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Structural Evidence for Effectiveness of Darunavir and Two Related Antiviral Inhibitors against HIV-2 Protease

Andrey Y. Kovalevsky^{1,*}, John M. Louis³, Annie Aniana³, Arun K. Ghosh⁴, and Irene T. Weber^{1,2,†}

¹Department of Biology, Molecular Basis of Disease Program, Georgia State University, Atlanta, Georgia 30303, USA.

²Department of Chemistry, Molecular Basis of Disease Program, Georgia State University, Atlanta, Georgia 30303, USA.

³Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda MD 20892.

⁴Departments of Chemistry and Medicinal Chemistry, Purdue University, West Lafayette, IN 47907, USA.

Abstract

No drugs have been targeted specifically for HIV-2 infection despite the increasing prevalence worldwide. The antiviral HIV-1 protease inhibitor darunavir, and the chemically related GRL-98065 and GRL-06579A, were designed with the same chemical scaffold and different substituents at P2 and P2' to optimize polar interactions for HIV-1 protease. These inhibitors are also effective antiviral agents for HIV-2 infected cells. Therefore, crystal structures of HIV-2 protease complexes with the three inhibitors have been solved at 1.2 Å resolution to analyze the molecular basis for their antiviral potency. Unusually, the crystals were grown in imidazole and zinc acetate buffer, which formed interactions with the protease and the inhibitors. Overall, the structures were very similar to the corresponding inhibitor complexes of HIV-1 protease with RMSD of 1.1 Å on main chain atoms. The majority of hydrogen bond and weaker C-H...O interactions with inhibitors were conserved in the HIV-2 and HIV-1 protease complexes, except for small changes in interactions with water or disordered side chains. Small differences were observed in the hydrophobic contacts for the darunavir complexes, which agreed with relative inhibition of the two proteases. These near-atomic resolution crystal structures verify the inhibitor potency for HIV-1 and HIV-2 proteases and will provide the basis for future development of antiviral inhibitors targeting HIV-2 protease.

Keywords

drug resistance; aspartic protease; darunavir (TMC114)

* current address: Bioscience Division, MS M888, Los Alamos National Laboratory, Los Alamos NM 87545; E-mail: ayk@lanl.gov.

†To whom correspondence should be addressed. E-mail: iweber@gsu.edu.

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INTRODUCTION

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) are the two etiological causative agents of AIDS. HIV-1 is observed worldwide, while HIV-2 is more prevalent in Western Africa.^{1,2,3} However, HIV-2 is slowly and persistently spreading to the other parts of the world.^{4,5} Individuals infected with HIV-2 progress to the disease more slowly, they are likely to remain asymptomatic for a longer period of time and have lower viral loads compared to those with HIV-1.^{6,7,8} An alarming trend in the AIDS pandemic is the emergence of dual HIV-1/HIV-2 infections, which have been poorly characterized.⁹ Moreover, no drugs have been designed specifically for HIV-2. The available anti-HIV-1 drugs have been used to treat HIV-2-infections.^{10,11} Currently, the most effective AIDS treatment is HAART (Highly Active Antiretroviral Therapy), which includes combinations of inhibitors of indispensable viral enzymes, such as the protease (PR).¹²

There are 10 FDA-approved HIV-1 protease inhibitors (PIs) today. Their successful development is a paradigm for structure-assisted drug design.^{13,14} The PIs are competitive inhibitors that bind in the active site cavity of HIV-1 PR and block hydrolysis of the viral Gag and Gag-Pol polyproteins, resulting in immature and non-infectious virions. The PIs have been used in therapy for patients with HIV-2 and showed moderate and generally weaker activity compared to that for HIV-1.^{10,11,15,16,17,18} HIV-1 PR (PR1) and HIV-2 PR (PR2) share about 50% sequence identity and very similar overall structures.^{19,20} However, the wild-type PR2 sequence harbors multiple substitutions associated with multi-drug and cross-resistance of HIV-1 to the current PIs.²¹ The presence of these resistance mutations in PR2 and weaker activity of several PIs against HIV-2 suggest that developing new drugs to specifically target PR2 will be beneficial and counteract the expansion of HIV-2 infections.

The structures of PR-inhibitor complexes will be vital for the development of inhibitors selective for HIV-2 PR. However, few structures of PR2-ligand complexes are known. Only one structure is available for the enzyme complex with an FDA-approved PI (indinavir).²⁰ Other reported structures contain a substrate analog²² and several different peptidic inhibitors^{23,24,25}. Darunavir and the new generation of PIs have been designed to target drug-resistant HIV-1 PR variants and retain potency against HIV-2 infection. Therefore, high resolution crystal structures of these PIs in complex with HIV-2 PR would further improve our understanding of their potency and have value for development of better drugs in the battle with AIDS.

