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Inhibition of HIV-1 replication by a peptide dimerization inhibitor of HIV-1 protease

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Abstract

Peptides based on the amino (N) and carboxy (C)-terminal regions of human immunodeficiency virus type-1 (HIV-1) protease and on the C-terminus of p6* can inhibit HIV-1 protease activity by preventing dimerization. We developed a peptide dimerization inhibitor, P27, that included these domains and a cell permeable domain derived from HIV-1 Tat. P27 inhibited wild type (WT) and protease inhibitor (PI)-resistant HIV-1 protease (IC_{50}: 0.23–0.32 μM). Kinetic and biochemical assays confirmed that P27 inhibits protease dimerization. Fluorescein-labeled peptide accumulated in MT-2 cells and protected acutely infected MT-2 cells from HIV-1-induced cytotoxicity (IC_{50}: 5.1 μM). P27 also inhibited p24 accumulation from H9 and U937 cells chronically infected with WT or PI-resistant HIV-1. Immunoblot analysis on the supernatants and infected cells revealed a block in virus release by P27 rather than an inhibition of polyprotein processing. However, inhibition of p55 Gag processing by active-site inhibitors was enhanced when combined with P27, suggesting that P27 can affect protease function in maturing virions. Although P27 was rationally designed to block dimerization of the mature HIV-1 protease, the effects of P27 on HIV-1 replication may be related to partial inhibition of Gag-Pol processing leading to a disruption in virus release.
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Keywords: HIV-1; Protease; Dimer; Peptide; Inhibitor; Polyprotein

1. Introduction

A number of HIV-1 protease inhibitors (PIs) are in clinical use for the treatment of HIV-1 infection (Deeks and Volberding, 1997; Flexner, 1998; Misson et al., 1997). These inhibitors target the active site of the protease, bind with very high affinity and prevent the production of infectious virions by infected cells. However, viral resistance to these inhibitors develops in patients as a result of HIV-1 protease mutations both near and far from the active site (Condra et al., 1995; Flexner, 1998). Certain combinations of these mutations can result in cross-resistance to more than one PI (Cabana et al., 1999; Condra et al., 1995; Deeks and Volberding, 1997; Misson et al., 1997). After long-term treatment with PIs, the protease can acquire ten or more mutations and yet still remain sufficiently active to produce infectious virus (Romano et al., 2002; Schmit et al., 1996; Shafer et al., 1998; Yoshimura et al., 1999). Furthermore, mutations in Gag that alter the natural protease cleavage sites can contribute to high-level PI resistance (Gatanaga et al., 2002). For these reasons, there is a need for new strategies to contain viral replication. One active area of research has focused on alternative mechanisms to inhibit protease activity (Oxford et al., 1989).

The HIV-1 protease is an obligate dimer and therefore one potential target for protease inhibitors is the dimer interface (Babé et al., 1992, 1995; Davis et al., 1997, 2003; Schramm et al., 1999; Shultz and Chmielewski, 1999; Weber, 1990; Zutshi and Chmielewski, 2000). The interface consists of four inter-

Abbreviations: HIV-1, human immunodeficiency virus type-1; WT, wild type; PI, protease inhibitor; CPD, cell permeable domain; CPP, cell permeable peptide; RP-HPLC, reverse phase high performance liquid chromatography; PBS, phosphate-buffered saline

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locking anti-parallel beta strands with two strands contributed from each monomer. These beta strands include the first five N terminal amino acids and the last five C terminal amino acids of each 99 amino acid protease monomer and contribute 75% of the Gibbs free energy of dimer stabilization (Todd et al., 1998). Our group has previously shown that reversible oxidation of a sulfur-containing amino acid at the dimer interface of HIV-1 or HIV-2 protease completely inhibits protease activity by blocking dimer formation (Davis et al., 1997, 2000, 2003). This data established the sensitive nature of protease dimerization to modification of the dimer interface and its potential value as a target for protease inhibitor development. Peptides mimicking the interface strands were the first compounds postulated as potential inhibitors of dimerization (Weber, 1990). The individual N and C terminal peptides can block protease activity and it was found that linking these two regions together in a single peptide using a glycine linker was even more effective (Babé et al., 1992). Others have developed peptides with different length synthetic linkers between variations of the N and C terminal peptides or potent lipopeptide dimerization inhibitors of the protease (Dumond et al., 2003; Shultz and Chmielewski, 1999; Ulysse and Chmielewski, 1998).

New methods have recently been developed for effectively delivering peptides and proteins into cells that involve attaching polycationic peptide sequences to peptides and proteins of interest (Fawell et al., 1994; Futaki et al., 2001, 2003; Richard et al., 2003; Vives et al., 1997). These polycationic peptides have been found to readily cross cell membranes in an energy-independent fashion (Futaki et al., 2001). One type of delivery involves the use of a highly basic region of the HIV-1 Tat protein that was initially found to facilitate the entry of the HIV-1 Tat protein into cells (Vives et al., 1997). Although there is uncertainty surrounding the exact mechanism by which these carrier peptides and their cargo enter and accumulate in living cells (Lundberg et al., 2003; Richard et al., 2003), several groups have demonstrated biological activity of the cargo peptides or proteins carried into the cells. Such activity has been demonstrated for inhibitors of beta cell death (Bonny et al., 2001), human catalase (Jin et al., 2001), a PKC agonist peptide (Chen et al., 2001), superoxide dismutase (Park et al., 2002), p53 (Takenobu et al., 2002), hsp70 (Wheeler et al., 2003), and a von Hippel-Lindau suppressor peptide (Datta et al., 2001). Others have described the delivery of biologically active proteins fused to a cell permeable domain (CPD) from HIV-1 Tat into the tissues of mice following injection (Schwarze et al., 1999). In this report, we describe the development of an HIV-1 protease peptide dimerization inhibitor containing a CPD from HIV-1 Tat and its effects on HIV-1 replication in acutely and chronically infected T-cells.

