for 3 hours, and reweighed. The latter weight was subtracted from the former and divided by the number of individuals in the sample to give the ash-free dry weight per larva or juvenile. Ash-free dry weights for adult individuals and for daily egg-mass production from paired adults were similarly obtained.
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Rational Design of Peptide-Based HIV Proteinase Inhibitors

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A series of peptide derivatives based on the transition-state mimetic concept has been designed that inhibit the proteinase from the human immunodeficiency virus (HIV). The more active compounds inhibit both HIV-1 and HIV-2 proteinases in the nanomolar range with little effect at 10 micromolar against the structurally related human aspartic proteinases. Proteolytic cleavage of the HIV-1 *gag* polyprotein (p55) to the viral structural protein p24 was inhibited in chronically infected CEM cells. Antiviral activity was observed in the nanomolar range (with one compound active below 10 nanomolar) in three different cell systems, as assessed by p24 antigen and syncytium formation. Cytotoxicity was not detected at 10 and 5 micromolar in C8166 and JM cells, respectively, indicating a high therapeutic index for this new class of HIV proteinase inhibitors.

DURING THE REPLICATION CYCLE of HIV, *gag* and *gag-pol* gene products are translated as polyproteins. These are subsequently processed by a virally encoded proteinase to yield structural proteins of the virus core (p17, p24, p9, and p7), together with essential viral enzymes including the proteinase itself (*1*). On the basis of its primary amino acid sequence (*1*), its inhibition by pepstatin (*2*), and its crystal structure (*3*), HIV-1 proteinase has been classified as an aspartic proteinase that functions as a homodimer (*4*). This enzyme was first suggested as a potential target for AIDS therapy by Kramer *et al.* (*5*) when it was shown that a frameshift mutation in the proteinase region of the *pol* gene prevented processing of the *gag* polyproptein precursor. In this report we describe the rational design of a series of potent, selective inhibitors of HIV proteinase that show powerful antiviral activity against HIV-1 in vitro combined with low cytotoxicity to the host cell lines.

Although HIV proteinase can cleave a number of specific peptide bonds (*6*), it is unusual in being able to cleave the Phe-Pro and Tyr-Pro sequences found in *gag* and *gag-pol* gene products. Since the amide bonds of Pro residues are not susceptible to cleavage by mammalian endopeptidases, we reasoned that this could provide a basis for the rational design of HIV proteinase inhibitors selective for the viral enzyme.

Our strategy was based on the transition-state mimetic concept, an approach that has been used successfully in the design of potent inhibitors of other aspartic proteinases. Transition-state mimetics include hydroxyethylene isosteres (*7*), phosphinic acid (*8*), reduced amide (*9*), statine types (*10*), and hydroxyethylamine mimetics (*11*). Since the reduced amide **I** and the hydroxyethylamine **II** structures (Fig. 1) most readily accommodate the imino acid moiety characteristic of Phe-Pro and Tyr-Pro in retroviral substrates, we chose to study these structural types. During our studies we, and others (*12, 13*), have discovered that compounds containing the reduced amide function are relatively poor inhibitors. In contrast, we now report that compounds incorporating the hydroxyethylamine moiety are very potent and highly selective inhibitors of HIV proteinase.

Compounds based on the *pol* fragment Leu^{165}-Ile^{169}, containing the transition state

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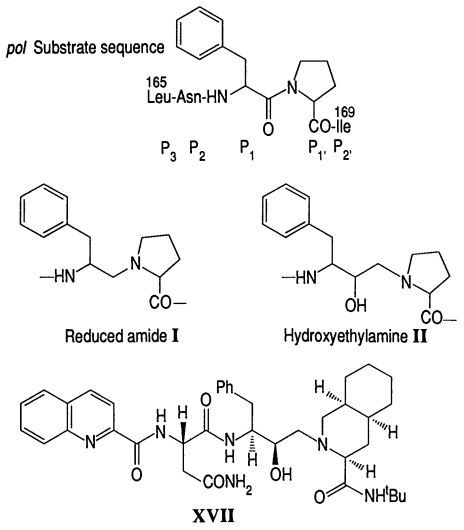

