



NMR and MD studies combined to elucidate inhibitor and water interactions of HIV-1 protease and their modulations with resistance mutations

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Abstract

Over the last two decades, both the sensitivity of NMR and the time scale of molecular dynamics (MD) simulation have increased tremendously and have advanced the field of protein dynamics. HIV-1 protease has been extensively studied using these two methods, and has presented a framework for cross-evaluation of structural ensembles and internal dynamics by integrating the two methods. Here, we review studies from our laboratories over the last several years, to understand the mechanistic basis of protease drug-resistance mutations and inhibitor responses, using NMR and crystal structure-based parallel MD simulations. Our studies demonstrate that NMR relaxation experiments, together with crystal structures and MD simulations, significantly contributed to the current understanding of structural/dynamic changes due to HIV-1 protease drug resistance mutations.

Keywords Drug design · HIV-1 · Protease · Inhibitor · NMR · MD · Crystal structures

HIV-1 protease structure, dynamics and inhibitor interactions

The human immunodeficiency virus type-1 (HIV-1) protease is a homodimeric aspartyl protease, with 99 amino acids comprising each subunit. The protease is indispensable in the HIV-1 life cycle as it processes the Gag and Gag-Pol polyproteins to yield individual mature proteins and thus is a target for antiviral drug design (Pearl and Taylor 1987; Copeland and Oroszlan 1988; Kohl et al. 1988; Oroszlan and Luftig 1990). Structural studies have revealed distinct protease structures for the inhibitor-free and bound forms (Wlodawer et al. 1989; Navia et al. 1989; Erickson et al. 1990; Spinelli et al. 1991; Murthy et al. 1992; Baldwin et al. 1995; Yamazaki et al. 1996). Following the determination of these crystal structures, various molecular dynamics

(MD) simulations and related calculations were performed to understand the dynamics of protease on various times scales (Harte et al. 1992; York et al. 2002; Harrison and Weber 1994; Collins et al. 1995; Silva et al. 1996; Rick et al. 1998). Results of MD simulations have been complemented with NMR relaxation experiments to obtain a better understanding of dynamical aspects of wild-type protease in solution, especially for highly flexible flap regions and β -hairpin structure that are dynamic on a wide range of time scales (Nicholson et al. 1995; Tjandra et al. 1996; Ishima et al. 1999; Freedberg et al. 2002).

HIV-1 protease is a molecular target that has proven structure-based drug design to be effective (Wlodawer and Erickson 1993; Erickson and Burt 1996; Walch et al. 1998), with ten FDA approved protease inhibitors developed to date (Arts and Hazuda 2012). However, drug resistance mutations can severely hamper the long-term effectiveness of the inhibitors (Ridky and Leis 1995; Lee et al. 1998; Shafer et al. 2000; Quinones-Mateu et al. 2008), motivating many structural studies to elucidate the molecular mechanisms by which mutations confer drug resistance (King et al. 2004; Surleraux et al. 2005a; Altman et al. 2008; Ghosh et al. 2008; Nalam and Schiffer 2008; Galiano et al. 2009; Weber and Agniswamy 2009; Van Marck et al. 2009; Koh et al. 2010; Nalam et al. 2010; Ghosh et al. 2015; Roche et al. 2015), as well as various simulations

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(Perryman et al. 2004; Foulkes et al. 2006; Galiano et al. 2009; Dirauf et al. 2010; Ragland et al. 2014; Yu et al. 2015). These studies elucidated direct and remote mutation effects on inhibitor interaction with the protease. Similarly, a significant number of thermodynamic studies of protease-inhibitor interactions have been conducted over the past two decades, showing that both binding entropy and enthalpy became more favorable in the second-generation inhibitors, compared to the first-generation inhibitors (Luque et al. 1998; Todd et al. 1998; Velazquez-Campoy et al. 2000, 2001; Prabu-Jeyabalan et al. 2002; Velazquez-Campoy et al. 2003; Vega et al. 2004; King et al. 2004; Ohtaka and Freire 2005; Layten et al. 2006; Bandyopadhyay and Meher 2006; Foulkes et al. 2006; King et al. 2012; Foulkes-Murzycki et al. 2013). These structural, thermodynamic, and dynamics studies have provided insights into the molecular mechanisms by which protease mutations confer resistance, but our understanding has not reached a degree to predict future resistance mutations or their impact. Drug-resistance is not limited to HIV-1 protease but is a common mechanism of escape for viruses and bacteria, and the lessons that we learn should be generally applicable.

Current opportunities in studying HIV-1 protease involve leveraging the plethora of data that exist on resistance mutations and determine the relational biophysical mechanism by which these changes confer resistance. Our interest has been to understand the interdependence of drug resistance mutations, i.e., to elucidate how a set of mutations affects the structure and dynamics of HIV-1 protease and the thermodynamics of inhibitor-protease interactions. Toward this aim, our laboratories, Ishima and Schiffer, have closely collaborated to characterize the biochemical and structural features of HIV-1 protease and drug resistant variants. Here, we summarize our efforts to combine solution NMR (Ishima laboratory) with structural studies and *parallel* MD (*pMD*) simulations (Schiffer laboratory) to understand the resistance mechanisms of HIV-1 protease. Various comparisons of NMR-derived parameters with those calculated from MD simulations have been published (Lienin and Bruschiweiler 2000; Case 2002; Showalter and Bruschiweiler 2007; Wong and Case 2008; Robustelli et al. 2012). However, our aim with this review is not a comparison of these parameters, but instead a description of how our NMR and MD data were utilized to examine variation in the protease and inhibitor structure and dynamics, as well as impact on water structure and stability.

