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In Vitro Anti-Human Immunodeficiency Virus (HIV) Activities of Transition State Mimetic HIV Protease Inhibitors Containing Allophenylnorstatine

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Transition state mimetic tripeptide human immunodeficiency virus (HIV) protease inhibitors containing allophenylnorstatine [(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] were synthesized and tested for activity against HIV in vitro. Two compounds, KNI-227 and KNI-272, which were highly potent against HIV protease with little inhibition of other aspartic proteases, showed the most potent activity against the infectivity and cytopathic effect of a wide spectrum of HIV strains. As tested in target CD4$^+$ ATH8 cells, the 50% inhibitory concentrations of KNI-227 against HIV type 1 LAI (HIV-1_{LAI}), HIV-1_{RF}, HIV-1_{MN}, and HIV-2_{ROD} were 0.1, 0.02, 0.03, and 0.1 μM, respectively, while those of KNI-272 were 0.1, 0.02, 0.04, and 0.1 μM, respectively. Both agents completely blocked the replication of 3′-azido-2′,3′-dideoxythymidine-sensitive and -insensitive clinical HIV-1 isolates at 0.08 μM as tested in target phytohemagglutinin-activated peripheral blood mononuclear cells. The ratios of 50% cytotoxic concentrations to 50% inhibitory concentrations for KNI-227 and KNI-272 were ~2,500 and >4,000, respectively, as assessed in peripheral blood mononuclear cells. Both compounds blocked the posttranslational cleavage of the p55 precursor protein to generate the mature p24 Gag protein in stably HIV-1-infected cells. The *n*-octanol–water partition coefficients of KNI-227 and KNI-272 were high, with log $P_{o/w}$ values of 3.79 and 3.56, respectively. Degradation of KNI-227 and KNI-272 in the presence of pepsin (1 mg/ml, pH 2.2) at 37°C for 24 h was negligible. Current data warrant further careful investigations toward possible clinical application of these two novel compounds.

Human immunodeficiency virus type 1 (HIV-1) encodes a virus-specific aspartic protease which is essential for its replication. The HIV protease mediates crucial proteolytic processing of viral protein precursors at a late stage in the replication of the virus and thus represents a virus-specific target for therapy for HIV infection.

The design of HIV-1 protease inhibitors based on the transition state mimetic concept has led to the generation of a variety of peptide derivatives highly active against viral replication in vitro (5, 17, 20, 22, 37). These active agents contain a nonhydrolyzable, dipeptide isostere such as hydroxyethylene (20, 22, 41) or hydroxyethylamine (34, 37) as an active moiety which mimics the putative transition state of the aspartic protease-catalyzed reaction. Twofold (C_2) symmetric inhibitors of HIV protease represent another class of potent HIV protease inhibitors which were created by Erickson et al. on the basis of the three-dimensional symmetry of the enzyme active site (5). If HIV protease inhibitors are successfully developed as therapeutic agents for AIDS treatment, the use of HIV protease inhibitors in combination with agents that have different antiretroviral mechanisms (e.g., 3′-azido-2′,3′-dideoxythymidine [AZT], 2′,3′-dideoxyinosine [ddI], and 2′,3′-dideoxycytidine) will likely enhance the efficacy and/or reduce the toxic effects of each drug. Indeed, we have observed synergism against HIV-1 between certain C_2 symmetric HIV inhibitors and AZT (13). However, there are several hurdles in developing HIV protease inhibitors as drugs for therapy for AIDS. These include relatively short plasma half-life, poor oral bioavailability, and the technical difficulty of scale-up synthesis (21).

In the current study, we designed and synthesized a variety of tripeptide HIV protease inhibitors containing a unique unnatural amino acid, allophenylnorstatine [Apns; (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid], with a hydroxymethylcarbonyl isostere (11, 12, 24–26, 30) as an active moiety, and tested these compounds for activity against a wide spectrum of HIV strains in vitro. The Apns moiety should serve as an effective transition state mimetic upon interaction with HIV protease.

We have identified two compounds containing Apns, KNI-227 and KNI-272, with potent antiretroviral activity against HIV. We have confirmed that both block the post-translational cleavage of the p55 precursor protein to gener-

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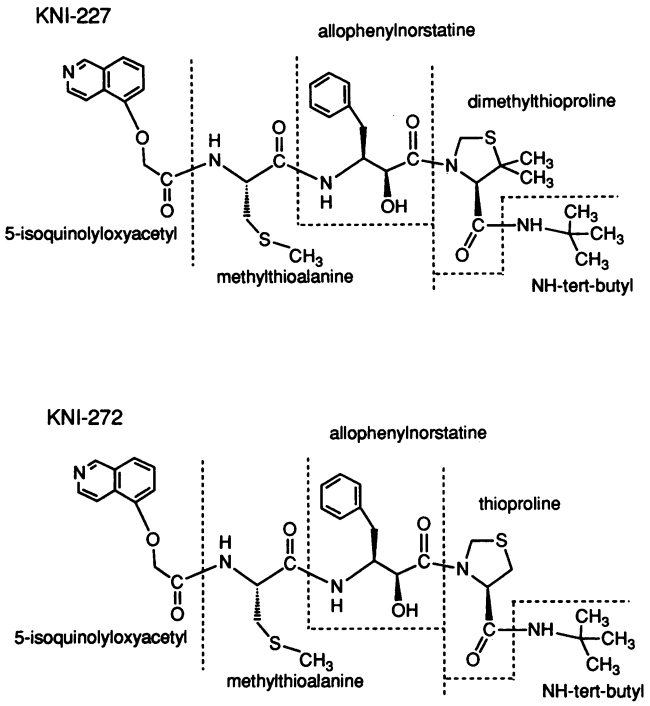

