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Probing Structural Changes among Analogous Inhibitor-Bound Forms of HIV-1 Protease and a Drug-Resistant Mutant in Solution by NMR

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Abstract

In the era of state-of-the-art inhibitor design and high-resolution structural studies, detection of significant but small protein structural differences in the inhibitor-bound forms is critical to further developing the inhibitor. Here, we probed differences in HIV-1 protease (PR) conformation among darunavir and four analogous inhibitor-bound forms, and compared with a drug-resistant mutant using NMR chemical shifts. Changes in amide chemical shifts of wild-type (WT) PR among these inhibitor-bound forms, Δ CSP, were subtle but detectable and extended over 10 Å away from the inhibitor-binding site, asymmetrically between the two subunits of PR. MD simulations revealed differential local hydrogen bonding as the molecular basis of this remote asymmetric change. Inhibitor-bound forms of the drug resistant mutant also showed a similar long-range Δ CSP pattern. Differences in Δ CSP values of the WT and the mutant (Δ CSPs) were observed at the inhibitor binding site and the surrounding region. Comparing chemical shift changes among highly analogous inhibitors and Δ CSPs effectively eliminated local environmental effects stemming from different chemical groups and enabled exploiting these sensitive parameters to detect subtle protein conformational changes, to elucidate asymmetric and remote conformational effects upon inhibitor interaction.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information. A pdf file that contains the following data is available free of charge: Figure S1, filtered NOESY strips that were used for subunit assignments and the corresponding NOE positions in the structure; Figure S2, Schematic diagram of resonance assignments of HIV-1 PR bound to DRV; Table S1, assigned backbone chemical shifts of WT PR bound to DRV (BMRB accession number 27378); Table S2, assigned backbone chemical shifts of Flap+ PR bound to DRV (BMRB accession number 27377).

Keywords

Drug design; HIV-1; Protease; Inhibitor; NMR; MD; Structural Biology

INTRODUCTION

In this era of state-of-the-art highly potent inhibitor design, detailed structural evaluation of drug-protein interactions is needed to increase the sensitivity in structure-based drug design. Especially, during the *late* stages of inhibitor optimization or in the analysis of drug-resistance mutations, slight changes in inhibitor chemical moieties appear to not impact overall intermolecular interactions or the target protein structure upon binding, but still often can change the resistance profile or inhibitory activity.^{1–5} This observation has been largely based on X-ray crystallographic analyses of inhibitor-protein interactions and MD simulations. Solution NMR spectroscopy is largely untapped to detect structural differences of highly analogous inhibitor-bound forms of a protein despite tremendous increase in NMR sensitivity in the last 20 years.⁶

Indeed, NMR relaxation or line-shape analysis has been successfully used to detect significant differences in dynamics between apo-form variants of a protein but not for high-affinity inhibitor-bound forms.^{7–10} There are a couple of reasons for the lack of sensitivity to detect the differences in inhibitor-bound forms. First, protein structure is expressed by ensemble of conformations^{11–15}, which may mask small conformational difference among similar protease-inhibitor complexes in solution. Second, model free analysis, to characterize dynamics using a single set of three relaxation parameters, can be easily affected by model-dependent errors, molecular anisotropy, and relaxation other than the dipolar coupling and chemical shift anisotropy.^{8, 16–18}

We have previously studied dynamics of HIV-1 protease (PR) and the drug resistant mutant Flap+, which contains L10I, G48V, I54V, and V82A mutations (Figure 1a)^{8, 9}. Crystal structures have shown that differences in the darunavir (DRV)-bound forms between WT and Flap+ were significant but small: Root-Mean-Square-Deviation (RMSD) of backbone C α atoms of WT bound to four known inhibitors (amprenavir, atazanavir, DRV, Tipranavir) exhibited less than 0.2 Å difference between PR and Flap+.¹⁹ In addition, molecular dynamics (MD) simulations have elucidated conformational differences between DRV-bound forms of WT and Flap+, but not at the level to be seen in the MD-derived backbone order parameters.⁹ NMR relaxation method alone was not sufficiently sensitive to detect if such conformational changes persisted solution, either. DRV equilibrium dissociation constant, K_d, of Flap+ (26 ± 10 pM) is 5 times weaker than that of WT (4.5 ± 2 pM), and the thermodynamics of binding exhibited an extreme entropy-enthalpy compensation on the

order of 10 kcal/mol compared to WT PR, with entropically-driven binding in contrast to WT binding which is driven by both favorable entropy and enthalpy.¹⁹ Overall, although inhibitor-binding thermodynamics suggested differences in conformational response of Flap + PR upon inhibitor binding compared to WT, experimental evidence of conformational differences between Flap+ and WT PR in the inhibitor bound forms in solution has been missing.

