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Ultra-high Resolution Crystal Structure of HIV-1 Protease Mutant Reveals Two Binding Sites for Clinical Inhibitor TMC114

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SUMMARY

TMC114 (darunavir) is a promising clinical inhibitor of HIV-1 protease (PR) for treatment of drug resistant HIV/AIDS. We report the ultra-high 0.84Å resolution crystal structure of the TMC114 complex with PR containing the drug resistant mutation V32I (PR_{V32I}), and the 1.22 Å resolution structure of a complex with PR_{M46L}. These structures show TMC114 bound at two distinct sites, one in the active-site cavity and the second on the surface of one of the flexible flaps in the PR dimer. Remarkably, TMC114 binds at these two sites simultaneously in two diastereomers related by inversion of the sulfonamide nitrogen. Moreover, the flap site is shaped to accommodate the diastereomer with the *S*-enantiomeric nitrogen rather than the one with the *R*-enantiomeric nitrogen. The existence of the second binding site and two diastereomers suggest a mechanism for the high effectiveness of TMC114 on drug resistant HIV and the potential design of new inhibitors.

Keywords

HIV-1 protease; drug resistance; darunavir; allosteric binding site; ultra-high resolution crystal structure; enantiomer

Two decades of research have yielded about twenty antiretroviral drugs and different drug regimens for treatment of HIV-1 infection.¹ Highly active antiretroviral therapy (HAART), in which a cocktail of drugs containing HIV-1 reverse transcriptase (RT) and protease (PR) inhibitors is administered to patients,^{2,3} has dramatically improved the survival of infected people and transformed the previously deadly disease into a treatable medical condition in many cases.

HIV-1 protease inhibitors (PIs) were developed successfully by structure-guided drug design.⁴ They work by blocking the activity of PR to process the viral polypeptides Gag and Gag-Pol into the structural and enzymatic proteins during the final stages of viral particle maturation. The Food and Drug Administration (FDA) has approved eight PIs and ten PI combinations to be used in HAART. Nonetheless, the drug resistant strains develop quickly due to the infidelity

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of the viral RT, and PR variants that are multi-drug and cross-resistant to some or all the available PIs are selected.^{5,6} Consequently, drug resistance has become a central problem in the treatment of HIV infection, and new therapeutic agents are being developed that inhibit drug resistant mutants as well as the wild type PR.⁷

TMC114 (darunavir) is an exceedingly potent antiviral agent designed to inhibit HIV-1 PR by binding at its active site.⁸ It is highly effective against various subtypes of HIV-1, including many drug resistant strains^{9,10} Recently, it was approved by the FDA for treatment of drug resistant HIV (www.fda.gov/bbs/topics/NEWS/2006/NEWS01385.html). TMC114 is a non-peptidic transition-state analog, with the chemical structure as shown below. It differs from its closest

chemical analog, amprenavir,⁴ by the presence of the bis-THF moiety. The design rationale was to increase the number of favorable interactions with main chain atoms of PR. This objective has been achieved as evidenced by crystallographic studies on wild type PR and mutant complexes with TMC114^{11,12} and by quantum chemical calculations of interaction energies of TMC114, amprenavir, or nelfinavir with wild type PR.¹³

No TMC114-specific resistant mutations in PR have been reported to date, so our approach has been to study PR mutants with a single substitution mutation that renders resistance to the other clinical inhibitors. Recently, we reported the analysis of TMC114 complexes with PR containing single multi-drug resistant mutations D30N, I50V, V82A, I84V and L90M.^{11,14} TMC114 adapted to the PR structural changes due to V82A and I84V mutations, with the K_i values increasing by no more than four times. In the case of PR_{L90M} TMC114 had even better inhibition than for the wild type enzyme. On the other hand, TMC114 exhibited less effective inhibition of PR_{D30N} and PR_{I50V}, where the K_i values were increased 12-17 times compared to the value for the wild type PR.¹⁴

Here, we focus on the multi-drug resistant mutations V32I and M46L.⁶ The mutation V32I is observed in about 20% of isolates from patients treated with amprenavir, in 3-6% of patients treated with ritonavir, lopinavir, atazanavir and indinavir, and in about 4% of those on a multi-PI regimen.⁶ It confers intermediate level resistance to amprenavir, ritonavir and indinavir. The mutation M46L is selected for resistance to seven of the eight FDA approved clinical PIs, and is a major mutation arising during treatment with indinavir.¹⁵ Structurally, V32I alters a residue in the substrate-binding site and can directly contribute to the drug resistance by unfavorable interactions with an inhibitor because isoleucine is larger than valine. Conversely,

