Potent Inhibition of HIV-1 Replication by Novel Non-peptidyl Small Molecule Inhibitors of Protease Dimerization*

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Dimerization of HIV-1 protease subunits is essential for its proteolytic activity, which plays a critical role in HIV-1 replication. Hence, the inhibition of protease dimerization represents a unique target for potential intervention of HIV-1. We developed an intermolecular fluorescence resonance energy transfer-based HIV-1-expression assay employing cyan and yellow fluorescent protein-tagged protease monomers. Using this assay, we identified non-peptidyl small molecule inhibitors of protease dimerization. These inhibitors, including darunavir and two identified non-peptidyl small molecule inhibitors of protease dimerization at concentrations of as low as 0.01 μM and blocked HIV-1 replication with IC50 values of 0.0002–0.48 μM. These agents also inhibited the proteolytic activity of mature protease. Other approved anti-HIV-1 agents examined except tipranavir, a CCR5 inhibitor, and soluble CD4 failed to block the dimerization event. Once protease monomers dimerize to become mature protease, mature protease is not dissociated by this dimerization inhibition mechanism, suggesting that these agents block dimerization at the nascent stage of protease maturation. The proteolytic activity of mature protease that managed to undergo dimerization despite the presence of these agents is likely to be inhibited by the same agents acting as conventional protease inhibitors. Such a dual inhibition mechanism should lead to highly potent inhibition of HIV-1.

Highly active antiretroviral therapy has had a major impact on the AIDS epidemic in industrially advanced nations. However, eradication of human immunodeficiency virus, type 1 (HIV-1) does not appear to be currently possible, in part due to the viral reservoirs remaining in blood and infected tissues. Moreover, a number of challenges have been encountered, which include various adverse effects, only partial and limited immunologic restorations achieved, and occurrence of various cancers as consequences of survival elongation with highly active antiretroviral therapy (1). Moreover, such limitations of highly active antiretroviral therapy are exacerbated by the development of drug-resistant HIV-1 variants (2). Thus, the identification of new classes of antiretroviral drugs that have one or more unique mechanisms of action and produce no or minimal adverse effects remains an important therapeutic objective.

Dimerization of HIV-1 protease subunits is an essential process for the acquisition of proteolytic activity of HIV-1 protease, which plays a critical role in the maturation and replication of the virus (3, 4). Thus inhibition of protease dimerization by chemical reagents is likely to abolish proteolytic activity and inhibit HIV-1 replication. However, for possible development of HIV-1 protease dimerization inhibitors, better understanding of the nature and dynamics of protease dimerization is crucial. The monomer subunits are connected by polar and non-polar interactions to form the dimer. Hydrophobicity of Leu-89, Leu-90, and Ile-93 and several other residues have been considered important in the folding of a protease monomer as well as in dimer stabilization (5, 6). For a systematic analysis of the conserved network of hydrogen bonds, termed “fireman’s grip,” Strisovsky et al. (7) have mutated the active site Thr-26 to a Ser, Cys, or Ala and have shown that T26A substitution reduced protease dimer stability, thus virtually nullifying the proteolytic activity of protease. Indeed, in our present study, T26A substitution effectively disrupted protease dimerization (see below), corroborating the results by Strisovsky et al. The flexibility of monomeric and dimeric HIV-1 protease and the feasibility of a stable protease monomer have also been studied...
by computational simulation (8, 9). There are four anti-parallel β-sheets involving the N and C termini of both monomer subunits and they contribute close to 75% of the dimerization energy (10), explaining at least in part why DRV failed to dissociate mature protease dimer (see below). The termini interface has been explored as a dimerization inhibition target by several groups (11–13). We have also recently reported that certain peptides containing the dimer interface sequences amino acids 1–5 and amino acids 95–99 blocked HIV-1 infectivity and replication (14). However, to the best of our knowledge, no evidence of direct dimerization inhibition by such compounds has yet been documented.

In the present study, we developed an intermolecular fluorescence resonance energy transfer (FRET)-based HIV-1-expression assay that employed cyan and yellow fluorescent protein-tagged HIV-1 protease monomers. Using this assay, we identified a group of non-peptidyl small molecule inhibitors of HIV-1 protease dimerization. These inhibitors, including the recently approved protease inhibitor (PI) darunavir (DRV) as well as two experimental protease inhibitors (PIs), blocked protease dimerization at concentrations of as low as 0.01 μM and blocked HIV-1 replication in vitro with IC_{50} values of 0.0002–0.48 μM. These agents also inhibited the proteolytic activity of mature HIV-1 protease. Another PI, tipranavir (TPV), active against HIV-1 variants resistant to multiple PIs, also blocked protease dimerization, although all other existing FDA-approved anti-HIV-1 drugs examined in the present study failed to block the dimerization. The present report represents the first demonstration that non-peptidyl small molecule agents can disrupt protease dimerization.

**EXPERIMENTAL PROCEDURES**

**Generation of FRET-based HIV-1 Expression System**—Cyan fluorescent protein (CFP)- and yellow fluorescent protein (YFP)-tagged HIV-1 protease constructs were generated using BD Creator® DNA cloning kits (BD Biosciences, San Jose, CA). First, Xhol/HindIII fragments from pCR-XL-TOPO vector containing the HIV-1 protease-encoding gene excised from pHIV-1NL4-3 was inserted into the pDNR-1r (donor vector containing the HIV-1 protease-encoding gene excised from pHIV-1NL4-3) with a primer pair: CFPYFP-5Ala-F (5'-GGC GGC CCC TAG GAA AAA GG-3′) and CFPYFP-5Ala-R (5'-ACT AAT GGG AAA CTT GTA CAG CTC GTC CAT GCC G-3′), and a downstream proviral DNA fragment containing the 5'-DNA fragment of reverse transcriptase (RT) and Smal site from pHIV-1NLsma (17), which had been created to have a Smal site by changing two nucleotides (2590 and 2593) of pHIV-1NLsma with a primer pair: 5'-TTG CCC ATT AGT CCT ATT GAG ACT GTA-3′ and 5'-CCA GAA ATC TTG AGT TCT CTT-3′, and the three DNA fragments (100 ng each) in a 20-μl reaction solution. Thermal cycling was carried out as follows: 94 °C for 3 min, followed by 35 cycles of 94 °C for 50 s, 53 °C for 50 s, and 72 °C for 2 min, and finally by 72 °C for 15 min. The amplified PCR products were cloned into pCR-XL-TOPO vector according to the manufacturer's instructions (Gateway Cloning System, Invitrogen). PCR products were generated with pCR-XL-TOPO vector as templates, followed by digestion by both Apal and Smal, and the Apal-Smal fragment was introduced into pHIV-1NLsma (17), generating pHIV-PR_{WT}^{CFP} and pHIV-PR_{WT}^{YFP}, respectively.

