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The Binary Protease Inhibitor, Darunavir, Has a High Genetic Barrier to the Emergence of Resistant HIV-1 Variants

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Introduction

Dimerization of HIV protease monomer subunits is essential for protease's acquisition of proteolytic activity, which plays a crucial role in HIV replication. We have identified a group of compounds including darunavir (DRV) and tipranavir (TPV), which inhibit the catalytic activity and dimerization process of HIV protease (Figs. 1 & 3). In the present study, we examined the effects of various amino acid substitutions within protease on its dimerization process and attempted to elucidate the mechanisms of the emergence of HIV variants resistant against DRV and TPV.

Methods

To assess whether wild-type and mutant protease monomer subunits dimerize in the presence or absence of a test compound, the intermolecular fluorescence resonance energy transfer (FRET)-based HIV-expression assay that employs cyan and yellow fluorescent protein-tagged HIV protease monomer subunits (FRET-HIV system) was used (Fig. 2). A variety of amino acid substitutions were made to HIV protease and such a mutant protease was introduced to the FRET-HIV system.

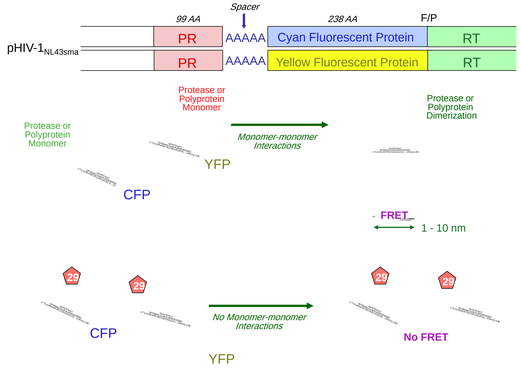
Results

A single mutation (P1A, Q2A, T4A, D25N, D30N, or N98A) allowed protease to undergo dimerization, which DRV effectively inhibited, suggesting that these amino acids are not significantly involved in the binding of DRV to the monomer subunit (Fig. 4). We found that four specific mutations, V32I, L33F, I54M and I84V, are present in common among mutations in various highly DRV-resistant clinical HIV isolates and newly generated DRV-resistant laboratory HIV variants (Table 1 & Fig. 5). Each of the four mutations allowed dimerization, which DRV effectively blocked (Fig. 6). DRV also blocked dimerization of protease carrying V32I/L33F, V32I/I84V, V32I/L33F/I84V, or V32I/L33F/I54M. However, DRV failed to block the dimerization of protease containing all four mutations (Fig. 7), suggesting that these four mutations are associated with the loss of DRV's dimerization inhibition activity in clinical setting. In contrast, TPV failed to block the dimerization of protease containing either of three mutations (L24M, L33I, L33F), all of which are often seen in TPV-resistant clinical HIV variants (Fig. 8). No single mutation (L24M, L33F, I54M, or I84V) conferred resistance to DRV or TPV on HIV as examined in the conventional p24 production inhibition assay (Table 2).

Figure 1 Structures of darunavir and tipranavir

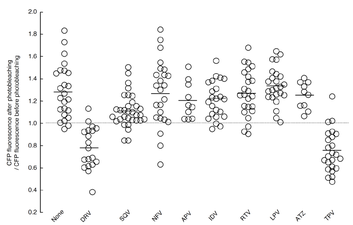
Darunavir Tipranavir

Figure 2 FRET-based HIV-1 expression system



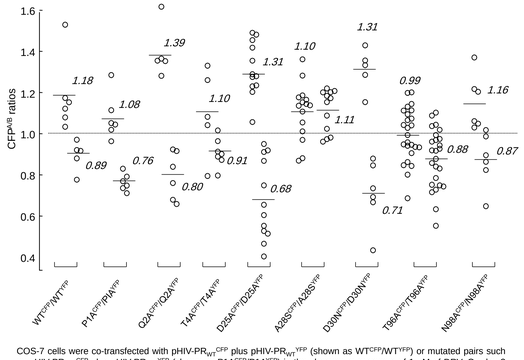
Several plasmids encoding full-length molecular infectious HIV-1 (HIV-1_{NL4-3}) clones producing CFP- or YFP-tagged protease using the PCR-mediated recombination method were prepared. A linker consisting of five alanines was inserted between protease and fluorescent protein. A phenylalanine-proline 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. If certain AA substitutions such as D29N (shown below) are introduced, protease subunits do not get dimerized and no FRET occurs in this system.

Figure 3 Inhibition of HIV-1 protease dimerization by DRV and TPV



COS7 cells were co-transfected with pPR_{WT}^{CFP} and pPR_{WT}^{YFP} in the presence of various anti-HIV-1 protease inhibitors at concentration of 1 μM. After 72 hr, cultured cells were examined in the FRET-HIV-1 assay system using confocal microscopy Fluoview FV500 confocal laser scanning microscope, and $CFP^{A/B}$ ratios were determined and plotted. The mean of these ratios obtained are shown as bars.

Figure 4 Dimerization profiles of single protease mutants in the presence of DRV



COS-7 cells were co-transfected with $pHIV\text{-}PR_{WT}^{CFP}$ plus $pHIV\text{-}PR_{WT}^{YFP}$ (shown as WT^{CFP}/WT^{YFP}) or mutated pairs such as $pHIV\text{-}PR_{P1A}^{CFP}$ plus $pHIV\text{-}PR_{P1A}^{YFP}$ (shown as $P1A^{CFP}/P1A^{YFP}$) in the absence or presence of 1 μM of DRV. On day 3 after transfection, $CFP^{A/B}$ ratios were determined. Average $CFP^{A/B}$ ratio that is greater than 1 signifies a protease dimer, whereas a ratio that is less than 1 signifies disruption of protease dimerization.