M46L alters a residue in the flexible PR flap (residues 43-58) and is not in direct contact with an inhibitor bound in the active-site cavity, although the main-chain atoms of Met46 form hydrogen bonds with substrate analogs¹⁶. The active dimer of HIV-1 PR employs the two flaps to enclose the substrate or inhibitor within the active-site cleft. The PR flap is believed to be essential for substrate or inhibitor recognition and delivery to the active site.¹⁷ Hence, the M46L mutation can influence the binding of inhibitor indirectly either by reducing the hydrophobic interactions during the binding process, or by strengthening interactions with a substrate.

We describe crystallographic analysis of the effects of TMC114 on mutants PR_{V32I} and PR_{M46L}. The crystal structures have been determined at 0.84Å and 1.22Å resolution for PR_{V32I}-TMC114 and PR_{M46L}-TMC114 complexes, respectively. The first ultra-high resolution structure of a PR-inhibitor complex showed two molecular species at 60% and 40% occupancy. The higher occupancy conformer has TMC114 bound at two distinct sites: the active site cavity and a second, new site on the surface of one of the flaps, while the lower occupancy conformer showed TMC114 only in the active site cavity. These results suggest an alternative mechanism for the effectiveness of TMC114 against many clinical drug resistant isolates of HIV-1 and may provide a distinct target for the design of novel inhibitors that bind to the second site on the flap.

RESULTS

Crystallographic analysis

The crystal structures of PR_{V32I} and PR_{M46L} drug resistant mutants complexed with the inhibitor TMC114 were solved in the space group P2₁2₁2₁ as summarized in Table 1. The asymmetric units contain the PR dimer and the residues in the two subunits are labeled 1-99 and 1'-99' (Fig. 1a). The crystals diffracted to the ultra-high resolution of 0.84 Å for PR_{V32I}-TMC114 and near-atomic resolution (1.22 Å) for PR_{M46L}-TMC114. The final R-factors are 11.7% and 13.1% for PR_{V32I}-TMC114 and PR_{M46L}-TMC114, respectively. There was clear electron density for all atoms of the protease, the inhibitor and solvent molecules in the two structures. The 2F_O-F_C electron density map had distinct peaks for each non-hydrogen atom in the ultra-high resolution structure of PR_{V32I}-TMC114, whereas the near-atomic resolution structure showed lower, less distinct peaks for atoms (Fig. 1b and 1c). The average atomic B-factor values were approximately two-fold lower for the PR_{V32I}-TMC114 complex (Table 1) indicating the higher accuracy of atomic positions for this structure. The high resolution of the diffraction data allowed modeling of two shells of solvent, including more than 200 water molecules, chloride anions and dimethylsulfoxide (DMSO) molecules.

Of particular interest is the fact that TMC114 is found not only inside the active-site cleft, as observed in other structures,^{11,14,18} but also on the protein surface in the flap region (Fig. 1a and 1d). The inhibitor bound in the active site of PR_{V32I}-TMC114 and PR_{M46L}-TMC114 structures has two alternate conformations related by a 180° rotation and occupancies of 60/40%. TMC114 shares the second surface binding site with the solvent DMSO molecule, with the occupancies refined to 60 and 40%, respectively. Remarkably, TMC114 has different configurations when bound on the surface and in the active-site cavity. The amide nitrogen of the sulfonamide moiety has a pyramidal configuration and is chiral due to the presence of three chemically different substituents and a lone electron pair. The sulfonamide nitrogen has the *R*-enantiomeric configuration when TMC114 is bound in the active-site cavity, but an *S*-enantiomeric configuration in the flap binding site (Fig. 1e). The two diastereomers are related by the nitrogen inversion, a well-known geometrical change of a pyramidal nitrogen atom. The presence of two enantiomers of a ligand bound to different sites in a protein molecule is unusual.

