| Title | Evaluation of novel protease inhibitors against darunavirresistant variants of HIV type 1 |
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| Author(s) | Inoue, Mari / Oyama, Daiki / Hidaka, Koushi / Kameoka, Masanori |
| Citation | FEBS Open Bio, 7(1):88-95 |
| Issue date | 2017-01 |
| Resource Type | Journal Article / 学術雑誌論文 |
| Resource Version | publisher |
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| DOI | 10.1002/2211-5463.12160 |
| JaLCDOI | |
| URL | http://www.lib.kobe-u.ac.jp/handle_kernel/90003907 |

PDF issue: 2018-11-04
Evaluation of novel protease inhibitors against darunavir-resistant variants of HIV type 1

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Keywords
antiretroviral therapy; HIV-1; lentiviral vector; phenotypic drug susceptibility test; protease inhibitor

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(Received 30 September 2016, revised 2 November 2016, accepted 5 November 2016)

doi:10.1002/2211-5463.12160

HIV disease became a manageable chronic disease since combination antiretroviral therapy (cART) was introduced as the standard treatment regimen. However, the emergence of drug-resistant viruses is a major problem associated with cART. A phenotypic drug susceptibility test using a lentiviral vector was established and applied to evaluate new protease inhibitors (PIs). Lentiviral vectors representing a wild-type (WT-lentivector) and darunavir (DRV)-resistant HIV type 1 (HIV-1) (DRVr-lentivector) were generated. Nine clinically approved protease inhibitors (PIs) inhibited the transduction ability of WT-lentivector similar to their inhibitory effects on the replication of WT HIV-1. Three new PIs reduced the transduction ability of WT- and DRVr-lentivector, suggesting that these PIs may be the candidates as novel antiretroviral drugs against drug-resistant variants of HIV-1.

HIV-1 is a major causative agent of AIDS. Combination antiretroviral therapy (cART), in use since the mid-1990s, has improved the quality of life and life expectancy of HIV-infected individuals, and HIV/AIDS has become a manageable chronic disease [1,2]. However, the emergence of drug-resistant viruses has become a major problem associated with cART [3]. Therefore, the development of new antiretroviral drugs is considered a critical issue. There are six classes of antiretroviral drugs: entry inhibitors, fusion inhibitors, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, integrase strand transfer inhibitors, and protease (PR) inhibitors (PIs). These drugs are designed against different crucial stages of the HIV-1 life cycle [4].

HIV-1 PR is a viral enzyme essential for the life cycle of HIV-1. It cleaves immature Gag and Gag-Pol polyproteins to create the mature protein components of an infectious HIV-1 virion [5]. This step is essential for the replication of HIV-1. PIs were designed to target viral PR and prevent virion maturation mediated by viral PR. Mortality and morbidity associated with HIV has decreased dramatically since 1996, when PIs were introduced into cART [6]. Darunavir (DRV), the most recently developed PI, is characterized by its high genetic barrier to resistance [7–10]; however, the emergence of DRV-resistant HIV-1 has now been reported [11–13].

There are two kinds of tests for the evaluation of HIV-1 drug resistance: genotypic and phenotypic drug susceptibility tests. Genotypic drug susceptibility tests

Abbreviations
APV, amprenavir; AIDS, acquired immunodeficiency syndrome; ATV, atazanavir; BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; BSL, biosafety level; cART, combination antiretroviral therapy; DRV, darunavir; EDC, N-ethyl-N-[3-(dimethylamino)propyl]carbodiimide; HIV, Human immunodeficiency virus; IC50, 50% inhibitory concentration; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; PI, protease inhibitor; PR, protease; RTV, ritonavir; SQV, saquinavir; TOF-MS, time-of-flight mass spectrometry; TPV, tipranavir; WT, wild-type.
have advantages of being faster and cheaper compared with phenotypic drug susceptibility tests. However, this test estimates drug resistance indirectly and sometimes shows erroneous results if the mutation is complicated [14]. In contrast, phenotypic drug susceptibility tests enable the evaluation of drug resistance directly [14,15]. Thus, phenotypic tests are more useful in combination with genotypic test [16]. One disadvantage of phenotypic drug resistance tests is that they need to be conducted in a biosafety level (BSL) 3 laboratory, because in many countries, experiments using HIV-1 are required to be conducted in BSL3 laboratories.