Fig. 1. Sequence of pol substrate and structures of inhibitors.

moiety PheΨ[CH(OH)CH_2N]Pro in place of the Phe^{167}–Pro^{168} scissile bond, were used to determine the minimum sequence required for potent inhibition (Table 1). Weak inhibitory activity was observed with a protected dipeptide (compound **III**). However, amino-terminal extension by one amino acid residue resulted in 40-fold improvement in potency (compound **IV**). Extension at the amino terminus or carboxyl terminus or both did not result in further improvement in potency (compounds **VI**, **VII**, and **VIII**). A preference for *R* stereochemistry at the hydroxyl-bearing carbon atom was initially indicated by compounds **IV** and **V**, which were prepared from precursors of known chirality. This effect was more dramatically confirmed with compounds **XIV** and **XV**.

Having identified compound **IV** as being of the minimum size required for potent inhibition, we systematically explored the structural requirements for optimal binding at each subsite. More than 100 compounds were synthesized in which the steric and electronic properties of each side chain and terminal substituent were individually modified. The most important findings were that a large hydrophobic pocket at P_3 was inferred from the high potency shown by compounds **X** and **XI**. At the P_2 subsite no improvement over asparaginyl was found, although the β-cyanoalanyl and *S*-methylcysteinyl analogues, compounds **XIII** and **XVI**, respectively, displayed comparable potencies. Similarly, at P_1 no improvement was found over the benzyl side chain of Phe. The most marked improvements in potency were achieved by varying the imino acid at $P_{1'}$, highly effective replacements for prolyl being piperidine-2-(*S*)-carbonyl (compound **XII**) and (*S*,*S*,*S*)-decahydro-isoquinoline-3-carbonyl (compound **XVII**). At the carboxyl terminus tert-butyl ester could be replaced by a tert-butylamide group without significant change in potency (compounds **IV** and **IX**), but no better replacement was identified.

Incorporating combinations of preferred side chains into individual molecules resulted in the generation of several very potent inhibitors of both HIV-1 and HIV-2 proteinases, for example, compounds **XIV** and **XVII**. The inhibition constant K_i for compound **XVII** at *p*H 5.5 was 0.12 n*M* against HIV-1 proteinase, and binding to HIV-2 proteinase was even stronger ($K_i < 0.1$ n*M*). It was important to confirm that inhibitors optimized in an assay using a small synthetic substrate were also effective in blocking the cleavage of the natural *gag* polyprotein substrate. In a mixed bacterial lysate assay, compound **XVII** at 1 μ*M* completely inhibited the processing of HIV-1 *gag* polyprotein by HIV-1 and HIV-2 proteinases as assessed by immunoblot analysis (Fig. 2A). Moreover, this compound was highly selective, causing less than 50% inhibition of the human aspartic proteinases renin, pepsin, gastricsin, cathepsin D, and cathepsin E (*14*) at a concentration of 10 μ*M*. In addition, compound **XVII** at a concentration of 10 μ*M* had no effect on representative proteinases from the serine, cysteine, and metallo classes [such as human leucocyte elastase, bovine cathepsin B, and human collagenase, respectively (*15*)].

The antiviral activities of compounds in this series correlated well with their potencies as proteinase inhibitors (Table 2). The most potent antiviral activity was observed with compound **XVII**, which had a 50% inhibition concentration (IC_{50}) of 2 n*M* against HIV-1 (strain RF) in C8166 cells as assessed by measurement of viral antigen (p24) in the culture medium. When the multiplicity of infection was increased tenfold from 10 to 100 $TCID_{50}$ (median tissue culture infectious dose) units per 2×10^5 cells, there was only a marginal effect on the IC_{50} values for compounds **IV** and **XVII** (Table 2). In this test system the IC_{50} for azidothymidine (AZT) was in the range 3 to 30 n*M*, which is similar to the IC_{50} for AZT reported (*16*) against HIV isolates from untreated AIDS or ARC patients using a plaque assay (10 to 50 n*M*) or p24 antigen levels (<30 n*M*). The antiviral potencies of compounds **IV**, **XIV**, and **XVII** were confirmed in JM cells infected with the HIV-1