Structural and dynamic effects of drug resistance mutations

We have focused on a biophysically fascinating multi-drug resistant HIV-1 protease variant (termed Flap+) containing L10I/G48V/I54V/V82A (King et al. 2012) mutations that

occur simultaneously in sequences from patients undergoing drug therapy. The drug resistance mutations in Flap+ are located in the flap region (residue 45–55), P1 loop (residue 79–84) and a region remote from the inhibitor interaction site, (Fig. 1) (Shafer et al. 2000). Our previous thermodynamic analysis of inhibitor binding to Flap+ indicated entropy-enthalpy compensation: compared to wild-type, Flap+ protease exhibits extremely large and opposite changes in the entropy and enthalpy of binding with several FDA approved inhibitors. These changes however did not drastically alter the binding free energy (King et al. 2012). Although crystal structures of Flap+ in complex with inhibitors revealed packing rearrangements around the bound inhibitor (King et al. 2012), these were not sufficient to explain the extreme entropy-enthalpy compensation. Moreover, the individual mutations at the flap region, G48V or I54V, did not cause any significant entropy-enthalpy compensation, suggesting interdependent effects of the mutations (Foulkes-Murzycki et al. 2013).

As a first step in characterizing the entropy-enthalpy compensation observed for Flap+, we compared the dynamics of the inhibitor-free forms of Flap+ and WT protease using NMR and a series of twenty 100 ns MD simulations (Cai et al. 2012). As expected, the two proteins exhibited differences in their dynamics: both MD and NMR data consistently indicated a more open flap region in Flap+ than in WT (Fig. 2a) in the inhibitor-bound form of the enzyme. Using double electron-electron resonance (DEER) EPR, the distance between spin-labels at residue 55 in the two subunits was measured to be 26 to 48 Å (Galiano et al. 2007, 2009). Our MD simulations of WT protease indicated a similar distribution for this distance with a mean of 35.8 Å (σ 2.7 Å). For Flap+, the mean distance increased to 36.9 Å with a