In this study, we established a HIV-1 phenotypic drug susceptibility test using a lentiviral vector system that is capable of being conducted in a BSL2 laboratory, and applied the test to evaluate the antiretroviral activity of new PIs against wild-type (WT-lentivector) and DRV-resistant lentiviral vectors (DRV r-lentivector) as well as against chimeric lentiviral vectors containing Gag and PR of HIV-1 CRF01_AE strains prevalent in Southeast Asia (AE-Gag/PR-lentivector).

Materials and methods

Cells

The 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS.

Lentiviral vector constructs

HIV-1 containing four amino acid substitutions, V32I (amino acid substitution from valine (V) to isoleucine (I) at position 32)/L33F/I54M/V82I or V32I/L33F/I54M/I84V were reported as high-level DRV-resistant variants [17]. Therefore, to generate DRV V-lentivectors, four amino acid substitutions were introduced into the PR-encoding gene of pCMV-AR8.91 [18], a second-generation packaging plasmid of an lentiviral vector, by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA), essentially as described previously [19]. In addition, to generate AE-Gag/PR-lentivector, pCMV-AR8.91 genes encoding HIV-1 Gag and PR (Gag/PR genes) were replaced with the gene fragments of HIV-1 CRF01_AE strains, as follows. The unique restriction enzyme recognition sites for BssHII at the N terminus of the Gag gene and for ClaI at the C terminus of the PR gene were introduced into pCMV-AR8.91 by site-directed mutagenesis. CRF01_AE Gag/PR gene fragments were amplified from pNL4-3-derived proviral DNA containing CRF01_AE Gag/PR genes, pNL-Luc-E’-Gag/PR45, and pNL-Luc-E’-Gag/PR62 [20], using primers containing BssHII and ClaI recognition sites. After the enzyme digestion, CRF01_AE Gag/PR gene fragments were inserted into pCMV-ΔR8.91 containing BssHII and ClaI recognition sites.

Protease inhibitors

Indinavir (IDV), ritonavir (RTV), nelfinavir (NFV), amprenavir (APV), saquinavir (SQV), atazanavir (ATV), lopinavir (LPV), tipranavir (TPV), and DRV were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. In addition, new PIs 1–11 were synthesized as described in a previous report on allophenylnorstatine-containing peptidomimetics [21]. Briefly, the starting amines including 2-methylbenzylamine, 2,6-dimethylbenzylamine, or β-methallylamine were coupled with Boc-(R)-5,5-dimethylthiazolidine-4-carboxylic acid using benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) or N-ethyl-N’-[3-(dimethylamino)propyl]carbodiimide hydrochloride. Removal of the protecting group using HCl/dioxane and coupling with Boc-forms of (2S,3S)-3-amino-2-hydroxy-4-phenylbutyric acid (allophenylnorstatine), valine, (2S,3’R)-tetrahydrofuranylglutamic acid, (2S)-2-aminoxybutyric acid, tert-leucine, 3-methylleucine, or carboxylic acids including 7-methoxybenzofuran-2-carboxylic acid, 4-(methylbenzylcarbamyl)formic acid, and 2-(methylbenzylcarbamyl)formic acid were repeated to give the crude products. Compound 8 was prepared in a similar manner to pseudosymmetric inhibitors [22]. All of the products were purified by preparative HPLC with > 95% purity (Table S1) and identified by time-of-flight mass spectrometry (TOF-MS) (Table S2). In addition, HIV PR inhibitory activity of the test compounds was determined based on the inhibition of a substrate (DABCYL-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS) cleavage using recombinant HIV-1 PR (Table 1). The chemical structures of 11 new PIs are shown in Fig. 1.