Table 1. HIV-1 and HIV-2 proteinases were cloned, expressed, and purified as previously described (*2*, *25*–*27*). Proteinase activity and its inhibition was assayed with the protected heptapeptide succinyl.Val.Ser.Leu.Asn.Tyr.Pro.Ile.isobutylamide as substrate. Quantification of substrate cleavage was achieved by measuring the production of H.Pro.Ile.isobutylamide by the spectrophotometric assay of amino-terminal proline. Substrate (0.68 m*M*) was dissolved in 125 m*M* citrate buffer, *p*H 5.5, containing 0.125 mg/ml Tween 20. Inhibitor solution (10 μl) and proteinase (10 μl) were added to 80 μl of buffered substrate. Digestion was carried out at 37°C for a fixed period of time, usually 3 hours, and the reaction terminated by the addition of 1 ml of color reagent [isatin (30 μg/ml) and 2-(4-chlorobenzoyl)-benzoic acid (1.5 mg/ml) in 10% acetone in ethanol]. The solution was heated in a boiling water bath for 15 min, and the pigmented residues were redissolved in 1 ml of 1% pyrogallol in 33% water in acetone. The optical density of the solution was measured at 599 nm. Compounds were synthesized by standard procedures and were characterized by thin-layer chromatography (silica gel), reversed-phase high-performance liquid chromatography, ^{1}H nuclear magnetic resonance mass spectroscopy, melting point, and, where possible, elemental analysis. Details of syntheses and characterization will be reported separately. Abbreviations: Z, benzyloxycarbonyl; Pheψ[CH(OH)CH_2N]Pro indicates replacement of the imide group (CON <) in the Phe.Pro. peptide bond by the hydroxyethylamine moiety, that is, CH(OH)CH_2N < where the hydroxy function has the configuration indicated in the table; ^{t}Bu, *tert*-butyl; *BN*, β-naphthoyl; QC, quinoline-2-carbonyl; PIC, piperidine-2(*S*)-carbonyl; CNA, β-cyanoalanyl; SMC, *S*-methyl-cysteinyl; and DIQ, (4a*S*, 8a*S*)-decahydro-3(*S*)-isoquinolinecarbonyl.

Compound number	Stereochemistry at –CHOH–	Inhibitor structure	IC_{50} (n*M*) HIV-1	IC_{50} (n*M*) HIV-2
III	*R**	Z.Pheψ[CH(OH)CH_2N]Pro.O^{t}Bu	6500	
IV	*R*	Z.Asn.Pheψ[CH(OH)CH_2N]Pro.O^{t}Bu	140	330
V	*S*	Z.Asn.Pheψ[CH(OH)CH_2N]Pro.O^{t}Bu	300	
VI	*R**	Z.Leu.Asn.Pheψ[CH(OH)CH_2N]Pro.O^{t}Bu	600	
VII	*R**	Z.Asn.Pheψ[CH(OH)CH_2N]Pro.Ile.NH^{t}Bu	130	
VIII	*R**	Z.Leu.Asn.Pheψ[CH(OH)CH_2N]Pro.Ile.NH^{t}Bu	750	
IX	*R*	Z.Asn.Pheψ[CH(OH)CH_2N]Pro.NH^{t}Bu	210	
X	*R*	BN.Asn.Pheψ[CH(OH)CH_2N]Pro.O^{t}Bu	52	50
XI	*R*	QC.Asn.Pheψ[CH(OH)CH_2N]Pro.O^{t}Bu	23	
XII	*R*	Z.Asn.Pheψ[CH(OH)CH_2N]PIC.NH^{t}Bu	18	
XIII	*R*	Z.CNA.Pheψ[CH(OH)CH_2N]PIC.NH^{t}Bu	23	
XIV	*R*	QC.Asn.Pheψ[CH(OH)CH_2N]PIC.NH^{t}Bu	2	9.5
XV	*S*	QC.Asn.Pheψ[CH(OH)CH_2N]PIC.NH^{t}Bu	470	
XVI	*R*	QC.SMC.Pheψ[CH(OH)CH_2N]PIC.NH^{t}Bu	12	15
XVII	*R*	QC.Asn.Pheψ[CH(OH)CH_2N]DIQ.NH^{t}Bu	<0.4	<0.8

*More active diastereo-isomer; assigned *R*-stereochemistry but not proven.