FIG. 1. Structures of KNI-227 and -272.

ate the mature p24 Gag protein and have characterized other biological properties of these compounds relevant to possible clinical application.

MATERIALS AND METHODS

Reagents. Twenty-nine different polypeptide HIV protease inhibitors containing Apns as a transition state mimetic were synthesized by the solid-phase method based on the *t*-butyloxycarbonyl strategy as previously described (15, 26) or by the solution method (24, 25). Detailed methods for the synthesis of these compounds have been described elsewhere (24–26). All synthesized compounds were >98% pure as assessed by high-performance liquid chromatography (HPLC). Structures of selected compounds are depicted in Fig. 1 and Table 1. AZT, or zidovudine, was purchased from Sigma (St. Louis, Mo.), while ddI, or didanosine, was supplied by Karl Flora, Developmental Therapeutics Program, National Cancer Institute.

Viruses and cells. HIV-1_{LAI} and HIV-2_{ROD} were pelleted by ultracentrifugation from the culture supernatants of HIV-1_{LAI} (also known as HIV_{IIIB})-producing H9 cells (2, 33, 42) and HIV-2_{ROD}-producing CEM cells (4) and were prepared to contain 7.39×10^{10} and 2.6×10^{11} virus particles per ml of fresh culture medium, respectively. Two clinical HIV-1 strains, HIV-1_{ERS104} (AZT-sensitive strain) and HIV-1_{ERS205} (AZT-insensitive strain) (39), were isolated by coculturing peripheral blood mononuclear cells from two different patients with AIDS. Briefly, 10^6 peripheral blood mononuclear cells obtained from patients with AIDS were cultured with an equal number of a healthy volunteer's peripheral blood mononuclear cells which had been activated with phytohemagglutinin (PHA-PBM) and cultured in the presence of exogenous interleukin-2 (50 U/ml; Amgen, Thousand Oaks, Calif.), and the culture supernatants collected on day 7 of culture were titrated and used as the source of infectious virions.

The 50% tissue culture infectious dose ($TCID_{50}$) per milliliter of cell-free HIV-1-containing supernatants was determined by the endpoint titration method as previously described by Leland and French (19). A cloned human T-cell leukemia virus type I-transformed $CD4^+$ T-cell line (ATH8) (28) or a cloned tetanus-toxoid-specific $CD4^+$ T-cell line (TM11) (27) was used as a target cell line for infection by HIV-1_{LAI}, whereas PHA-PBM served as target cells for clinical HIV-1 isolates.

HIV cytopathic effect inhibition assay. The HIV cytopathic effect inhibition assay was performed as previously described (28). Briefly, target $CD4^+$ T cells (ATH8) (2×10^5) were exposed to 3,000 to 4,000 $TCID_{50}$s of HIV-1_{LAI} for 1 h and 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 (human, purified; Advanced Biotechnologies Inc., Silver Spring, Md.) and 50 U of recombinant interleukin-2 per ml (Amgen), and the cells were incubated at 37°C in 5% CO_2-containing humidified air. In the case of HIV-2 infection, ATH8 cells were exposed to 8,000

TABLE 1. Antiviral activities of selected HIV protease inhibitors against HIV-1_{LAI} in ATH8 cells

Compound	Structure[a]						IC_{50} (μM)	TC_{50} (μM)
	P_3	P_2	P_1	P_1'	P_2'	P_3'		
Substrate[b]	H- Phe-	Asn-	Phe-	Pro-	Ile-	Val- NH_2		
KNI-091	Z-	Asn-	Apns-	Pro-	Ile-	NHChm	17	>20
KNI-102	Z-	Asn-	Apns-	Pro-	$NHBu^t$		1.1	>20
KNI-153	Qc-	Asn-	Apns-	Pro-	$NHBu^t$		1.0	>20
KNI-144	Noa-	Asn-	Apns-	Pro-	$NHBu^t$		0.9	>20
KNI-154	Noa-	Asn-	Apns-	Thz-	$NHBu^t$		0.5	>50
KNI-174	Noa-	Asn-	Apns-	Dmt-	$NHBu^t$		0.4	30
KNI-225	Noa-	Mta-	Apns-	Dmt-	$NHBu^t$		0.2	7
KNI-170	Noa-	Msa-	Apns-	Dmt-	$NHBu^t$		0.1	16
KNI-227	iQoa-	Mta-	Apns-	Dmt-	$NHBu^t$		0.1	40
KNI-272	iQoa-	Mta-	Apns-	Thz-	$NHBu^t$		0.1	>50

[a] Chm, cyclohexylmethyl; Dmt, L-5,5-dimethylthiazolidine-4-carboxylic acid (dimethylthioproline); iQoa, 5-isoquinolyloxyacetyl; Msa, L-methanesulfonylalanine; Mta, L-methylthioalanine; Bu^t, *t*-butyl; Noa, naphthyloxyacetyl; Qc, quinoline-2-carbonyl; Thz, L-thiazoline-4-carboxylic acid (thioproline); Z, benzyloxycarbonyl.