Drug susceptibility test

To generate the luciferase reporter lentiviral vector, 293T cells (4 × 10^6 cells in a 100 mm dish) were transfected with pCMV-AR8.91 or the derivative, a lentiviral vector plasmid, pLenti CMV Puro LUC (w168-1) (Addgene, Cambridge, MA, USA) [23] and a vesicular stomatitis virus G protein expression plasmid, pHiT/G [24] using FuGENE HD Transfection Regent (Promega, Madison, WI, USA). Seventeen hours after transfection, 293T cells were transferred into 96-well plate and serially diluted PIs were added in each well. Thirty-one hours later, 293T cells, seeded as target cells 24 h prior to the infection in 96-well plate, were infected with lentiviral vector in the culture supernatant of plasmid-transfected cells. Twenty-four hours after infection, luciferase activity in infected cells was measured using a Steady-Glo Luciferase Assay System.
Table 1. Inhibitory activity of PIs against a synthetic substrate.

| Compound | % Inhibition at 5 nM (SD) | \( K_i \) (pM) |
|----------|--------------------------|-----------------|
| 1        | > 99                     | 107 (± 45)       |
| 2        | > 99                     | –               |
| 3        | > 99                     | –               |
| 4        | > 99                     | –               |
| 5        | > 99                     | –               |
| 6        | > 99                     | –               |
| 7        | 98.1 (± 1.9)             | –               |
| 8        | 91.7 (± 2.0)             | –               |
| 9        | > 99                     | –               |
| 10       | > 99                     | 110 (± 14)      |
| 11       | 97.2 (± 1.2)             | –               |
| DRV      | > 99                     | 306 (± 29)      |

HIV protease inhibitory activity of the test compounds was determined based on the inhibition of a substrate (DABCYL-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS) cleavage using recombinant HIV-1 protease (a kind gift from Dr. M. Adachi). In the inhibition assay, 65 \( \mu \)L of 2-(N-morpholino)ethanesulfonic acid (MES)-NaOH buffer (pH 5.5) was mixed with 5 \( \mu \)L of the inhibitor (100 nM) dissolved in dimethylsulfoxide and 20 \( \mu \)L of HIV-1 protease (200 ng·mL\(^{-1}\)) in AcOH buffer (pH 5.0). The reaction was initiated by addition of 10 \( \mu \)L of a substrate solution. Production of cleaved EDANS substrate was monitored (excitation 335 nm/emission 500 nm) for 15 min at 37 °C, then, the kinetic slope of was calculated with or without the inhibitor. Average of the percent inhibition was obtained from more than three experiments. \( K_i \) value was determined by testing under several inhibitor concentrations fitting the velocity to Morrison’s equation of tight binding inhibitor.

(Promega) with an LB962 microplate luminometer (Berthold, Bad Wildbad, Germany). Drug susceptibility was evaluated as a reduction in luciferase activity in infected cells. The 50% inhibitory concentration (IC\(_{50}\)) of PIs for suppressing the transduction of luciferase gene was calculated from the dose–response curve using a standard function of GRAPHPAD PRISM 5 software (GraphPad Software, San Diego, CA, USA).

Statistical analysis

Statistical analysis was carried out using sss Statistics Ver. 23 (IBM, Armonk, NY, USA) with Dunnett’s \( t \) test within a one-way ANOVA experimental design or with an unpaired \( t \) test.

Results

Establishment of a phenotypic drug susceptibility test using a lentiviral vector system

A lentiviral vector containing a luciferase reporter gene was generated from a molecular cloned, WT HIV-1, pNL4-3 [25]; therefore, it could be applied to monitor the efficiency of the single-round replication cycle of WT HIV-1. Namely, the production of lentiviral vector from plasmid-transfected 293T cells could be used for monitoring the late stage of HIV-1 replication, including the viral life-cycle steps of RNA transcription, protein translation, viral assembly, and the release of the progeny virus. In addition, the transduction ability of the luciferase gene by the infection of a lentiviral vector to target cells could be used for monitoring the early stage of HIV-1 replication, including the viral life-cycle steps of viral entry into cells, encapsidation, reverse transcription, integration, RNA transcription, and protein translation. To evaluate the susceptibility of lentiviral vectors to PIs, nine clinically approved PIs were tested for their inhibitory effect on the transduction of the luciferase gene by WT-lentivector. The results showed that IC\(_{50}\) values of seven PIs, SQV, RTV, IDV, APV, ATV, TPV, and DRV, for reducing the transduction ability of WT-lentivector, were similar to those for inhibiting the single-round replication of WT, luciferase reporter HIV-1 in our previous report (Table 2) [20]. Two PIs, NFV and LPV, showed relatively higher and lower IC\(_{50}\) values against WT-lentivector than against WT HIV-1, respectively, suggesting that the susceptibility of WT-lentivector to these PIs was somewhat different from WT HIV-1 (Table 2). Nevertheless, these results suggested that this phenotypic drug resistance assay was useful for evaluating the antiviral activity of PIs, although with some caution for potential differences in the susceptibility to PIs between lentiviral vector and HIV-1.

