PHA and 5 μM AZT and cultured for 7 days. Half of culture medium was changed with an equal amount of fresh culture medium every 7 days. The concentration of AZT was kept the same throughout the culture period. The amount of p24 Gag protein present in the culture medium was determined by RIA. All assays were performed in quadruplicate. Data are expressed by the concentration that inhibited p24 Gag protein production by 50% (IC_{50}) and 90% (IC_{90}).

3. Results

3.1. Antiviral activity of KNI-272 combined with AZT or ddI

We first asked whether KNI-272 exerted an antagonistic or synergistic antiviral activity against HIV-1 in vitro when used in combination with AZT or ddI. Table 1 shows representative data on the antiviral activity of KNI-272 combined with AZT. Using PHA-PBM as target cells, the IC_{50} value of AZT against HIV-$1_{ERS104pre}$ was ~ 0.016 μM. The IC_{50} value of KNI-272 was ~ 0.08 μM, consistent with previously published potency of KNI-272 against AZT-sensitive and -insensitive clinical HIV-1 isolates as tested in PHA-PBM (Kageyama et al., 1993). The concentrations of all 3 drugs were substantially low and, indeed, we did not see any significant toxicity in the concentrations of all drugs when used alone or in combinations (data not shown).

The data shown in Table 1 were further analyzed for possible synergy when two drugs were combined by using the COMBO program package. Table 2 shows results of combined use of KNI-272 with AZT and those of KNI-272 with ddI, illustrating the

parameter estimates resulting from the COMBO analyses of two different experiments. Fits were done in two ways: (1) considering KNI-272 as the potentiator; and (2) considering AZT as the potentiator. Synergy corresponds to mutual potentiation. Of most interest 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 and PI = +1 indicates that the drug is as effective through its potentiating (or antagonistic) activity as it is through its intrinsic activity. As expected, the estimates of IC_{50} shown in Table 2 for the dideoxynucleosides and protease inhibitor were essentially the same regardless of which was considered as a potentiating agent. As indicated by the *P*-values shown, none of the values for PI were significantly different from zero, in the direction of either potentiation or antagonism.

A useful derived parameter is PC_{50}, 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. The parameters determined indicated no evidence of antagonism or synergy between KNI-272 and AZT or ddI.

Fig. 2a graphically illustrates the relationships for the potentiation of KNI-272 antiviral activity by AZT. As assessed by COMBO analyses, AZT was found to be essentially additive (not to be synergistic or antagonistic) with KNI-272. This was also the case when KNI-272 was combined with ddI (Fig. 2b).

3.2. In vitro antiretroviral activity of KNI-272 against HIV-1 in PHA-PBM and R-PBM

The infectivity and replication of HIV-1 is affected by the state of cell activation (Stevenson et al., 1990; Zack et al., 1990; Bukrinsky et al., 1992; Zack et al., 1992).

Table 1
In vitro antiretroviral activity of KNI-272 and AZT in combination against HIV-1 [a]

AZT (μM)	KNI-272 (μM)						
	0	0.0004	0.002	0.01	0.05	0.25	1.25
0	0 [b]	10±12	13± 8	21± 5	28±8	98±0.05	99±0.01
0.0006	26±5	31± 3	36± 6	30± 3	39±2	ND [c]	ND
0.0032	29±1	35± 5	36±14	38±10	54±1	ND	ND
0.016	52±6	51± 4	54± 1	53± 1	68±4	ND	ND
0.08	81±8	83± 3	85± 2	84± 1	93±4	ND	ND
0.4	99±0.03	ND	ND	ND	ND	ND	ND
2	100±0	ND	ND	ND	ND	ND	ND

[a] Percent p24 Gag protein production inhibition was determined by the following equation: % inhibition = 100 ×[1 −(the amount of p24 Gag protein produced in the presence of drug/the amount of p24 Gag protein produced in the absence of drug)]. PHA-PBM were exposed to a 200 $TCID_{50}$ dose of HIV-$1_{ERS104pre}$ and the cultured supernatants were harvested on day 7 of culture as described in Materials and methods. This experiment was conducted twice and comparable data were obtained: one set of such data are shown here. All assays were performed in duplicate. Values shown represent the mean values (±1 S.D.) of percent p24 Gag protein inhibition.