Recently, a series of HIV-1 PR inhibitors were generated to incorporate modifications at P1' and P2' moieties of the DRV scaffold.² The analogue inhibitors (here, UX = U2, U3, U7, or U10) share the same core scaffold but differ at either or both of the R¹ and R² groups, which correspond to the P1' or P2' moieties of substrates (Table 1, and Figure 1b and 1c).² All these DRV analogues were highly potent picomolar inhibitors of WT PR, with inhibition constants, K_is, undistinguishable from DRV within the experimental error of the assay, and the crystal structures of PR bound to these inhibitors revealed almost identical three-dimensional structures, with 0.11–0.16 Å RMSD of backbone C_α atoms.² Inhibition constant of these UX inhibitors for Flap+ were similar to those of WT, except for U10.² Although MD simulations exhibited asymmetrical conformational changes around the active site among inhibitor-bound forms of PR,²⁰ it is unclear whether or not any structural differences may be experimentally detected when the ensemble of structures *in solution* are compared among these analogous inhibitor-bound forms or between the WT and Flap+ PR.

Here, we used NMR chemical shifts to investigate how specific changes of inhibitor moieties or drug resistance mutations impact PR structure and binding characteristics. While NMR chemical shift is a sensitive probe to characterize molecular interactions,^{21–26} inhibitor modification or mutation itself alters the electron shielding of the observing nuclei. To overcome this issue and tease out the structural changes, we selected highly analogous inhibitors and analyzed only the differences in chemical shifts, Δ CSP, of the two inhibitor-bound forms. DRV and UX analogues (Table 1, and Figure 1a).² Taking the difference Δ CSP aims to negate most of the electron shielding effect from the inhibitors to the PR resonances, likely except those within immediate proximity of the modified moiety, and therefore to reflect changes in the PR conformation caused by the difference in the specific chemical groups of the inhibitors. Further, comparing differences in Δ CSPs between WT and Flap+,

Δ CSP, eliminated the direct effect of mutations on chemical shifts to elucidate the conformational response originating from the inhibitor chemical groups between the WT and Flap+ (Figure 1b). In addition, the observed Δ CSP and Δ CSP values were analyzed in combination with the previous structural and MD simulation analysis.^{9, 19, 20}

The NMR chemical shift analysis, Δ CSP, elucidated among analogue-bound PR conformations subtle but detectable long-distance effects, over 10 Å, across the remote single α -helix of the protein. This long-range effect is asymmetric between the two subunits of PR, when R² group is modified (P2' moiety changes), which primarily interacts with subunit B. This asymmetric conformational change is consistent with recent MD simulations.²⁰ Comparing WT and Flap+, Δ CSP was detected at sites remote from the inhibitor, including residues 75–78, which comprise a β -sheet extending to a loop region. In contrast, Δ CSP was less significant at the active site and in the α -helix region, compared to Δ CSP, suggesting that the inhibitor effects to these regions in Flap+ similarly responded

to those in WT. These effects were detected above the background variations in the ensemble of PR conformations present in solution. Overall, NMR chemical shift analysis indicates that even subtle changes in an inhibitor moiety in highly potent tight-binding inhibitors can propagate over the target PR structure, causing asymmetric effects that can differ between WT and drug resistant variants.

EXPERIMENTAL SECTION

Protease Expression, Purification and NMR Sample Preparation

Plasmid containing the HIV-1 PR gene, coding the protein's 99 amino acid residues (PQITLWKRPL VTIRIGGQLK EALLDTGADD TVIEEMNLPG KWKPKMIGGI GGFIVRQYD QIPIEAGHK AIGTVLVGPT PVNIIGRNLL TQIGATLNF) was purchased from DNA2.0 (Newark, CA). Although the construct contains four mutations (Q7K, L33I, C67A, C95A) to reduce autoproteolysis and cysteine thiol oxidation,^{27, 28} it is called WT PR to distinguish Flap+ variant in this study. Differences in amino acid sequence of this WT from the construct used in inhibition experiments^{2,19} are only L33I, C67A, and C95A. For consistency with previous studies, the WT amino acid sequence in this study contains L63P polymorphism.^{2, 19} Construct Flap+ contains additional mutations L10I/G48V/I54V/V82A, which was also prepared and verified by DNA sequencing. Each plasmid was transformed and expressed in Escherichia coli strain BL21-(DE3) competent cells with T7 expression system (Agilent Technology, Santa Clara, CA). To produce ¹⁵N and ¹⁵N/¹³C labeled proteins, cells were grown using minimal media containing ¹⁵NH₄Cl and ¹⁵NH₄Cl/¹³C₆-glucose, respectively. ¹⁵N-labeled perdeuterated protein was also prepared by culturing E. coli using minimal media with 99.9% D₂O content containing ¹⁵NH₄Cl. Proteins were purified using the previously published protocol and stored at -80 °C.^{29, 30} Protein samples for NMR experiments were prepared by the dialysis protocol³¹ to yield the final protein concentration at ~250 μM (as a monomer) in a buffer containing 20 mM sodium phosphate pH 5.8 and with 5% D₂O. Inhibitor bound samples were prepared by adding a 2:1 molar ratio of inhibitor to refolded protein. Previously prepared inhibitors were used in the current study.²