2. Materials and methods

2.1. Reagents

Peptides were obtained from Sigma Genosys (The Woodlands, TX). All peptides were synthesized using standard Fmoc chemistry and all peptides contained a C terminal amide. Fluorescein-labeled peptides were labeled at the N-terminus. Each peptide was received as a lyophilized powder (>90–95% purity) and stored frozen at −20 °C as a 1–2 mM solution in sterile PBS. Wild type (WT) HIV-1 protease (sequence derived from an untreated patient infected with HIV-1) and two resistant mutants termed R1 and $R1_{F95C}$ (R1 sequence derived from a heavily PI-treated HIV-1 infected patient) (Yoshimura et al., 1999) were prepared and refolded using the methods described previously (Davis et al., 1996) and stored at −70 °C at 0.6–3.5 mg ml^{-1}. Protease concentrations were determined spectrophotometrically (ε_{280} 12,300 M^{-1} cm^{-1} for HIV-1 protease) and the proteases were >95% pure based on RP-HPLC analysis.

2.2. Fluorimetric HIV-1 protease assay and kinetics

The fluorescent HIV-1 protease substrate (RE-(EDANS)-SQNYPIVQK-(DABCYL)-R) was obtained from Molecular Probes Inc. (Eugene, OR). Substrate and buffer were pre-warmed at 37 °C for at least 20 min before use. For screening and time-dependent studies, the protease (25 nM final concentration) and peptides were pre-incubated in the manufacturer's recommended assay buffer (10 μl) (100 mM Na acetate, 1 M NaCl, 1 mM EDTA, 1 mM DTT, 10% DMSO and 1 mg ml^{-1} BSA pH 4.7) at 37 °C for up to 30 min and then added to 90 μl of warmed substrate solution (final concentration 40 μM) to initiate the reaction. The total assay volume was 100 μl. Fluorescence was monitored for at least 10 min in a fluorescence microplate reader (FMAX, Molecular Devices, Sunnyvale, CA) with 355 nm excitation and 460 nm emission filters. The resulting progress curves were analyzed by non-linear regression using a first-order exponential fit to obtain initial velocities and determine IC_{50}s (BatchKi, BioKin Ltd., Pullman, WA). Kinetic analysis was performed using the Zhang and Poorman equation, which measures the dissociative inhibition of dimeric enzymes (Zhang et al., 1991). For these experiments HIV-1 protease was used at concentrations from 0.79 to 3.15 nM (active dimer) and substrate was used at a concentration of 5 μM. To initiate the reactions, the enzyme was added to buffer with or without the inhibitors, and activity was monitored for 30 min. Under the assay conditions indicated above, the substrate K_m was determined to be 21 ± 2 μM. E_0/k_{exp} as a function of k_{exp} was plotted at the different enzyme concentrations tested. Each enzyme concentration with and without inhibitor was run in triplicate and the resulting progress curves were analyzed by non-linear regression using a first-order exponential fit to obtain initial velocities from BatchKi (Pullman, WA). The concentration of active protease indicated above was determined by active site titration (Tomasselli et al., 1990) and fit to the Morrison equation using Prism (San Diego, CA) (Kuzmic et al., 1992).

2.3. Fluorescent microscopy

MT-2 cells grown in 10% fetal calf serum in RPMI were incubated for 1 h with N-terminal fluorescein labeled peptide PQITLRKKRRQRRRPPQVSFNFATLNF (F-P27A) or N-terminal fluorescein labeled cell permeable peptide RKKRRQRRRPPQVSFNF (F-CPP) at 30 μM in media containing 10%

serum. Following incubation, the cells were washed three times with fresh media, stained with trypan blue to assess cell viability and observed under phase contrast and fluorescence modes to assess uptake of fluorescein labeled peptides.

2.4. Generation of recombinant WT and PI-resistant HIV-1 molecular clones

Determination of nucleotide sequences of HIV-1 was performed as previously described (Yoshimura et al., 1999). In brief, pre-therapy HIV-1_{A01} (WT) and post-therapy HIV-1_{A2} (PI-resistant) clinical HIV-1 strains were isolated by culture from the peripheral blood mononuclear cells (PBMC) of an HIV-1 infected patient. The HIV-1 isolates were passed once in PHA-stimulated PBMC (PHA-PBMC) obtained from an HIV-1 negative donor. High molecular weight DNA was extracted from the PHA-PBMC harboring HIV-1_{A01} or HIV-1_{A2} using Instagene Matrix (Bio-Rad, Hercules, CA). The HIV-1_{A01} WT pre-therapy protease sequence contained four polymorphic amino acid substitutions when compared to the subtype B WT sequence (I15V, E35D, N37D, and I93L) but did not contain any primary resistance PI mutations as defined by Shafer et al. (2000). The HIV-1_{A2} sequence had nine mutations as compared to the pre-therapy sequence (L10I, N37E, K45R, I54V, L63P, A71V, V82T, L90M, and C95F), including three that are described as primary resistance mutations (I54V, V82T, and L90M) and that are known to confer resistance to multiple PIs (Kuiken et al., 2004; Shafer et al., 2000). The patient from whom HIV-1_{A2} was obtained had a high viral load in spite of taking anti-HIV therapy (treatment failure) at the time that this resistant protease sequence was the predominant viral isolate and was shown to be highly resistant to a variety of potent active site HIV-1 protease inhibitors in clinical use (Yoshimura et al., 1999). The entire protease-encoding region of HIV-1_{A01} and HIV-1_{A2} was amplified with *Taq* DNA polymerase from Perkin-Elmer Life Sciences (Shelton, CT) using the following two primer pairs: the forward primer1 (Kapa1) 5′-GCA GGG CCC CTA GGA AAA AGG GCT GTT GG-3′ and the reverse primer1 (PR12) 5′-CTC GTG ACA AAT TTC TAC TAA TGC-3′ and the forward primer2 (Kapa3) 5′-CAG GGC CCC TAG GAA AAA GGG CTG TTG GAA ATG TGG-3′, the reverse primer2 (Ksma4) 5′-GGG CCA TCC ATC CCG GGC TTT AAT TTT ACT GG-3′. The forward primer contained an *Apa*I site and the reverse primer contained an *Xma*I site. The PCR products were purified with PCR Select III column (5 Prime ‡ 3 Prime Inc., Boulder, CO) and subjected to molecular cloning using Original TA Cloning Kit (Invitrogen, Carlsbad, CA), followed by sequence determination using an Applied Biosystems model 373 automated DNA sequencer.