**Analysis of Inter- and Intra-molecule Interactions of Protease Subunits**—Analysis of inter- and intra-molecule interactions of protease subunits was conducted by employing the crystal structure of DRV with HIV-1 protease (PDB ID: 2IEN). Hydrogens were added and minimized using the OPLS2005 force field with constraints on heavy atom positions. The calculation was performed using MacroModel 9.1 from Schrödinger, LLC. Hydrogen bonds were assigned when the following distance and angle cut-off was satisfied: 3.0 Å for H–A distance; D-H-A angle >90°; and H-A-B angle >60° where H is the hydrogen, A is the acceptor, D is the donor, and B is a neighbor atom bonded to the acceptor. The representative distance between the termini of two monomers was determined by analyzing the protease-DRV crystal structure (PDB ID: 2IEN). The distance between the α carbons at the N termini and C termini is around 0.5 nm, whereas the distance between the α carbons of the N termini ends of two monomers is around 1.8 nm.

**FRET Procedure**—COS7 cells plated on EZ view cover-glass bottom culture plate (Iwaki, Tokyo) were transfected with the indicated plasmid constructs using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions in the presence of various concentrations of each compound, cultured for 72 h, and analyzed under Fluoview FV500 confocal laser scanning microscope (Olympus Optical Corp., Tokyo) at room temperature. The fluorescence intensity was continuously scanned using a confocal imaging system (FLUOVIEW microscope) equipped with a scanning microscope (Olympus Optical Corp., Tokyo) at room temperature.
Potent HIV-1 Inhibition and Protease Dimerization Inhibition

When the effect of each compound was analyzed by FRET, test compounds were added to the culture medium simultaneously with plasmid transfection.

The results of FRET were determined by quenching of CFP (donor) fluorescence and an increase in YFP (acceptor) fluorescence (sensitized emission), because part of the energy of CFP is transferred to YFP instead of being emitted. This phenomenon can be measured by bleaching YFP, which should result in an increase in CFP fluorescence. This technique, also known as acceptor photobleaching, is a well-established method of determining the occurrence of FRET (18–21). Dequenching of the donor CFP by selective photobleaching of the acceptor YFP was performed by first obtaining YFP and CFP images at the same focal plane, followed by illuminating for 3 min the same image at a wavelength of 488 nm with a laser power set at the maximum intensity to bleach YFP and re-capturing the same CFP and YFP images. The changes in the CFP and YFP fluorescence intensity in the images of selected regions were examined and quantified using Olympus FV500 Image software system (Olympus Optical Corp.). Background values were obtained from the regions where no cells were present and were subtracted from the values for the cells examined in all calculations. For each chimeric protein, the data were obtained from at least three independent experiments. Digitized image data obtained from the experiment were prepared for presentation using Photoshop 6.0 (Adobe Systems, Mountain View, CA). Ratios of intensities of CFP fluorescence after photobleaching to CFP fluorescence prior to photobleaching (CFP/CFP ratios) were determined. It was well established that the CFP/CFP ratios of >1.0 indicate that association of CFP and YFP-tagged proteins occurred, and it was interpreted that the dimerization of protease subunits occurred. When the CFP/CFP ratios were <1, it indicated that the association of the two subunits did not occur, and it was interpreted that protease dimerization was inhibited.

Non-peptidyl Small Molecule Compounds—Seven non-peptidyl small molecule compounds were synthesized in a convergent manner by coupling an optically active P2 ligand and an (R)-hydroxyethylamino sulfonamide isostere (22). Synthetic methods for TMC126 and DRV have been previously described (22, 23). Detailed synthetic methods for the other four compounds will be described elsewhere. TPV was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health.

Dual Luciferase Assay—Dual luciferase assay was established using the CheckMate™ Mammalian Two-Hybrid System (Promega Corp., Madison, WI). Briefly, BamHI/KpnI fragments from pCR-XL-TOPO vector containing the HIV-1 protease (PR<sub>WT</sub>)-encoding gene excised from pHIV-1NL4-3 was inserted into the pACT vector and pBIND vector that had been digested with BamHI and KpnI, generating pACT-PR<sub>WT</sub> and pBIND-PR<sub>WT</sub>, which produced an in-frame fusion of wild-type HIV-1 protease downstream of the VP16 activation domain and GAL4 DNA-binding domain, respectively. COS7 cells were co-transfected with pACT-PR<sub>WT</sub>, pBIND-PR<sub>WT</sub>, and pG5Luc in the absence or presence of 0.1 or 1.0 μM DRV in white 96-well flat bottom plates (Corning, NY), cultured for 48 h, and the intensity of firefly luminescence (Fluc) and <i>Renilla</i> luminescence (Rluc) was measured with TR717 microplate luminometer (Applied Biosystems) according to the manufacturer’s instructions. DRV was added to the culture medium simultaneously with plasmids to be used. Fluc/Rluc intensity ratios were determined with co-transfection of pACT-PR<sub>WT</sub>, pBIND-PR<sub>WT</sub>, and pG5Luc in the absence of DRV, serving as maximal values. Fluc/Rluc intensity ratios determined with co-transfection of a pACT vector, a pBIND vector, and pG5Luc served as minimal (background) values. Relative response ratios (RRR) were determined using the following formula: 

$$\text{RRR} = \frac{\text{negative control Fluc/Rluc}}{\text{positive control Fluc/Rluc}} - \frac{\text{control Fluc/Rluc}}{\text{positive control Fluc/Rluc}}$$