[b] The mean amount of p24 Gag protein in the no drug control was 478 (±56) ng/ml as determined in octaplicate.

[c] ND, not determined.

Table 2
Summary of antiviral data on combination of KNI-272 and AZT or ddI as assessed by COMBO analyses

Calculation [a]	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 [b]	Sum of squared deviations/degree of freedom [c]
A	AZT	KNI-272	0.02 ± 0.002	0.074 ± 0.009		0.23	0.32 ± 0.59	0.59	0.003
B	AZT	KNI-272	0.02 ± 0.002	0.075 ± 0.007	0.082		0.24 ± 0.19	0.20	0.003
A	ddI	KNI-272	1.73 ± 0.17	0.056 ± 0.004		-0.084	-0.67 ± 0.96	0.49	0.004
B	ddI	KNI-272	1.79 ± 0.14	0.057 ± 0.004	-7.1		-0.25 ± 0.32	0.43	0.004

[a] A, potentiation of drug 1 by drug 2; B, potentiation of drug 2 by drug 1.
[b] Two-tailed *P*-values for the null hypothesis of additivity.
[c] Sum of squared weighted deviations per degree of freedom in the model fit with the response variable (p24) normalized to the interval 0 to 1. This is a heuristic indicator of goodness of fit to the model.

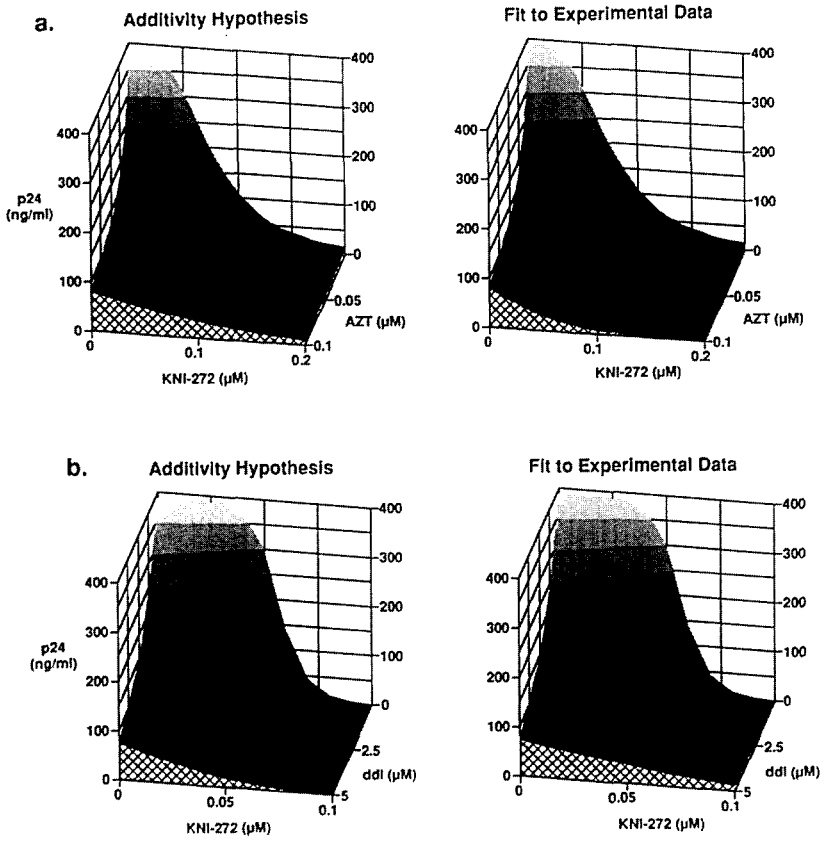

Fig. 2. Synergistic or additive interactions of KNI-272 when combined with AZT or ddI. The amounts of p24 Gag protein production were determined in PHA-PBM as described in the legend to Table 1. Upper and lower panels were for KNI-272 plus AZT and KNI-272 plus ddI, respectively. The left panels represent surfaces of response corresponding to the null hypothesis that drug interactions are additive. The right panels represent best-fits to the data. A concave curvature indicates a trend toward synergy. See Table 2 for detailed parameter values and statistics. Calculations were performed for the effect of KNI-272 on AZT or ddI.