For the generation of a recombinant infectious molecular clone harboring the above-described WT virus protease sequence (rHIV-$_{A01}$), the PCR products obtained as above were digested with two enzymes *Apa*I and *Sma*I, and the obtained fragments were introduced into pHIV-1_{NLSma} which was designed to have a *Sma*I site by changing two nucleotides (2590 and 2593) of pHIV-1_{NL4-3}. We also generated an infectious molecular clone carrying a cysteine at position 95 plus the other substitutions described for rHIV-1_{A2} (rHIV-1_{A2F95C}) by starting with HIV-1_{A2} and utilizing site directed mutagenesis with the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) with forward primer (95TGC1) 5′-GAC TCA GAT TGG CTG CAC TTT AAA TTT TC-3′ and a reverse primer (95TGC2) 5′-GAA AAT TTA AAG TGC AGC CAA TCT GAG TC-3′. The PCR products were introduced into pHIV-1_{NLSma}, eventually generating rHIV-1_{A2F95C}. An HIV-1 isolate containing these protease resistance mutations was shown previously to be highly resistant to the protease inhibitors ritonavir, saquinavir, amprenavir, and indinavir (Yoshimura et al., 1999). Determination of the nucleotide sequences of newly generated plasmids confirmed that each clone had the desired mutations but no unintended mutations. Each recombinant plasmid was transfected into COS-7 cells with LipofectAmin (Life Technologies, Gaithersburg, MD) and infectious virions rHIV-1_{A01} and rHIV-1_{A2F95C} were harvested 48 h after transfection and stored at −80 °C until use.

2.5. Virus replication and cell viability assays

For studies on the effect of these peptides on acute infection, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to determine the ability of compounds to protect MT-2 cells from the cytopathic effect of HIV-1_{LAI}. MT-2 cells (4×10^3 cells in 200 μl complete medium per well) grown in RPMI-based culture medium with10% fetal calf serum (HyClone, Logan, UT) with 50 U ml^{-1} penicillin and 50 mg ml^{-1} streptomycin were incubated with 100 $CCID_{50}$ of HIV-1_{LAI} for 2 h and then added to 96-well plates containing the test compounds giving final concentrations from 0 to 50 μM. Lopinavir, a potent active-site inhibitor, was used as a positive control. Seven days later, 100 μl of medium was removed and MTT solution (10 μl, 7.5 mg ml^{-1}) was added to each well. The plates were incubated at 37 °C for 2 h and then each well treated with 100 μl of acidified isopropyl alcohol containing 4% (v/v) TritonX-100 to dissolve the formazan crystals. The absorbance at 570 nm was then determined in a microplate reader (Spectramax). Each assay was performed in duplicate. In this assay, the cytopathic effect of HIV-1 requires several rounds of infection and therefore is susceptible to inhibitors that act on the late stages of viral replication. The concentration of peptide required to inhibit HIV-1 induced cytotoxicity by 50% was calculated from the plot of peptide concentration versus the percent viable cells compared to the untreated HIV-1 infected control. The concentration of peptide that induced 50% cytotoxicity in uninfected cells was calculated from the plot of peptide concentration versus the percent viable cells in uninfected cells treated only with PBS.

Chronically infected cell lines were prepared by infecting H9 cells with wild type (WT, rHIV-1_{A01}) or PI-resistant virus (rHIV-1_{A2F95C}). One million cells were infected with 100 ng p24 in 1 ml of media and incubated at 37 °C for 2 h and then additional media added (15 ml) and cells maintained and passed every 5 days. The infected cells were maintained for several passages before use in inhibitor assays. Cells were grown in RPMI 1640 media (Life Technologies) containing 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 4 mM L-glutamine,

50 U ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin (Life Technologies). Chronically infected U937 cells were prepared in the same way by infection with WT, rHIV-1$_{A01}$. H9 cells or U937 cells chronically infected with HIV-1 were washed two times with media and then plated at 250,000 cells ml^{-1} (1 ml well^{-1}) and incubated for 2 h prior to treatment. The peptides dissolved in sterile PBS were added to wells (2.5–20 μM) and each treatment was done in triplicate. Controls were treated with an equal volume of sterile PBS. In some experiments, peptides were used in combination with active-site inhibitors to determine the effect on polyprotein processing. Three days after treatment, the supernatants were sampled for HIV-1 p24 antigen in the media using the Retrotek p24 ELISA from ZeptoMetrix Corporation that picks up the mature form of the p24 viral antigen (Buffalo, NY). The effect of the peptides on cell viability was assessed using the CellTiter-Glo^{TM} Luminescent Cell Viability Assay that determines the level of cellular ATP (Promega, Madison, WI). Luminescence was read on the Victor2 luminometer from Perkin-Elmer (Boston, MA).

2.6. p24 immunoblot analysis of virus and HIV-1 infected cells

After sampling the media for use in p24 determination, the cells were pelleted and the supernatant was harvested. The cells were washed twice with PBS and then lysed with100 μl lysis buffer per million cells (lysis buffer: 0.75% triton X-100, 300 mM NaCl, 50 mM Tris–HCl, pH 7.4, 0.4% DMSO and 0.5× of Halt protease inhibitor cocktail from Pierce (Rockford, IL)). Immunoblot analysis of the cells was carried out on 2 μg of total protein. Following removal of the cells by centrifugation the virus was pelleted from the supernatant by ultracentrifugation at 45,000 rpm and washed once with cold PBS containing 10 μM of a potent HIV-1 protease inhibitor, ritonavir (to prevent any additional polyprotein processing taking place during the washing steps). The viral pellets were lysed and resuspended in 25 μl SDS sample buffer. Viral samples of equal volume (4 μl) were then run on SDS gels and transferred to nitrocellulose. Blots were then analyzed for the presence of Gag and processed forms of Gag, including p24, with a monoclonal p24 antibody (Advanced Bioscience Laboratories, Kensington, MD) as described previously (Davis et al., 1999).

3. Results

3.1. HIV-1 protease interface peptides with a cell permeable domain (CPD) from HIV-1 tat inhibit the HIV-1 protease

In order to evaluate HIV-1 protease dimerization inhibitors as potential inhibitors of HIV-1 replication, we designed various peptides containing sequences previously reported to inhibit protease dimerization (Babé et al., 1992; Ishima et al., 2003; Shultz and Chmielewski, 1999; Ulysse and Chmielewski, 1998) with a 13 amino acid cell permeable peptide (CPP), (RKKRRQRRRPPQV) containing the CPD from HIV-1 Tat (RKKRRQRRR) (Brooks et al., 2005; Fawell et al., 1994; Vives et al., 1997).