Drug Susceptibility Assay—The susceptibility of HIV-1<sub>LAI</sub> to various drugs and their cytotoxicity were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (24). Briefly, MT-2 cells (2 × 10<sup>4</sup>/ ml) were exposed to 100 50% tissue culture infectious doses (TCID<sub>50</sub>) of HIV-1<sub>LAI</sub> in the presence or absence of various concentrations of drugs in 96-well microculture plates and cultured at 37 °C for 7 days. After 100 μl of the medium was removed from each well, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (10 μl, 7.5 mg/ml in phosphate-buffered saline) was added to each well, followed by incubation at 37 °C for 4 h. After incubation, 100 μl of acidified isopropanol containing 4% (v/v) Triton X-100 was added to each well, to dissolve the formazan crystals, and the optical density was measured in a kinetic microplate reader (V<sub>max</sub> Molecular Devices, Sunnyvale, CA). All assays were performed in duplicate or triplicate. In some experiments, MT-2 cells were chosen as target cells in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, because these cells undergo greater HIV-1-elicited cytopathic effects than MT-4 cells.

Enzyme Kinetics—The chromogenic substrate Lys-Ala-Arg-Val-Nle-paranitro-Phe-Glu-Ala-Nle-amide (Sigma) was used to determine the kinetic parameters (25, 26). Wild-type protease, at final concentrations of 160–190 nM, was added to various concentrations of substrate (100–400 μM) maintained in 50 mM sodium acetate (pH 5.0), 0.1 M NaCl, 1 mM EDTA, and assayed by monitoring the decrease in absorbance at 310 nm using a Varian Cary 100Bio UV-visible spectrophotometer. The <i>k<sub>cat</sub></i> and <i>K<sub>m</sub></i> values were obtained employing standard data fitting techniques for a reaction obeying Michaelis-Menten kinetics. The data curves were fitted using SigmaPlot 8.0.2 (SPSS Inc., Chicago, IL). The active enzyme concentrations were calculated from the intercept of the linear fit to the IC<sub>50</sub> versus [S] plots with the IC<sub>50</sub> axis. The <i>K<sub>v</sub></i> values were obtained from the IC<sub>50</sub> values estimated from an inhibitor dose-response curve with the spectroscopic assay using the equation <i>K<sub>v</sub></i> = (IC<sub>50</sub> − [E]/2)/(1 + [S]/<i>K<sub>m</sub></i>), where [E] and [S] are the protease and substrate concentrations, respectively (27). The <i>K<sub>v</sub></i> values were measured at four to five substrate concentrations. The measurement was repeated at least three times to produce the average values.

Assay for Effects of Darunavir on Dimerized Mature Protease—To examine whether a representative dimerization inhibitor, DRV, could dissociate mature protease that had already been
dimerized, COS7 cells were co-transfected with a pair of plasmids encoding HIV-PRWT-CFP and HIV-PRWT-YFP and exposed to a protein synthesis inhibitor cycloheximide (CHX, 50 μg/ml, Sigma) at 24, 48, 72, and 96 h of culture following transfection. When the CFPA/B ratios determined were >1.0, it was determined that HIV-1 protease had been generated and dimerization had occurred. The production of HIV-1 was monitored every 24 h following transfection by determining levels of p24 Gag protein produced into culture medium as previously described (24).

RESULTS

Generation of FRET-based HIV-1 Expression Assay—The basic concepts of the intermolecular FRET-based HIV-1-expression assay (FRET-HIV-1 assay) are shown in Fig. 1. Within a plasmid (pHIV-1NL4-3), which encodes a full-length molecular infectious HIV-1 clone, the gene encoding a CFP was attached to the downstream end (C terminus) of the gene encoding an HIV-1 protease subunit through the flexible linker (five alanines), generating pHIV-1NL4-3/CFP (Fig. 1A).

Within the other plasmid (pHIV-1NL4-3), the gene encoding a YFP was attached to the downstream end of protease-encoding gene in the same manner, generating pHIV-1NL4-3/YFP. Both CFP and YFP were designed to have phenylalanine and proline in the connection with RT so that the protease is cleaved from RT when two subunits dimerize and the dimerized protease acquires enzymatic activity. Fig. 1B illustrates that HIV-1 virions generated in COS7 cells transfected with pHIV-1NL4-3/CFP contained CFP-tagged protease and those in COS7 cells transfected with pHIV-1NL4-3/YFP contained YFP-tagged protease as measured by Western blot analysis using lysates of pelleted virions. The CFP- and YFP-tagged proteases were visualized by SuperSignal WestPico Chemiluminescent Substrates using polyclonal anti-GFP antiserum and ECL anti-rabbit IgG peroxidase-linked species-specific whole antibody. 

Fig. 1C illustrates the fluorescence images of co-transfected cells prior to and after acceptor photobleaching. COS7 cells plated on EZ View cover-glass bottom culture plate were transfected with two plasmids, pPRWT-CFP and pPRWT-YFP, using Lipofectamine, cultured for 72 h, and analyzed under a Fluoview FV500 confocal laser scanning microscope. Both PRWT-CFP and PRWT-YFP proteins were visualized prior to photobleaching. Note that photobleaching of the cells dramatically reduced YFP fluorescence with a YFPA/B ratio of 0.17 and increased CFP emission with a CFPA/B ratio of 1.38, signifying the dimerization of both YFP- and CFP-tagged protease subunits. D, ratios of the emission intensities before and after photobleaching. Fluorescence intensities of each cell transfected with two plasmids, pPRWT-CFP and pPRWT-YFP, were measured before and after photobleaching, and ratios of the emission intensities before and after photobleaching (CFPA/B ratios) were determined, and plotted. The CFPA/B ratio values were 1.24 ± 0.11 (n = 23), whereas the YFPA/B ratio values were 0.47 ± 0.09 (n = 23). The mean of these ratios are shown as bars.