Therefore, antiviral activity may also be exerted differently depending on the state of activation of target cells. Indeed, different triphosphorylation profiles of certain dideoxynucleoside reverse transcriptase inhibitors have recently been demonstrated in R-PBM and PHA-PBM (Gao et al., 1993, 1994). Such differential phosphorylation profiles of anti-HIV-1 nucleoside analogs may have practical relevance in the design of combination chemotherapy for HIV-1 infection. Hence, we asked if KNI-272 exerted differential antiviral activity in R-PBM and PHA-PBM.

Representative data shown in Fig. 3 illustrate that KNI-272 exerted its potent anti-HIV-1 activity in both PHA-PBM and R-PBM. When determined in PHA-PBM, the average IC_{50} and IC_{90} values of KNI-272 were 0.09 and 0.31 μM, respectively (Table 3). When determined in R-PBM, the average IC_{50} and IC_{90} values of KNI-272 were

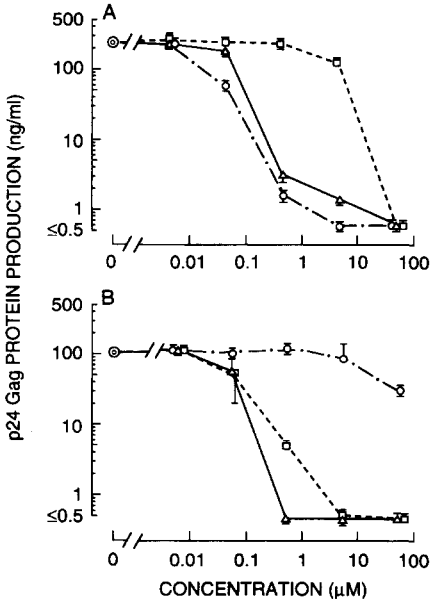

Fig. 3. Suppression of HIV-1 replication in PHA-PBM and R-PBM by KNI-272, AZT, and ddI. PHA-PBM (A) and R-PBM (B) were preincubated with various concentrations of KNI-272 (triangles), AZT (circles), or ddI (squares), exposed to HIV-1, and cultured in the continuous presence of the drug. The supernatants of PHA-PBM were collected on day 7 of culture while the supernatant of R-PBM was collected on day 28 (see Materials and methods). The amounts of p24 Gag protein production in the culture supernatants were determined by an RIA. The data shown represent geometric means of quadruplicate determinations with one standard deviation.

Table 3
Anti-HIV-1 activity of KNI-272 in resting and PHA-activated PBM

Donor	Cells	IC_{50} (μM)			IC_{90} (μM)		
		KNI-272	AZT	ddI	KNI-272	AZT	ddI
A	Resting	0.16	15	0.18	0.42	> 50	2.1
	PHA-activated	0.11	0.26	16	0.48	2.3	40
B	Resting	0.07	23	0.06	0.33	> 50	0.4
	PHA-activated	0.17	0.02	4.9	0.39	0.3	32
C	Resting	0.02	18	ND	0.05	47	ND
	PHA-activated	0.01	0.03	ND	0.05	0.37	ND
Means	Resting	0.08	19	0.12	0.27	ND	1.25
	PHA-activated	0.09	0.10	10	0.31	0.99	36

The IC_{50} and IC_{90} values of KNI-272 when tested in resting and PHA-activated PBM were determined as described in Materials and methods. All target cell populations were exposed to an 800 $TCID_{50}$ dose of HIV-1. Note that each set of resting PBM and PHA-PBM was from the same healthy donor. The values shown represent the means of quadruplicate determinations. ND, not determined.