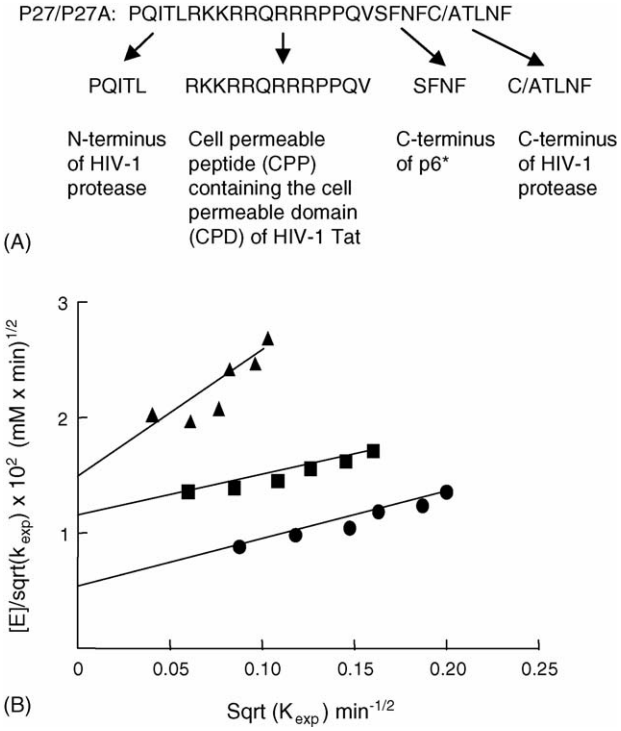

Fig. 1. Amino acid sequence and distinct domains for peptide 27 (P27) and 27A (P27A) (A) and the Zhang–Poorman plot for the inhibition of HIV-1 protease by P27A (PQITLRKKRRQRRRPPQVSFNFATLNF) or acetyl pepstatin (B). E represents the active enzyme concentration determined by active site titration and K_{exp} represents the rate of the reaction $K_{exp} = v/[s]$. (●), No inhibitor; (■), P27A 20 μM; (▲), acetyl pepstatin 20 nM.

These peptides were then screened for inhibition of protease activity at 50 μM using a commercially available fluorimetric HIV-1 protease assay. Peptide 27 (P27) (PQITLRKKRRQRRRPPQVSFNFCTLNF) designed as shown in Fig. 1A was the most effective inhibitor in this assay and when tested in a peptide based RP-HPLC protease assay carried out as described previously (Davis et al., 1997). At 50 μM, P27 inhibited the protease by more than 90%. With the use of various truncations of P27 it was found that the cell permeable peptide (CPP) (RKKRRQRRRPPQV) alone was inactive while removal of the SFNFCTLNF sequence (containing the C terminal four amino acids of p6 and the C-terminal five amino acids of protease) resulted in a substantial loss in activity (data not shown). Replacement of the cysteine in P27 with alanine to create P27A had little effect on its inhibitory activity and therefore P27A was used in kinetic studies to avoid any potential problems that could arise from cysteine oxidation. To verify that this peptide functioned as a dimerization inhibitor of the protease we carried out the Zhang and Poorman kinetic analysis used for studying dissociative inhibitors of dimeric enzymes (Zhang et al., 1991). If a compound acts as a dimerization inhibitor of the HIV-1 protease, the resulting Zhang–Poorman plot gives a line with a slope similar to that obtained for the untreated control but with a different intercept. A non-dissociative inhibitor, such as an active-site inhibitor, yields a line with a different slope from the untreated control. As shown in Fig. 1B, inhibition of HIV-1 protease by P27A resulted in a line with a similar slope to the untreated buffer control (P27A slope

3.5 ± 0.41 (average ± standard deviation), versus the control slope 4.1 ± 0.42) but with a significantly different *Y*-intercept (P27A *y*-intercept 1.2 ± 0.05, control *y*-intercept 0.54 ± 0.05). The results are consistent with P27A acting as a dimerization inhibitor. By contrast, use of the active-site inhibitor, acetyl pepstatin, resulted in a line with a significantly different slope (slope 10.9 ± 3.1) as compared to the untreated enzyme, which is indicative of a non-dissociative inhibitor (Fig. 1B). As determined from the *y*-intercepts, the K_i for P27A was calculated to be 17 ± 3 μM. We also found that unlike that seen with an active site inhibitor, inhibition of HIV-1 protease activity by P27 and P27A was dependent on the time of pre-incubation with the enzyme and inversely dependent on enzyme concentration (data not shown), both of which are characteristic of dimerization inhibitors (Zhang et al., 1991) (data not shown).

3.2. P27 and P27A inhibit PI resistant HIV-1 protease

We also assessed the ability of P27 and P27A to inhibit a PI resistant form of the HIV-1 protease. The resistant protease, designated R1, carries eight PI-resistant mutations (L10I, I15V, I54V, L63P, A71V, V82T, L90M, and I93L) (Muzammil et al., 2003; Ridky and Leis, 1995) and shows high-level resistance to a number of different active site PIs in in vitro resistance assays (Yoshimura et al., 1999). R1 also has a C95F mutation at the dimer interface, therefore a PI-resistant protease without the mutation at position 95 ($R1_{F95C}$) was also created to determine if the mutation near the dimer interface affected the activity of P27 and P27A. The IC_{50}'s for P27 and P27A were determined for the WT, R1, and $R1_{F95C}$ protease using the fluorescence-based HIV-1 protease assay and the values are reported in Table 1. P27 and P27A were both effective inhibitors of WT and drug-resistant proteases with IC_{50}'s ranging from 0.23 to 0.58 μM. Overall, P27 was about two-fold more active than P27A although there was little difference in the potency of the two peptides between WT and resistant proteases (Table 1). Interestingly, P27 and P27A had similar activity toward the PI-resistant protease with or without the C95F mutation (Table 1).