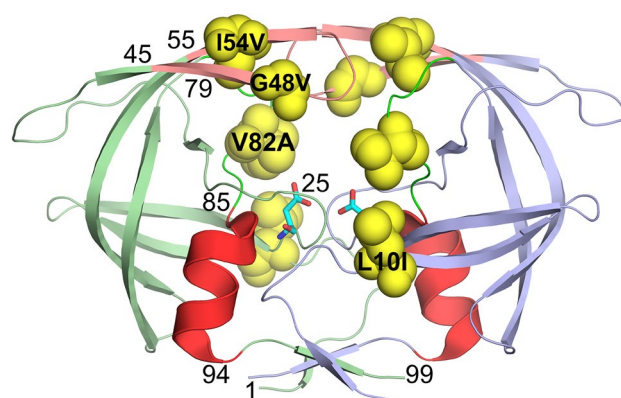


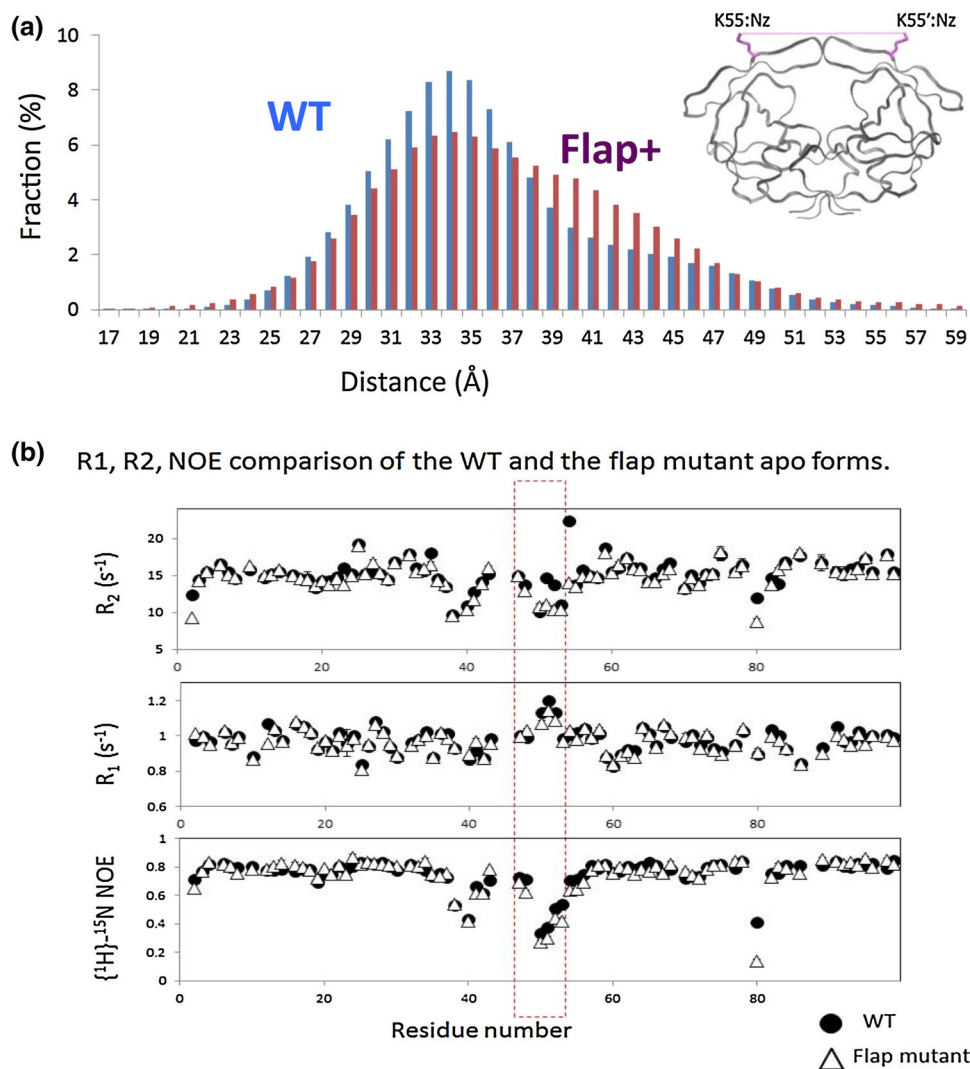
Fig. 1 HIV-1 protease structure, showing the locations of residues L10, G48, I54 and V82 (yellow spheres) that are mutated in Flap+. Two subunits, A and B, are depicted in green and light blue, respectively. The flap region (residues 45–55), the P1 loop (residues 79 to 84) and the α -helix (residues 85 to 94) are highlighted in pink, green and red. Key residue numbers are indicated in small font. The structure was generated using PDB: 1T3R (Surleraux et al. 2005b)

wider distribution (σ 4.0 Å) (Fig. 2a) (Cai et al. 2012). In NMR experiments, a lower $^{15}\text{N}\{-^1\text{H}\}$ NOE and less chemical exchange (R_{ex}) for the residues in the flap region were observed for Flap+ compared to WT protease (Cai et al. 2012), indicating faster dynamics of the flap region (Fig. 2b), presumably due to less flap–flap interaction (Ishima and Louis 2008). Thus, both NMR and MD indicated differential dynamics of the flap region in the Flap+ variant due to resistance mutations.

We next examined Flap+ and WT protease in inhibitor darunavir (DRV)-bound form, again combining MD simulations with ^{15}N NMR relaxation data (Cai et al. 2014). The flaps of protease are known to be mobile in the unliganded form and closed upon inhibitor binding (Wlodawer et al. 1989; Navia et al. 1989; Erickson et al. 1990; Spinelli et al. 1991; Yamazaki et al. 1996; Nicholson et al. 1995; Tjandra et al. 1996; Ishima et al. 1999; Freedberg et al. 2002). Our MD and NMR data were consistent with this, revealing a basically closed conformation of Flap+ and WT in the DRV-bound forms. A total of

20 parallel fully solvated MD trajectories of 100 ns each were generated, enabling rigorous statistical analysis of dynamic changes between the two protease variants. MD simulations further indicated that the whole backbone of protease is more rigid in Flap+ compared to WT in DRV-bound form ($p < 0.05$; Wilcoxon rank-sum test), with average $\text{C}\alpha$ root-mean-squared fluctuations (RMSFs) decreasing to 1.30 from 1.43 Å with a dispersion of 0.50 and 0.53, respectively (Cai et al. 2014). This RMSF difference was observed throughout the protein except for the active site region, suggesting a change in the protein's overall rigidity but not in the internal motion of a specific region. However, the generalized-order parameter, S^2 , determined using amide longitudinal relaxation rate (R_1), transverse relaxation rate (R_2) and $^{15}\text{N}\{-^1\text{H}\}$ NOE, was consistent with those calculated from the MD trajectories, except for residues in the loop and β -turn regions, and did not detect such dynamical differences between the two proteins. This may be primarily because, unlike RMSF that does not have any directionality, S^2 reflects only rotational motion. In addition,

Fig. 2 **a** Distribution of distances between the nitrogen atoms in the amino group of residue K55 and K55' side chain, calculated from MD trajectories for WT (blue) and Flap+ (purple) protease; **b** NMR relaxation data for WT (filled circles) and Flap+ protease (open triangles). The red dashed rectangle indicates the flap region. The figures were adapted from (Cai et al. 2012). Reprinted with permission Cai et al. (2012). From Copyright 2012 American Chemical Society



although the overall dynamics are slightly different between Flap+ and WT protease in the DRV-bound forms, S^2 derived from NMR relaxation may not sensitively detect this difference because such overall relaxation change does not necessarily affect S^2 but also affect the rotational correlation time.