NMR Data Acquisition and Analysis

All the NMR experiments were performed using Bruker Avance 600 MHz or 900 MHz NMR spectrometer, as described below, equipped with a cryogenic probe at 20 °C. Backbone resonance assignments were performed as follows. First, ¹⁵N and ¹³C signals for DRV, U3 and U10 bound forms of WT PR were carried out using an HNCA spectrum.^{32, 33} Next, 3D ¹⁵N half-filtered-NOESY experiment that detects NOE from non-¹⁵N labeled proton to ¹⁵N-labeled proton was performed using ²H/¹⁵N labeled sample.³⁴ These NOE data were used to identify whether signals were from either subunit A or B in complex with DRV, of which signal positions were identified based on published data.³⁵ Last, amide ¹H and ¹⁵N signals of PR bound to U2 and U7 were assigned by comparing them with those of others. Through all the assignments, the same relative orientation of inhibitors against subunit A or B was used, defined in the U2, U3, U7, and U10-PR crystal structures (PDB: 3O9A, 3O9B, 3O9F, and 3O9I, respectively). Similar assignment strategy was taken for the Flap+: HNCAs for the DRV-bound form and U10-bound form and 3D ¹⁵N half-filtered-

NOESY experiment for the DRV-bound form were recorded to assign the inhibitor-bound spectra of the Flap+ PR.

Comparison of WT PR chemical shifts bounds to DRV, U2, U3, U7 and U10 was carried out using the hetero-nuclear correlation spectra. The combined chemical shift changes, CSP, bound to DRV and to different inhibitors were calculated as normalized chemical shift differences of ^1H and ^{15}N .

$$\Delta\text{CSP} = \sqrt{(\delta_H^{\text{DRV}} - \delta_H^{\text{UX}})^2 + [(\delta_N^{\text{DRV}} - \delta_N^{\text{UX}}) \times \frac{\gamma_N}{\gamma_H}]^2} \quad (1)$$

where, γ_N and γ_H are gyromagnetic ratios of nitrogen and proton respectively. Similar comparisons of chemical shifts were performed among the UX inhibitors, by replacing DRV with one of UX inhibitors in equation (1).

A similar comparison of Flap+ chemical shifts bounds to DRV, U2, U3, U7 and U10 was carried out by recording ^1H - ^{15}N correlation spectra. CSPs between these UX inhibitor-bound Flap+ and the DRV-bound Flap+ were calculated using equation (1). CSP was calculated using CSPs determined between DRV and UX bound forms, denoted as $^{\text{WT}}\text{CSP}_{\text{DRV-UX}}$ and $^{\text{Flap+}}\text{CSP}_{\text{DRV-UX}}$, for WT PR and Flap+, respectively,

$$\Delta\Delta\text{CSP}_{\text{WT-Flap+}} = ^{\text{WT}}\Delta\text{CSP}_{\text{DRV-UX}} - ^{\text{Flap+}}\Delta\text{CSP}_{\text{DRV-UX}} \quad (2)$$

Here, individual subtractions, $^{\text{WT}}\text{CSP}_{\text{DRV-UX}}$ and $^{\text{Flap+}}\text{CSP}_{\text{DRV-UX}}$, are expected to detect relative changes in chemical shifts of the two inhibitor bound forms, while ΔCSP detects difference in CSP between WT and Flap+.