Evaluation of the antiretroviral activity of new PIs against WT-lentivector

We next investigated the inhibitory effects of 11 newly designed PIs against the transduction of the luciferase gene by WT-lentivector. Among 11 PIs tested, nine new PIs showed strong antiretroviral activity in reducing the transduction ability of WT-lentivector (Table 3). In particular, five PIs, 1, 2, 4, 5, and 10, inhibited luciferase expression with IC\(_{50}\) values less than 2 nM (Table 3). The values of five new PIs were similar to that of DRV, the most recently developed PI, in reducing the transduction ability of WT-lentivector. Two PIs, 3 and 7, also inhibited luciferase expression with low IC\(_{50}\) value (2.09 nM) (Table 3); however, 2-methylbenzyl structure at the P2' position of 3 and 7 was potentially associated with relatively low antiretroviral activity of PIs [21]. Therefore, we concluded that five PIs, 1, 2, 4, 5, and 10, may be candidates of new antiretroviral agents.
Drug susceptibility of DRV*-lentivectors to DRV

We evaluated the susceptibility of DRV*-lentivectors containing V32I/L33F/I54M/V82I and V32I/L33F/I54M/I84V mutations to DRV. The results showed that the IC50 values of DRV against the DRV*-lentivectors were significantly higher than that for the WT-lentivector (Table 4). The IC50 values of DRV against the V32I/L33F/I54M/V82I and V32I/L33F/I54M/I84V mutants were 12.66 ± 0.88 and 112.08 ± 27.72 nM with resistance of 15- and 133-fold, respectively. These results suggested that the susceptibility of these two DRV*-lentivectors to DRV were decreased as expected by the introduction of four amino acid substitutions.

Antiretroviral activities of new PIs against DRV*-lentivectors

We next investigated the antiretroviral activity of five new PIs, 1, 2, 4, 5, and 10, against DRV*-lentivectors. Compounds 1, 2, and 10, showed significantly lower IC50 values in reducing the transduction ability of DRV*-lentivectors (Table 4). In addition, DRV*-lentivectors containing the V32I/L33F/I54M/V82I and V32I/L33F/I54M/I84V mutations were more susceptible to 1 and 10 than WT-lentivector. In contrast, 4 and 5 showed higher IC50 values against DRV*-lentivectors than 1, 2, and 10. In particular, the IC50 values of 4 and 5 against the V32I/L33F/I54M/I84V mutant

Fig. 1. Chemical structures of 11 new protease inhibitors.

| 1 | 2 | 3 |
|---|---|---|
| 4 | 5 | 6 |
| 7 | 8 | 9 |
| 10 | 11 |

| 11 | 12 | 13 |

Fig. 1. Chemical structures of 11 new protease inhibitors.
Antiretroviral activity of the PIs against WT-lentivector was examined. All data are presented as mean ± standard error of six independent experiments. The 50% IC\textsubscript{50} values of nine PIs for reducing the transduction ability of WT-lentivector were compared with those for inhibiting the replication of WT HIV-1 reported previously [20]. Unpaired t test was used, and the IC\textsubscript{50} value against WT-lentivector was considered significantly different from that against WT HIV-1 for P values < 0.05 (*).