Here, we report near-atomic resolution (1.2 Å) crystal structures of PR2 in complex with three antiviral inhibitors, darunavir, GLR-06579A and GRL-98065 (Figure 1). Darunavir (DRV), designed to target drug resistant PR1 by introducing more hydrogen bonds with main chain PR atoms,²⁶ is the latest addition to the pool of clinical PIs. It is highly potent against multi-PI-resistant virus and recombinant viruses of different subtypes.^{27,28,29} The structural basis for the tight binding of DRV has been demonstrated in the wild-type PR as well as in several highly PI-resistant mutants.^{30,31,32,33} The chemical scaffold of DRV was used in the design of the two antiviral inhibitors GRL-06579A and GRL-98065, which have modified end groups (Figure 1). On one end, the aniline moiety is changed to hydroxymethylphenylene in GRL-06579A and to 1,3-benzodioxole in GRL-98065. The unique bis-THF group of DRV remains in the chemical structure of GRL-98065, while a novel substituent hexahydrocyclopenta[b]furan was introduced in the GRL-98065. All three PIs are effective antiviral agents for both HIV-1 and HIV-2. DRV was as potent towards HIV-2 as it was against HIV-1 infected cells.^{27,28,34} However, DRV showed 17-fold increased kinetic inhibition for HIV-2 compared to HIV-1 PR.³⁵ GRL-06579A and GRL-98065, on the other hand, showed about 10-fold decreased inhibition of HIV-2 relative to HIV-1 infected cells.^{34,36} However, no crystal structures have been obtained to date of the HIV-2 PR in complex with these PIs.

Our structural analysis has two major goals: i) providing the structural evidence for the selected PIs' effectiveness against HIV-2 PR; ii) establishing a reference point for optimization of the inhibitors in order to develop better antiviral agents for HIV-2.

RESULTS

Crystallographic Analysis

Crystal structures of the wild-type PR2 in complex with antiviral inhibitors DRV, GRL-06579A and GRL-98065 were solved in space group C2 with isomorphous unit cell dimensions, as indicated in Table 1. The asymmetric units contained the PR2 dimer with bound inhibitor, and the residues in the two subunits are labeled 1-99 and 1'-99' (Figure 2). Crystals of the three complexes diffracted to very similar resolutions of 1.18-1.20 Å. The final R-factors were 13.6%, 15.6% and 14.2% for PR2-DRV, PR2-GRL06579A and PR2-GRL98065, respectively. There was clear electron density for all the atoms of PR2, inhibitor, solvent molecules and ions in all the structures. The high resolution of the data allowed the refinement of anisotropic *B*-factors, two shells of solvent, including ~ 200 water molecules, ~ 10 zinc cations, ~ 10 chloride anions and a sodium cation, and geometrically restrained hydrogen atoms in each of the structures. The inhibitors adopt an extended conformation with a single orientation in the PR2 active site cavity, as shown by the electron density in Figure 3. Alternate conformations were modeled for residues when clear electron density was observed for both positions of the side chains. In general, alternate conformations for side chains were observed for residues on the surface of the protein. Additionally, some residues in the active site cavity of PR2 showed alternate conformations of their side chains. Such residues in the active site cleft of the enzyme have an intrinsic disorder, which cannot be attributed to the presence of two inhibitor conformations as observed in the majority of PR1 complexes with DRV and similar inhibitors.^{30,32,33} Asp30 and Ile32' showed alternate side chain conformations in at least one of the structures.

Notably, there were 9, 10 and 11 zinc cations coordinated to PR2 residues in the complexes with DRV, GRL-06579A and GRL-098065, respectively. The binding of zinc cations is similar in all three studied complexes. Only Zn401 had 100 % occupancy in every structure. Mostly, the metal ions were coordinated to carboxylic oxygens of Asp or Glu side chains. Other zinc ligands were imidazole molecules, chloride anions and water. The Asp30 side chain was coordinated alternatively to two zinc cations with relative occupancies of 60/40%. One Zn²⁺ (60%) had an η^1 -OOC coordination to the carboxylate of Asp30', while the other (40%) was chelated by the carboxylate (an η^2 -OOC coordination) (Figure 4a). In the PR2-GRL98065 structure the Asp30 side chain in the other subunit also binds to a zinc cation, while Asp30 is metal-free in the other two complexes. The other Zn²⁺ ions had 50% occupancy. Interestingly, one metal binding site was found in a surface groove between Trp6' and Lys7', where the metal coordinates to imidazole, two Cl⁻ ions and an H₂O and has no coordination with the protein atoms (Figure 4b).