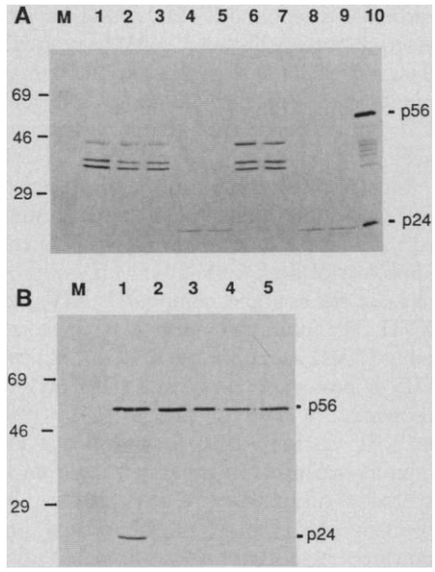

Fig. 2. Inhibition of proteinase-mediated *gag* p24 production by compound **XVII**. (**A**) Immunoblot analysis of *gag* p24 immunoreactive products derived from an in vitro *Escherichia coli* mixed lysate assay (*26*). Lane M, prestained marker proteins in kilodalton; lane 1, incubation of *gag* lysate alone; lanes 2 to 4, incubation of *gag* lysate with HIV-1 proteinase in the presence of compound **XVII** at concentrations of 10 μ*M* (lane 2), 1 μ*M* (lane 3), and 0.1 μ*M* (lane 4); lane 5, incubation of *gag* lysate with HIV-1 proteinase in the absence of compound; lanes 6 to 9, same as lanes 2 to 5 but with HIV-2 proteinase. In lane 10 an extract of HIV-1 infected cells is used to illustrate the migration of *gag* p56 and p24. The *E. coli* strains used and the methods of preparation of recombinant protein lysates have been previously described (*26*). Lysates were incubated for 2 hours at 37°C in 50-μl reactions in 125 m*M* citrate buffer, *p*H 5.5, containing 0.0125% Tween 20 followed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with a *gag* p24 monoclonal antibody (MRC ADP 313). (**B**) Immunoblot analysis of *gag* p24 immunoreactive proteins in CEM cells chronically infected with HIV-1 (strain HTLV IIIB) after treatment with compound **XVII**. Lane M, prestained marker proteins (kilodalton); lane 1, cells in the absence of compound **XVII**; lanes 2 to 5, cells incubated in the presence of compound **XVII** at a concentration of 1 μ*M* (lane 2), 100 n*M* (lane 3), 10 n*M* (lane 4), and 1 n*M* (lane 5). CEM-IIIB cells in exponential growth were washed twice with growth medium (RPMI 1640 containing 10% fetal bovine serum) and resuspended at 2×10^5 cells per milliliter in growth medium. After culture for 24 hours at 37°C in the presence or absence of compound **XVII**, the cells were washed and cultured for a further 24 hours with drug as before. Cell viability was 95% for all samples as determined by trypan blue exclusion. After a further wash in phosphate-buffered saline, aliquots of 10^5 cells were lysed for assay by SDS-PAGE and immunoblotting with the *gag*-3 p24 monoclonal antibody (*29*).

strain GB8 (*17*) by quantitation of syncytium formation. Mean IC_{50} values of 450 n*M* ($n = 2$), 10 n*M* ($n = 2$), and 2.5 n*M* ($n = 5$), respectively, were obtained. Dose-response characteristics for **XVII** by syncytial count assay (Table 3) showed good correlation with the extracellular levels of p24 core antigen. Dideoxycytidine (ddC) had good antiviral activity in the JM cell system (IC_{50} syncytial assay, 36 n*M*; p24 antigen, 24 n*M*), but AZT was inactive at concentrations up to 100 μ*M*. This was explained by very poor phosphorylation of AZT in these cells (*18*).