Table 3. Antiretroviral activity of new PIs against WT-lentivector.

| Compound | IC\textsubscript{50} (nM) |
|----------|-----------------|
| 1        | 1.94 ± 0.60     |
| 2        | 1.16 ± 0.25     |
| 3        | 2.09 ± 0.38     |
| 4        | 1.09 ± 0.06     |
| 5        | 0.86 ± 0.20     |
| 6        | 2.26 ± 0.54     |
| 7        | 2.09 ± 0.10     |
| 8        | 42.53 ± 3.92*   |
| 9        | 2.20 ± 0.18     |
| 10       | 1.71 ± 0.39     |
| 11       | 6.80 ± 1.33*    |

Antiretroviral activity of PIs against WT-lentivector was examined. All data are presented as the mean ± standard error of six independent experiments. Dunnett’s t test was performed, and the IC\textsubscript{50} value of a new PI was considered significantly different from that of DRV in reducing the transduction ability of WT-lentivector for P values < 0.05 (*).

Table 4. Antiretroviral activity of PIs against DRV\textsuperscript{r} -lentivectors.

| Compound | IC\textsubscript{50} (nM) |
|----------|-----------------|
| DRV      | 12.66 ± 0.88    |
| 1        | 0.18 ± 0.02*    |
| 2        | 0.08 ± 0.00*    |
| 4        | 2.15 ± 0.33*    |
| 5        | 4.85 ± 0.74*    |
| 10       | 0.30 ± 0.07*    |

Antiretroviral activity of PIs against DRV\textsuperscript{r} -lentivectors containing V32I/L33F/I54M/V82I and V32I/L33F/I54M/I84V mutations was examined. All data are presented as the mean ± standard error of more than six independent experiments. Dunnett’s t test was performed, and the 50% IC\textsubscript{50} value of a new PI was considered significantly different from that of DRV at reducing the transduction ability of a DRV\textsuperscript{r}-lentivector for P values < 0.05 (*).

Discussion

Phenotypic drug susceptibility testing using HIV-1 must be conducted in a BSL3 laboratory. Although DRV has a high genetic barrier to resistance, the emergence of DRV-resistant viruses has been reported and will presumably become a major problem in the future. In this study, we established a phenotypic drug susceptibility test that is capable of being conducted in a BSL2 laboratory by using a lentiviral vector system. In addition, the test was applied to evaluate the antiretroviral activity of new PIs against WT and DRV-resistant HIV-1.

Among the 11 new PIs tested, 1, 2, 4, 5, and 10 showed high antiretroviral activities against WT-lentivector (Table 3). New PIs tested in this study contain allophenylnorstatine with hydroxymethylcarbonyl isoster. The carbonyl group and the hydroxyl proton of allophenylnorstatine forms a hydrogen bond with the proton on the aspartic acid residue at amino acid position 25 and the deprotonated aspartic acid residue at the position 125, the catalytic residues of HIV-1 PR.
In addition, 1, 2, and 10 contain tetrahydrofuranylglycine at the P2 position [27], whereas 4 and 5 contain oxamide at the P3 position, which has two carbonyl oxygen atoms as hydrogen bonding acceptors. The tetrahydrofuranyl oxygen makes a hydrogen bond with an aspartic acid residue at amino acid position 30 (D30) of HIV-1 PR [27]. Compounds 1 and 2 contain benzofurancarbonyl-tetrahydrofuranylglycine in the P3-P2 units, whereas 4 and 5 contain oxalylvaline in these units. By comparing the antiretroviral activity of the two groups, benzofurancarbonyl-tetrahydrofuranylglycine in the P3-P2 units are suggested to confer stronger antiretroviral activity. This might be because benzofurancarbonyl-tetrahydrofuranylglycine in the P3–P2 units improves the balance of hydrophilic and hydrophobic moieties in the PI molecule, which presumably reflects the better membrane permeability. In addition, 10 was designed to replace valine at the P2 position of 4 with tetrahydrofuranylglycine; therefore, it contained oxalyl-tetrahydrofuranylglycine in the P3–P2 units. Because 10 showed a strong antiretroviral activity, oxalyl-tetrahydrofuranylglycine in the P3–P2 units may be acceptable for maintaining the antiretroviral activity of the PI.