0.08 and 0.27 μM, respectively (Table 3). These data suggest that KNI-272 was comparably active against the virus in both R-PBM and PHA-PBM in vitro. In contrast, AZT blocked the infectivity and replication of HIV-1 at 0.99 μM by 90% as examined in PHA-PBM, while 47 μM or higher concentrations of AZT were required to bring about the same level of antiviral activity in R-PBM (Table 3). On the contrary, ddI suppressed HIV-1 replication at 1.25 μM by 90% in R-PBM, while in PHA-PBM a much higher concentration, 36 μM, was required to yield the same level of activity (Table 3). These data are consistent with previously published biochemical data regarding the differential phosphorylation of dideoxynucleoside analogs (Gao et al., 1993, 1994).

4. Discussion

In this study, we demonstrate that the anti-HIV-1 activity of KNI-272, a conformationally constrained HIV protease inhibitor, was additive, but not synergistic or antagonistic, in vitro when combined with AZT or ddI, as analyzed with the COMBO program package. There have been several reports that certain HIV protease inhibitors including A77003 (Kageyama et al., 1992), SK&F 108922 (Lambert et al., 1993), and saquinavir (Ro 31-8959) (Craig et al., 1994) exert synergistic (or greater than additive) antiviral activity against HIV-1 in vitro when used in combination with dideoxynucleosides. It seems unlikely that protease inhibitors significantly alter the intracellular anabolic phosphorylation of dideoxynucleosides or physiologic nucleosides. It is believed that HIV protease inhibitors block a stage of HIV-1 replication cycle different from that targeted by dideoxynucleoside analogs; therefore, they may bring about a synergistic antiviral effect (Kageyama et al., 1992; Lambert et al., 1993; Craig et al., 1994). It is not clear why KNI-272 failed to show synergistic effect against the virus in this study when used in combination with AZT or ddI. There could be several explanations for this: (1) different HIV protease inhibitors, different assay systems, and different HIV-1 strains employed; (2) different endpoints used; and (3) the analytical method employed to determine the interaction of drugs (the Combo package used in this study is relatively more stringent than several other analytical methods) (Ashorn et al., 1990b; Bunow and Weinstein, 1990; Weinstein et al., 1990).

In the present study, KNI-272 blocked the replication of HIV-1 effectively both in resting PBM (R-PBM) and PHA-activated PBM (PHA-PBM). This was not surprising, since KNI-272, unlike dideoxynucleoside analogs, does not require intracellular modification (i.e., phosphorylation) to exert its antiviral activity. However, this observation may be of significance in the design of combination chemotherapy. In this regard, it should be noted that neither AZT nor ddI affects the other's pharmacokinetics in vivo (Collier et al., 1993; Yarchoan et al., 1994) or triphosphorylation profile (Gao et al., 1994). Moreover, White et al. conducted a detailed kinetic analysis of the interaction of AZTTP (the putative active metabolite of AZT) and ddATP (the putative active metabolite of ddI), and concluded that there is no synergistic inhibition of HIV-1 RT by these two triphosphates, suggesting that AZTTP and ddATP do not synergize in the cytoplasm of a single cell (White et al., 1993). Nevertheless, a more favorable antiviral

activity has been seen in patients receiving a simultaneous regimen of AZT plus ddI than in those receiving an alternating regimen of the two drugs, as assessed by circulating CD4 counts, p24 levels, body weight (Yarchoan et al., 1994) and viremia levels (Kojima et al., 1995). In this regard, our recent analyses of the phosphorylation of various dideoxynucleoside analogs suggest that AZT is preferentially phosphorylated and that higher AZTTP/TTP ratios are achieved in activated, dividing cells than in resting cells, while ddI is preferentially phosphorylated and higher ddATP/dATP ratios are achieved in resting cells than in activated cells (Gao et al., 1994). In the present study, we demonstrated that, as expected from the phosphorylation patterns of AZT and ddI described above, AZT was more potent against HIV-1 in activated cells, while ddI was more active against the virus in resting cells. If the complementary activity of AZT and ddI contributed to the apparent in vivo synergism of these two drugs (Yarchoan et al., 1994), combinations of AZT plus KNI-272 and KNI-272 plus ddI, in both of which complementary antiviral activity is expected in vivo situation where both resting cells and dividing cells exist, may exert more than additive antiviral activity in patients.