[b] The amino acid sequence shown represents the peptide sequence designed on the basis of two HIV protease substrates, p17/p24 and transframe protein-protease segments, as previously described (26).

$TCID_{50}$s of HIV-2_{ROD}. In experiments in which chronically HIV-1_{RF}-infected or HIV-1_{MN}-infected H9 cells (H9/RF, H9/MN) lethally γ-irradiated (12,000 rads) were used as a source of infectious virions, ATH8 cells (2×10^5) were cocultured with 2.5×10^3 H9/RF cells or 10^4 H9/MN cells. Control cells were treated similarly but were not exposed to the virus. Each drug was added to the culture after the viral exposure, and the cells were continuously exposed to the drug in culture. At various time points in culture, viable cells were counted in a hemocytometer under the microscope by the trypan blue dye exclusion method. Data were subjected to further analysis only when the number of viable cells exposed to the virus and cultured was less than 20% of those of control virus-unexposed and drug-unexposed cells. The antiviral activity and cytotoxicity of a given compound are expressed as 50% inhibitory concentration (IC_{50}) and 50% toxic concentration (TC_{50}), respectively.

Experiments done by using TM11 cells were performed in a way similar to that of the experiments using ATH8 cells, although 6,000 $TCID_{50}$s of HIV-1_{LAI} were used as a virus inoculum.

Determination of HIV-1 Gag protein production by PHA-PBM. HIV-1 Gag protein production by PHA-PBM was quantified as previously described (13). Briefly, cells (5×10^5/2 ml) were preincubated with drugs for 2 h, exposed to a clinical isolate of HIV-1 preparation, HIV-1_{ERS104} or HIV-1_{ERS205}, and cultured in the presence or absence of the drug. On day 3 in culture, 50% of the culture medium for PHA-PBM was replaced with an equal amount of fresh medium. Cells were continuously exposed to the same concentrations of each drug. The amounts of p24 Gag protein in culture medium on day 7 were determined by radioimmunoassay (Du Pont, NEN Research Products, Boston, Mass.).

HIV protease inhibition assay. Inhibition constants of KNI-174, KNI-227, and KNI-272 were determined by a fluorometric assay with the fluorogenic substrate Arg-Glu (EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Lys(DABCYL)-Arg (kindly provided by Grant Krafft, Abbott Laboratories). Typically, 390 μl of 0.125 M 2-(*n*-morpholino)ethanesulfonic acid–NaOH buffer, pH 6.2, containing 1.2 M $(NH_4)_2SO_4$, 6.25 mM dithiothreitol, and 0.625 mg of bovine serum albumin per ml (all reagents from Sigma), was mixed with 4 μl of inhibitor dissolved in dimethyl sulfoxide–1.6 μl (0.04 to 0.12 pmol) of titrated HIV-1 protease (concentrations, 0.1 to 0.3 nM; supplied by Krzysztof Appelt, Agouron Pharmaceuticals). The mixture of protease and inhibitor was preincubated for 4 min at 37°C, and the reaction was initiated by addition of 4 μl of a 500 μM substrate solution in dimethyl sulfoxide. Increase in fluorescence intensity at the emission maximum of 487 nm (excitation wavelength was 349 nm) was recorded as a function of time. The data were analyzed with the mathematical model for tight-binding inhibitors (43) in which the concentration of inhibitor is less than or approximately equal to the enzyme concentration. The data were fitted by nonlinear regression analysis to the equation

$$V = \frac{1V_0}{2E_t}\left(\left\{\left[K_i\left(1+\frac{S}{K_m}\right)+I_t-E_i\right]^2+4K_i\left(1+\frac{S}{K_m}\right)E_i\right\}^{1/2}-\left[K_i\left(1+\frac{S}{K_m}\right)+I_t-E_t\right]\right)$$

with program Enzfitter (Version 1.05), where V is the initial velocity with an inhibitor; V_0 is the measured initial velocity in the absence of the inhibitor; the substrate K_m is estimated to be 22 μM; and S, E_t, and I_t are the concentrations of substrate, active enzyme, and inhibitor, respectively. I_t values were varied over the ranges 25 to 400 pM for KNI-272 and KNI-227 and 25 to 250 pM for KNI-174.