Cytotoxicity studies with compound **XVII** in C8166 and JM cells have used a variety of cell viability markers: ^{14}C protein hydrolysate and [^{3}H]thymidine uptake, MTT reduction (*19*), and cell growth. These have given TD_{50} (toxic dose) values in the range from 5 to 100 μ*M*; that is, at least 2000-fold greater than the concentration required for antiviral activity.

In clinical HIV infection, a constant burden on the patient will be the continued release of mature virus from infected cells. Accordingly, CEM cells chronically infected with HIV-1 strain IIIB were cultured in the presence of compound **XVII**; inhibition of *gag* p24 formation was demonstrated at inhibitor concentrations as low as 1 n*M* (Fig. 2B), indicating a blockade in *gag* processing and viral maturation. This mode of action has been supported by preliminary electron microscopic examination of these cells, which indicated a marked increase in the number of immature extracellular virions in the presence of drug. In similar experiments in H9 cells chronically infected with HIV-1 (RF strain) and treated with compound **XIV**, SDS–polyacrylamide gel electrophoresis analysis of radioimmunoprecipitation products from cell-free virions showed an absence of p24 antigen with the accumulation of p55. Under these conditions, cell-free infectivity in the presence of compound **XIV** at 10 μ*M* was reduced by >3 $\log_{10}$ units, whereas AZT (10 μ*M*) had no effect (*20*).

Recent studies (*21, 22*) have demonstrated antiviral activity with inhibitors of HIV proteinase and shown that antiviral activity is associated with inhibition of *gag* and *gag-pol* processing. These data have added further support to the hypothesis first advanced by Kramer *et al.* (*5*) that HIV proteinase is a viable target for therapeutic intervention of HIV infection. We have also observed inhibition of *gag-pol* processing by compounds **XIV** and **XVII** in a whole cell, baculovirus system (*23*). Our choice of using a hydroxyethylamine transition-state mimetic incorporating an analog of the amide bond of Phe.Pro., rather than hydroxyethylene isosteres (*21, 22*), has resulted in inhibitors of HIV proteinases of considerably enhanced potency and, more importantly, a very high level of selectivity. The high selectivity associated with our compounds may result in a reduced potential for toxicity, and the low affinity for aspartic proteinases in the gut, namely pepsin, gastricsin, and cathepsin E, may enhance oral absorption. The enhancement in antiviral activity of compound **XVII** compared with the better hydroxyethylene isosteres (approximately 100-fold) parallels

Table 2. C8166 cells, a CD4$^+$ T lymphoblastoid cell line, were grown in RPMI 1640 medium containing 10% v/v fetal bovine serum, and harvested for experiment in log phase growth. Cultures were incubated for 90 min at 37°C with HIV-1 (strain RF) at a level of 10 $TCID_{50}$ units per 2×10^5 cells or 100 $TCID_{50}$ units (values in parentheses) after which cells were washed three times in phosphate-buffered saline, Dulbecco A (PBS), to remove unadsorbed virus. The cells were resuspended in growth medium, and cultured at a density of 2×10^5 cells per 1.5 ml in 6-ml Falcon culture tubes, with or without test drug, for 72 hours at 37°C, at an atmosphere of 5% CO_2. HIV antigen (p24) was measured in the supernatant using a commercial enzyme-linked immunosorbent assay (ELISA, (Coulter Electronics Limited, Luton, U.K.) (*28*). Antiviral activity was expressed as the inhibitor concentration required to reduce HIV antigen levels by 50% (IC_{50}). The cytotoxic effects of test compounds were assessed by incubating uninfected C8166 cells with drug under the same conditions as above. After 72 hours the cells were washed with PBS then resuspended in growth medium containing ^{14}C protein hydrolysate (Amersham). The cells were harvested after 12 hours and ^{14}C incorporation measured. Results are expressed as the drug concentration required to reduce ^{14}C incorporation by 50% (TD_{50}) compared with untreated controls. Similar experiments were performed for compound **XVII** using [^{3}H]thymidine incorporation and MTT reduction (*19*) to assess cell viability.