In the NMR part of the above studies, we optimized our analysis method to enable a direct comparison of NMR-derived S^2 values of the two proteins, WT and Flap+: we used “same-model selection” (Cai et al. 2012), which decreases the model’s dependency on S^2 . Specifically, when the dataset for a particular residue in one protein, either WT or Flap+, was not fit by a standard model with two parameters (S^2 and a correlation time for internal motion, τ_i) but was instead fit using a three-parameter model that contained an additional parameter (either chemical exchange, R_{ex} , or an extended motion (Mandel et al. 1995); data is usually explained with only one of the two but not both), the dataset for that residue in the other protein was also analyzed using the same three-parameter model. Note, even if the relaxation rates of two proteins are intrinsically the same, the associated errors can differ and these differences affect model selection. Thus, our application of same-model selection for a given residue minimized model-dependent artifacts in the comparison of two closely related proteins.

Overall, our integrated interpretation of NMR and MD studies revealed that resistance mutations alter the flap dynamics in the inhibitor-free form, and MD suggested a more rigid Flap+ compared to WT protease. Although the inhibitor has an asymmetric chemical structure, the observed dynamical differences between WT and Flap+ were largely limited to symmetric conformational changes. Additional NMR methods may provide more comprehensive information than ^{15}N relaxation alone, particularly $\text{C}\alpha$ NMR relaxation experiments that provide rotational orientation information orthogonal to ^{15}N probes (LeMaster and Kushlan 1996; Richarz et al. 2002; Sun et al. 2013); cross-correlated relaxation experiments that probe dihedral angle information (Yang et al. 1998; Kloiber and Konrat 2000; Wang et al. 2003; Lundstrom et al. 2005); and methyl side chain dynamics that are sensitive to hydrophobic packing (LeMaster and Kushlan 1996; Muhandiram et al. 1995; Skrynnikov et al. 2001; Korzhnev et al. 2004; Lundstrom et al. 2007). Similarly, as described below, other dynamic changes not captured in our analysis, particularly involving the water structure, may have a role in the observed entropy–enthalpy compensation of inhibitor binding.

Efforts to identify the effect of inhibitor chemical moiety changes

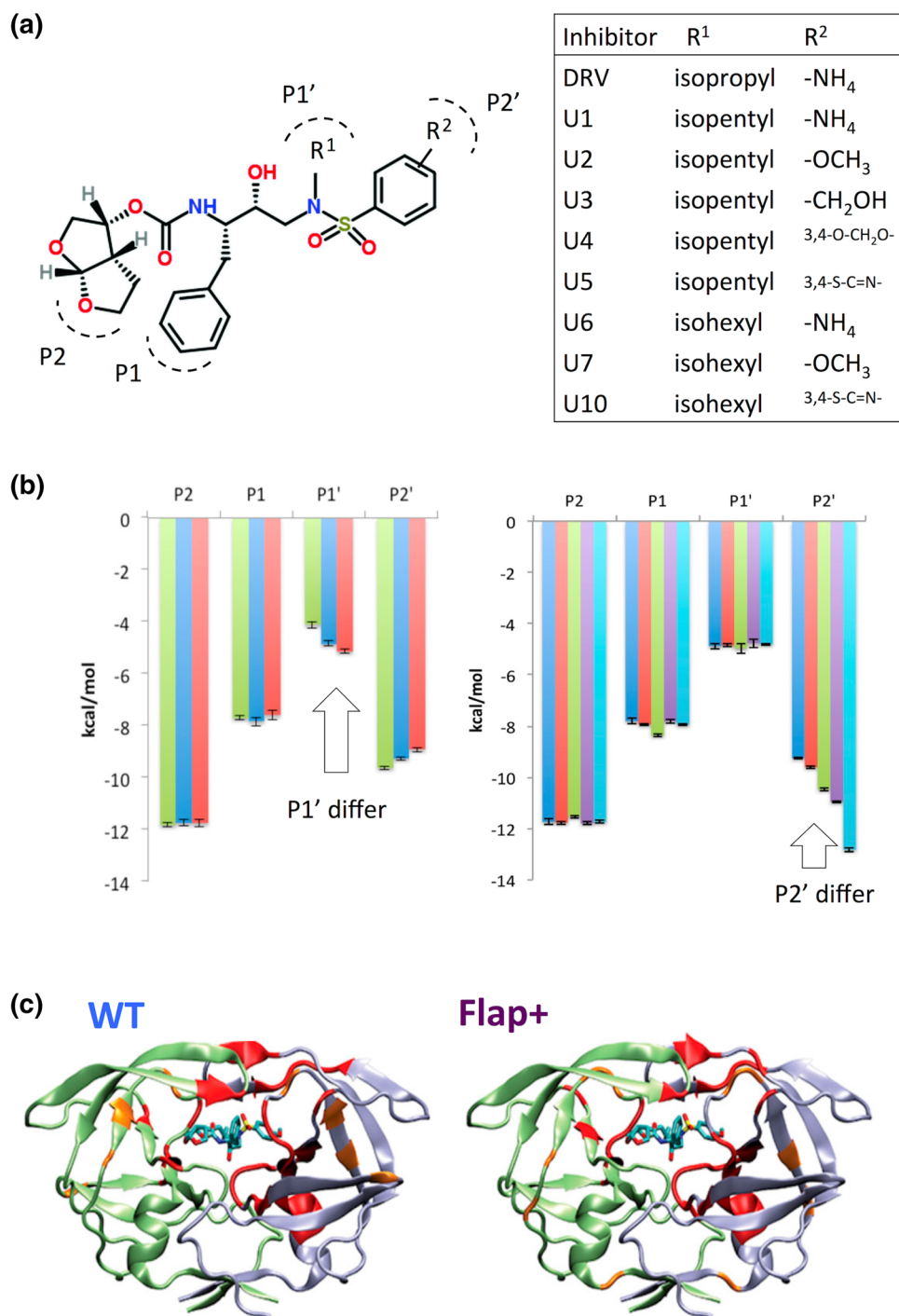
To gain more site-specific information about inhibitor interactions with protease, we systematically changed the chemical moiety of inhibitors: designed a series of DRV analogs