PR2 Interactions with Inhibitors

The new clinical inhibitor DRV, and the investigational antiviral inhibitors GRL-06579A and GRL-98065, (Figure 1) were designed to be effective against multi-drug resistant HIV-1 PR variants by introducing more hydrogen bonds with the main chain atoms of the enzyme relative to those of older PIs. Therefore, these interactions are likely to be conserved in the HIV-2 PR-PI complexes. The PR2 interactions with inhibitor are reported in terms of conventional hydrogen bonds (O-H...O and N-H...O), unconventional hydrogen bonds (C-H...O), C-H... π contacts, and water... π interactions, as described previously.^{32,37}

Conventional hydrogen bonds—The hydrogen bond interactions of PR2 with the three PIs are illustrated in Figure 5. The central part of the inhibitors is the common chemical scaffold that forms conserved interactions with PR2 residues Asp25 and 25', Gly27, and water mediated interactions with Ile50 and 50'. The hydroxyethylene OH interacts closely with the carboxylate side chains of catalytic Asp25 and Asp25'. The OH group makes two short contacts of ~ 2.5-2.6 Å to the outer Oδ2 of Asp25 and the inner Oδ1 of Asp25' in PR2-DRV (Figure 6a). The latter distance is elongated to 2.8 Å in PR2-GRL98065. The other two distances to the inner Oδ1 of Asp25 and the outer Oδ2 of Asp25' are significantly longer at 2.8-3.2 Å in the three structures. The sulfonamide oxygen and the urethane carbonyl form almost symmetric hydrogen bonds to the flap water, which mediates the interactions with the amides of Ile50 and Ile50'. The O...O distances are 2.7-2.9 Å, and the amides are 2.9-3.0 Å away from the flap water in the three structures (Figure 5). The other common hydrogen bond is formed between NH of urethane and the main chain carbonyl of Gly27' at 3.1-3.3 Å distance.

The P2 groups show a conserved hydrogen bond with the amide of Asp29' (Figure 5). The P2 group is bis-THF in DRV and GRL-98065 and forms hydrogen bonds to the main chain amides of Asp29' and Asp30' with N...O distances in the 2.9-3.2 Å range, which are similar to those observed in the wild-type and mutant HIV-1 PR-DRV structures.³³ The P2 group of GRL-06579A has a single oxygen atom that forms a single hydrogen bond of 2.8 Å with the amide of Asp29', instead of the three interactions of bis-THF in the other two PIs.

The three different P2' substituents, an aromatic amine in DRV, an aromatic hydroxymethylene in GRL-06579A, and a benzodioxole group in GRL-98065, form N-H...O or N-H...N hydrogen bonds with the main chain amide of Asp30 at distances of 3.1-3.4 Å (Figure 5 and 6b). The aniline in PR2-DRV has a weak hydrogen bond with the main chain carbonyl of Asp30 (distance of 3.4 Å), while this interaction is absent in other two structures. In addition, the aniline has a water-mediated interaction with the side chain carboxylate of Asp30, whereas a direct hydrogen bond forms in the PR2-GRL06579A and PR2-GRL98065 structures. In PR2-GRL06579A and PR2-GRL98065 an additional water-mediated interaction connects the P2' group to the main chain amides of Asp29 and Gly48, respectively (Figure 5).