Alternate conformations were modeled for 50 and 19 residues in the PR_{V32I}-TMC114 and PR_{M46L}-TMC114 crystal structures, respectively. Owing to the ultra-high resolution data for PR_{V32I}-TMC114, alternate conformations for main-chain as well as side-chain atoms were observed for many amino acid residues. On the contrary, the lower resolution data for PR_{M46L}-TMC114 resulted in less apparent disorder for the main-chain atoms; only the peptide bond connecting residues Ile50 and Gly51 has two alternate conformations. In the PR_{V32I}-TMC114 structure the main-chain and side-chain atoms of residues 23-25, 30-32, 47-52 and 22'-25', 30', 32'-33', 47'-55' have two alternate conformations, with the occupancies refined to 60 and 40%, the same as the relative populations of the two inhibitor conformations.

The high quality and 0.84 Å resolution of the X-ray data permit the decomposition of the PR_{V32I}-TMC114 structure into two distinct conformers with 60% and 40% occupancy. The remarkable conclusion is that two different molecular species have co-crystallized together; the 60% occupancy species has two inhibitor molecules bound to the protease at the active-site and the surface of the flap, while the other conformer has a single inhibitor bound in the active-site and DMSO occupies the surface site.

Effect of mutations on TMC114 binding in the active-site cavity of HIV-1 protease

TMC114 forms a variety of interactions inside the active-site cavity. On average about a hundred different contacts are made, including:

- a. strong O-H...O hydrogen bonds – normal distances are in the 2.6-3.0 Å range¹⁹;
- b. moderately strong N-H...O and N-H...N hydrogen bonds – normal distances are in the 2.8-3.2 Å range¹⁹;
- c. c) weaker C-H...O contacts – contacts are considered good when the distances are of 3.0-3.7 Å²⁰;
- d. C-H... π interactions - the distance to any atom of a π -system has to be < 4.0 Å, provided C-H is not in the aromatic ring plane²¹;
- e. the weakest van der Waals interactions such as C-H...H-C – when distances of 3.8-4.2 Å the interactions are attractive, while at distances of < 3.6 Å they are repulsive²².

Although the V32I mutation introduces a bigger side chain next to the inhibitor, potentially reducing the size of the active-site cavity, in fact the inhibitor loses some favorable interactions with the protease, especially in its minor conformation, rather than gaining unfavorable contacts (Figure 3). A similar effect is observed in the PR_{M46L}-TMC114 structure, even though M46L has no direct contacts with the TMC114 molecule that occupies the active site cavity. The interactions are described below separately for the major and minor molecular species in the two mutant complexes.

The major conformation of TMC114 in the active site of PR_{V32I} and PR_{M46L} has interactions that are similar to those for the inhibitor in the wild type PR structure, except for the differences noted below. In PR_{V32I} C-H... π contacts between the aniline π -system of the inhibitor and the side chains of residues Ala28', Ile32' and Ile50 are preserved and the distances between non-hydrogen atoms of 3.4-3.8 Å are comparable to those calculated in the PR structure with residues Ala28, Val32 and Ile50'. However, a direct hydrogen bond of the NH...O type with a distance of 2.7 Å from the aniline NH₂ group to a carboxylate oxygen of Asp30 is replaced by a weaker water-mediated interaction in PR_{V32I}, with the distances NH₂...H₂O...OOC (Asp30') of 3.0 Å and 2.7 Å, respectively. Additionally, an unconventional hydrogen bond C α -H...O between Gly49' C α and an oxygen of the sulfonamide moiety is weaker in the PR_{V32I} structure with a distance of 3.3 Å, which is significantly longer than the 2.9 Å observed in PR.

A quite symmetric pattern of hydrogen bonds is observed between the central OH group of TMC114 and the two Asp25 and 25' residues (Fig. 2a) for the major and minor conformations of the inhibitor in PR-TMC114 co-crystal and for the major conformation of TMC114 in PR_{V32I} complex (Fig. 2b). However in PR_{M46L}-TMC114, a strong asymmetry is apparent in the similar hydrogen-bond network (Fig 2c). The OH...OOC distances are 2.4, 2.8 Å and 2.9, 3.1 Å with Asp25' and Asp25, respectively. However, we cannot rule out the possibility that some of these differences may be an artifact of the lower resolution (1.3 Å) of the wild type complex. The minor form in PR_{V32I}-TMC114 has an asymmetric hydrogen-bond network of the catalytic Asp 25 and 25' similar to that seen for the major conformation in PR_{M46L}-TMC114 (Fig. 2b). However, the minor inhibitor conformation in PR_{M46L}-TMC114 has four very similar hydrogen bonds to the catalytic aspartates.