FIGURE 1. FRET-based HIV-1 expression system. A, generation of FRET-based HIV-1 expression system. Various plasmids encoding full-length molecular infectious HIV-1 (HIV-1NL4-3) clones producing CFP- or YFP-tagged protease using the PCR-mediated recombination method were prepared. A linker consisting of five alanines was inserted between protease and fluorescent protein. A phenylalanine-proline site (F/P) that HIV-1 protease cleaves was also introduced between the fluorescent protein and RT. Shown are structural representations of protease monomers and dimer in association with the linker atoms and fluorescent proteins. FRET occurs only when the fluorescent proteins are 1–10 nm apart. B, expression of CFP- and YFP-tagged wild-type HIV-1 protease. To confirm the presence of HIV-1 protease tagged to fluorescent protein in COS7 cells transfected with a plasmid encoding HIVWT-CFP, HIVA28S-CFP, HIVWT-YFP, or HIVA28S-YFP, Western blot analysis was performed using lysates of pelleted virions. The CFP- and YFP-tagged proteases were visualized by SuperSignal WestPico Chemiluminescent Substrates using polyclonal anti-GFP antiserum and ECL anti-rabbit IgG peroxidase-linked species-specific whole antibody. pLPYFP denotes the lysates of cells transfected with a plasmid encoding only YFP. The lysates of COS7 cells transfected with a plasmid encoding HIVWT, and those of untreated COS7 cells serve as controls. C, fluorescence images of co-transfected cells prior to and after acceptor photobleaching. COS7 cells plated on EZ View cover-glass bottom culture plate were transfected with two plasmids, pPRWT-CFP and pPRWT-YFP, using Lipofectamine, cultured for 72 h, and analyzed under a Fluoview FV500 confocal laser scanning microscope. Both PRWT-CFP and PRWT-YFP proteins were visualized prior to photobleaching. Note that photobleaching of the cells dramatically reduced YFP fluorescence with a YFPA/B ratio of 0.17 and increased CFP emission with a CFPA/B ratio of 1.38, signifying the dimerization of both YFP- and CFP-tagged protease subunits. D, ratios of the emission intensities before and after photobleaching. Fluorescence intensities of each cell transfected with two plasmids, pPRWT-CFP and pPRWT-YFP, were measured before and after photobleaching, and ratios of the emission intensities before and after photobleaching (CFPA/B ratios) were determined, and plotted. The CFPA/B ratio values were 1.24 ± 0.11 (n = 23), whereas the YFPA/B ratio values were 0.47 ± 0.09 (n = 23). The mean of these ratios are shown as bars.
examined in Western blotting. The HIV-1 virions produced were capable of infecting CD4+ MT-4 cells when the cells were exposed to the supernatant of the transfected COS7 cells (data not shown), indicating that the expressed tagged protease was enzymatically and virologically functional. In the cytoplasm of COS7 cells co-transfected with pHIV-1NL4-3CFP and pHIV-1NL4-3YFP, a CFP-tagged protease subunit interacts and dimersizes with a YFP-tagged protease subunit, and CFP and YFP get close because the termini are separated by only 0.5 to 1.8 nm in the dimeric form of protease (note: the representative distance was determined by analyzing the protease-DRV crystal structure (PDB ID: 2IEN)). A focused laser beam excitation of CFP (triggered by helium-cadmium laser) results in rapid energy transfer to YFP, and most of the fluorescence photons are emitted by YFP (28). If the dimerization is blocked, the average distance between CFP and YFP becomes larger, the energy transfer rate is decreased, and the fraction of photons emitted by YFP is lowered.

To help us interpret the energy transfer efficiency quantitatively, we used the acceptor photobleaching technique, in which the change in CFP emission quenching is measured by comparing the value before and after selectively photobleaching YFP, which prevents problems associated with variable expression levels. In this acceptor photobleaching approach, when FRET occurs, the fluorescence of the CFP donor increases after bleaching the YFP acceptor chromophore, which is recognized as a signature for FRET (18). Thus, the analysis of the change in CFP fluorescence intensity in the same specimen regions, before and after removal of the acceptor, directly relates the energy transfer efficiency to both donor and acceptor fluorescence. Fig. 1C illustrates representative images of co-transfected cells prior to and after YFP photobleaching, showing that, following photobleaching, YFP fluorescence of YFP-tagged wild-type protease subunit (PRWT YFP) was decreased, whereas CFP fluorescence of PRWT CFP increased.

To further help us evaluate the energy transfer efficiency, we determined the ratios of cyan fluorescence intensity, determined with a confocal laser scanning microscope, after photobleaching over that before photobleaching (hereafter referred to as CFP/A/B ratios). We also determined YFP/A/B ratios in the same manner. If the CFP/A/B ratios are >1.0, it is thought the energy transfer (or FRET) took place (18), signifying that dimerization of PRWT CFP and PRWT YFP subunits occurred. Fig. 1D shows that in the co-transfected COS7 cells (n = 23), the CFP/A/B ratios were all >1.0 (CFP/A/B ratios, 1.24 ± 0.11; YFP/A/B ratios, 0.47 ± 0.09), demonstrating that dimerization of protease subunits occurred.

Changes in Fluorescence Emission with Amino Acid Substitutions in Protease—First, it was determined whether the above-described FRET-HIV-1 assay could be used to detect the disruption of HIV-1 protease dimerization. Five amino acids at the N terminus and those at the C terminus have been shown to be critical for protease dimerization (29). As shown in Fig. 2A, two protease monomer subunits are connected by four antiparallel β-sheets involving the N and C termini of both subunits. It is of note that mature dimerized protease has as many as 12 hydrogen bonds in this N- and C-terminal region. Thus, we introduced a Pro-1 to Ala substitution (P1A), Q2A, I3A, T4A, L5A, T96A, L97A, N98A, or F99A substitution into the replication-competent HIV-1NL4-3 and found that I3A, L5A, T96A, L97A, and F99A disrupted protease dimerization, although other substitutions did not disrupt the dimerization.