Unconventional C-H...O hydrogen bonds and C-H...π interactions—Other interactions, including C-H...O and C-H...π contacts, are vital for tight binding of the inhibitors to the PR2. C-H...O interactions occur at C...O distances of less than 3.7 Å,³⁸ while favorable C-H...π contacts occur at C...C distances of less than 4.0 Å when the aliphatic carbon is not in the same plane as the aromatic ring.³⁹ The strength of a C-H...O contact increases in the order of the donor group as CH₃<CH₂<CH, provided other substituents remain the same. Also, the strength of the C-H...O interaction depends on the electronegativity of the atoms connected to the C-H. Therefore, the strongest C-H...O hydrogen bond donor would be the CaH₂ of Gly in a protein, and the aliphatic and aromatic CH groups in the inhibitors. Based on the above description, each inhibitor makes several moderately strong C-H...O contacts involving the P2 and sulfonamide moieties, and a number of weaker interactions formed by these and other parts of the PIs. The strongest (based on distance and electronegativity) unconventional C-H...O hydrogen bonds of 3.0-3.3 Å are the C-H_{P2}...O_{Gly48'} formed by the P2 bis-THF or hexahydrocyclopentafuran and carbonyl oxygen of Gly48', and the CaH_{Gly49}...O=S formed by the Gly49 Ca and sulfonyl oxygen (Figure 7). The P2 groups of the PIs have weak interactions with residues at positions 28', 29' and 30', while the sulfonamide also interacts with Cβ of Ile50' and terminal CH₃ groups of Ile50' and Ile84 in all three structures, with the distances of 3.3-3.6 Å. Other notable C-H...O interactions are formed by aromatic CH of P2' and P1 phenylalanine moieties with main chain carbonyl groups of Gly48 (3.2-3.3 Å in PR2-GRL06579A and PR2-GRL98065, and 3.6 Å in PR2-DRV) and Gly27' (3.4-3.5 Å in all structures), respectively; and by CH of the central hydroxyethylene with the outer Oδ2 of Asp25' of 3.4 Å in all three structures. Finally, the unique conformationally rigid 1,3-

benzodioxole of GRL-98065 forms a strong C-H...O interaction of 2.9 Å to the carboxylate of Asp30, whilst this interaction is not possible for the other two inhibitors which contain shorter P2' groups (Figure 5b).

The three PIs have similar aromatic moieties, consisting of benzene rings in the P1 phenylalanine and P2' parts of the molecules, and show practically identical C-H... π interactions in all three complexes. For instance, C γ 2 of Ile82 is positioned to make very symmetric C-H... π contacts with all six aromatic carbon atoms of P1 with the distances of 3.4-3.9 Å. The second aromatic system of the inhibitors, the benzene ring of P2', has a number of contacts with residues Ala28, Ile32 and Ile50'. The interactions with Ala28 are as short as 3.3-3.4 Å in PR2-DRV and PR2-GRL98065 structures, while they are significantly elongated and weakened (3.7-3.8 Å) in PR2-GRL06579A. The contacts with Ile32 and Ile50' are comparable among the three structures and the closest contacts are 3.5-3.6 Å. Thus, the hydrophobic C-H... π interactions are largely conserved in the three PR2-PI structures due to the similarities between the aromatic groups, while the conventional and unconventional hydrogen bonding is disparate for DRV, GRL-06579A and GRL-98065 owing to differences in hydrogen bond functionalities in these PIs.

Other solvent-mediated PR2-inhibitor interactions—A conserved water molecule is present in all three structures that links the inhibitor to the amide of Asp29 by means of O-H... π interactions with the benzene ring of P2' groups and a hydrogen bond with the main chain of PR2 (Figure 5). The water... π distances are shorter for GRL-98065 and DRV at 3.1 Å and 3.3 Å, respectively, while the distance increases to 3.6 Å for GRL-06579A. The N-H...O hydrogen bonds of this water molecule are identical in the three structures, with the distances of 2.9-3.0 Å.

The zinc ions provide unique interactions that have not been observed in other HIV PR-inhibitor structures. No metal or halide ions have been identified in close proximity to inhibitor molecules in the PR active site cavity in previous structures. In these new PR2-PI complexes, Zn²⁺ cations coordinate with the PR2 residues Asp30 and Asp30' and chloride ions near the inhibitors (Figure 4a). In PR2-DRV and PR2-GRL06579A these contacts are confined to the atoms of the P2 bis-THF and hexahydrocyclopentafuranyl, respectively. In PR2-GRL98065, such contacts are also observed for the P2' group, due to the presence of Zn cations in both subunits at opposite sides of the active site cleft. The C...Zn and C...Cl distances are 3.8-4.0 Å. In fact, zinc alone has been shown to inhibit HIV-1 PR.⁴⁰ So it is possible that zinc interactions with PR have a role in HIV infected cells.