It is of note that KNI-272 virtually completely blocked the replication of HIV-1 when tested in resting PBM as target cells at 0.5 μM and beyond (Fig. 3). The self-assembly of HIV protease and the subsequent protease-mediated proteolysis of viral precursor proteins are generally believed to occur late in the replicative cycle of HIV. Therefore, one can assume that HIV-1 can infect resting PBM even in the presence of a high concentration of KNI-272, and a certain level of p24 Gag protein would be ultimately detected in the long-term culture. Hence, the observed complete inhibition of HIV-1 replication by high concentrations of KNI-272 in R-PBM raises 3 possibilities: (1) all the infected cells were immediately destroyed by the virus; (2) in the presence of high concentrations of KNI-272, there were not a sufficient number of HIV-1-infected resting cells to produce a detectable amount of p24 Gag protein; and (3) KNI-272 blocked HIV-1 replication not only in late stages, but also in early stages of the viral replicative cycle. The first possibility seems unlikely since R-PBM infected in the presence of high concentrations of AZT (5 μM) survived HIV-1 infection and produced a significant level of p24 (Fig. 3A). The second possibility is more likely. Indeed, several HIV protease inhibitors have been shown to virtually completely block the replication of HIV-1 in vitro and no detectable p24 antigen has been found in culture systems using dividing target cells (Jacobsen et al., 1992; Kageyama et al., 1992, 1993; Kort et al., 1993; Lambert et al., 1993). It should be noted, however, that the number of resting cells infected with HIV-1 in the presence of high concentrations of a protease inhibitor will have to be determined somehow, which would be a topic of future research. In regard to the last possibility, Oroszlan and his coworkers have reported that HIV-1 protease may play a role in the early stages of HIV-1 infection, either in stabilizing full-size unintegrated cDNA or in cleavage of the nucleocapsid protein, which may be required for the proper formation of the preintegration complex and/or for its transport to the nucleus (Nagy et al., 1994). Our observation may be in support of this hypothesis.

In the present study, all target cells were exposed to HIV-$1_{ERS104pre}$ for 8 h (note that the cells were not washed), then exposed to PHA and 5 μM AZT. The production of detectable amounts of p24 after PHA activation proves that HIV-1 infection was established under this condition. After PHA activation, however, two modes of HIV-1

infection were likely to happen: (1) virions in the initial virus inoculum, which remained infectious in the culture, could effectively and continuously infect (as yet uninfected) cells; and (2) virions produced by the cells, which were infected at resting state started producing infectious virions which could continuously infect (as yet uninfected) cells. These two modes of continuous infection could obscure the antiviral activity exerted against the virus when the cells were at the resting state since p24 Gag protein production was used as an endpoint. In the present study, in order to block these two modes of continuous infection after PHA activation, 5 μM AZT was added with PHA. Thus, the detected p24 Gag protein should reflect the magnitude of viral replication in PBM that were infected by HIV-1 when they were in the resting state, and the observed inhibition of p24 Gag protein production should reflect drug inhibition of viral infection before PHA stimulation. In this regard, Malley et al. have recently reported that ddI (and ddC as well), which has proven to be highly potent against HIV-1 in resting cells (Table 3) failed to fully inhibit viral replication in resting PBM (Malley et al., 1994). In their study, however, AZT was not added in culture after PHA activation; therefore, conceivably, virions left in culture (even after washing) started to infect (as yet uninfected) cells, which could have resulted in obscuring the antiviral activity of ddI exerted against the virus when the cells were in the resting state.

Taken together, the present data suggest that KNI-272 may well have a role in combination therapy with certain dideoxynucleosides. The combination could also decrease toxicity of each drug since the toxicity profiles of the two agents are likely to differ. Further investigation in the direction of clinical application of KNI-272 is warranted.

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