Several amino acid substitutions outside the N and C termini have also been known to play a role in HIV-1 protease dimerization. Ishima and Louis and their colleagues have demonstrated that the introduction of T26A, D29N, D29A, or R87K to HIV-1 protease disrupts the dimer interface contacts and destabilizes protease dimer, causing the inhibition of protease dimerization (30–32). Fig. 2 (B and C) shows the locations of intermolecular hydrogen bonds formed by such amino acids between two monomer subunits. The hydrogen bond interactions between two subunits occur between Asp-29 and Arg-8’, Arg-87 and Leu-5’, Leu-24 and Thr-26’, and Thr-26 and Thr-26’. There are also intra-molecular hydrogen bond interactions between Asp-29 and Arg-87 as shown in Fig. 2 (B–D). Thus, mutations in those amino acids were introduced into HIV-PRWT CFP and HIV-PRWT YFP, generating HIV-PRT26A CFP, HIV-PRT26A YFP, HIV-PRD29N CFP, HIV-PRD29N YFP, HIV-PRD29A CFP, HIV-PRD29A YFP, HIV-PRR87K CFP, and HIV-PRR87K YFP. Co-transfection of COS7 cells with a pair of CFP- and YFP-tagged protease-carrying HIV-1-encoding plasmids demonstrated that these four amino acid substitutions disrupted protease dimerization (the average CFP/A/B ratios were all <1.0; Fig. 2E). Substitutions of two amino acids adjacent to Asp-29 were also introduced, generating HIV-PRT26A CFP, HIV-PRD29N YFP, HIV-PRD30N CFP, and HIV-PRD30N YFP. Both A28S and D30N are known primary amino acid substitutions, conferring resistance to TMC126 and nelfinavir on HIV-1, respectively (33, 34). The fact that A28S- or D30N-containing HIV-1 is infectious and replication-competent indicates that these two amino acid substitutions would not disrupt protease dimerization. HIV-1 virions generated in COS7 cells transfected with HIV-PRD28S CFP and HIV-PRD28S YFP were confirmed to contain CFP-tagged protease and YFP-tagged protease in Western blotting, respectively (Fig. 1B). As expected, neither substitution disrupted the dimerization as examined in the FRET-HIV-1 assay (Fig. 2E). Another mutation D25A, which is adjacent to Thr-26 and is known to abrogate replicative activity of HIV-1 (35), failed to disrupt protease dimerization, indicating that the inability of D25A mutation-carrying HIV-1 to replicate is not due to dimerization disruption, but due to the loss of proteolytic activity of dimerized HIV-1 protease. Analysis of these data indicated that the FRET-HIV-1 assay system is a reliable tool to evaluate agents for their potential to inhibit protease dimerization.

Inhibition of Protease Dimerization by Non-peptidyl and Peptidyl Compounds—After establishing the validity of the FRET-HIV-1 assay to detect protease dimerization inhibition, we evaluated various newly generated non-peptidyl small molecule agents, including the currently available anti-HIV-1 drugs for their ability to inhibit protease dimerization in a blind manner, where agents examined were identified only under code in conducting the FRET-HIV-1 assay. Six different non-peptidyl small molecule agents (GRL-0036A, GRL-06579A (26), TMC126 (33), GRL-98065 (36), DRV (24), and brecanavir (BCV) (37); M, ranging from 547 to 704 (Fig. 3)) were found to disrupt protease...
FIGURE 2. Critical amino acid residues for protease dimerization. A, four antiparallel β-sheets involving the N and C termini of both monomer subunits. Two HIV-1 protease monomer subunits are connected by four antiparallel β-sheets involving the N and C termini of both monomer subunits. It is of note that mature dimerized protease has as many as 12 hydrogen bonds in this N- and C-terminal region, and these interactions seem to be critical for dimer formation. A monomer subunit is shown by the green ribbon, and the other monomer subunit is shown by the red ribbon. A molecule disrupting these inter-protease hydrogen bond contacts can also disrupt their dimerization. B and C, intermolecular hydrogen bonds between two HIV-1 protease monomer subunits. The figure shows the intermolecular hydrogen bonds between two protease monomer subunits. The hydrogen bond interactions between protease monomer A (shown as green ribbon) and monomer B (shown in red ribbon) are Asp-29 to Arg-8, Arg-87 to Leu-5, Leu-24 to Thr-26, and Thr-26 to Thr-26. The corresponding amino acids of monomer B also form hydrogen bonds with monomer A (i.e. Asp-29 to Arg-8, etc.). Intra-molecular hydrogen bond interaction between Asp-29 and Arg-87 is shown by white dotted lines. The residues forming critical intermolecular contacts between two monomer subunits are shown by atom color types (C, gray; N, blue; O, red; and H, white). Only polar hydrogens are shown. The residues of chain A are labeled green, and those of chain B are labeled red. This provides a structural explanation to the FRET experimental data, which show that mutations Leu-5, Asp-29, Thr-26, and Arg-87 prevent formation of a protease dimer. D, potential binding sites of the small molecule dimerization inhibitors. The figure shows one of the potential binding sites of the dimerization inhibitors. Asp-29, Arg-87, and Thr-26 are spatially close enough to form binding interactions with the dimerization inhibitor and prevent the other protease monomer from interacting with these residues. E, changes in emission intensity ratios upon amino acid substitution. COS7 cells were co-transfected with a pair of HIV-PRCFP and HIV-PRYFP carrying wild-type protease or protease with one amino acid substitution, and CFP/YFP ratios were determined. The CFP/YFP ratio value for PRNCFP/PRNYPF was 1.17 ± 0.18 (mean ± S.D.); PRVCFP/PRVYPF, 1.13 ± 0.18; PRDAF/PRDYFP, 1.26 ± 0.14; PRDAF/PRVYPF, 0.77 ± 0.10; PRKCFP/PRKYFP, 1.07 ± 0.14; PRCKCFP/PRCKYPF, 0.84 ± 0.13; PRKCP/PRKYPF, 1.13 ± 0.10; PRKCP/PRKYPF, 0.84 ± 0.15; PRKCP/PRKYPF, 0.76 ± 0.19; PRKCP/PRKYPF, 1.17 ± 0.15; PRKCP/PRKYPF, 0.84 ± 0.10; PRKCP/PRKYPF, 0.94 ± 0.10; PRKCP/PRKYPF, 0.77 ± 0.19; PRKCP/PRKYPF, 1.21 ± 0.16; and PRKCP/PRKYPF, 0.88 ± 0.13. All the experiments were conducted in a blind fashion. The CFP/YFP ratio that is >1 signifies a protease dimer, whereas a ratio that is <1 signifies disruption of protease dimerization. Note that the residue (such as Ile-3 or Asp-29) whose mutation resulted in disruption of dimerization had an inter-molecular hydrogen bond contact with the other protease monomer as shown in A–C. The mean value of the ratios is shown as bars.
dimerization at concentration of 1 \( \mu M \) in the assay (Fig. 4A). All of these agents had potent inhibitory activity against HIV-1 protease with \( K_i \) values of 29, 3.5, 10, 14, 16, and 6.8 \( \mu M \), respectively, as examined in the assay previously described (25, 26), and were highly potent against HIV-1 \( \text{LAI} \) in acute HIV-1 infection assays using target CD4\(^{+}\) MT-2 cells (24) with \( I_C_{50} \) values of 0.0002–0.005 \( \mu M \) (Table 1). In addition to small molecule agents, we examined various peptides in the FRET-HIV-1 assay. A 27-amino acid peptide containing the dimer interface sequences amino acids 1–5 and amino acids 95–99 (P27: PQITLRKKRRQPPQVFATLNF), which blocks HIV-1 infectivity and replication (14), also inhibited protease dimerization as examined in the FRET-HIV-1 expression assay. Another peptide P9 (RKRRQRRPPQVFATN) that lacks the dimer interface sequences and is not active against HIV-1 (14) failed to inhibit protease dimerization in the FRET-HIV-1 assay. These data again corroborated the utility of the assay to evaluate protease dimerization.