Comparison of high resolution HIV-2 and HIV-1 PR-inhibitor structures

It is instructive to compare the interactions of the individual inhibitors in the active sites of PR1 and PR2 in order to understand the specificity differences of the two enzymes and to design modified PIs with better affinity for the PR2. Moreover, PR2 can be viewed as a multiple mutant of PR1 having mutations such as V32I, M46I, and I47V that are common in drug resistant variants of PR1. The active site cavities have only three amino acid differences of Val/Ile at residues 32, 47 and 82; PR1 has Val32, Ile47 and Val82, while PR2 has Ile32, Val47 and Ile82. Residues 32 and 47 form part of the S2 and S2' subsites, while residue 82 contributes to the S1 and S1' subsites. The inhibitors share similar chemical structures, except for the groups at P2 and P2'. DRV and GRL-98065 have the same bis-THF group at P2, while P2' is aniline in DRV and 1,3-benzodioxole group in GRL-98065. Hence, the inhibitor interactions with the two enzymes are expected to show more differences in the S2 and S2' subsites due to the presence of Val/Ile32 and Ile/Val47 and the different P2 and P2' groups. The following PR1 structures were chosen for the comparative analysis: PR1-DRV (2IEN at 1.30 Å resolution),³⁰ PR1-GRL06579A (2HB3 at 1.35 Å)³⁶ and PR1-GRL98065 (2Z4O at 1.60 Å resolution).

³⁴ In two of the structures, the inhibitors were found in two conformations in the active site cleft, related by a 180° rotation, with occupancies of 55/45% in PR1-DRV and 60/40% in PR1-GRL98065. Therefore, for complete structural analysis the PR1 interactions involving both orientations of the inhibitors will be compared with those made by the inhibitors in the PR2 structures.

PR2-DRV vs. PR1-DRV—The two structures superimpose with the RMSD of 1.1 Å for all main-chain atoms. As expected, the characteristic aspartic protease triplets of Asp-Thr-Gly (residues 25-27 and 25'-27') buried inside the active site cavity are the most structurally conserved regions, showing shifts of ~ 0.1 Å between PR1 and PR2. The largest structural variations, on the other hand, are observed for residues 35-44 and 35'-44' in the surface loops, where the shifts are as dramatic as ~ 6 Å.

The PR-DRV hydrogen bond network is conserved in both structures, with essentially identical hydrogen bond distances for most interactions. However, differences are observed in the interactions with Asp30. In PR1, the P2' aromatic NH₂ group of DRV (55% occupied conformation) forms three hydrogen bonds to Asp30 main- and side-chain atoms. The shortest contact is with the carboxylate oxygen of Asp30 of 2.7 Å. Similarly, the 45% populated DRV orientation has a 2.9 Å hydrogen bond to the side-chain of Asp30'. On the other hand, in the PR2-DRV structure the direct hydrogen bond is substituted by a water-mediated interaction NH₂...H₂O...OOC of Asp30 with the corresponding distances of 3.2 Å and 2.3 Å. Furthermore, the solvent site in PR2 is only 50 % occupied, implying that the interaction occurs only half of the time and therefore is most likely unimportant for the overall inhibitor binding. Thus, DRV lacks one direct hydrogen bond in PR2-DRV relative to its binding with PR1. Other hydrophilic interactions, *i.e.* C-H...O contacts, are changed insignificantly when DRV binds to PR2 relative to the PR1-DRV complex. However, relative to the 55% DRV orientation in the PR1 complex a number of hydrophobic C-H... π interactions of the inhibitor's aromatic systems with residues Ile32, Val47, Pro81, Ile82, and Ile50' are significantly elongated in PR2-DRV structure by 0.3-0.4 Å (Figure 8a-b) suggesting diminished strength, although other contacts with Leu23, Ala28 are preserved. These interactions are more similar to those of the minor 45% DRV conformation in PR1-DRV, where only C-H... π contacts with residues 50 and 82' are about 0.4 Å shorter than the corresponding distances with 50' and 82 in the PR2-DRV complex. The fact that these hydrophobic interactions are altered in PR2-DRV can be explained by multiple substitutions of V32I, I47V, and V82I relative to PR1, which will alter the shape of the active site cavity.

PR2-GRL06579A vs. PR1-GRL06579A—The two structures superimpose with the RMSD of 1.1 Å similar to the previously discussed complexes of DRV. Again, as observed for the DRV complexes, the dramatic shifts of > 5 Å in the positions of residues in the two GRL-06579A structures are confined to the surface residues. Unlike the DRV complexes, the interactions of GRL-06579A with the residues of PR1 and PR2 are essentially identical. The only exception is that GRL-06579A forms a direct hydrogen bond to the carboxylate side chain of Asp30 in PR2, instead of the water-mediated contact for GRL-06579A in the PR1 complex. However, these interactions are made with partially occupied Asp 30 carboxylates in both structures, and may be less critical for the inhibitor binding to the two proteases.