Table 1 Reported DRV resistance associated mutations

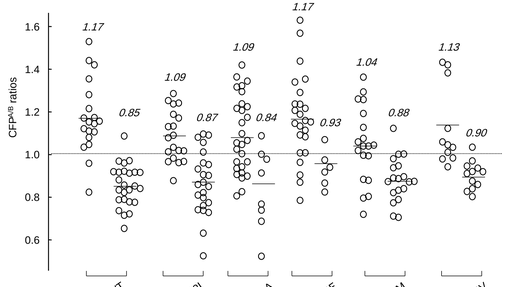
DRV resistance associated mutations	References
V11I, V32I, L33F,I47V, I50V, I54L/M, G73S, L76V, I84V, and L89V	De Meyer *et al.* Antivir Ther 2006; 11:S83.
	Mitsuya *et al.* J Infect Dis. 196:1177-9, 2007
V32I, L33F, I47V, I54L, and L89V	De Meyer *et al.* AIDS Res Hum Retroviruses. 24:379-88, 2008
V32I, I50V, I54L, I54M, L76V, and V82F	Marck *et al.* J Virol. 83; 9512-9520, 2009
V32I, L33F, I54M, V82I, and I84V	Koh *et al.* unpublished data

Multiple protease mutations associated with diminished darunavir/r virological response were identified from several publications and observations

Figure 5 AA changes conferring DRV resistance on HIV-1

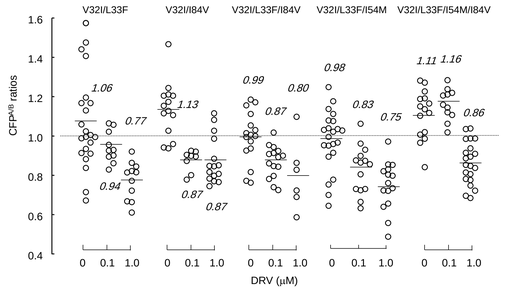
Locations of amino acid substitutions associated with HIV-1's DRV resistance including V32I, L33A/F, I54M and I84V. The location of Asp29 (D29), which is known to be an essential amino acid for dimerization, is also shown.

Figure 6 Profiles of DRV's dimerization inhibition of protease carrying a single AA substitution



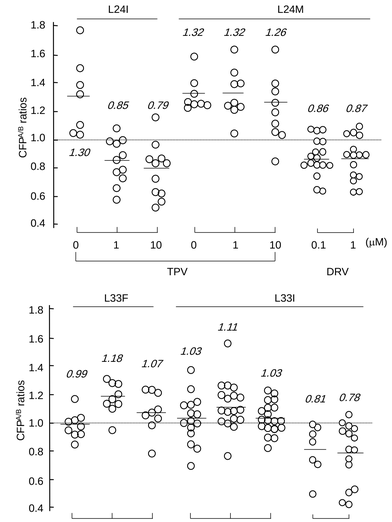
COS7 cells were co-transfected with a pair of $HIV\text{-}PR^{CFP}$ plus $HIV\text{-}PR^{YFP}$ carrying wild-type protease or a single AA substitution such as V32I, L33F, I54M or I84V, each of which was found to be associated with the development of HIV-1 resistance to DRV, in the presence o 1μM DRV, further cultured, and the $CFP^{A/B}$ ratios were determined. Note that none of the AA substitutions introduced blocked the dimerzation of protease.

Figure 7 Profiles of DRV's dimerization inhibition of protease carrying combined AA substitutions



COS7 cells were co-transfected with a pair of $HIV\text{-}PR^{CFP}$ plus $HIV\text{-}PR^{YFP}$ carrying combined AA substitutions such as V32I/L33F, V32I/I84V, V32I/L33F/I84V, V32I/L33F/I54M, or V32I/L33F/I54M/I84V. The COS-7 cells were further cultured in the continuous presence of 0, 0.1, and 1 μM DRV and the $CFP^{A/B}$ ratios were determined at the conclusion of the 3-day period of culture.

Figure 8 Impact of L24M, L33F, and L33I on dimerization inhibition by DRV and TPV



COS7 cells were co-transfected with a pair of $HIV\text{-}PR^{CFP}$ plus $HIV\text{-}PR^{YFP}$ carrying L24M, L33F, and L33I. The COS-7 cells were further cultured in the continuous presence of 0, 1, 10 μM of TPV and 0.1, and 1 μM of DRV and the $CFP^{A/B}$ ratios were determined at the conclusion of the 3-day period of culture.

Table 2 Sensitivity of infectious clones against DRV and TPV

Infectious clone	EC_{50} ± SDs, μM (fold change) DRV	TPV
HIV_{NL4-3}	0.0031 ± 0.0002	0.33 ± 0.01
HIV_{NL4-3}^{L24M}	Not done	0.029 ± 0.003(0.09)
HIV_{NL4-3}^{L33F}	0.0028 ± 0.0008 (0.9)	0.32 ± 0.01 (1)
HIV_{NL4-3}^{I54M}	0.0026 ± 0.0001 (0.8)	0.33 ± 0.01 (1)
HIV_{NL4-3}^{I84V}	0.0035 ± 0.0001 (1)	0.33 ± 0.02 (1)

MT-4 cells ($1x10^5$ /ml) were exposed to 100 $TCID_{50}$ of infectious molecular HIV-1 clones and the inhibition of p24 Gag protein production by the drug was used as an endpoint on day 7 culture.All assays were performed in triplicate, and the values shown are representative of three independent experiments.

Conclusions

The present data show that the protease dimierization inhibition activity of DRV is in operation in its clinical use and that DRV has a high genetic barrier to the emergence of DRV-resistant HIV-1 variants.