MD Simulations

Previously performed MD simulations were used to analyze the NMR-detected distal effect in the α -helix region.²⁰ In brief, co-crystallized structures (PDB: 1T3R, 3O9A, 3O9B, 3O9F, and 3O9I)^{2, 36} with DRV, U2, U3, U7 and U10 were prepared for MD simulation. All crystallographic waters were included; however, all crystallization buffer molecules were removed. The Protein Preparation Wizard from Schrödinger^{37, 38} was used to add hydrogen atoms, add missing side chains, determine and optimize protonation states of residues and ligands and check for structural violations such as overlapping atoms. Additionally, the hydrogen-bonding network was optimized by determining the optimal orientation of polar hydrogen atoms and sampling water orientations. Following this preparation, systems were minimized using OPLS2005 force field with restraints on heavy atoms but allowing hydrogen atoms to freely rotate. Desmond was used for all molecular dynamics simulations using the OPLS2005 force field. Production MD simulations were performed at 27 °C and 1 bar for a total of 300 ns over 3 trajectories, using NPT ensemble, Langevin thermostat and MTK barostat. Backbone RMSF of DRV and UX bound forms of WT PR were calculated for all the UX bound forms, using the coordinates from snapshots taken every 100 ps.²⁰ Variation of the RMSF values was assessed by taking average and standard deviation of the

values for all the UX bound forms. The frequencies of hydrogen bonding in the helix region encompassing residues I85 to T91 and the surrounding residues in chain A were subtracted from B. The errors in hydrogen bonding frequencies were computed using block averaging over the 3 trajectories. For Asp side chain, due to inter conversion of R87/D29:OD1 and R87/D29:OD2 hydrogen probabilities, their average value was used.

RESULTS

NMR chemical shift assignment of inhibitor-bound PR subunits

Backbone amide chemical shifts of the HIV-1 WT PR bound to U2, U3, U7 and U10 were compared with those of DRV, by recording the ^1H - ^{15}N correlation spectra of these complexes (Figure 2a). The PR construct used for the NMR experiments contains four mutations (Q7K, L33I, C67A, C95A) to reduce autoproteolysis and cysteine thiol oxidation,^{27, 28} and is referred to as WT PR in this study (see detailed sequence information in the Materials and Methods). As expected, the observed patterns of DRV and UX-bound PR ^1H - ^{15}N correlation spectra are highly similar, reflecting similarity among the chemical structures of the inhibitors. The number of signals observed in these spectra are larger than that observed from PR in the apo-form,³⁹ as apo-PR is a two-fold symmetrical homodimer with overlapping (degenerate) signals, but upon binding the asymmetrical inhibitor, some of the PR signals shift and split into two due to different local chemical environments experienced by the residues in the two PR subunits.

To assign PR backbone signals, first, a conventional HNCA experiment using the PR–DRV complex was performed. Approximately, one-third of amide ^1H - ^{15}N cross peaks split upon binding DRV due to different electron shielding of residues in subunit A versus B. Two backbone traces were completely and separately assigned for three segments of the protein sequence. A 3D ^{15}N half-filtered-NOESY spectrum was recorded to differentiate whether these segments belonged to subunit A or B (Figure S1). Through these signal assignments, each amide signal in the ^1H - ^{15}N HSQC spectrum was sequentially assigned, resulting in three assignment categories: (a) only one degenerate signal stemming from both subunits, (b) two split signals but not segmentally assigned to either subunit A or B, and (c) two split signals that were assigned to subunit A or B (Table S1). In the latter category, three segments were unequivocally assigned to subunit A or B: Segment 1, residues 22 to 38; Segment 2, residues 42 to 61; Segment 3, residues 82 to 90 (Figure S2). These regions overlapped with residues interacting directly with DRV in the crystal structure: the active site (residues 25 to 30), flap (residues 44 to 57), and the P1-loop (residues 79 to 84).¹⁹ Signal assignments of complexes of PR with U2, U3, U7 and U10 exhibited ^1H - ^{15}N spectra very similar to that with DRV. Note that lack of assignment to subunit A or B is not due to insufficiency of experimental data but is a natural consequence of identical or similar within the resolution of ^{13}C chemical environments of the flanking residues in the two subunits, and correspond to regions located distal from the inhibitor binding site. The same procedure was followed to obtain similar backbone assignments for the Flap+ PR (Figure 2b and Table S2).