Radioimmunoprecipitation. Metabolic labeling of cells with [^{35}S]methionine and immunoprecipitation were performed as previously described (18, 29). Briefly, after a preincubation with 10 μM HIV protease inhibitors in 30 ml of culture medium for 24 h and subsequent methionine starvation for 10 min, 10^7 cells were incubated with 0.1 mCi of [^{35}S]methionine for 30 min. The radiolabeled cells were washed and further cultured in 15 ml of fresh culture medium for up to 48 h. At the end of the chase, cells were washed, pelleted, and lysed with 1 ml of lysing buffer containing 0.75% Triton X-100, 300 mM NaCl, 50 mM Tris (pH 7.4), 10 μg of leupeptine per ml (Sigma), 10 μg of aprotinine per ml (Sigma), and 25 μM *p*-nitrophenyl-*p'*-guanidinobenzoate (Sigma). Viruses produced in the supernatant were lysed with a 10% volume of 10× lysing buffer. Each lysate was precleaned twice with protein G-Sepharose beads for 2 h and then mixed with 50 μl of anti-p24 rabbit antiserum (a kind gift of Anthony DeVico and M. G. Sarngadharan) coupled to protein G-Sepharose beads overnight. Antigen-antibody complex was dissociated with 100 μl of Laemmli's buffer containing 50 mM Tris, 1% sodium dodecyl sulfate, 10% glycerol, and 3% 2-mercaptoethanol. The samples (100 μl) were then subjected to 12% polyacrylamide gel electrophoresis and visualized by autoradiography.

Partition coefficient determination. *n*-Octanol–water partition coefficients were determined by a microshake-flask procedure, as previously described (7, 38). Briefly, 1 mg of the given compound dissolved in 1 ml of buffer-saturated *n*-octanol was thoroughly mixed with 1 ml of *n*-octanol-saturated (pH 7.0) 0.01 M potassium phosphate buffer in a 2-ml Mixxor apparatus (Genex Corp., Gaithersburg, Md.) at 24 to 26°C. The phases were separated and centrifuged individually, and the relative concentration of sample in a 50-μl aliquot of each phase was determined by HPLC analysis. The partition coefficient was calculated by dividing the absolute area of the appropriate integrator peak from the *n*-octanol phase by that of the buffer phase. The values were expressed as log $P_{o/w}$ and were determined from two independent experiments.

Acid stability and resistance to pepsin digestion. KNI-227 and KNI-272 (both 0.1 mM) were incubated in pH 1 solution (0.1 N HCl and 50 mM KCl) at 37°C. At various time intervals, aliquots were neutralized with the same volume of 0.1 N NaOH solution and analyzed by HPLC. To test resistance to pepsin, the compounds were incubated with pepsin A (1 mg/ml, from porcine stomach mucosa; EC 3.4.23.1; Sigma) in 0.2 M citrate solution (pH 2.2) at 37°C, neutralized with 0.1 N NaOH, and monitored by HPLC. A synthetic nonapeptide (H-Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-NH_2, representing HIV-1 Gag protein, amino acids 128 to 136) served as a control substrate.

RESULTS

Antiviral activity of HIV protease inhibitors tested against HIV-1_{LAI} in ATH8 cells. Table 1 shows activities of 10 selected protease inhibitors against a laboratory HIV-1 strain, HIV-1_{LAI}, with $CD4^+$ ATH8 cells as target cells. The prototypic inhibitor, KNI-091, in which P_3-Phe was replaced

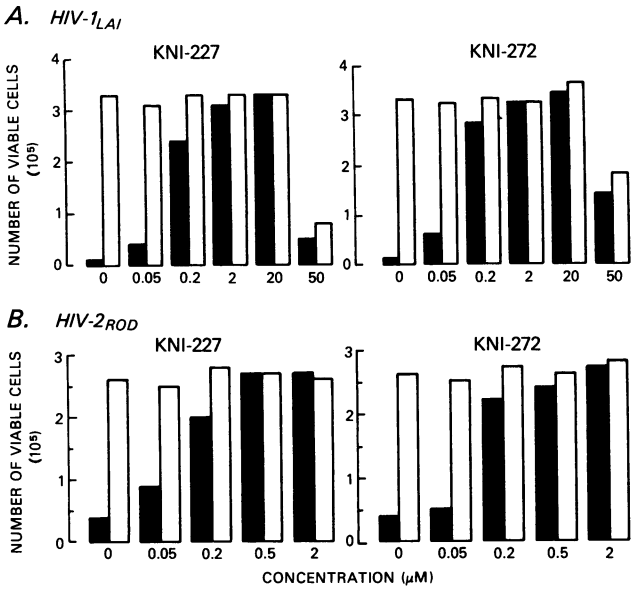

FIG. 2. Inhibition of the cytopathic effect of HIV-1 and HIV-2 by HIV protease inhibitors KNI-227 and -272. ATH8 cells (2×10^5) were exposed to 3,000 $TCID_{50}$s of HIV-1 (A) and 8,000 $TCID_{50}$s of HIV-2 (B) in the presence or absence of various concentrations of an HIV protease inhibitor, KNI-227 or -272 (solid column). Control cells were not exposed to the virus (open column). On day 7 (HIV-1) and day 6 (HIV-2), the total viable cells were counted.