We generated DRV⁻lentivectors by the introduction of amino acid substitutions. In a previous report, molecular cloned HIV-1 mutants HIV-1NL4-3 and HIV-1NL4-3 showed DRV resistance of 11-fold and 205-fold, respectively, relative to the parental NL4-3 [17]. DRV⁻lentivectors containing the V32I/L33F/I54M/V82I and V32I/L33F/I54M/V82I mutations showed DRV resistance of 15- and 133-fold, respectively, suggesting that we could generate DRV⁻lentivectors that showed high levels of DRV resistance. Compounds 1, 2, 4, 5, and 10 showed antiretroviral activity against the V32I/L33F/I54M/V82I mutant; however, only 1, 2, and 10, but not 4 and 5, showed activity against the V32I/L33F/I54M/V82I mutant (Table 4). The IC₅₀ values of 4 and 10 against the V32I/L33F/I54M/V82I mutant were 45.4 ± 15.3 and 1.21 ± 0.37 nM, respectively, indicating that antiretroviral activity of 10 was 37.5-fold higher over that of 4. It is conceivable that replacement of the valine residue (present in 4 and 5) with tetrahydrofuranylglycine (present in 1, 2, and 10) at the P2 position of the PIs contributed to the stronger antiretroviral activity against the V32I/L33F/I54M/V82I mutant. The side chain of valine at the P2 position of 4 and 5 interacts with the S2 pocket of HIV-1 PR by a hydrophobic interaction only; therefore, the affinity of PIs to the structurally altered S2 pocket of DRV-resistant PR might not be maintained relative to that of WT PR. In contrast, an oxygen atom in P2 tetrahydrofuranylglycine of 1, 2, and 10 formed a hydrogen bond with the amide NH of D30 on the backbone of HIV-1 PR in addition to the hydrophobic interaction with the S2 pocket; therefore, it might be able to interact stably with the structurally altered S2 pocket of DRV-resistant PR. The V32I/L33F/I54M/V82I mutant was rather more susceptible to 2 and 10 than WT-lentivector. We consider that the structure of the five-membered ring of tetrahydrofuran might fit better to the structurally altered S2 pocket of DRV-resistant PR than to WT PR; therefore, the V32I/L33F/I54M/V82I mutant was more susceptible to 2 and 10 than WT-lentivector.

New PIs tested in this study were designed to contain oxalyl at the P3 position, which interacts with PIs indirectly through hydrogen bonding with a bridging water molecule. These PIs are considered to be able to interact stably with drug-resistant PR containing a structurally altered S2 pocket. However, our results suggested that tetrahydrofuranylglycine at the P2 position, rather than oxalyl at the P3 position, is important for the strong antiretroviral activity of PIs such as 1, 2, and 10 against DRV⁻lentivectors. Therefore,
further tests are required on the importance of the tetrahydrofuranylglycine residue at the P2 position for the antiretroviral activity of new PIs.

According to a previous report [20], AE-Gag/PR-recombinant HIV molecular clones were significantly less susceptible to clinically approved PIs than WT virus. In particular, AE-Gag/PR62 showed nearly or more than 10-fold resistance to nine PIs [20]. Our results demonstrated that 1 and 10 were more potent against AE-Gag/PR-lentivectors than clinically approved PIs. HIV-1 is characterized by extensive genetic heterogeneity. Therefore, the development of new antiretroviral drugs that are capable of inhibiting the replication of several subtypes and circulating recombinant forms of HIV-1 is required. Our results suggested that new PIs tested in this study could inhibit the replication of not only subtype B viruses prevalent in Western countries but also CRF01_AE viruses prevalent in Southeast Asian countries [28].

In conclusion, we show here that our HIV-1 phenotypic drug susceptibility test, which can be conducted in a BSL2 laboratory, is able to evaluate new PIs and may be applicable for use with various HIV-1 strains. We believe that this phenotypic drug susceptibility test will be useful in the development of novel antiretroviral drugs. In addition, new PIs, 1 (KNI-1657), 2 (KNI-1715), and 10 (KNI-5003) found in the present study may have the potential to be developed for clinical use in a future study.