Chemical shift perturbations among UX-bound forms of WT PR

To assess the effect of inhibitor chemical moiety modifications on PR structure, the difference in the assigned chemical shift for a given residue was calculated between UX inhibitor bound forms. The amide chemical shifts are highly sensitive probes of the local electronic environment, and the differences thereof report on the structural changes due to binding of inhibitors. Overall, most of the chemical shift perturbations, CSPs, between two UX-bound forms of WT PR were relatively small (< 0.025 ppm). This was expected because the CSPs reflect chemical shift differences caused by relatively minor modification of chemical groups of UX-inhibitors. Clustered CSPs (> 0.025 ppm) corresponded to three major regions around the site where the inhibitors bind: the active site, flap region, and the P1-loop region, regardless of the inhibitor (Figure 3). Interestingly, CSPs were observed even for residues located over 10 Å away from the binding site, in the single α -helix of PR. For example, the amides of residue 89 and 90 in this α -helix are located over 12 Å away from the inhibitor. Such remote CSP cannot be explained by ring-current effects from the inhibitor because the maximum ring-current effect estimated over a 12 Å distance is less than 0.015 ppm,^{40,41} which is much smaller than the observed CSP. Therefore, the CSP for these residues remote from the inhibitor was most likely due to structural effects that propagated through the helix or through the environment in which the helix is located.

To dissect how modifying the inhibitor P2' moiety affects PR conformation, CSPs between U2 and U3-bound forms and between U7 and U10-bound forms were calculated (Figure 3a and 3b). Compounds U2 and U3 possess the same isopentyl P1' moiety but different P2' moiety. Compounds U7 and U10 also share the same P1' moiety, an isohexyl with an additional methyl group, but possess yet different P2' moiety. Significant CSPs between U2 and U3-bound forms were observed at the active site, flap and terminal α -helix regions in both subunits A and B, but were more pronounced in subunit B compared to A (Figure 3a). Similarly, CSPs between U7 and U10-forms follow the same pattern of chemical shift changes, with more CSPs in subunit B than subunit A (Figures 3b). The more pronounced CSPs in subunit B compared to A due to the modification of the P2' moiety is not surprising considering that this moiety mainly interacts with residues in subunit B (Figure 1a).

Next, CSPs between U2 and U7-bound forms were calculated to dissect how the P1' moiety affects PR conformation (Figure 3c). Inhibitors U2 and U7 possess the same P2' moiety, 4-methoxybenzene, but different P1' moiety; the P1' moiety has one additional methyl group in U7, compared to U2. Finally, comparison of the U3 and U10 bound forms (Figure 3d), which have distinct P1' and P2' moieties, revealed differences based on the combination of the previous comparisons: pronounced CSP changes in both A and B subunit residues. The U3 and U10 comparison verifies the above observations for the individual moiety changes, and indicates compounded structural effects due to modification of both moieties at the same time. Taken together, these chemical shift analyses reveal that there is an asymmetric effect on the PR structure that extend to residues remote from the binding site upon modification of the inhibitors.

Molecular basis of the long-distance asymmetric effect obtained from MD simulation data

The molecular basis of the distal asymmetric change in the α -helix region detected by the NMR chemical shifts was investigated by analyzing the previously obtained MD simulation trajectories of the PR bound to the UX inhibitors.²⁰ These trajectories were analyzed in detail before to reveal subsite changes in the inhibitors when bound to PR, but not for PR backbone dynamics. Backbone fluctuations (RMSFs) of U2-bound WT PR for residues with the asymmetric effect were relatively low ($< 0.5 \text{ \AA}$) in both A and B subunits, indicating that these regions (active-site, P1-loop, and the α -helix) are relatively rigid (Figure 4a). The frequencies of intra-helix backbone hydrogen bonds calculated from the MD data, including L89/Q92, L89/I93, L90/I93, L90/G94, were almost identical between subunits A and B (Figure 4b). In contrast, the frequency of side chain hydrogen bonds both within the α -helix and with residues surrounding the helix, such as N88/D29, N88/T31, N88/T74, and N88/Q92 (Figure 4c), were higher in subunit B compared to subunit A (Figure 4b). The latter observation indicates that the helix in subunit B experiences a different local environment than that in subunit A. Consistently, RMSF values for residues 16–18 and 60–70 surrounding the helix in subunit B were slightly lower than those in subunit A (Figure 4a). Taken together, MD data indicate that the CSPs occur at relatively rigid regions of the PR, and the conformational perturbations propagate to the remote helix via varying tertiary interactions determining the helix environment in the two subunits.