The minor (40%) species shows larger differences in the TMC114-protease interactions in the mutant complexes (Figure 3). Interestingly, the minor and major conformations of TMC114 show different binding in the active site cavity of PR_{V32I}, presumably related to the asymmetric hydrogen-bond network with Asp25 and 25'. On the other hand, the analogous asymmetry of the interactions of the major conformation of TMC114 with the catalytic aspartates in PR_{M46L}-TMC114 has little effect on its overall interactions with the protein. Therefore, it is surprising that the minor conformations in both mutant structures have similar absent or weaker interactions. A good hydrogen bond between NH₂ of the aniline and O=C of Asp30 of ~ 3.2 Å in PR-TMC114 is completely absent in the mutant structures where the corresponding distances are more than 4.3 Å. Similarly, only a single C-H...O contact (3.1-3.3 Å) of the bis-THF part with the main-chain carbonyl of Gly48' remains in the PR_{V32I} and PR_{M46L} complexes, whereas two such interactions with the distances of 2.9-3.4 Å are present in the wild-type structure. In addition, another C-H...O contact between C_α-H of Gly49 and an O of the sulfonamide, which can be considered a good non-conventional hydrogen bond with the distance of 2.8-2.9 Å in PR-TMC114, is considerably longer with the distances of 3.2 Å and 3.6 Å in PR_{M46L} and PR_{V32I}, respectively. Furthermore, the aniline moiety of TMC114 lacks the aforementioned C-H...π interactions with residue 32 in both mutant complexes, since the corresponding C...C distances are > 4.4 Å. Finally, repulsive short van der Waals interactions are introduced in the mutant structures that include 3.4-3.5 Å contacts involving one methyl group of the *iso*-butyl moiety of TMC114 and the side-chain atoms of Val82.²² Analogous contacts in PR-TMC114 are attractive van der Waals interactions with the distances of 3.9 Å.

Second binding site for TMC114 on the protease surface

A second TMC114 molecule was found on the protein surface in the major conformer of the PR_{V32I} and PR_{M46L} complexes. The larger part of the inhibitor molecule is positioned in a groove located in the flap region of the protease (Fig. 4a). The groove is formed by the residues Glu35', Trp42', Pro44'-Met46'(or Leu46'), Lys55'-Arg57' and Val77'-Pro79', and is less evident on the other protease subunit. The smaller part of the TMC114 molecule that is outside the groove consists of the phenyl and bis-THF groups that face two symmetry-related protein molecules. A similar, although shallower, groove is present in the PR-TMC114 structure where a 50% occupancy glycerol molecule is bound (Fig. 4b). Thus, these structures suggest that the groove can open up to accommodate TMC114.

TMC114 has a larger number and significantly stronger interactions with the residues in the groove formed by the flap than with the residues of the symmetry-related protein molecules. A network of hydrogen bonds is formed between the aniline, sulfonamide and carbamate moieties of the TMC114 and the main- and side-chain atoms of PR_{V32I} or PR_{M46L} (Fig. 5a). Two direct hydrogen-bonds of 2.9-3.3 Å are made with the main-chain of Lys45' and side-chain of Arg57' in the two mutant structures (aniline NH₂...O=C of Lys45' and S-O...H-N of Arg57'). Additionally, there are interactions mediated by two water molecules that involve the

central OH group and the carbamate carbonyl oxygen atom of the inhibitor and the side chain amino groups of Lys55' and Arg57' of the mutants, with the distances in the 2.8-3.1 Å range (Fig. 5a). The binding of TMC114 at the flap is also supported by other weaker interactions, such as non-conventional C-H...O hydrogen bonds. Two C-H...O interactions connect the main-chain carbonyl oxygens of Val56' and Val77' with the aniline and the iso-butyl group of TMC114, respectively. The distances are comparable in both mutant structures (3.2-3.3 Å). A slightly weaker C-H...O interaction exists between the sulfonamide oxygen and C γ of Arg57', with 3.4 Å separation between heavy atoms in PR_{V32I} and PR_{M46L}. Moreover, the aniline group of TMC114 is bound more tightly in the groove by C-H... π interactions with side chains of Pro44' and Lys55' and interatomic distances as short as 3.4 Å. Similar van der Waals contacts of 3.9-4.0 Å are found with residue 46' in the V32I and M46L mutant structures. Hence, remarkably, the M46L mutation, though forming a part of the second binding site, does not alter the inhibitor binding in this site.