3.3. Fluorescein-labeled P27A enters living MT-2 cells

To verify that P27 could be taken up by living cells, we treated MT-2 cells with N-terminal fluorescein-labeled P27A. As a positive control, a fluorescein labeled version of CPP (F-CPP) was also tested. When MT-2 cells were treated with F-CPP for 1 h in media containing 10% serum and imaged under fluorescence microscopy, more than half the cells showed accumulation of the peptide (Fig. 2). Only living cells were fluorescent while none of the dead cells, stained with trypan blue, accumulated the F-CPP peptide. In addition, not all living cells took up the peptide providing evidence that the fluorescence was not a result of non-specific binding to the cells. Similarly, treatment of cells with fluorescein labeled P27A (F-P27A) led to the accumulation of the peptide in the cells (Fig. 2). Again, only living cells accumulated the peptide while dead cells did not. The extent of peptide accumulation appeared to be lower for F-P27A than F-CPP, perhaps due to a tendency of F-P27A to aggregate. Aggregates of F-P27A can be seen in the phase contrast image but they do not fluoresce presumably due to self-quenching of the fluorescein (Fig. 2). Nevertheless, these studies indicated that the peptide accumulated in MT-2 cells and therefore could be potentially useful as an inhibitor of HIV-1 replication.

3.4. P27 protects MT-2 cells from HIV-1 induced cytotoxicity and inhibits p24 accumulation in the supernatant of chronically infected H9 and U937 cells

The ability of P27 and control peptides to block acute HIV-1 infection was assessed in MT-2 cells. MT-2 cells were infected for 2 h with $HIV\text{-}1_{LAI}$ and then added to media containing the test peptides. The ability of the peptides to block HIV-1-induced cytotoxicity was determined by measuring the viability of the cells 7 days later using the MTT assay. Two control peptides, CPP (RKKRRQRRRPPQV) and CPP-SFNF (RKKRRQRRRPPQVSFNF), that were inactive against the protease were also tested in the assay and compared to P27. CPP and CPP-SFNF were inactive in the acute infection assay when tested up to 50 μM (Fig. 3A and B). However, P27 (PQITLRKKRRQRRRPPQVSFNFCTLNF) protected cells from HIV-1 induced cytotoxicity in a dose-dependent manner with an IC_{50} of 5.1 μm (Fig. 3C). The positive control lopinavir had an IC_{50} of 2 nM (Fig. 3D). The inhibition of virus-induced toxicity by a number of different peptides evaluated in this assay correlated directly with their ability to inhibit protease activity. Although P27A also had some activity at low concentrations of the peptide, it was toxic to uninfected MT-2 cells at concentrations (>5 μM) similar to those required by P27 to provide greater than 50% protection (data not shown).

The effect of P27 on HIV-1 replication was also investigated in H9 cells chronically infected with a WT or a PI-resistant HIV-1 clone. Chronically infected cells that continually produce HIV-1 were washed and then treated with 5 μM of each

Table 1
IC_{50} values for P27 and P27A using the HIV-1 protease fluorimetric assay

Peptide	Sequence	IC_{50} (μM)		
		WT[a]	R1[b]	$R1_{F95C}$[c]
P27	PQITLRKKRRQRRRPPQVSFNFCTLNF	0.37	0.23	0.28
P27A	PQITLRKKRRQRRRPPQVSFNFATLNF	0.58	0.51	0.53

[a] WT: Wild type HIV-1 protease.
[b] R1: Drug resistant HIV-1 protease containing F95.
[c] $R1_{F95C}$: Drug resistant HIV-1 protease containing C95.

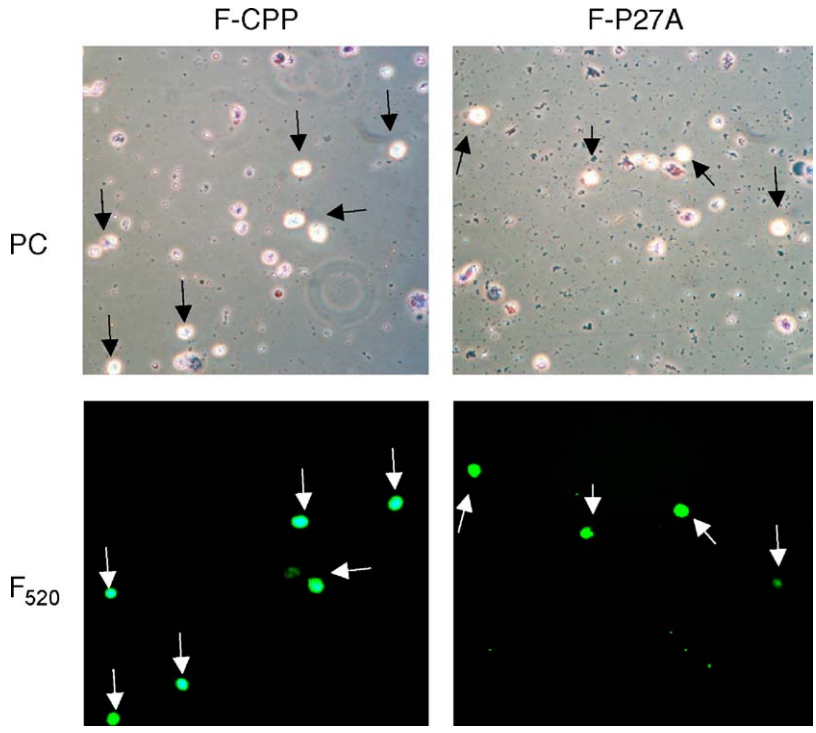

Fig. 2. Cellular accumulation of fluorescein-labeled peptides containing the cell permeable domain of HIV-1 Tat. MT-2 cells were treated with 30 μM F-CPP (F-RKKRRQRRRPPQV), or 30 μM F-P27A and then incubated for 1 h. The cells were stained with trypan blue to assess viability and then visualized under phase contrast (PC) and fluorescent (F_{520}) microscopy. Shown is a single image for each treatment as seen under PC or F_{520}. Arrows indicate living cells showing accumulation of fluorescent peptides. Note that none of the dead cells (stained with trypan blue in phase contrast image) showed accumulation of the peptides.