To test the robustness and reproducibility of the FRET-HIV-1 assay data, we determined the CFPA/B ratios in a total of 143 COS7 cells transfected with pPR\(_{\text{WT}}\)CYP and pPR\(_{\text{WT}}\)YFP plasmids and cultured in the presence or absence of 1 \( \mu M \) DRV for 3 days on 11 different occasions. In the presence of DRV, only 7 (4.9\%) of 143 cells had the ratios of slightly more than 1.0, whereas all the rest (95.1\%) had values of <1.0 \((n = 143; \text{average of } 0.73 \pm 0.22) \) (Fig. 4B). The CFPA/B ratios determined in the absence of DRV were mostly >1.0 \((n = 172, \text{average of } 1.21 \pm 0.17) \). We next examined whether a dose response in the dimerization inhibition could be seen when the cells were exposed to various concentrations of DRV. As shown in Fig. 4C, DRV effectively inhibited protease dimerization at concentrations of 0.1 \( \mu M \) and above, whereas the average CFPA/B ratio was slightly >1.0 at 0.01 \( \mu M \), and no dimerization inhibition was seen at 0.001 \( \mu M \). These data show that the inhibition by DRV was roughly dose-responsive up to 0.1 \( \mu M \). In addition, we examined a TMC126-congener GRL-0026A (Fig. 3) that is substantially less potent than TMC126 against HIV-1 with \( I_C_{50} \) of 0.48 \( \mu M \) (Table 1), along with TMC126 and BCV for their dose response dimerization inhibition in the FRET-HIV-1 assay and found that the inhibition was similarly dose-responsive (Fig. 4D).

None of the FDA-approved Anti-HIV-1 Drugs Except TPV Blocks Dimerization—We asked whether other currently approved PIs blocked protease dimerization in the FRET-HIV-1 assay. None of the seven PIs (saquinavir, nelfinavir, amprenavir, indinavir, ritonavir, lopinavir, and atazanavir) inhibited protease dimerization at 1 \( \mu M \) concentration, whereas the control DRV clearly inhibited the dimerization as shown in Fig. 4E. Considering that DRV is generally more potent against HIV-1 \( \text{in vitro} \) than most currently existing PIs (24), four PIs (saquinavir, amprenavir, nelfinavir, and atazanavir) were examined in the FRET-HIV-1 assay at a higher concentration, 10 \( \mu M \), however, none of these four PIs inhibited protease dimerization (Fig. 4F). Interestingly, TPV, which has been shown to provide more favorable virological and immunological responses in patients who have received extensive previous antiretroviral treatment than an optimized background regimen when
TABLE 1
Antiviral activity and enzyme inhibition of protease dimerization inhibitors

| Drug          | IC<sub>50</sub> | K<sub>i</sub> |
|---------------|----------------|-------------|
| GRL-0036A     | 0.005 ± 0.002  | 29          |
| GRL-06579A    | 0.0014 ± 0.0008 | 3.5        |
| GRL-98065     | 0.0004 ± 0.0001 | 14         |
| TMC126        | 0.0003 ± 0.0001 | 10         |
| DRV           | 0.0034 ± 0.0005 | 16         |
| BCV           | 0.0002 ± 0.0001 | 6.8        |
| GRL-0026A     | 0.48 ± 0.04    | ND*         |
| TPV           | 0.10 ± 0.04    | ND*         |

*ND, not determined.