The phenyl and bis-THF groups are directed away from the groove in the flap and toward two symmetry-related protease molecules, while a third protease molecule is situated on top of the groove and above the inhibitor (Fig. 5b). These groups make only a few hydrophobic contacts and no hydrogen bonds with residues of PR_{V32I} or PR_{M46L}. These symmetry-related interactions of the phenyl and bis-THF moieties are similar in both mutant structures. The π -system of the phenyl substituent is in the close vicinity of Arg41 from the first symmetry-related protease molecule and forms interactions of 3.4-3.7 Å with the main-chain amide and side-chain C β atoms. The side-chain of Arg41 past the C β atom is highly disordered, indicating the weakness of the protease/inhibitor intermolecular binding in this region. Similarly, bis-THF interacts with the indole group of Trp6 of the second symmetry-related molecule by C-H... π contacts with the distances of 3.4-3.8 Å. The side chain of Trp6 is also parallel to and about 3.5 Å away from the inhibitor's carbamate moiety and therefore can participate in π - π stacking interactions. Another set of C-H... π contacts involves residue Gly94' of the third symmetry-related protease and the aniline group. These interactions are very similar in PR_{V32I} and PR_{M46L} with distances of 3.4-3.6 Å.

Binding of the second inhibitor molecule induces conformational changes in the protease

When the mutant structures are superimposed onto the PR-TMC114 structure (PDB code 1S6G)¹¹ the overall main-chain root-mean-square deviation (rmsd) is 0.6 Å for both PR_{V32I} and PR_{M46L}. The rmsd value is analogous to that for comparison of HIV PR structures with different unit cells,^{14,23} where the largest differences were observed for surface residues not involved in the inhibitor-protease interactions. However, the PR_{V32I} and PR_{M46L} complexes show dramatic conformational disparities relative to PR-TMC114 that go beyond the changes usually observed on comparison of closely related structures in different unit cells (Fig. 6a). The largest differences are in the conformations of the broad surface loop of residues 34-43, where the atomic shifts reach 5 Å compared with the position in the wild type structure. This large change of the flexible loop in the mutants is likely due to the close contacts with the symmetry-related surface-bound TMC114, in particular with Arg41. The second largest differences in the conformation of the proteases are evident for the flap residues that form the surface TMC114 binding site (Fig. 6b). The main-chain atoms of residues 44'-46' shift by 1.2-2.4 Å towards the inhibitor in the mutant complexes and form hydrogen bonds (*i.e.*, aniline NH₂ ...O=C of Lys45') and hydrophobic interactions, while the side-chain of Arg57' moves by 3.5 Å, breaking its salt-bridge interactions in the PR and forming hydrogen bond interactions with TMC114. The residues that form hydrophobic interactions are shifted either toward the TMC114, like Pro44', or slightly away from it, like Lys55' and Trp42', and therefore either form good C-H... π contacts (Pro44' and Lys55') or avoid unnecessary close interactions with the sulfonamide group (Trp42'). The changes of the protease atoms in the other areas that are in contact with TMC114 do not exceed 0.4 Å, indicating much weaker inhibitor interactions.

These structural changes confirm that TMC114 binding on the protease surface is mostly confined to the flap area. The protease adjusts in this area to accommodate the drug, while other symmetry-related interactions are mostly due to the crystal packing. Interestingly, the configuration of TMC114 also adjusts since the S-enantiomer is bound at this surface site.

Enzyme kinetics and inhibition

The kinetic parameters of protease-catalyzed hydrolysis were measured for wild-type PR and the two mutants PR_{V32I} and PR_{M46L} using the chromogenic substrate that represents the CAp2 cleavage site of the HIV-1 Gag precursor (Table 2). PR and PR_{V32I} showed essentially the same k_{cat}/K_m , while PR_{M46L} showed only 50% of the PR value. The lower activity of PR_{M46L} is primarily due to an approximately three-fold increase in the K_m value.