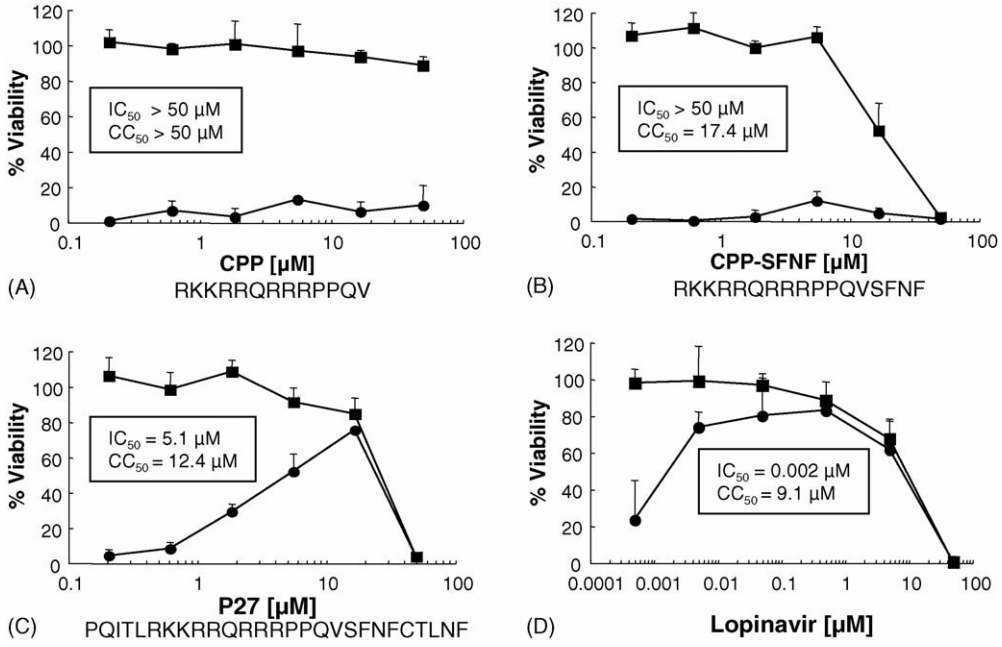

Fig. 3. Inhibition of the cytopathic effect of HIV-1 by the P27 protease dimerization inhibitor and control peptides. MT-2 cells were exposed to 100 $TCID_{50}$ of $HIV\text{-}1_{LAI}$ for 2 h (●) and then incubated in the presence of 0.21, 0.62, 1.85, 5.56, 16.7 or 50 μM of (A) CPP (RKKRRQRRRPPQV), (B) CPP-SFNF (RKKRRQRRRPPQVSFNF), (C) P27 (PQITLRKKRRQRRRPPQVSFNFCTLNF) or (D) in the presence of the active site protease inhibitor lopinavir at 0.0005, 0.005, 0.05, 0.5, 5 or 50 μM. Viability was assessed using the MTT assay 7 days after infection. Cellular toxicity of the compounds was also assessed in MT-2 cells not exposed to HIV-1 (■). The calculated 50% inhibitory concentrations (IC_{50}) and the 50% cell cytotoxic concentrations (CC_{50}) are indicated within each figure. The values plotted are the average of samples run in triplicate and the error bars show the positive standard deviation.

peptide and the level of mature capsid protein (p24) in the supernatant was determined 4 days later. P27 (5 μM) inhibited p24 production by approximately 50% in cells infected with wild type HIV-1 while having little effect on cell viability. The CPP and CPP-SFNF peptides, however, had little or no effect on p24 accumulation (Fig. 4A). At 5 μM, P27 inhibited p24 production in cells infected with PI-resistant virus by 60%, while 5 μM ritonavir was only weakly active (Fig. 4B). The inhibition of p24 by the different peptides paralleled their ability to inhibit protease activity (Fig. 4C) suggesting that P27 likely affects some aspect of viral maturation involving protease. In addition, dose-dependent inhibition of p24 production was observed when chronically infected U937 cells were treated with P27A (Fig. 5). At 5 μm, P27A inhibited p24 production by more than 85%, while having little effect on cell viability (Fig. 5).

3.5. Western blot of virus and infected cells treated with P27

To further characterize the antiviral effect of P27, immunoblot analysis was carried out on viral supernatant from chronically infected H9 cells treated with the peptides. As expected, ritonavir treatment, used as a positive protease inhibitor control, resulted in a decrease in p24 and an increase in p55 Gag and p165 Gag-Pol in the virus released from infected cells (Fig. 6A). Treatment of cells with P27 also led to a dose-dependent decrease in p24 (Fig. 6A) comparable to that from the p24 ELISA. However, it did not result in a corresponding increase in p55 Gag. At the same time, treatment with P27 did lead to the accumulation of unidentified high molecular weight proteins detected with the p24 antibody, suggesting a partial inhibition of Gag-Pol processing (Fig. 6A). These results suggested that P27 might inhibit p24 accumulation in the media by blocking virus release, perhaps as a result of disordered Gag-Pol processing. To investigate this possibility, we analyzed the presence of viral proteins in the infected cells treated with P27 by p24 immunoblot. As expected, treatment with ritonavir inhibited polyprotein processing within cells as indicated by the complete absence of a p24 band and the presence of unprocessed forms of p55 Gag (Fig. 6B). By contrast, treatment of cells with P27 resulted in increased levels of p24 within the cells (Fig. 6B). Very similar results were obtained from the supernatant and cell lysates of cells infected with PI-resistant virus (data not shown).

3.6. P27 enhances the effect of potent active-site inhibitors used at suboptimal concentrations

We next analyzed the effect of a combination of suboptimal concentrations of P27 and potent active-site inhibitors on HIV-1 replication in cells infected with wild type HIV-1. As shown in Fig. 7, treatment of cells with 1 μM ritonavir, saquinavir or KNI-272 resulted in the partial inhibition of p55 Gag processing. The addition of 1 μM P27 with 1 μM of each of these active-site inhibitors resulted in a further inhibition of p24 production as evidenced by a decrease in p41 Gag and an increase in p55 Gag and with ritonavir and saquinavir p165 Gag-Pol precursors (Fig. 7). The enhanced block of polyprotein processing was observed with all three of the active-site inhibitors tested, and indicated that suppression of protease activity can be observed with P27 at least when administered with a partially suppressive concentration of an active site inhibitor.