PR2-GRL98065 vs. PR1-GRL98065—Identically to the other comparisons the RMSD for superimposing the two complexes is 1.1 Å. The majority of interactions changes by 0.4 Å or less, which is probably insignificant due to the lower resolution (1.6 Å) of the PR1-GRL-98065 structure. Yet, unexpectedly, the inhibitor has small differences in polar interactions with the PR2 residues compared to the PR1. The aromatic P2' group is connected to the main-chain amide of Gly48 by means of a water-mediated contact involving one of the oxygen atoms in PR2-GRL-98065 (O...H₂O...HN distances are 3.2 Å and 3.4 Å, respectively), while this water

molecule is shifted 0.8 Å towards Gly48 and away from the major inhibitor orientation in PR1-GRL98065 (Figure 8c). Interestingly, the P2' group bends by 0.5 Å toward the H₂O in PR1, but not enough to achieve such hydrogen bonding as in PR2 complex. In contrast, the minor conformation of the inhibitor forms a good hydrogen bond with the similarly positioned water on the opposite side of the active site cleft and the O...H₂O...HN Gly48' distances are both 3.0 Å.³⁷ Additionally, in the PR2 complex the P2 bis-THF group makes an extra weak C-H...O contact with an oxygen atom of the Asp30' carboxylate of 3.6 Å, but the interaction is absent (> 4.0 Å) in PR1 complex for both inhibitor conformations. Unlike in DRV complexes of the two proteases, GRL-98065 forms very comparable hydrophobic interactions with residues of PR1 and PR2. To reiterate, although some small geometrical changes can be discerned in the inhibitor binding to PR2 relative to PR1, they may not be significant due to the lower resolution and the presence of two inhibitor conformations in the PR1-GRL98065 crystal structure.

DISCUSSION

Rational or structure-assisted drug design has become a cornerstone in research efforts to provide efficient therapies for many diseases. Currently, HIV-1 PR inhibitors are almost exclusively designed with the help of structural information acquired from PR1-PI complexes. In contrast, despite the persistent spread of HIV-2 infection, no HIV-2 specific drugs have been designed. The major goal of contemporary drug design of HIV-1 PR inhibitors is doubleheaded: 1) to achieve equal potency for the wild-type PR and drug resistant variants, and 2) to achieve high threshold to resistance of the PIs, *i.e.* PR variants specifically selected for the resistance to such PIs do not appear for a prolonged period of therapy. The three PIs studied here are excellent examples. DRV, FDA-approved in 2006, was designed to maximize the number of hydrogen bonds with the main-chain atoms of the PR1.²⁶ DRV was shown to be highly potent against a variety of drug resistant PR1 variants. The same chemical scaffold was used in the design of the new antiviral inhibitors GRL-06579A and GRL-98065. All three inhibitors are effective against HIV-2 *in vitro*, although the latter two PIs inhibit HIV-2 replication with IC₅₀ values that are about 10-fold higher than those for HIV-1, while DRV retains similar potency on both viruses.^{34,36} Interestingly, the relative potency on HIV-1 and -2 *in vitro* is reflected by only small structural differences observed between PR1-PI and PR2-PI complexes. The complexes are similar and show significant differences mostly in hydrophobic interactions, such as C-H...π, with PIs. The Val/Ile32, Ile/Val47 and Val/Ile82 are the only substitutions that distinguish the active site cavities of PR1 and PR2. The three residues are involved in C-H...π interactions with P2' and P1 substituents of the inhibitors. Residues 32 and 47 have complementary changes in their side chain length, each either loses or gains a methyl group, and therefore do not significantly alter the overall size of S2' pocket. The side chain of Val82 becomes the bulkier Ile82 in PR2, which may reduce the size of the active site cleft. Yet, the Ile82 side chain simply rotates away from the P1 phenyl ring of the inhibitors avoiding unfavorable interactions, and forms van der Waals contacts with the terminal nitrogen of Arg8 in all three PR2 complexes. Whereas, in the PR1 complexes the Val82 side chains have two alternate conformations forming van der Waals contacts with the inhibitor. Nonetheless, in PR2-DRV a number of C-H...π contacts with residues 32, 47, 81, 82, and 50' are significantly elongated compared with their distances in the PR1-DRV complex, consistent with recent calorimetric studies³⁵ showing the DRV inhibition of PR2 was 17-fold weaker than for PR1. In contrast, GRL-06579A and GRL-98065 showed small differences in polar interactions with the two enzymes, however, these altered interactions involve water or protease side chains with alternate conformations. These structural changes do not appear to agree with the relative antiviral potency on HIV-1 and -2. Moreover, other physiological factors will affect the *in vitro* antiviral potency of the PIs.