Acknowledgements

We are sincerely grateful to Prof. Y. Kiso for providing valuable advice and to Mr. Y. Toda, Ms. H. Igami, and Ms. H. Ikeda for synthesizing some of the new PIs. Protease inhibitors, APV, ATV, DRV, IDV, LPV, NFV, RTV, SQV, and TPV were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This work was supported in part by a Grants-in-Aid for Scientific Research (KAKENHI) from the Japan Society for the Promotion of Science (JSPS); and the program of the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) from Ministry of Education, Culture, Sport, Science and Technology in Japan, and Japan Agency for Medical Research and Development (AMED). The manuscript was proofread by Medical English Service, Kyoto, Japan.

Author contributions

MK conceived and supervised the study; MI and MK designed experiments; MI and DO performed experiments; KH provided new tools and reagents; MI, KH, and MK analyzed the data; MI wrote the manuscript; DO, KH, and MK made manuscript revisions.

References

1 Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ and Holmberg SD (1998) Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N Engl J Med 338, 853–860.
2 Mocroft A, Vella S, Benfield TL, Chiesi A, Miller V, Gargalianos P, d’Arminio Monforte A, Yust I, Bruun JN, Phillips AN et al. (1998) Changing patterns of mortality across Europe in patients infected with HIV-1. EuroSIDA Study Group. Lancet 352, 1725–1730.
3 Wensing AM, Calvez V, Gunthard HF, Johnson VA, Paredes R, Pillay D, Shafer RW and Richman DD (2015) 2015 Update of the drug resistance mutations in HIV-1. Top Antivir Med 23, 132–141.
4 Arts EJ and Hazuda DJ (2012) HIV-1 antiretroviral drug therapy. Cold Spring Harb Perspect Med 2, a007161.
5 Kohl NE, Emini EA, Schleif WA, Davis LJ, Heimbach JC, Dixon RA, Scolnick EM and Sigal IS (1988) Active human immunodeficiency virus protease is required for viral infectivity. Proc Natl Acad Sci U S A 85, 4686–4690.
6 Swanstrom R and Erona J (2000) Human immunodeficiency virus type-1 protease inhibitors: therapeutic successes and failures, suppression and resistance. Pharmacol Ther 86, 145–170.
7 Ghosh AK, Dawson ZL and Mitsuya H (2007) Darunavir, a conceptually new HIV-1 protease inhibitor for the treatment of drug-resistant HIV. Bioorg Med Chem 15, 7576–7580.
8 Koh Y, Nakata H, Maeda K, Ogata H, Bicler G, Devasamudram T, Kincaid JF, Boross P, Wang YF, Tie Y et al. (2003) Novel bis-tetrahydrofuranyluorothane-containing nonpeptidic protease inhibitor (PI) UIC-94017 (TMC114) with potent activity against multi-PI-resistant human immunodeficiency virus in vitro. Antimicrob Agents Chemother 47, 3123–3129.
9 De Meyer S, Azijn H, Surleraux D, Jochmans D, Tahri A, Pauwels R, Wiggerinck P and de Bethune MP (2005) TMC114, a novel human immunodeficiency virus type 1 protease inhibitor active against protease inhibitor-resistant viruses, including a broad range of clinical isolates. Antimicrob Agents Chemother 49, 2314–2321.
10 King NM, Prabu-Jeyabalan M, Nalivaika EA, Wiggerinck P, de Bethune MP and Schiffer CA (2004) Structural and thermodynamic basis for the binding of
TMCI114, a next-generation human immunodeficiency virus type 1 protease inhibitor. J Virol 78, 12012–12021.

11 Lambert-Nicolot S, Flandre P, Canestri A, Peytavin G, Blanc C, Agher R, Soulie C, Wirden M, Katlama C, Calvez V et al. (2008) Factors associated with the selection of mutations conferring resistance to protease inhibitors (PIs) in PI-experienced patients displaying treatment failure on darunavir. Antimicrob Agents Chemother 52, 491–496.

12 Descamps D, Lambert-Nicolot S, Marcelin AG, Peytavin G, Roquebert B, Katlama C, Yeni P, Felices M, Calvez V and Brun-Vezinet F (2009) Mutations associated with virological response to darunavir/ritonavir in HIV-1-infected protease inhibitor-experienced patients. J Antimicrob Chemother 63, 585–592.