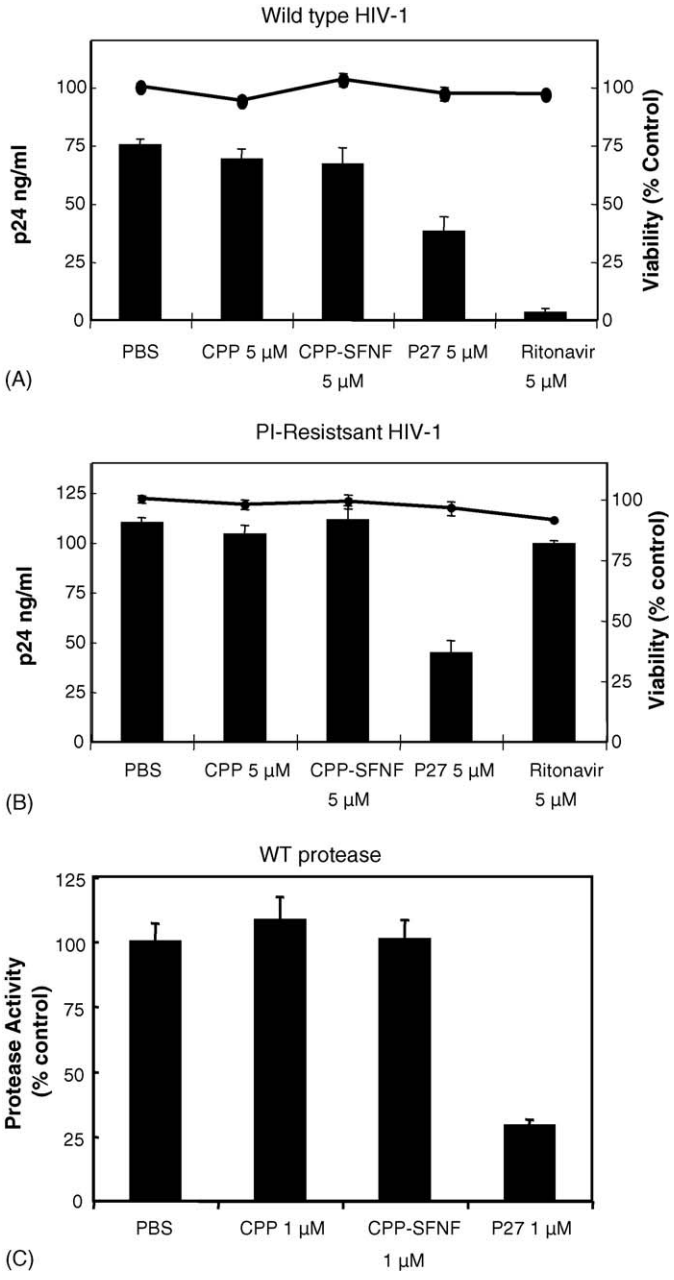

Fig. 4. P27 inhibits p24 production by chronically infected cells and inhibits HIV-1 protease activity. H9 cells chronically infected with WT (rHIV-1_{A01}) (A) or PI-resistant (rHIV-1_{A2F95C}) virus (B) were treated with 5 μM CPP (RKKRRQRRRPPQV), 5 μM CPP-SFNF (RKKRRQRRRPPQVSFNF), 5 μM P27 or the active site protease inhibitor ritonavir (5 μM) as a positive control and incubated for 3 days in triplicate wells. The supernatants were analyzed for p24 antigen (ng ml^{-1}) (—) and the cells were assessed for viability (Y2-axis) (●), as described in Section 2. The results represent the average ± the standard deviation for samples run in triplicate. In (C) protease was treated with 1 μM of each peptide and incubated for 30 min. Enzyme activity was measured as described in Section 2. The results represent the average ± the standard deviation for samples run in triplicate.

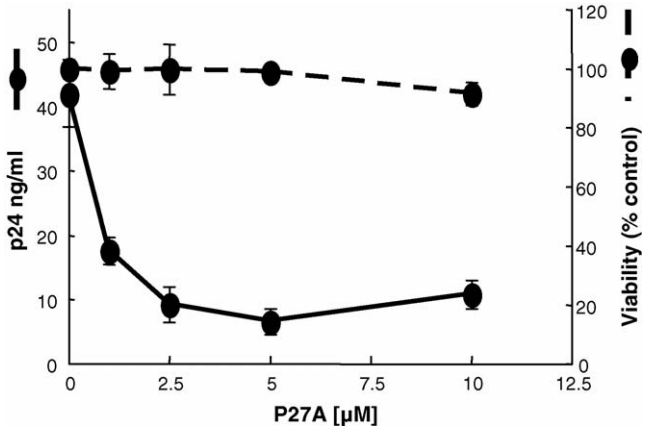

Fig. 5. Dose-dependent inhibition of p24 production by P27A in chronically infected cells. U937 cells chronically infected with wild type HIV-1 were treated in triplicate with P27A (0, 1, 2.5, 5, and 10 μM) and incubated for 4 days. The supernatants from cells were analyzed for p24 antigen (—) and the cells were assessed for viability (- - -). The values represent the average ± the standard deviation.

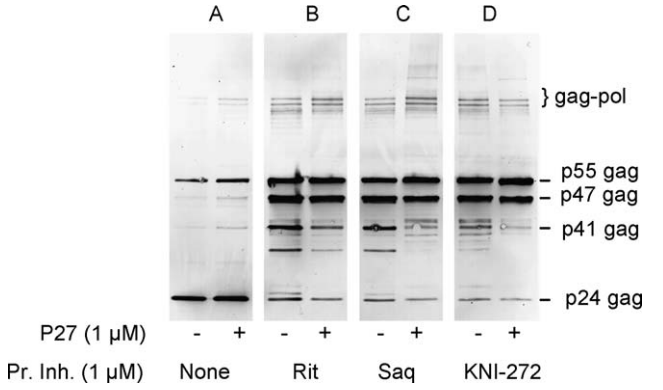

Fig. 7. Enhanced inhibition of viral polyprotein processing by active site inhibitors when used in combination with P27. H9 cells chronically infected with WT HIV-1 (rHIV-1_{A01}) were treated without (−) or with (+) P27 at 1 μM, in the presence of no treatment (none), 1 μM ritonavir (Rit), 1 μM saquinavir (Saq) or 1 μM kynostatin 272 (KNI-272). Three days after treatment, the supernatant was harvested and pelleted virus was analyzed by Western blot with a monoclonal antibody to p24.