Compound number	HIV-1 proteinase inhibition	Antiviral activity	Cytotoxicity C8166 cells
	IC_{50} (n*M*)	IC_{50} (n*M*)	TD_{50} (n*M*)
IV	140	300* (350)	$>10^5$
IX	210	400	$>10^5$
X	52	130	$>10^4$
XI	23	110	$>10^5$
XIV	2	17*	$>10^5$
XVI	12	13*	$>10^4$
XVII	<0.4	2* (4)	$>10^4$

*Mean values of at least four separate experiments. Other values mean of two experiments.

Table 3. Dose-response for compound **XVII** against JM cells infected with HIV-1 (GB8).

XVII (n*M*)	Mean syncytial count	p24 Antigen (ng/ml)
3	25 (19%)	0.88 (2.8%)
1	50 (31%)	1.56 (5.7%)
0.3	71 (53%)	2.65 (9.8%)
0.1	137 (102%)	16.0 (54%)
0.0*	134 (100%)	27.0 (100%)

*Drug free.

a similar enhancement in HIV proteinase inhibition. In contrast to AZT and other inhibitors of the reverse transcription process (*24*), proteinase inhibitors are effective against chronically infected cells. The high level of antiviral activity and low cytotoxicity for compound **XVII** provides a high therapeutic index.

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15. Human leukocyte elastase assays made use of enzyme purified from leukocytes of patients with chronic granulocytic leukemia; methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-*p*-nitro-anilide was the substrate at *p*H 8. Bovine cathepsin B (Sigma Chemical Co., Ltd.) was assayed at *p*H 6.0 with benzoylarginine-*p*-nitroanilide as substrate. Collagenase partially purified from human synovial fibroblast culture medium was assayed by the method of B. Johnson-Wint [*Anal. Biochem.* **104**, 174 (1985)].
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Immunobiology and Pathogenesis of Hepatocellular Injury in Hepatitis B Virus Transgenic Mice

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The role of the immune response to hepatitis B virus (HBV)–encoded antigens in the pathogenesis of liver cell injury has not been defined because of the absence of appropriate experimental models. HBV envelope transgenic mice were used to show that HBV-encoded antigens are expressed at the hepatocyte surface in a form recognizable by major histocompatibility complex (MHC) class I–restricted, $CD8^+$ cytotoxic T lymphocytes specific for a dominant T cell epitope within the major envelope polypeptide and by envelope-specific antibodies. Both interactions led to the death of the hepatocyte in vivo, providing direct evidence that hepatocellular injury in human HBV infection may also be immunologically mediated.

THE HBV IS AN ENVELOPED, CIRCULAR, double-stranded DNA virus that causes acute and chronic liver disease and hepatocellular carcinoma (*1*). The mechanisms responsible for HBV-induced hepatocellular injury are not well understood (*2*). Although HBV-specific T cells are present in the peripheral blood and intrahepatic lymphocyte populations in HBV vaccine recipients (*3, 4*) and patients with chronic hepatitis (*5*), there is no definitive evidence that these responses are involved in the destruction of infected hepatocytes in this disease. The narrow host range of HBV and its nontransmissibility in routine cell culture systems have prevented the use of conventional approaches to this question. The minimal model for T cell–mediated liver cell injury requires hepatocellular synthesis of HBV gene products; the appropriate processing, association, and surface expression of these products with major histocompatibility determinants; the induction and expansion of a cellular immune response specific to MHC-associated HBV peptides; and the susceptibility of the primary hepatocyte to T cell–mediated cytolysis.

We developed an HBV transgenic mouse model system to analyze the immunopathogenesis of HBV-induced liver disease (*6*). The HBV (subtype ayw) envelope region that contained the preS(1), preS(2), and HBs antigens (HBsAg) was ligated to mouse albumin regulatory sequences and introduced into unicellular inbred (B10.D2) mouse embryos (*7*). Transgenic mouse lineage 107-5 [official designation Tg (Alb-1, HBV) Bri66] expresses noncytotoxic amounts of the HBV large and major envelope polypeptides in the hepatocytes (*7*). This lineage does not develop spontaneous liver disease and is immunologically tolerant to the preS and HBs antigens (*7*). Donor B10.D2 mice were immunized with a recombinant vaccinia virus (vHBs4) that contained the coding region for the HBV major envelope polypeptide. Transgenic recipients of primed spleen cells from these

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