with the isosteric benzyloxycarbonyl (Z) and P_3'-Val was replaced with the cyclohexylmethylamine group, showed only moderate inhibition of the cytopathic effect of the virus, with an IC_{50} of 17 μM, and exhibited substantial toxicity at 20 μM and beyond. KNI-102, in which P_3'-Val was deleted and P_2'-Ile was replaced by the isosteric *t*-butylamine, was synthesized. This compound showed quite potent activity against the virus compared with the prototype KNI-091. The IC_{50} and TC_{50} were 1.1 and >20 μM, respectively. When KNI-102 was further modified, having P_3-Phe replaced with a 5-isoquinolyloxyacetyl group, P_2-Asn replaced with L-methylthioalanine, and P_1'-Pro replaced with L-dimethylthioproline or L-thioproline, generating KNI-227 and KNI-272, respectively, the most potent antiretroviral activity and the least toxicity were observed (Fig. 2A). KNI-154, KNI-174, and KNI-170 also had preferable profiles of antiretroviral activity and cytotoxicity.

Antiviral activities of selected KNI compounds against primary HIV-1 isolates including an AZT-insensitive strain. Five HIV protease inhibitors selected on the basis of their favorable antiviral activity were examined in an assay system using a primary HIV-1 isolate (AZT-sensitive strain $HIV\text{-}1_{ERS104}$) as a source of infectious virions and PHA-PBM as a target cell population (Fig. 3A). In this assay system, both KNI-227 and KNI-272 showed the most potent antiviral activity and completely suppressed viral replication at ~0.08 μM with an IC_{50} of 0.02 μM. KNI-170 also showed a potent antiviral activity with an IC_{50} of 0.02 μM. The TC_{50}s obtained for KNI-154, KNI-170, KNI-174, KNI-227, and KNI-272 were 77, 19, 32, 49, and >80 μM, respectively, as assessed by [^{3}H]thymidine incorporation assay. These data produced relatively high TC_{50}/IC_{50} ratios, 2,500 and >4,000 for KNI-227 and KNI-272, respectively. The high TC_{50}/IC_{50} ratios are comparable to those reported for several other classes of potent HIV inhibitors (5, 13, 14, 22, 37, 41). The TC_{50}/IC_{50} ratios of KNI-154, KNI-170, and KNI-174 were lower, at 700, 1,000, and 300, respectively.

KNI-227 and KNI-272 also showed potent antiviral activity against an AZT-insensitive primary HIV-1 strain,

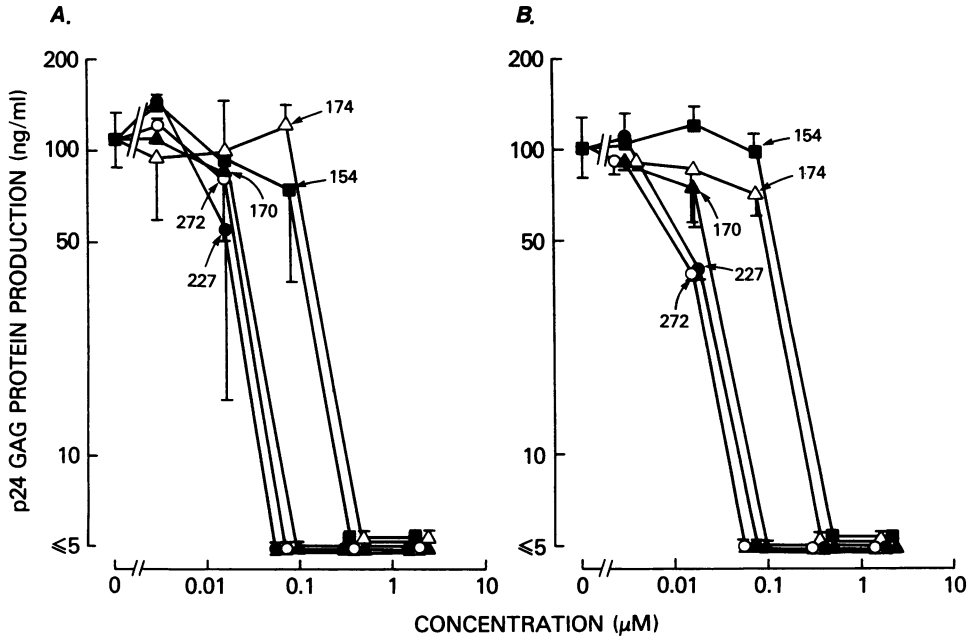

FIG. 3. Suppression of HIV-1 replication in PHA-PBM by HIV protease inhibitors KNI-154, -170, -174, -227, and -272. Normal PHA-PBM were exposed to a primary HIV-1 isolate, AZT-sensitive strain $HIV\text{-}1_{ERS104}$ (100 $TCID_{50}$s) (A) or AZT-insensitive strain $HIV\text{-}1_{ERS205}$ (200 $TCID_{50}$s) (B), and cultured in the presence of various concentrations of each of the HIV protease inhibitors. The amount of p24 Gag protein released into culture medium was determined by radioimmunoassay.