4. Discussion

One of the major driving forces behind the development of dimerization inhibitors is the emergence of viral resistance in patients taking currently available active site HIV-1 protease inhibitors. Mutations rarely occur at the dimer interface of the protease (Kuiken et al., 2004; Svicher et al., 2005), suggesting that dimerization inhibitors could be effective against drug resistant mutants acquired during long-term use of active-site inhibitors. Furthermore, a potential advantage of the dimer interface as a target for inhibitor development is that the interface sequences are part of substrate cleavage sites within Gag-Pol and are also involved in direct protein–protein interactions to form the dimer interface (Todd et al., 1998). These two features put constraints on the development of protease interface mutations that could yield resistance. In some cases, however, resistant HIV-1 has been reported to have a C95F mutation at the dimer interface (Ceccherini-Silberstein et al., 2004; Svicher et al., 2005; Yoshimura et al., 1999). P27 and P27A were active against WT HIV-1 protease and PI-resistant HIV-1 protease both with and without the C95F mutation. Interestingly, P27 was more effective against the drug-resistant proteases than WT protease. It has previously been reported that resistance mutations that occur away from the interface in the protease (V82F, V82F/I84V, V82T/I84V, and L90M) can decrease dimer stability making the protease more susceptible to dimerization inhibitors (Xie et al., 1999). The PI-resistant proteases utilized in these experiments contained the V82T and L90M mutations that have been reported to decrease dimer stability (Xie et al., 1999), and this may make these proteases more susceptible to certain dimerization inhibitors as compared to the WT proteases.

P27 inhibited HIV-1-induced cytotoxicity of MT-2 cells and was minimally toxic to uninfected MT-2 cells at the protective concentrations. However, peptides that lacked the N and C terminal interface sequences of the HIV-1 protease did not protect against HIV-1-induced cytotoxicity in the MT-2 assay.

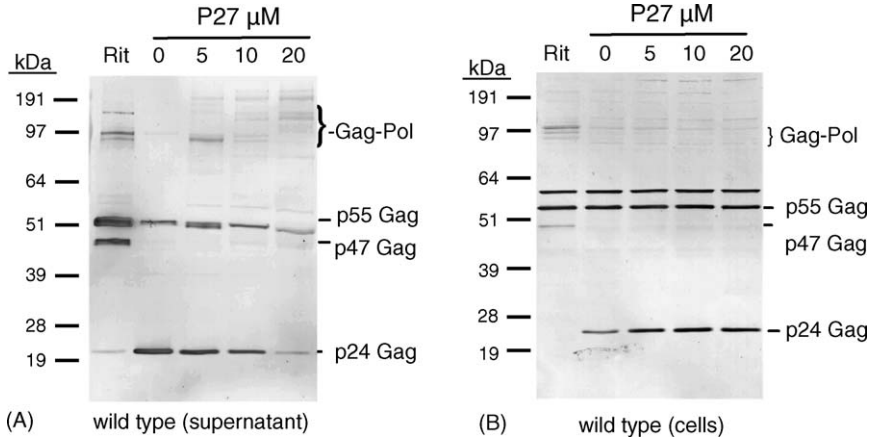

Fig. 6. Effect of P27 on viral polyprotein processing in HIV-1 infected cells. H9 cells chronically infected with WT HIV-1 (rHIV-1_{A01}) were treated with P27 at 0, 5,10 or 20 μM, or ritonavir (10 μM) for 3 days and then supernatants (A) and cells (B) were analyzed by Western blot using a monoclonal p24 antibody. The band detected just above p55 in the immunoblot of the cell lysates is a cross-reactive protein from the cell lysates and is not found in released virus. The locations of the Gag-Pol-related and p55 Gag related proteins (p55, p47 and p24) are indicated.

The requirement for the interface sequences suggests that the effect is related to a specific effect on some aspect of viral protease activity. P27 was also effective in blocking p24 production from WT and PI-resistant HIV-1 infected cells at concentrations near the K_i determined for protease inhibition. Interestingly, P27 was more effective in blocking p24 production from PI-resistant HIV-1 infected cells than from WT-infected cells, and this parallels the effect of P27 on WT and PI-resistant proteases. Surprisingly, the decrease in p24 could not be clearly attributed to a block in p55 Gag processing. Instead, the data is more consistent with P27 inhibiting the release of virus from the infected cells. Others have reported that HIV-1 assembly and release is highly dependent on the proper timing and activity of HIV-1 protease, and that partial inhibition of protease activity can lead to major defects in the assembly and production of HIV-1 particles (Hill et al., 2005; Kaplan et al., 1993, 1994; Karacostas et al., 1993; Krausslich, 1991). Thus, P27 may be partially affecting protease activity in infected cells, in turn resulting in an inhibition of viral release. Although Western blots of the virus released from P27 treated cells did not show significant increases in p55 Gag, the virus did contain proteins larger than p55 Gag that were detectable with the p24 antibody and that appear to be related to Gag-Pol. These protein bands were not detected in control virus or virus treated with peptides that were inactive on the protease. The nature of these proteins is currently under investigation.

P27 was designed to block protease dimerization, and the activity of this and related peptides in infected cells paralleled the activity against HIV protease (this article and Davis et al., unpublished observations). HIV-1 protease functions at a number of steps during HIV-1 particle maturation. During the initial stages of Gag-Pol processing, Gag-Pol polyproteins dimerize and the precursor-embedded protease then accomplishes the initial cleavages of Gag-Pol as intramolecular events (Pettit et al., 2004). It is only when the protease dimer is cleaved out that it can manifest trans cleavages of Gag and Gag-Pol. Active-site inhibitors are quite potent against the mature, free protease dimer, but are as much as 10,000 times less active toward the embedded, immature protease (Pettit et al., 2004). We hypothesize that a dimer inhibitor like P27 may be equally or even more effective against the immature protease embedded in Gag-Pol than against the mature, free protease dimer, and that the activity of P27 in HIV-infected cells results primarily from partial inhibition or dysregulation of initial Gag-Pol processing. This would be consistent with the results obtained from experiments using P27 in combination with active site inhibitors, where an increase in the inhibition of Gag and Gag-Pol processing was observed. Additional studies are underway to test this hypothesis.

Combination therapy for HIV-1 infection has proven quite successful for the treatment of AIDS and newly developed protease inhibitors would likely be used in combination with other approved drugs. These data suggest that understanding the precise mechanism by which P27 affects polyprotein processing and prevents viral release may lead to the development of more active dimerization inhibitors and new strategies for HIV-1 antiviral therapy.

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