Chemical shift perturbations in drug-resistant Flap+ PR variant

To determine if the perturbations detected in WT PR are conserved in a drug resistant variant and how the inhibitor binding to Flap+ might differ from that to WT, CSPs between two UX-bound forms were also obtained for Flap+. CSPs of Flap+ exhibited similar profiles to those of WT: clustered CSPs ($> 0.025 \text{ ppm}$) were detected at the active site, flap region, and the P1-loop region, regardless of the inhibitor (Figure 5). CSPs between U2 and U3-bound forms and between U7 and U10-bound forms, possessing the same P1' moiety but different P2' moiety, exhibited more pronounced CSPs in subunit B compared to A (Figure 5a and 5b). In contrast, CSPs between U2 and U7-bound forms, possessing the same P2' moiety, but different P1' moiety, exhibited more pronounced CSPs in subunit A compared to B (Figure 5c). CSPs between U3 and U10-bound forms were significant in both subunits (Figure 5d).

Overall, the patterns of perturbations due to inhibitor moiety modifications and residues that exhibited significant CSPs in the drug resistant Flap+ were similar to those of WT PR (Figure 6). In both Flap+ and WT PR, P2' moiety difference affects mostly CSPs in subunit B compared to A (Figure 6a and 6b, versus 6e and 6f), while P1' moiety difference affects mostly CSPs in subunit A compared to B (Figure 6c and 6h). Although almost identical CSP patterns to those of WT are observed in Flap+ PR, the magnitude of the CSPs of Flap+ in subunit A in U2-U7 comparison (Figure 5c) was larger than those of the WT (Figure 3c), which may suggest altered conformational response between WT and Flap+.

Difference in chemical shift perturbations between Flap+ and WT PR

As Flap+ harbors multiple mutations compared to WT PR, direct chemical shift comparison would be dominated by changes in electron shielding due to these mutations and hinder the detection of any structural changes. To eliminate the effect of mutations in comparison, we used $^{WT}CSP_{DRV-UX}$ (Figure 7a) and $^{Flap+}CSP_{DRV-UX}$ (Figure 7b), and calculated

$CSP_{WT-Flap+}$ (Figure 7c), using Eq. (2), for each DRV-UX comparison. Since CSP subtracts intrinsic chemical shifts for each protein, $CSP_{WT-Flap+}$ negates the electron shielding difference caused by side chain differences between WT and Flap+ and elucidates relative conformational response originating from CSP between the WT and Flap+ variants. Even so, note that $CSP_{WT-Flap+}$ does not reflect entire conformational changes between WT and Flap+ PR but reports relative conformational response to inhibitor modification between the two variants,

Although both WT and Flap+ PR exhibited similar CSP profiles to each other (Figure 7a and 7b; Figure 8a and 8b), indeed, there were detectable $CSP_{WT-Flap+}$ for residues in the flap region, the P1-loop, including residues 75–78, which comprise a β -sheet extending to the loop region (Figure 7c and 8c). However, the resulting $CSP_{WT-Flap+}$ values indicated no changes at the active site or the distal α -helix region, indicating the perturbations at the active site and the propagated long-range effects were the same in the two variants. Overall, conformational changes of Flap+ upon inhibitor binding differ from the WT at the inhibitor interaction sites and the subsequent region of the P1-loop, but do not extend to the entire PR.

DISCUSSION

HIV-1 PR is essential for viral maturation and has been an important therapeutic target for the treatment of AIDS.^{42–51} Although most recent PR inhibitor approved by the FDA for clinical use, darunavir (DRV), binds the wild-type (WT) PR extremely tightly with a picomolar inhibition constant,⁵² PR inhibitors have limited long-term effectiveness in curbing the emergence of drug resistance as single therapy.^{53, 54} The molecular mechanism underlying the development of drug resistance, especially the structural basis for viral adaptation, has been widely studied using various computational and biophysical approaches.^{55–58} In this study, we explored chemical shift analysis to investigate how PR structure adapts to accommodate changes in specific chemical moieties among analogous inhibitors sharing the common DRV scaffold.

CSP values of WT and Flap+ PR variants were measured in response to changes in the chemical moieties at the P1' and P2' positions of the inhibitor. Overall, although the chemical structures of the inhibitors are highly analogous and the moiety changes are rather subtle, small but significant conformational changes were detected both near and distal from the inhibitor-binding site. These fluctuations propagated throughout the enzyme in a subunit-asymmetric manner. Our results revealed that the R² group, corresponding to the P2' moiety at the inhibitor, has asymmetrical structural effects not only around this moiety but also throughout subunit B. Based on the structure (Figure 1b), this asymmetric conformational effect is explained by the direct interaction of the R² group with subunit B, and is consistent with previous MD studies (Figure. 6).²⁰