HIV-1_{ERS205} (39) (Fig. 3B). We, therefore, prioritized KNI-227 and KNI-272 for further detailed studies of other critical biological properties for possible clinical application.

Anti-HIV spectrum of KNI-227 and KNI-272. Two compounds, KNI-227 and KNI-272, were tested against a variety of HIV strains including HIV-1_{RF}, HIV-1_{MN}, and HIV-2_{ROD} in an assay system using the $CD4^+$ ATH8 cells as target cells. The IC_{50} and IC_{90} of KNI-227 against HIV-1_{RF} were 0.02 and 0.3 μM, respectively, while those of KNI-272 were 0.02 and 0.2 μM, respectively. The IC_{50} and IC_{90} of KNI-227 against HIV-1_{MN} were virtually comparable, with 0.03 and 0.09 μM, respectively, while those of KNI-272 were 0.04 and 0.1 μM, respectively.

When tested against an HIV-2 strain, HIV-2_{ROD}, the IC_{50} and IC_{90} of KNI-227 were 0.1 and 0.4 μM, respectively, while those of KNI-272 were 0.1 and 0.5 μM, respectively (Fig. 2B). These data indicated that both KNI-227 and KNI-272 had antiretroviral activity against a wide variety of HIV strains.

We also tested KNI-227 and KNI-272 for activity against HIV-1_{LAI} in normal clonal helper T cells, TM11 cells. These compounds brought about virtually complete protection against the cytopathic effect of HIV-1_{LAI} at about 0.1 μM. Both compounds had no detectable toxicity at up to 10 μM (data not shown).

Inhibition of HIV protease activity by KNI-174, KNI-227, and KNI-272. The K_i values for KNI-174, KNI-227, and KNI-272 were estimated by a continuous fluorometric assay to be 6.8, 2.3, and 5.5 pM, respectively. These estimated values are approximately 1,000 times lower than the IC_{50}s reported previously (24). This discrepancy can be explained by the fact that apparent IC_{50}s will exceed actual K_i values for tight-binding inhibitors when the enzyme concentration is higher than the K_i value. In general, the lower the K_i, the greater will be the discrepancy. In addition, K_i values for HIV protease inhibitors are known to be lower at higher ionic strengths (3).

Inhibition of the viral protease-mediated processing of the Gag protein precursor by KNI-227 and KNI-272. We then asked whether KNI-227 and KNI-272 could block the viral protease-mediated processing of the p55 Gag protein precursor in the cytoplasm of chronically HIV-1-infected cells. In HIV-1_{LAI}-infected cells radiolabeled in the absence of the compounds, the formation of mature protein, p24, was readily detectable in 24 h both in cell lysates and in culture supernatants (Fig. 4). In addition to p24 Gag protein, a slightly larger molecule, which appeared to represent p24 Gag protein with an additional 14 amino acids at the carboxyl terminus was also detected in the cell lysate (22). At 48 h, this slightly larger molecule disappeared and only one strong signal representing p24 Gag protein was identified (Fig. 4). However, when the cells were cultured in the presence of 10 μM of either of the compounds, there was essentially no detectable p24 formation seen in both the cell lysates and the culture supernatants harvested after 48 h of culture. Despite the lack of p24 Gag protein formation, an expected reciprocal increase in the amount of the p55 precursor protein in CEM/HIV-1_{LAI} cells incubated with the drug was not obvious. This was probably due to an overlap of cellular protein(s) which was cross-reactive with the antiserum used in this study and/or a rather low reactivity of the antiserum against the p24 antigenic epitopes on the p55 precursor protein. It was noted, however, that, although p55 precursor protein was not detected and only p24 Gag protein was detected in the culture medium of the control drug-unexposed CEM/HIV-1_{LAI} cells, p55 was readily identified and