by altering the functional groups at the P1’ and P2’ positions, i.e., R¹ and R² groups (Fig. 3a) (Nalam et al. 2013). All ten analogs synthesized, with two different R¹ groups and five different R² groups, had similar pico-molar inhibition constants to WT protease but not to resistant variants (Nalam et al. 2013).

To identify how the individual inhibitor–protein contact site affects the interdependence of resistance mutations throughout the protein, we generated a series of 100 ns $p\text{MD}$ trajectories starting from high-resolution structures of protease co-crystallized with DRV or the ten DRV analogs. The $p\text{MD}$ trajectories were analyzed in terms of correlated fluctuations, van der Waals (vdW) contact energies, and electrostatic interactions between the inhibitor and protease to illuminate subsite interdependence (Paulsen et al. 2017). The two protease monomers had differential cross-correlations with inhibitor fluctuations due to the asymmetry of the inhibitor, in agreement with analysis of crystal structures (Nalam et al. 2013; Paulsen et al. 2017). The cross-correlation analysis also revealed that monomer containing the P2 moiety, but not the P2’ moiety, was always highly correlated with the inhibitor motions. The most striking feature revealed is that inhibitor modification at P1’ significantly impacted van der Waals contacts at the P2’ subsite (Fig. 3b, left), but the reverse was not true: alterations at P2’ did not influence P1’ subsite (Fig. 3b, right). Analysis of the electrostatic interactions further revealed that hydrogen bond formation with the catalytic residue was affected by modifications at P1’, regardless of the P2’ moiety. These $p\text{MD}$ results indicated specific couplings between the subsites where the inhibitor binds, suggesting inhibitor moieties cannot necessarily be independently optimized as their interactions with the protease active site are interdependent.

By NMR, we elucidated how modification of each inhibitor chemical moiety affects protease residues outside of the inhibitor-binding pocket (Khan et al. 2018). The amide chemical shift differences, ΔCSPs , which are a sensitive parameter for detecting remote conformational changes, were compared among protease complexes with DRV and four selected analog inhibitors. Since ΔCSP is generated by subtraction of the chemical shifts of one inhibitor-bound form of protease from that of another inhibitor-bound form, it ideally removes any intrinsic chemical shift effect and extracts changes due to protein structural/dynamic differences and/or chemical moiety differences. We determined the uncertainty of ΔCSP by calculating the 20%-trimmed mean ΔCSP among the homologous inhibitor-bound forms of the protease, which was 0.017 (± 0.003) ppm, and assessed ΔCSP above 0.025 ppm (Khan et al. 2018). Overall ΔCSP patterns between the WT and Flap+ were predominantly similar to each other, except for a few sites that had a key effect due to mutation (Fig. 3c) (Khan et al. 2018). Importantly, ΔCSPs obtained upon subtraction

Fig. 3 **a** Chemical structure of DRV and its analogs with substitutions at P1' and P2' moieties, **b** van der Waals contact energies for P1' analogs (DRV, U1 and U6) and those for P2' analogs (U1–U5), **c** differences in Δ CSP between DRV and UX-bound forms (here, X=2, 3, 7, 10; orange and red, Δ CSP > 0.05 ppm for WT (left) and Flap+ (right), Δ CSP > 0.05 ppm for WT (left) and Flap+ (right). **a** Only the analog inhibitors that are presented in this review article are listed. **b** The figure was adapted from the reference (Paulsen et al. 2017). Reprinted with permission Paulsen et al. (2017). Copyright 2017 American Chemical Society. **c** Residues that exhibit Δ CSP > 0.05 ppm explicitly either to subunit A or B are red-highlighted while degenerated residues that exhibit Δ CSP > 0.05 ppm in two subunits are orange-highlighted. **c** The figure was adapted with permission from “Khan et al. (2018)”. Copyright 2018 American Chemical society. Structures of the UX inhibitors are in the reference