Based on the comparison of the crystal structures of PR2-DRV, PR2-GRL06579A and PR2-GRL98065 it is obvious that the unique bis-THF moiety incorporated into DRV and GRL98065

chemical structures is a better choice than the hexahydrocyclopentafuran of GRL-06579A, due to the presence of two, rather than one, hydrogen bond forming oxygen. At the other end of the PI, the P2' aniline in DRV is substituted by hydroxymethylphenylene or benzodioxole in GRL-06579A or GRL-98065, respectively. The P2' group has gained water-mediated contacts with Asp29 or Gly48 for GRL-06579A and GRL-98065, respectively. Thus, these P2' substituents are expected to be superior to the aniline in DRV. Indeed, GRL-98065 has an EC₅₀ value of 10-fold lower than DRV for the inhibition of HIV-1. In order to further improve the binding of the inhibitors their P2' groups should be altered to introduce direct hydrogen bonds with main-chain amides of Asp29 and Gly48. This design goal is being pursued in the next generation of PIs.

MATERIALS AND METHODS

Expression, purification and crystallization of HIV-2 PR

The mature wild type PR2 (accession number AAU08233) was expressed using the pET11a vector in BL21 (DE3) host, purified and folded by a similar procedure as described for PR1.⁴¹ DRV, GRL-06579 and GRL-98065 were dissolved in dimethylsulfoxide for stock solutions. The crystals of PR2 complexed with the inhibitors were grown by the hanging-drop vapor diffusion method using 5:1 ratio of inhibitor to protein. Crystals of the three complexes grew in nearly identical conditions with a well solution of 0.6–0.7 M imidazole/0.12–0.14 M Zn(OAc)₂ buffer (pH = 5.75) and 1.25–2 M NaCl. Crystals in the form of long sticks appeared in two-three days and grew to average dimensions of 0.5×0.15×0.15 mm within two-three weeks. Similar conditions were explored for the crystallization of PR1 in complexes with the three inhibitors. However, the attempts were unsuccessful.

X-ray data collection

Crystals were transferred into a cryoprotectant solution containing the reservoir solution plus 25% (v/v) glycerol, mounted on a Litho-loop (Molecular Dimensions Ltd., Apopka FL, USA) and flash-frozen in liquid nitrogen. X-ray diffraction data were collected on the SER-CAT beamline of the Advanced Photon Source, Argonne National Laboratory at 90K using 0.8 Å wavelength. Data were processed in the monoclinic space group C2 using HKL2000.⁴² The low symmetry of the crystals only allowed the data completeness to reach ~90% for the three complexes.

Structure determination and refinement

The CCP4i suite of programs^{43,44} was used to obtain a molecular replacement solution and the starting model was the wild type HIV-1 PR complex with DRV (PDB code 2IEN) in case of each structure determination. The structures were refined using SHELX97⁴⁵ and refitted using O10⁴⁶ and Coot⁴⁷ programs. Alternate conformations were modeled for protease residues when obvious in the electron density maps. Anisotropic atomic displacement parameters (B-factors) were refined for all atoms including solvent molecules. Hydrogen atoms were added at the final stages of the refinement. The identity of ions and other solvent molecules from the crystallization conditions was deduced based on the shape and peak height of the 2F_O-F_C and F_O-F_C electron density, the potential hydrogen bond interactions and interatomic distances. The PR2-DRV, PR2-GRL06579A and PR2-GRL98065 crystal structures were refined with nine-to-twelve Zn²⁺ cations, seven-to-eight chloride anions, one sodium atom, six imidazole molecules coordinated to zinc ions and 195–209 water molecules including partial occupancy sites. Figures were made by Bobscript⁴⁸ and PyMol⁴⁹.

Accession codes

Protein Data Bank: Coordinates and structure factors have been deposited with the accession codes 3EBZ (HIV-2 PR-DRV complex), 3EC0 (HIV-2 PR-GRL06579A complex) and 3ECG (HIV-2 PR-GRL98065 complex).

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Abbreviations

HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; PR2, HIV-2 protease; PR1, HIV-1 protease; DRV, darunavir; RMSD, root mean square deviation.

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Figure 1.

The studied antiviral protease inhibitors. The groups P2-P2' are indicated by analogy to the standard nomenclature for peptide substrates.

Figure 2. PR2-DRV dimer structure. Two subunits (in green and magenta) are shown in a ribbon representation indicating the secondary structure. DRV is in ball-and-stick representation colored by atom type. Structures of PR2-GRL06579A and PR2-GRL98065 are similar.