The wild-type PR and mutants were assayed for inhibition by the clinical inhibitor TMC114. TMC114 shows sub-nanomolar inhibition of PR, while the relative K_i values are about 7 and 10-fold higher for the inhibition of PR_{V32I} and PR_{M46L}, respectively (Table 2). This decreased inhibition for PR_{V32I} and PR_{M46L} is consistent with the loss of interactions with TMC114 observed in the crystal structures (Figure 3d-3f). The PR_{V32I} and PR_{M46L} mutants are significantly more resistant to the inhibition by TMC114 than the PR_{V82A} and PR_{I84V} mutants employed in our previous study¹¹. Alternatively, TMC114 showed less effective inhibition of PR_{D30N} and PR_{I50V}.¹⁴

DISCUSSION

TMC114 is performing exceptionally well in clinical trials for treatment of HIV infection. It shows an outstanding resistance profile and high effectiveness against all the subtypes of HIV, ^{9,10} and has been approved as a salvage therapy for those patients who fail other drug regimens. Remarkably, there are no reports of resistant mutations in HIV-1 specifically selected by treatment with TMC114.

Similar to other drugs, TMC114 was designed to bind exclusively in the active-site cleft of the HIV-1 PR. Unexpectedly, the structures of PR_{V32I}-TMC114 and PR_{M46L}-TMC114 obtained with ultra-high 0.84 Å and near-atomic 1.22 Å resolution, respectively, have unequivocally shown that TMC114 binds at two sites: the active-site and a surface site on the flap. The subatomic resolution of the PR_{V32I}-TMC114 structure allowed us to definitively relate the major conformation of TMC114 in the active site and the surface flap site. Consequently, we concluded that two molecular species have co-crystallized: one has two TMC114 molecules bound to the protease, the PR_{V32I}-(TMC114)₂ species, and the other has just one TMC114 molecule in the active-site cavity and a DMSO solvent molecule in the second potential site on the protein surface. It is not unusual for an enzyme to have two binding sites for inhibitors, where one binds at the catalytic site and the other (allosteric) site is located in a different part of the protein molecule.²⁴ Detailed kinetic analysis may help to establish whether the second site for TMC114 has an inhibitory effect.

The crystal structures of PR_{V32I} and PR_{M46L} imply a biological role for the TMC114 binding site on the flap. Other types of compounds, like beta-lactams²⁵ or polyoxometalate anions,²⁶ have been demonstrated to inhibit the HIV-1 protease by exclusively binding to surface sites in the flap region. The part of TMC114 bound on the protease flap makes a number of strong stabilizing interactions to the main- and side-chain atoms of the protein. On the other hand, the rest of the inhibitor that contacts three symmetry-related protease molecules has only a few hydrophobic contacts with each of them. Brynda *et al.*²⁷ reported another PR structure with a peptide inhibitor bound to a similar second site. In contrast to TMC114, this peptide inhibitor binds with a number of direct hydrogen bonds and water-mediated contacts with all four surrounding PR molecules in the crystal. Notably, the peptide inhibitor forms only water-

mediated contacts with the flap residues, while direct hydrogen bonds are observed with other symmetry-related PR molecules. Thus, the authors concluded that the second site did not have any relevance for the inhibition of the PR. The analysis of the surface site in our PR_{V32I} and PR_{M46L} crystal structures suggests that TMC114 has significantly more interactions with the flap region than with the other parts of the protease. Another consideration is that the conformation of the protease in the flap site changes on binding of TMC114. In the complexes with PR_{V32I} and PR_{M46L} the residues in this flap site show substantial shifts from their positions in the wild-type PR that optimize the interactions with TMC114 (Fig. 6b). Finally, TMC114 has two different configurations related by the sulfonamide nitrogen inversion: an *S*-enantiomeric configuration when bound on the surface and an *R*-enantiomeric configuration in the active-site cavity of the protease. If the active-site bound inhibitor configuration is superimposed onto the TMC114 bound in the flap site, the aniline moiety clashes with protease residues (Fig. 4c). Thus, it is evident that the flap binding site is shaped to accommodate only the diastereomer with *S*-enantiomeric amide nitrogen. The other diastereomer with the *R*-enantiomeric amide nitrogen cannot bind at the flap site due to the steric collisions with the protease atoms. Therefore, both the protease and TMC114 adapt to form a complex with two bound inhibitors.

We therefore propose that the second TMC114 binding site observed in the structures of HIV-1 PR_{V32I} and PR_{M46L} mutants can explain the remarkable effectiveness of TMC114 on the drug resistant strains of HIV-1.