of shifts between UX-(X=2, 3, 7, 10) and DRV-bound forms were asymmetric for the two subunits, with long-distance effects, > 10 Å, across the remote single α -helix of the protein (Khan et al. 2018). Such long-range effects are not explained by direct inhibitor-origin chemical shift effects, but only by propagation of conformational changes (Fig. 3c). Differences in the inhibitor interactions of WT and Flap+ were further elucidated by taking the double Δ CSPs, $\Delta\Delta$ CSP, i.e. subtraction of Δ CSP of two inhibitors of WT

from that of Flap+ (Khan et al. 2018). $\Delta\Delta$ CSPs effectively eliminated local environmental effects stemming from different chemical groups and enabled exploiting these sensitive parameters to detect subtle conformational changes in the mutant relative to the wildtype protein. For example, although inhibitor binding produced significant Δ CSPs in both WT and Flap+, $\Delta\Delta$ CSPs of the two proteins indicated no differences between the proteins at the active site region, which is critical for catalytic activity (Khan et al. 2018).

Although the sensitive chemical shifts detected long-distance effects propagating to the distal helix, ^{15}N relaxation could not detect significant dynamic differences in the inhibitor-bound forms (Khan et al. 2018). Further, we did not conduct chemical shift calculations, because the inhibitors affect electron shielding of the proteases. As mentioned above, NMR relaxation (except for chemical exchange) reflects only spatial rotational motion and thus is sensitive to the local chain dynamics but not to displacement of secondary structural units. To understand the molecular mechanism behind the long-distance effects due to inhibitor modifications, we performed MD simulations of inhibitor-bound protease. The MD trajectories clearly demonstrated that occurrence (frequency) of *side chain* hydrogen bonds, both within and surrounding residues in the α -helix, differ among the inhibitor-bound forms, indicating that the conformational perturbations propagate to the remote helix via varying tertiary interactions of the helix in the two subunits (Khan et al. 2018). Although the determinants of ΔCSP are complex and their utility is limited (such as evaluation of the long-range effect only), ΔCSP was able to capture the differences among different inhibitor-bound forms, which was further evaluated by MD simulations.

To assess conformational differences at surrounding region of the inhibitor by NMR, ^{15}N -half filtered NOESY spectra of [$\text{U-}^2\text{H/U-}^{15}\text{N}$] inhibitor-bound protease were acquired, which detect NOEs between protease amide protons and inhibitor protons, as well as NOEs between amide protons and hydroxyl side chains or water protons (Persons et al. 2018). Comparison of the NOEs of the two analogous-inhibitor bound forms elucidated a similar NOE pattern of the conserved P2 site to each other, but with a difference in the P1' and P2' site, consistent with the previous MD data (Paulsen et al. 2017). However, since correlated motion between protein and ligand is difficult to investigate by NMR, we used half-filtered NOESY to assess the conformational similarity of the analogous inhibitor-bound forms. Taken together, comparison of analogous inhibitor-bound forms sensitively elucidated site-specific features of the asymmetric conformational changes of protease-inhibitor complexes by both NMR experiments and MD simulations.

Understanding effect of water structure and dynamics on inhibitor interactions

Water can significantly modulate entropy and enthalpy of binding in inhibitor–protein interactions (Lafont et al. 2007; Luque and Freire 2002). NMR, specifically water NOE, has previously shown the presence of long-lived water molecules trapped between inhibitor and protease (Wang et al. 1996). To analyze the water structure around DRV-bound protease, from the MD trajectories we calculated the water density,

occupancy and hydrogen bonds, and compared those of Flap+ with WT protease (Paulsen et al. 2017; Leidner et al. 2018). The analysis identified 145 symmetric water site pairs in the two subunits of WT protease, reflecting overall symmetry of the dimer structure, and 55 hydration sites that did not have a symmetry partner. The latter included a water positioned between residue 50 and inhibitor, named “flap water”, which was previously detected by NMR (Wang et al. 1996). Many of the high occupancy (>0.6) water positions were observed near the active site (Fig. 4). The water density and occupancy of DRV-bound Flap+ were similar to those of DRV–WT protease complex, reflecting an overall similar structure. However, occupancies and asymmetric hydration sites at the inhibitor–protease interface were significantly altered in DRV–Flap+ compared to WT complex. The occupancy of the flap water decreased from 90 to 82%, four water sites coordinating the inhibited conformation of protease were completely lost and the remaining had drastically reduced occupancy in Flap+ protease. Effect of changes in surrounding water on the entropy–enthalpy compensation is well known (Ryde 2014; Fox et al. 2018). Thus, these alterations in water structure stabilizing the inhibitor-bound form of protease likely contribute to potency loss and entropy–enthalpy compensation in inhibitor binding due to drug resistance mutations.