13 de Meyer S, Vangeneugden T, van Baelen B, de Peae E, van Marck H, Picchio G, Lefebvre E and de Bethune MP (2008) Resistance profile of darunavir: combined 24-week results from the POWER trials. AIDS Res Hum Retroviruses 24, 379–388.

14 Hanna GJ and D’Aquila RT (2001) Clinical use of genotypic and phenotypic drug resistance testing to monitor antiretroviral chemotherapy. Clin Infect Dis 32, 774–782.

15 Hanna GJ (2002) HIV-1 genotypic and phenotypic resistance. Clin Lab Med 22, 637–649.

16 Kuritzkes DR (2007) HIV resistance: frequency, testing, mechanisms. Top HIV Med 15, 150–154.

17 Koh Y, Aoki M, Danish ML, Aoki-Ogata H, Amano M, Das D, Shafer RW, Ghosh AK and Mitsuya H (2011) Loss of protease dimerization inhibition activity of darunavir is associated with the acquisition of resistance to darunavir by HIV-1. J Virol 85, 10079–10089.

18 Zufferey R, Nagy D, Mandel RJ, Naldini L and Trono D (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 15, 871–875.

19 Kitagawa Y, Kameoka M, Shoji-Kawata S, Iwabu Y, Mizuta H, Tokunaga K, Fujino M, Natori Y, Yura Y and Ikuta K (2008) Inhibitory function of adapter-related protein complex 2 alpha 1 subunit in the process of nuclear translocation of human immunodeficiency virus type 1 genome. Virology 373, 171–180.

20 Jinnopat P, Isaranckura-na-ayuthaya P, Utachee P, Kitagawa Y, de Silva UC, Siripanyaphinyo U, Kameoka Y, Tokunaga K, Sawanpanyalert P, Ikuta K et al. (2009) Impact of amino acid variations in Gag and protease of HIV type 1 CRF01_AE strains on drug susceptibility of virus to protease inhibitors. J Acquir Immune Defic Syndr 52, 320–328.

21 Hidaka K, Kimura T, Abdel-Rahman HM, Nguyen JT, McDaniel KF, Kohlhrenner WE, Molla A, Adachi M, Tamada T, Kuroki R et al. (2009) Small-sized human immunodeficiency virus type-1 protease inhibitors containing allophenynorstatine to explore the S2’ pocket. J Med Chem 52, 7604–7617.

22 Hidaka K, Kimura T, Hayashi Y, McDaniel KF, Dekhtyar T, Colletti L and Kiso Y (2003) Design and synthesis of pseudo-symmetric HIV protease inhibitors containing a novel hydroxymethylcarbonyl (HMC)-hydrazide isostere. Bioorg Med Chem Lett 13, 93–96.

23 Campeau E, Ruhl VE, Rodier F, Smith CL, Rahmberg BL, Fuss JO, Campisi J, Yaswen P, Cooper PK and Kaufman PD (2009) A versatile viral system for expression and depletion of proteins in mammalian cells. PLoS One 4, e6529.

24 Foucher RA, Meyer BE, Simon JH, Fischer U and Malim MH (1997) HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. EMBO J 16, 4531–4539.

25 Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A and Martin MA (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 59, 284–291.

26 Adachi M, Ohbara T, Kurihara K, Tamada T, Honjo E, Okazaki N, Arai S, Shoyama Y, Kimura K, Matsumura H et al. (2009) Structure of HIV-1 protease in complex with potent inhibitor KNI-272 determined by high-resolution X-ray and neutron crystallography. Proc Natl Acad Sci U S A 106, 4641–4646.

27 Ghosh AK, Thompson WJ, Holloway MK, McKee SP, Duong TT, Lee HY, Munson PM, Smith AM, Wai JM, Darke PL et al. (1993) Potent HIV protease inhibitors: the development of tetrahydrofuranylglycines as novel P2-ligands and pyrazine amides as P3-ligands. J Med Chem 36, 2300–2310.

28 Hemelaar J, Gouws E, Ghys PD and Osmanov S (2011) Global trends in molecular epidemiology of HIV-1 during 2000–2007. AIDS 25, 679–689.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. HPLC analysis of synthetic compounds.
Table S2. Time-of-flight mass spectrometry (TOF-MS) data of synthetic compounds.