To determine whether drug resistance mutations alter the response to inhibitor moiety modifications in the ensemble structures in solution, the chemical shift perturbations of WT PR were compared with inhibitor-bound forms of a drug resistant PR variant, Flap+. A significant $\text{CSP}_{\text{WT-Flap+}}$ over the entire flap region was detected, in agreement with previous MD simulations and X-ray crystal structure studies.^{9, 19, 20} In addition to the flap region, $\text{CSP}_{\text{WT-Flap+}}$ changes were detected for residues 75–78, which comprise a β -sheet extending to a loop region, indicating different conformational response between WT and Flap+ PR. In the crystal structures, RMSD of backbone C_α atoms for the four inhibitor-bound forms exhibited significant differences between WT and Flap+ PR in the flap region, and the region that includes P1-loop (residues 77 – 82),¹⁹ in agreement with the NMR

CSP detected. Hence, $\text{CSP}_{\text{WT-Flap+}}$ analysis presented here sensitively captures the difference in the binding environments in WT versus the drug-resistant PR, and reveal that the differences seen in the crystal structures persist in solution.

In contrast to these regions at the flaps and the surrounding regions, $\text{CSP}_{\text{WT-Flap+}}$ were insignificant in the active site region (Figure 7c), indicating that the drug resistance mutations do not impact the region including the active residue D25. This is in agreement with the requirement that drug resistant variants of PR still have to be catalytically active to allow viral propagation. Similarly, $\text{CSP}_{\text{WT-Flap+v}}$ values in the α -helix region were small while CSPs themselves were different between WT and Flap+ (Figure 7). Thus, conformational changes of Flap+ upon inhibitor binding differs from the WT at the inhibitor interaction sites and the subsequent region of the P1-loop, but do not extend to the entire PR. Given the locations of the four mutations in Flap+ PR (Figure 1c) relative to WT, it is not surprising that conformational differences between the two PRs are localized mostly at the inhibitor interaction site, but not for the α -helix region.

$\text{CSP}_{\text{WT-Flap+}}$ also showed variations among different DRV-UX forms (Figure 7c). DRV-U10 $\text{CSP}_{\text{WT-Flap+}}$ values (purple, Figure 7c) were more pronounced throughout the protein, compared to other DRV-UX forms (black, green, and blue in Figure 7c). Inhibition constant, K_i , of U10 to Flap+ is 0.126 ± 0.014 (nM), which is 5 times larger than those of other UX inhibitors to Flap+ and is over 80 times larger than that to WT PR.² The largest

$\text{CSP}_{\text{WT-Flap+}}$ detected for U10 may well be related to differences in inhibitor-binding modes leading to differences in the binding affinity, although the magnitude of the chemical shift does not directly relate to magnitude of conformational change and other factors such as the structure and dynamics of the apo form may also contribute to the inhibitor binding thermodynamics.^{8, 9} Even so, the results of DRV-UX $\text{CSP}_{\text{WT-Flap+}}$ are indicative of inhibitor specific changes in inhibitor-protein interactions for the drug resistant mutant.

Our results demonstrate that NMR chemical shift analysis is able to probe the conformational response of the target protein upon modification of bound inhibitor. While the PR–inhibitor structures were virtually indistinguishable within experimental error in the crystal state, CSP analysis, which compares analogous inhibitor-bound forms, revealed conformational response to the PR α -helix region that is extended over 10 Å away from the inhibitor-binding site in solution. More importantly, analysis of the differences in CSP,

CSP, was able to capture the change in this response due to drug resistance mutations by eliminating the common conformational changes, such as the motion in the α -helix region,

between the proteins. This analysis is significant and sensitive in that NMR relaxation could not previously identify this response.⁹ Although CSP and CSP analyses cannot reveal exactly what the protein conformational changes look like, the mere presence of such changes provides insight into a mechanistic understanding for communication between inhibitor binding and distal sites of the protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

NMR	nuclear magnetic resonance spectroscopy
DRV	darunavir
PR	protease
CSP	chemical shift perturbation

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Figure 1.

UX-bound PR structure, showing (a) location of residues L10, G48, I54, and V82 that are mutated in Flap+ PR, (b) positions of R¹ and R² group, which corresponds to the P1' or P2' moiety of the substrate,² and (c) protease sidechain interactions to the inhibitor. Two subunits, A and B, are depicted in green and light blue, respectively. Residue numbers of subunit A and B are labeled without and with the prime symbol, respectively. Inhibitor is in stick representation. Structures were generated using PDB: 3O9F (U7-bound WT PR).²

Figure 2.