To validate the MD-detected high-occupancy water positions, ^{15}N -half filtered NOESY spectra of [$\text{U-}^2\text{H/U-}^{15}\text{N}$] protease bound to DRV or to a DRV-analogue, U10, were recorded and assessed. NOEs both between protease amide protons and water protons as well as amide protons and inhibitor protons or hydroxyl side chains were analyzed (Persons et al. 2018). ^{15}N -half filtered NOESY spectra, together with water-NOE/ROE, suggested the presence of resident waters, including the flap water, near some amides in the inhibitor-bound protease. Since the time-scale of MD and NOESY differ, especially with regard to assessing water exchange, we cannot quantitatively compare water data from MD with that of NOESY. Nevertheless, some of the water positions found by MD were similar to the amides that exhibited water-amide NOEs, and validated the MD observations (Persons et al. 2018) (Fig. 5).

Summary and future directions

With the described studies, we aimed to combine NMR and MD data to gain a clearer view of how dynamics and structure modulate drug resistance, and obtain insights that may be more generally applicable possibly even beyond HIV-1 protease. We found a more opened flap-flap configuration in Flap+ compared to WT protease in the inhibitor-free form; this observation indicates burial of the hydrophobic flaps upon inhibitor interaction may correlate

Fig. 4 **a** Close-up views of hydration sites around the protease active site facing the aniline moiety of DRV. Active site residues are color coded as yellow: apolar, blue: polar, red: charged. **b** Mean occupancies. **c** Close-up views of hydration sites around the protease active site facing the bis-THF moiety of DRV. Active site residues are color coded as in **a**. **d** Mean occupancies Reprinted with permission from “Leidner et al. (2018)”. Copyright 2018 American Chemical Society

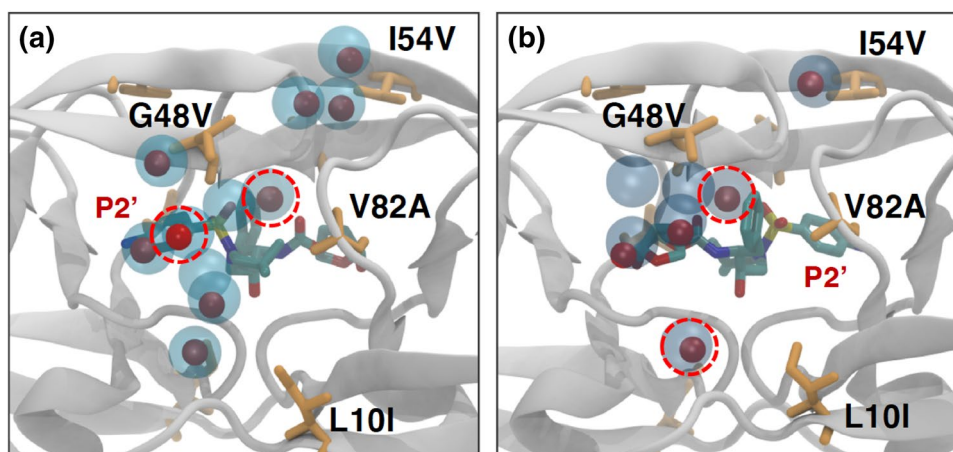
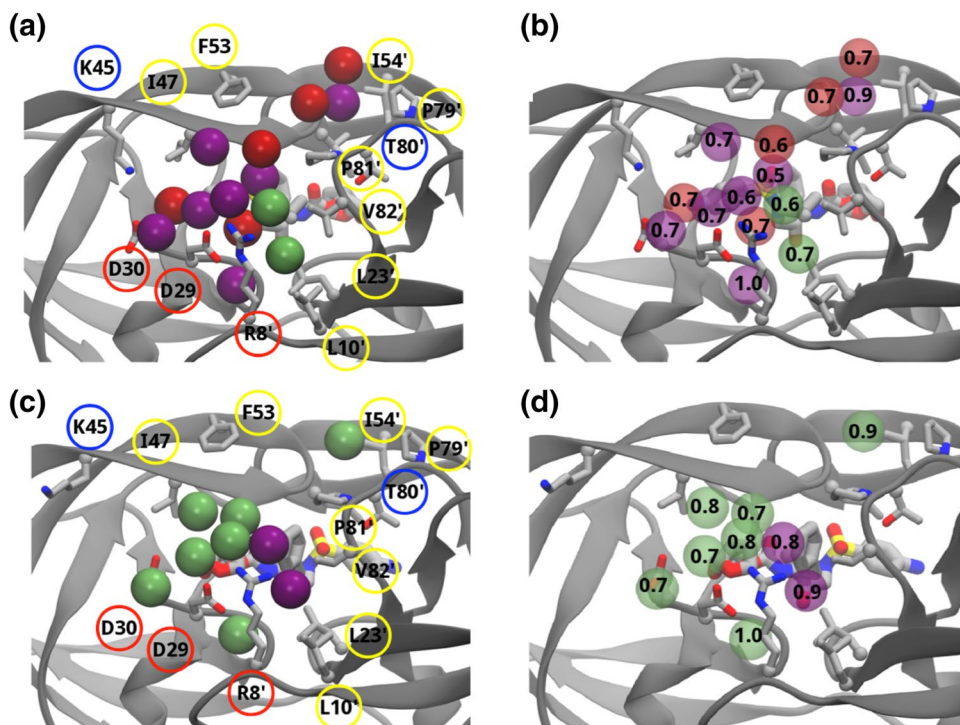