FIGURE 4. Inhibition of protease dimerization. A, inhibition of protease dimerization by non-peptidyl and peptidyl compounds. COS7 cells were exposed to each of the agents (1 μM of GRL-0036A, GRL-06579A, GRL-98065, TMC126, DRV, and BCV and 10 μM of P9 and P27) and subsequently co-transfected with pPR<sub>WT</sub> and pPR<sub>WT</sub>iferase. After 72 h, cultured cells were examined in the FRET-HIV-1 assay system using confocal microscopy Fluoview FV500 confocal laser scanning microscope, and CFP/YPF ratios were determined and plotted. The mean of these ratios obtained are shown as bars. B, distribution of the values of CFP<sub>KD</sub> ratio in the presence of 1 μM DRV. 143 cells obtained from 11 independent assays were examined, and CFP<sub>KD</sub> ratios determined were plotted. All the assays were conducted in a blinded manner. C, dose-responsive dimerization inhibition by DRV. COS7 cells were exposed to various concentrations of DRV, co-transfected with pPR<sub>WT</sub>iferase and pPR<sub>WT</sub>iferase, and A/B ratios were determined. D, dose-responsive dimerization inhibition by various non-peptidyl compounds. COS7 cells were exposed to various concentrations of GRL-0026A, TMC126, and BCV, co-transfected with pPR<sub>WT</sub>iferase and pPR<sub>WT</sub>iferase, and cultured for 72 h. At the end of the culture, CFP<sub>KD</sub> ratio values were determined. E, failure of seven clinically available protease inhibitors except DRV and TPV to inhibit the dimerization of PR<sub>WT</sub>iferase and PR<sub>WT</sub>iferase. COS7 cells were co-transfected with pPR<sub>WT</sub>iferase and pPR<sub>WT</sub>iferase in the presence of various anti-HIV-1 protease inhibitors at concentration of 1 μM, and A/B ratios were determined. F, failure of a high concentration of four clinically available protease inhibitors to inhibit HIV-1 protease dimerization. COS7 cells were co-transfected with pPR<sub>WT</sub>iferase and pPR<sub>WT</sub>iferase in the presence of four PIs (saquinavir, amprenavir, nevirapine, and efavirenz), CCR5 inhibitor aplaviroc, and soluble CD4, and A/B ratios were determined. G, protease dimerization inhibition by DRV on dual luciferase assay. COS7 cells were co-transfected with pACT-PR<sub>WT</sub>iferase, pBIND-PR<sub>WT</sub>iferase, and pGSLuc in the presence or absence of 0.1 or 1.0 μM of DRV, cultured for 48 h, and the intensity of firefly luminescence (Fluc) and Renilla luminescence (Rluc) was measured with TR717 microplate luminescence meter. DRV was added to the culture medium simultaneously with plasmids to be used. Fluc/Rluc intensity ratios were determined with co-transfection of pACT-PR<sub>WT</sub>iferase, pBIND-PR<sub>WT</sub>iferase, and pGSLuc in the absence of DRV, serving as maximal values.

FI

FIGURE 5. Darunavir does not dissociate once-dimerized protease in cells producing infectious HIV-1 virions. COS7 cells were co-transfected with two plasmids, pPR<sub>WT</sub>iferase and pPR<sub>WT</sub>iferase, exposed to CHX (50 μg/ml) in 24, 48, 72, and 96 h of culture. The cells were exposed to DRV on day 5 of culture. The production of HIV-1 was monitored every 24 h by determining levels of p24 Gag protein produced into culture medium. The values of the CFP<sub>KD</sub>/Rluc ratio were determined at various time points.
detected by day 2, but no significant increment in the production of p24 Gag protein was seen on those days subsequent to the addition of CHX. When the cells were exposed to CHX on days 3 and 4, greater amounts of Gag protein were seen (Fig. 5). The CFP/A/B ratios determined on days 4 and 5 of culture were all >1.0, signifying that HIV-1 protease had been generated and dimerization had occurred. On day 5, DRV (1 μM) was added to all the cultures described above and the CFP/A/B ratios were determined on day 6 of culture. The ratios remained >1.0 in all of the cultured COS7 cells (Fig. 5). These data strongly suggest that DRV does not dissociate mature protease once dimerized within the HIV-1-producing COS7 cells.

DISCUSSION

In the present study, we developed an intermolecular FRET-based HIV-1-expression assay (FRET-HIV-1 expression assay) that employed cyan and yellow fluorescent protein-tagged HIV-1 protease monomers. Using this assay, we identified a group of non-peptidyl small molecule inhibitors of HIV-1 protease dimerization (molecular weight, 547–704). Dimerization of HIV-1 protease subunits is an essential process for the acquisition of proteolytic activity of HIV-1 protease, which plays a critical role in the replication cycle of HIV-1. Hence, the inhibition of dimerization of HIV-1 protease subunits represents a unique target for potential intervention of HIV-1 replication. The strategy to target protease dimerization as a possible anti-HIV-1 modality has been explored (8, 11–13), and certain compounds have been reported as potential protease dimerization inhibitors. However, no direct evidence of dimerization inhibition by such compounds has been documented. The present report represents the first demonstration that non-peptidic small molecule agents can disrupt protease dimerization.

The structural feature that is in common to the four dimerization inhibitors (TMIC26 (33), GRL-98065 (36), DRV (24), and BCV (37)) is that all of these agents contain the structure-based designed privileged cyclic ether-derived non-peptidyl P2 ligand, 3(3R,3aS,5,6aR)-hexahydrofuranylmethane (bis-THF) and a sulfonamide isostere (22, 23). GRL-0036A and GRL-06579A (26) have bis-THF-related ligand instead of bis-THF. Crystallographic data of dimerized protease complexed with three dimerization inhibitors (GRL-98065 (36), TMIC26 (26), and DRV (40)) have revealed that bis-THF forms three tight hydrogen bond interactions with Asp-29 and Asp-30, two highly conserved catalytic site amino acids. We also observed that TPV has the ability to disrupt protease dimerization. TPV, which does not possess the bis-THF component, also has interactions with both Asp-29 and Asp-30 through its pyridinesulfonamide group, as shown in crystallographic analysis of a dimerized protease complexed with TPV (41). Thus, the inhibition of protease dimerization is not inherent only to the bis-THF component.