METHODS

Preparation of HIV-1 PR_{V32I} and PR_{M46L} mutants, purification and crystallization

An HIV-1 PR (Genbank HIVHXB2CG) clone with 5 mutations that reduce autoproteolysis (Q7K, L33I, L63I) and prevent cysteine-thiol oxidation (C67A and C95A), was used as the template to introduce the mutations V32I or M46L.²⁸ The PR mutants were expressed in *E. coli* and purified from the inclusion bodies, as described previously.^{28,29}

The crystals of the PR mutants complexed with TMC114, which was dissolved in dimethylsulfoxide, were grown by the hanging-drop vapor diffusion method using 10:1 and 2:1 ratio of the inhibitor to protein for PR_{V32I} and PR_{M46L}, respectively. Crystals of PR_{V32I} grew with a well solution of sodium acetate buffer (pH = 4.2-4.8) and 1-1.5M NaCl. PR_{M46L} crystals grew using well solution of sodium acetate buffer (pH = 3.8) and 25% NaCl.

Enzyme Kinetics

The chromogenic substrate Lys-Ala-Arg-Val-Nle-p-nitroPhe-Glu-Ala-Nle-amide (Sigma, St. Louis, MO) was used to determine the kinetic parameters. Wild-type or mutant PR at final concentrations of 160-190 nM were added to varying concentrations of substrate (100-400 μ M) maintained in 50mM sodium acetate pH = 5.0, 0.1M NaCl, 1mM EDTA, and assayed by monitoring the decrease in absorbance at 310 nm using a Varian Cary 100Bio UV-Vis spectrophotometer. k_{cat} and K_m 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_{50} vs. $[S]$ plots with the IC_{50} axis. The K_i values were obtained from the IC_{50} values estimated from an inhibitor dose-response curve with the spectroscopic assay using the equation $K_i = (IC_{50} - [E])/2 / (1 + [S]/K_m)$, where $[E]$ and $[S]$ are the PR and substrate concentrations, respectively.³⁰ The K_i values were measured at 4-5 substrate concentrations. The measurements were repeated at least three times to produce the average values given in Table 1.

X-ray diffraction data collection

Crystals were transferred into a cryoprotectant solution containing the reservoir solution plus 20-30% (v/v) glycerol, mounted on a nylon loop 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 0.8Å wavelength. Data were processed using HKL2000.³¹ A large rod-like crystal of the PR_{V32I} complex, with dimensions of 0.5×0.3×0.3 mm, diffracted X-rays to 0.84Å resolution and had mosaicity of 0.3°. The crystal of the PR_{M46L} complex had dimensions of 0.2×0.2×0.1 mm, and diffracted X-rays to 1.22Å resolution with mosaicity of 0.7°.

Structure determination and refinement

The CPP4i suite of programs^{32,33} was used to obtain a molecular replacement solution and the starting model was the wild type PR complex with peptide inhibitor KI2 (PDB code 1NH0), which is in the same space group. The structures were refined using SHELX97³⁴ and refitted using O 10³⁵ program. Alternate conformations were modeled for the inhibitor and 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 PR_{V32I} crystal structure was refined with three chloride anions, a 40% populated DMSO molecule and 258 water molecules including partial occupancy sites. The PR_{M46L} structure included two chloride anions, a 40% populated DMSO molecule and 212 water molecules including partial occupancy sites. Figures were made by Bobscript³⁶ and PyMol³⁷.

Accession codes

Protein Data Bank: Coordinates and structure factors have been deposited with the accession codes 1HS1 (PR_{V32I}-TMC114 complex) and 1HS2 (PR_{M46L}-TMC114 complex).

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Figure 1.

(a) PR_{V32I} dimer structure. Two subunits (in red and blue) are shown indicating the secondary structure. TMC114 is in ball-and-stick representation colored by atom type, and is bound in two sites. (b) and (c) The electron density ($2F_O - F_C$) for residues 55-60 in the PR_{V32I} and PR_{M46L} structures. Contour levels are 3.6σ for (b) and 2.4σ for (c). (d) The $2F_O - F_C$ electron density for TMC114 bound to the flap in PR_{V32I} contoured at 1.8σ . TMC114 has 60% occupancy, while the other 40% correspond to a DMSO solvent molecule, depicted in magenta. (e) Structures of TMC114 bound in the active site cavity (*R*-enantiomer) and in the flap region (*S*-enantiomer). The moieties in the box are related by reflection in a mirror and can be obtained by inversion of the sulfonamide nitrogen.