Figure 3. Omit $F_O - F_C$ electron density for the inhibitors (a) DRV, (b) GRL-06579A and (c) GRL-98065 in PR2 complexes contoured at 4.5σ .

Figure 4.

Zn binding sites. **a)** PR2-DRV showing one zinc site with Zn1 at 60% occupancy with van der Waals contacts with DRV, and Zn2 at 40% occupancy. **b)** PR2-GRL06579A showing zinc (50% occupied site) coordinating H₂O and Cl ions in groove between Trp6' and Lys7'. The binding of zinc is similar in all three studied complexes.

Fig. 5(a)

Fig. 5(b)

Fig. 5(c)

Figure 5. Polar Interactions between PR2 and Inhibitors. **a)** DRV, **b)** GRL-06579A and **c)** GRL-98065. Hydrogen bonds are indicated by dashed lines. The alternate conformation of the Asp30 side chain in PR2-GRL06579A is shown in magenta. The two shortest distances connecting the central OH group of each inhibitor with the catalytic Asp25 and 25' are indicated by green dotted lines. The O-H... π interaction between the aromatic system of P2 group and a water molecule is indicated by a dotted black line.

Figure 6.

a) 2F_O-F_C electron density and hydrogen bonds for DRV and the active site residues Asp25 and Asp25' in the PR2-DRV structure. Similar hydrogen bonds are seen in PR2-GRL06579A and PR2-GRL98065 structures. **b)** 2F_O-F_C electron density, C-H...O (magenta dotted lines) and hydrogen bond (black dashed line) interactions of GRL-98065 with Asp30. The densities are contoured at 3σ level.

Figure 7. Schematic representation of C-H...O unconventional hydrogen bond interactions in **a)** PR2-DRV, **b)** PR2-GRL06579A and **c)** PR2-GRL98065. Pink dotted lines indicate C-H...O contacts with distances in Å. The green dotted lines indicate the O-H...O hydrogen bonds of the catalytic Asp25/25'.

Fig. 8(a)

Fig. 8(b)

Fig. 8(c)

Figure 8.

Comparison of PR2 and PR1 complexes. a) Hydrophobic contacts of DRV in PR1 (green) and PR2 (magenta) for Val/Ile32, Ile/Val47 and Ile50 with the P2' group of DRV. b) Hydrophobic contacts of Pro81 and Val/Ile82 with the P1 phenyl group of DRV. Contacts are indicated by black (PR1) or magenta (PR2) lines with distances in Å. The major conformation of DRV is shown for PR1 complex. c) Water-mediated interactions of the P2' aromatic group of GRL-98065 and Gly48 in PR1 and PR2 complexes. PR2 complex is shown as cyan ball and stick with red water, and PR1 is in yellow bonds with purple water. The major conformation of GRL-98065 is shown for the PR1 complex. The minor conformation in PR1-GRL98065 (not shown) has two good hydrogen bond distances of 3.0 Å for the water interactions.

Table 1
Crystallographic data collection and refinement statistics.

	PR2-DRV	PR2-GRL06579A	PR2-GRL98065
Data collection			
Space group	C2	C2	C2
Unit cell dimensions:			
a, b, c (Å)	105.4, 30.8, 55.8	105.4, 30.9, 55.9	105.4, 31.0, 55.6
β , °	91.4	91.8	91.4
Resolution Range (Å)	50 – 1.20	50 – 1.18	50 – 1.18
	(1.24 – 1.20) [§]	(1.22 – 1.18) [§]	(1.22 – 1.18) [§]
Unique reflections ($I > 2\sigma(I)$)	51581 (42711)	53554 (43684)	51263 (40772)
$I / \sigma(I)$	22.3 (3.0)	18.7 (2.7)	21.2 (2.8)
R_{merge} (%)	4.7 (29.0)	5.5 (32.1)	4.8 (29.6)
Completeness (%)	91.7 (61.3)	89.7 (56.1)	86.5 (52.1)
Refinement			
Resolution range (Å)	10–1.20	10–1.18	10–1.18
R_{work} (%)	13.6	15.6	14.2
R_{free} (%)	18.1	20.6	18.8
No. of solvent molecules	210	195	203
<u>RMSD from ideality:</u>			
Bonds (Å)	0.013	0.011	0.012
Angle distance (Å)	0.032	0.030	0.030
<u>B-factors (Å²):</u>			
Main-chain	17.7	16.0	13.6
Side-chain	26.4	24.0	23.1
Inhibitor	13.2	12.4	14.5
Solvent	31.2	28.6	28.1

[§]The numbers in parentheses are given for the highest resolution shell