Most of the dimerization inhibitors we examined in this study exerted potent activity against PI-resistant protease in addition to their potent activity to wild-type HIV-1. DRV is potent against HIV-1NL4-3 variants exposed to and selected for resistance to saquinavir, indinavir, nelfinavir, and ritonavir (24). Crystal structures of HIV-1 protease with a single mutation (D30N, I50V, V82A, I84V, or L90M) complexed with DRV demonstrate that DRV not only binds to the same catalytic active site as it does for wild-type protease but also maintains hydrogen bond interactions with the backbone atoms of Asp-29 and Asp-30 (40, 42). GRL-06579A and GRL-98065 are also potent against multidrug resistant HIV-1 strains, and molecular modeling indicates that for multidrug-resistant clinical isolates, these inhibitors maintain many of the interactions to critical active site residues (26, 36). TPV, which is active against HIV-1 carrying multidrug-resistant protease, also maintains critical hydrogen bond interactions with backbone atoms in the catalytic active site of mutant protease (43).

It is of note that the D30N-carrying HIV-1 variant is infectious and replication-competent (34). Structural studies do not show any hydrogen bond interactions between two monomer proteases mediated through Asp-30, and the FRET-HIV-1 expression assay showed that D30N mutant did not disrupt protease dimerization. This suggests that Asp-30 is not a critical residue for disrupting protease dimerization, and the interaction of these inhibitors with Asp-30 is not linked to the observed dimerization inhibition. However, potential interactions of dimerization inhibitors such as DRV involving Asp-29 could be critical, because D29N and D29A mutations disrupted protease dimer formation (Fig. 2E). Our analysis using the FRET-HIV-1 expression assay also revealed that the introduction of T26A and R87K to HIV-1 protease disrupted protease dimerization (the average CFP/A/B ratios were all <1.0 (Fig. 2E)). If the protease monomer takes a configuration comparable to that in the dimerized protease, it is possible that the hydrogen bonding of the inhibitors with Asp-29, and/or Thr-26 and Arg-87, both of which are in the vicinity of Asp-29 and could be critical for dimerization, could be associated with the disruption of dimerization process through affecting the intermolecular and/or intramolecular hydrogen bond network (Fig. 2B–D). In this regard, Ishima et al. (30) have shown that a truncated protease monomer takes a configuration similar to the one in the mature dimerized protease; however, it is unknown whether the untruncated monomer subunit takes a similar mature configuration. Furthermore, it is not known as to what stage of protease maturation (before dimerization) the dimerization inhibitors reported here bind to the monomer subunit in.

Another possible mechanism of the dimerization inhibition by the agents reported here is that they might interact with another dimerization interface formed by an interdigitation of the N- and C-terminal portions of each monomer (residues 1–5 and 95–99 (Fig. 2A)). In this regard, when we introduced a Pro-1 to Ala substitution (P1A), Q2A, I3A, T4A, L5A, T96A, L97A, N98A, or F99A into the replication-competent HIV-1NL4-3, five substitutions (I3A, L5A, T96A, L97A, and F99A) produced the ratios of less than 1.0, strongly suggesting that most of the protease monomer subunit failed to dimerize with each of these five substitutions. These data confirmed the five amino acids at the N terminus and those at the C terminus are critical for protease dimerization (30–32). There are no polar interactions involving Q2A or T4A, so it is not surprising that
these mutations did not affect dimer formation. However, the failure of P1A and N98A to disrupt dimerization does not necessarily indicate that these amino acids are not critical for protease dimerization. It is possible that conversion to a residue other than alanine may disrupt dimerization.

In the present study, DRV failed to dissociate mature protease dimer (Fig. 5). It is of note that mature dimerized protease has as many as 12 hydrogen bonds in the N- and C-terminal regions, which may explain in part why DRV failed to dissociate two subunits of mature protease. These data also suggest that protease dimerization is inhibited before the association of two protease subunits occurs, probably when protease is in the form of nascent Gag-Pol polyprotein. However, the absence of structural data of nascent forms of protease subunit-containing polyprotein makes it difficult to conclusively predict how the dimerization inhibitors inhibit protease dimerization.

It is noteworthy that the D25N substitution, which is known to render HIV-1 protease enzymatically inactive (44), failed to disrupt dimerization (Fig. 2E), showing that catalytically inactive subunits are still capable of undergoing dimerization. This observation indicates that the dimerization inhibition is a differing event than the process that confers catalytic activity on two protease monomer subunits.

DRV has a potent activity against a wide spectrum of HIV-1 isolates, including highly multipeptide-inhibitor-resistant HIV-1 variants. The emergence of DRV-resistant HIV-1 seems to be substantially delayed both in vitro (45) and clinical settings (46, 47). One can speculate that DRV inhibits protease dimerization, leaving catalytically inert monomers, but if certain monomers escape from DRV and achieve the mature dimer form, DRV again blocks the proteolytic action of mature (wild-type and mutant) protease as a conventional protease inhibitor. This dual anti-HIV-1 function of DRV may explain why DRV is such a highly effective anti-HIV-1 therapeutic and differentiates it from many of the currently available protease inhibitors (46, 47). It is of note that the plasma concentrations of DRV achieved in those receiving DRV and ritonavir remain >2 μg/ml or ~3.66 μM (48). These concentrations substantially exceed the concentration of DRV effectively disrupting protease dimerization (0.1 μM in culture as shown in Fig. 4C). Hence, the dimerization inhibition by DRV should be in operation in the clinical settings. Furthermore, DRV could more efficiently disrupt protease dimerization in individuals with HIV-1 infection receiving DRV and ritonavir, because the protease expression levels upon transfection in this study appear to be considerably greater than the protease expression levels in vivo, considering that the p24 production levels could be as high as 500–1500 ng/ml by 5 days following transfection of COS7 cells with plasmids used in the FRET-HIV-1 expression assay. The inhibition of HIV-1 protease dimerization by non-peptidyl small molecule agents represents a unique mechanism of HIV-1 intervention, and the dual functional inhibitors reported here might serve as potential candidates as a new class of therapeutic agents for HIV-1 infection and AIDS. The present data should not only help design and examine agents that potentially inhibit HIV-1 protease dimerization but also should give new insights into the process and dynamics of HIV-1 protease dimerization per se.

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