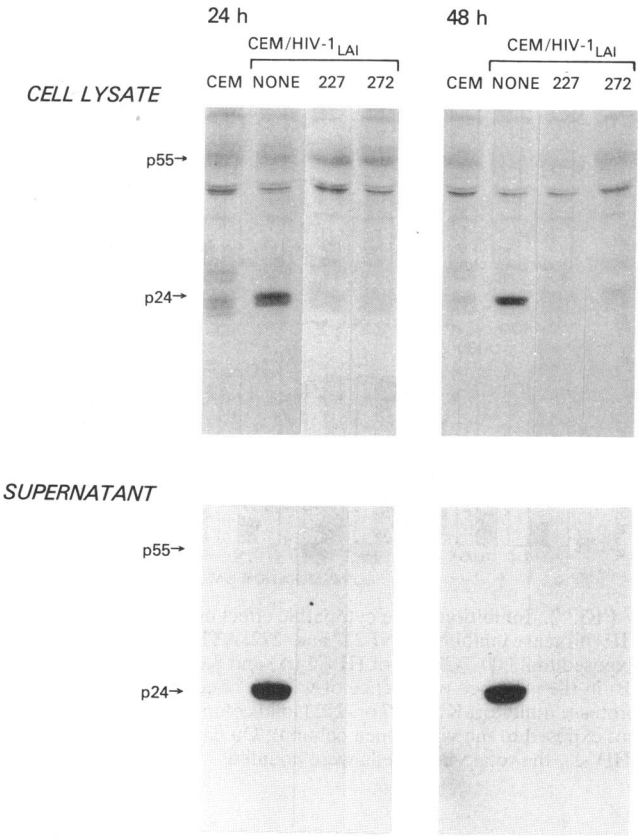

FIG. 4. Inhibition of the proteolytic activity of HIV-1 protease by KNI-227 and KNI-272 in stably HIV-1-infected CEM cells. Chronically HIV-1_{LAI}-infected CEM (CEM/HIV-1_{LAI}) cells were metabolically labeled with [^{35}S]methionine in the presence of KNI-227 or KNI-272, and a radioimmunoprecipitation assay was performed with rabbit antiserum reactive against HIV-1 p24 Gag protein. The formation of the mature p24 Gag protein was greatly reduced in the cells cultured with KNI-227 or KNI-272 in comparison with that in the control drug-unexposed (CEM/HIV-1_{LAI}) cells.

no p24 Gag protein was detected in the culture medium of the CEM/HIV-1_{LAI} cells cultured in the presence of KNI-227 or KNI-272.

Lipophilicity of selected protease inhibitors as assessed by *n*-octanol–water partition coefficients. We further asked whether KNI-174, KNI-227, and KNI-272 were lipophilic (hydrophobic) by determining *n*-octanol–water partition coefficients by a microshake-flask procedure and isocratic reverse-phase HPLC (7, 10). In general, the greater the values of log $P_{o/w}$ and column capacity factor, the higher the lipophilicity (hydrophobicity) of a given compound (7, 10). All three protease inhibitors examined had partition coefficients approximately 3,500 times greater than that of AZT. The column capacity factors confirmed the relative order of increasing lipophilicity among these inhibitors to be KNI-272 < KNI-227 ≈ KNI-174 (Table 2).

Stability of KNI-227 and KNI-272 in acidic conditions. When KNI-227 and KNI-272 were incubated in a hydrochloric acid solution (pH 1) at 37°C, both compounds were found to be stable and no degradation was detected even after 24 h (data not shown). This was also true when both compounds were incubated in the presence of pepsin A (1 mg/ml) in 0.2 M citrate solution (pH 2.2) at 37°C for 24 h. The control

TABLE 2. The *n*-octanol–water partition coefficients and column capacity factors of selected HIV protease inhibitors, KNI-174, -227, and -272

Compound	Log $P_{o/w}$[a]	Capacity factor (k' ± SD)[b]
KNI-174	3.77	3.74 ± 0.10
KNI-227	3.79	3.02 ± 0.08
KNI-272	3.56	1.72 ± 0.06
AZT[c]	0.052	ND
ddI[c]	−1.242	ND

[a] *n*-Octanol–water partition coefficients (log $P_{o/w}$) were determined by the microshake-flask technique (7, 38). Values represent means of two determinations. Column: Altex/Beckman 5-μm octyldecyl silane, 250 × 4.6 mm. Mobile phase: CH_3CN, 50% in 0.01 M phosphate (pH 7). Detector: Waters model 994 Photodiode Array at 220 nm; absorbance units, full scale, 0.05. Flow: 1 ml/min. Injection volume: 50 μl. t_o = 2.12 min. A reference compound, propylbenzene, gave a log $P_{o/w}$ value of 3.72 in this experiment.

[b] Capacity factor, a retention time parameter, was determined by the following formula: $k' = (t_r - t_0)/t_0$, where t_r is a sample retention time and t_0 is a retention time of unretained compound (7). Values represent means ± 1 standard deviation of three independent determinations. ND, not determined.

[c] Log $P_{o/w}$ values of AZT and ddI are shown for reference (38).

synthetic nonapeptide was completely digested under identical conditions (data not shown).

DISCUSSION

In the present study, we identified a series of tripeptide antiviral HIV protease inhibitors containing a unique unnatural amino acid, Apns [(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid], with a hydroxymethylcarbonyl isostere as a transition state mimetic. Normally, statine is considered to be a dipeptide (P1-P1′) transition state isostere. Apns, as used in this study, is probably acting as a P1 substitution only, with the thioproline acting as P1′, while in order to clarify this issue, refinement of the crystal structure of Apns is required.