An overlay of ^1H - ^{15}N correlation spectra acquired for HIV-1 (a) WT and (b) Flap+ PR in complex with inhibitors DRV (red), U2 (green), U3 (orange), U7 (black) and U10 (purple) in 20 mM sodium phosphate pH 5.8 and with 5% D_2O at 20 °C. Only positive signals in the correlation are shown.

Figure 3.

NMR backbone chemical shift perturbations (CSPs) of HIV-1 WT PR, determined as differences in amide nitrogen chemical shifts between (a) U2 and U3 bound forms, with the same P1' but different P2' moieties, (b) U7 and U10 bound forms, also with the same P1' but different P2' moieties, (c) U2 and U7 bound forms, with different P1' but the same P2' moieties, and (d) U3 and U10 bound forms, with different both P1' and P2' moieties. The symbols for data points reflect the assignment categories as described in Figure S2 legend.

Figure 4.

MD simulation analysis: (a) backbone RMSF of U2 bound form of PR in subunit A and B from MD simulations; (b) differences in the frequencies of hydrogen bonding during MD simulations, in the helix region encompassing residues I85 to T91 and the surrounding residues; (c) the close-up view of the PR structure around the helix region. In (a), only the RMSF for U2-bound form is shown for presentation purposes, with error bars representing the similarity of results among the other inhibitor-bound forms. In (b), values for subunit B were subtracted from those for subunit A. The error bars on the hydrogen bonding

frequencies are too small to visualize for most data points. In (c), the protease structures were generated using PDB: 3O9F and VMD software.

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Figure 5.

NMR backbone chemical shift perturbations (ΔCSP s) of HIV-1 Flap+ PR, determined as differences in amide nitrogen chemical shifts between (a) U2 and U3 bound forms, with the same P1' but different P2' moieties, (b) U7 and U10 bound forms, also with the same P1' but different P2' moieties, (c) U2 and U7 bound forms, with different P1' but the same P2' moieties, and (d) U3 and U10 bound forms, with different both P1' and P2' moieties. The symbols for data points reflect the assignment categories as described in Figure S2 legend

Figure 6.

Asymmetric changes in PR conformation between (a) U2 and U3 bound forms, (b) U7 and U10-bound forms, (c) U2 and U7-bound forms, and (d) U3 and U10 bound forms of WT PR and (e-h, right-hand side column) those of Flap+ PR variant, as detected by NMR CSPs in Figure 3 and 5. Two subunits, A and B, are depicted in green and light blue, respectively. Red or orange ribbons indicate CSP effect > 0.04 ppm, which is approximately above one standard-deviation higher than the average: Red, for residues that were assigned to either subunit A or B explicitly; Orange, for residues that were degenerate in the two subunits or

not segmentally separated. All panels were generated using PDB 3O9F (U7-bound WT PR).
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Figure 7.

NMR backbone chemical shift perturbations (ΔCSP s) of (a) WT PR (b) Flap+ determined as differences in amide nitrogen chemical shifts between the DRV-bound and each UX-bound form ($X = 2, 3, 7, 10$), and (c) a plot of $\Delta\Delta\text{CSP}$ between WT PR and Flap+. In both ΔCSP s and $\Delta\Delta\text{CSP}$ s were plotted with the following color coding; DRV-U2 (black), DRV-U3 (green), DRV-U7 (blue) and DRV-U10 (purple). Panels (a) and (b) are essentially the same as those in Figure 3 and 5 but shows only the residues that exist in all three panels, for comparison. The parentheses indicate data points that are out of the range of the plot.

Figure 8.

Asymmetric changes observed in (a) WT PR, $^{WT}CSP_{DRV-UX}$, (b) Flap+, $^{Flap+}CSP_{DRV-UX}$, and (c) the difference of the two, $^{CSP_{WT-Flap+}}$, as detected by NMR in Figure 7. Two subunits, A and B, are depicted in green and light blue, respectively. Red or orange ribbons indicate CSP effect > 0.05 ppm, which is approximately above one standard-deviation higher than the average: Red, for residues that were assigned to either subunit A or B explicitly; Orange, for residues that were degenerate in the two subunits or not segmentally separated. All panels were generated using PDB 3O9F (U7-bound WT PR).²

Table 1

The chemical structure of HIV-1 PR inhibitor DRV and analogue inhibitors, U2, U3, U7 and U10 used in this study.

Compound	R ¹	R ²
DRV	Isopropyl
U2	Isopentyl
U3	Isopentyl
U7	Isohexyl
U10	Isohexyl

^aU2, U3, U7 and U10 were previously named **10b**, **10c**, **11b**, and **11e**, respectively (adopted from reference²).