Figure 2. Hydrogen bonds between the central OH group of TMC114 and the catalytic Asp25 and Asp25'. The major conformation of TMC114 is colored by atom type, and the minor conformation is green. Interatomic distances are shown in Å (a) PR-TMC114 (PDB code 1S6G); the TMC114 conformations were refined with 55% and 45% occupancies. (b) PR_{V32I}-TMC114 and (c) PR_{M46L}-TMC114. The 2F_O-F_C electron density for the active site residues Asp25 and Asp25' is shown with the contour levels of 2.2σ. The alternate conformations have occupancies of 60% and 40%.

Figure 4.

(a) TMC114 at the flap binding site in PR_{V32I}, and (b) similar view in PR. TMC114 in the PR_{V32I} complex, and a glycerol molecule in PR-TMC114 are in a space-filling representation and colored by atom type. The protease is represented as a surface, and the residues forming the binding site are labeled. (c) Superposition of *R*-enantiomer (magenta) from the active-site cavity with the *S*-enantiomer (colored by atom type) bound in the flap site of PR_{V32I}. The aniline moiety of the *R*-enantiomer (indicated by arrow) collides with the protease residues, which would prevent it from binding in the flap site. The geometry is similar in PR_{M46L}.

Figure 5

. (a) Hydrogen bond network and C-H...O interactions of TMC114 bound in the flap site of PR_{V32I}. Hydrogen bonds are colored in red, C-H...O contacts are black, and distances are in Å. The interactions in the PR_{M46L} complex are very similar. (b) TMC114 bound to the surface site is surrounded by four protein molecules. The asymmetric unit consists of PR_{V32I} (blue) and two inhibitor molecules shown in yellow ball-and-stick representations. The symmetry related protease molecules are in cyan, orange and magenta.

Figure 6.

(a) The superposition of the PR_{V32I} and PR_{M46L} structures onto the wild type PR. (b) The residues (labeled) of the flap binding site for TMC114 have the largest differences. PR is colored by atom type, while PR_{V32I} and PR_{M46L} are colored in magenta and cyan, respectively. The atomic shifts (Å) are indicated by dashed arrows.

Table 1

Data collection and refinement statistics for PRV32I and PRM46L in complex with TMC114.

	PR _{V32I}	PR _{M46L}
Data collection		
Space group	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁
Unit cell dimensions a, b, c (Å)	28.70, 65.92, 92.53	28.9, 66.6, 93.1
Resolution Range (Å)	50–0.84 (0.87–0.84) [§]	50–1.22 (1.26–1.22) [§]
Unique reflections (obsvd. with I>2σ(I))	153847 (131172)	50541 (42888)
I/σ(I)	45.3 (2.0)	20.0 (3.1)
R _{merge} (%)	6.3 (35.8)	9.9 (31.9)
Completeness (%)	95.9 (63.3)	93.0 (73.2)
Refinement		
Data range for refinement (Å)	20–0.84	10–1.22
R _i (I > 2σ(I))	11.7	13.1
R _{work} (%)	12.4	14.0
R _{free} (%)	14.8	19.6
No. of solvent molecules	258	212
No. of obsvd. reflections/No. of refined params.	6.6	2.5
RMS deviation from ideality:		
Bonds (Å)	0.018	0.013
Angle distance (Å)	0.039	0.033
Main-chain	8.4	15.1
Side-chain	13.3	21.2
Inhibitor at active site, at flap's site	10.2, 12.1	17.3, 24.5
Solvent	26.3	31.5
Occup. of alternate conf. of TMC114 (%) in active site cavity	60/40	60/40
Occup. of TMC114/DMSO at 2nd site in flap	60/40	60/40

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

Table 2

Kinetic parameters from the spectrophotometric assay for hydrolysis of peptide Ac-KARVNle(Phe-p-NO₂) EANle-CO-NH₂ and inhibition by TMC114 of PR, PR_{V32I} and PR_{M46L}.

Protease	K_m , μM	k_{cat} , min^{-1}	k_{cat}/K_m , $\text{min}^{-1}\cdot\mu\text{M}^{-1}$	K_i , nM (Relative)
PR	106 ± 9	245 ± 10	2.3 ± 0.2	0.49 ± 0.13 (1)
PRV32I	90 ± 8	198 ± 6	2.2 ± 0.2	3.3 ± 0.2 (6.7)
PRM46L	286 ± 23	283 ± 11	1.00 ± 0.08	4.9 ± 0.4 (10)