Two compounds designated KNI-227 and KNI-272 were identified as having a highly potent inhibitory activity against HIV protease with little inhibition of other aspartic proteases such as human plasma renin (IC_{50}, >100,000 nM) and porcine pepsin (IC_{50}, >10,000 nM) (24). These two compounds exerted the most potent antiretroviral activity against a wide spectrum of HIV strains including HIV-1_{LAI}, HIV-1_{RF}, HIV-1_{MN}, an AZT-sensitive primary HIV-1 isolate, an AZT-insensitive HIV-1 variant, and an HIV-2 strain as tested in a variety of target cells. Such broad-spectrum anti-HIV activity is notable since HIV-1 has recently been shown to readily develop decreased sensitivity both in vitro and in vivo to certain nonnucleoside reverse transcriptase inhibitors such as tetrahydro-imidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1H)-one and -thione (TIBO) derivatives and nevirapine which do not show antiviral activity against HIV-2 (31, 35, 36). There is a growing body of data suggesting that the lack of activity of a given compound against HIV-2 may predict the relatively rapid loss of sensitivity of HIV-1 to that drug (1, 31). Thus, KNI-227 and KNI-272, both of which are active against HIV-2, may not allow HIV-1 to easily develop variants with decreased sensitivity to these compounds.

It is also worth noting that employing Apns may bring about relative ease in the synthesis of tripeptide protease inhibitors. This potential advantage is mainly due to the simple coupling procedures which are performed stepwise in solution. Furthermore, the condensing processes employ only reagents which are relatively easily available (15, 16, 26).

Both KNI-227 and KNI-272 were shown to block the formation of the mature core protein, p24 Gag protein, in stably HIV-1-infected CEM cells as assessed by radioimmunoprecipitation assay using rabbit anti-p24 Gag protein antiserum (Fig. 4). These data strongly suggested that KNI-227 and KNI-272 blocked the in vitro replication of HIV-1 by inhibiting the proteolytic activity of HIV protease. Moreover, it was noted that although the formation of p24 Gag protein was predominant in the supernatant of HIV-1-infected CEM cells, a substantial amount of the mature p24 Gag protein was also detected in the cell lysates. It is believed that retroviral virions are assembled proximally to the cell membrane as immature virion particles, composed of the external glycoprotein envelope, the genomic RNA, and viral polyproteins, and that, after budding from the cells, maturation of fully formed immature virion particles occurs with the action of the retroviral protease (21, 23). However, our observations suggest that the proteolytic activity of HIV-1 protease is operating both in the cytoplasm (before the retroviral budding) and in virion particles (after the budding), although mature virion particles that might have budded into vacuoles or enclosed sacs within the cell may have contributed to the detection of p24 Gag protein in the cell lysate. More careful studies are required.

HIV-1 actively replicates in the central nervous system (CNS) and often causes a variety of neurological disorders in patients with HIV-1 infection. Thus, the capacity of antiretroviral agents to penetrate into the CNS may constitute an important property of therapeutic agents for treatment of HIV-1 infection. We found that all three protease inhibitors tested, including KNI-227 and KNI-272, had substantially high levels of lipophilicity (hydrophobicity) as determined by *n*-octanol–water partition coefficients (Table 2). Log $P_{o/w}$ values were all greater than 3.5, which was significantly higher than that of AZT or ddI. It is thought that agents with log $P_{o/w}$ values of approximately 2 most effectively penetrate into the CNS (10). It should be noted, however, that a drug with high lipophilicity may exhibit increased neurotoxicity. It should also be noted that the principal determinants of entry of any agents into the CNS include lipophilicity, protein binding, and carrier systems (10, 32). Only in vivo studies can address the issue of CNS penetration of these compounds.

Both KNI-227 and KNI-272 are capable of blocking the activity of HIV protease in a pure enzyme assay. However, like renin inhibitors which have shown remarkably reduced activity when tested in the presence of plasma (6), Apns-containing protease inhibitors may also have decreased activity in plasma. In fact, we have recently observed that in the presence of higher concentrations of fetal calf serum (e.g., 50 to 80%), 6- to ~30-fold higher concentrations of KNI-272 were required to achieve the antiviral activity observed in the presence of 15% fetal calf serum (12a).

In general, the delivery of peptide analogs as pharmaceutical agents is problematic, as has been noted in the attempt to use peptide-based renin inhibitors as antihypertensives (8), because of poor aqueous solubility, poor oral bioavailability, and rapid clearance from the plasma by hepatobiliary excretion. Undoubtedly, these hurdles are common to any peptide-based HIV protease inhibitors. Like previously described transition state mimetic HIV protease inhibitors (22, 37), Apns-containing HIV protease inhibitors required lipophilic (hydrophobic) groups in both the N-terminal and C-terminal regions, for favorable binding interactions with HIV protease. Indeed, the incorporation of less lipophilic groups resulted in a substantial loss in antiretrovi-

ral activity (Table 1). The resulting low aqueous solubility, however, is likely to limit oral bioavailability. Although a nonaqueous formulation enhances oral absorption of the highly lipophilic cyclic peptide cyclosporin (9, 40), it is unclear whether a similar strategy would prove to be effective and practical for Apns-containing HIV protease inhibitors. In this work, we have also found that both KNI-227 and KNI-272 were highly stable in the presence of pepsin at acidic conditions.

Taken together, the current data suggest that two compounds, KNI-227 and KNI-272, represent potential antiretroviral drugs for therapy for HIV-1 infection. Further careful investigation in the direction of possible clinical applications of these compounds is warranted.

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