Fig. 5 Altered resident water positions (mean occupancy > 65%) for WT (transparent cyan spheres) versus Flap+ (solid red spheres) protease bound to DRV, in **(a)** an orientation in which P2' position is on left in the panel and **(b)** an orientation in which P2' position is on right in the panel (the same orientation as that in Fig. 3c). Hydrat-

ion sites of WT and Flap+ are shown as transparent cyan spheres and solid red spheres, respectively. Reprinted with permission from “Leidner et al. (2018)”. Copyright 2018 American Chemical Society. Water positions that are close to the amides that exhibited water-NOEs in WT protease are indicated by red-dashed circles

with more favorable entropy changes in inhibitor binding. For the inhibitor-bound forms, our MD simulations indicated that the whole backbone of DRV-bound protease is more rigid in Flap+ compared to WT (Cai et al. 2014). NMR experiments were qualitatively consistent with this result, given long-range Δ CSP but lack of $\Delta\Delta$ CSP in the active site region. Conservation of active site dynamics may be important for catalytic activity, as the active site

is the least affected due to resistance mutations and the mutated protease has to maintain activity.

Since MD and NMR have distinct but complementary advantages, with the parameters detected not necessarily overlapping, integration of these methods is ideal to obtain a comprehensive picture of the structure and dynamics of proteins in solution. This combined approach, together with additional complementary techniques such as elucidation

of binding thermodynamics, provides valuable information on the conformational dynamics aspects of drug resistance mechanisms that cannot be gained from crystal structures alone. Such integrative methods, together with other biophysical and virologic data, will aid our efforts to understand interdependent effects of drug resistance mutations. With more work addressing these complex mechanisms of interdependence, the information gained will hopefully lead to general principles that can guide the design of robust inhibitors and may be valuable for machine learning models to predict drug resistance in future.

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References

- Altman MD, Ali A, Reddy GS, Nalam MN, Anjum SG, Cao H, Chelappan S, Kairys V, Fernandes MX, Gilson MK, Schiffer CA, Rana TM, Tidor B (2008) HIV-1 protease inhibitors from inverse design in the substrate envelope exhibit subnanomolar binding to drug-resistant variants. *J Am Chem Soc* 130:6099–6113
- Arts EJ, Hazuda DJ (2012) HIV-1 antiretroviral drug therapy. *Cold Spring Harb Perspect Med* 2:a007161
- Baldwin ET, Bhat TN, Gulnik S, Liu B, Topol IA, Kiso Y, Mimoto T, Mitsuya H, Erickson JW (1995) Structure of HIV-1 protease with KNI-272, a tight-binding transition-state analog containing allophenylnorstatine. *Structure* 3:581–590
- Bandyopadhyay P, Meher BR (2006) Drug resistance of HIV-1 protease against JE-2147: I47V mutation investigated by molecular dynamics simulation. *Chem Biol Drug Des* 67:155–161
- Cai Y, Yilmaz NK, Myint W, Ishima R, Schiffer CA (2012) Differential flap dynamics in wild-type and a drug resistant variant of HIV-1 protease revealed by molecular dynamics and NMR relaxation. *J Chem Theory Comput* 8:3452–3462
- Cai Y, Myint W, Paulsen JL, Schiffer CA, Ishima R, Kurt Yilmaz N (2014) Drug resistance mutations alter dynamics of inhibitor-bound HIV-1 protease. *J Chem Theory Comput* 10:3438–3448
- Case DA (2002) Molecular dynamics and NMR spin relaxation in proteins. *ACC Chem Res* 35:325–331
- Collins JR, Burt SK, Erickson JW (1995) Flap opening in HIV-1 protease simulated by activated molecular-dynamics. *Nat Struct Biol* 2:334–338
- Copeland TD, Oroszlan S (1988) Genetic locus, primary structure, and chemical synthesis of human immunodeficiency virus protease. *Gene Anal Tech* 5:109–115
- Dirauf P, Meiselbach H, Sticht H (2010) Effects of the V82A and I54 V mutations on the dynamics and ligand binding properties of HIV-1 protease. *J Mol Model* 16:1577–1583
- Erickson JW, Burt SK (1996) Structural mechanisms of HIV drug resistance. *Annu Rev Pharmacol Toxicol* 36:545–571
- Erickson J, Neidhart D, VanDrie J, Kempf D, Wang X, Norbeck D, Plattner J, Rittenhouse J, Turon M, Wideburg N et al (1990) Design, activity, and 2.8 Å crystal structure of a C2 symmetric inhibitor complexed to HIV-1 protease. *Science* 249:527–533
- Foulkes JE, Prabu-Jeyabalan M, Cooper D, Henderson GJ, Harris J, Swanstrom R, Schiffer CA (2006) Role of invariant Thr80 in human immunodeficiency virus type 1 protease structure, function, and viral infectivity. *J Virol* 80:6906–6916
- Foulkes-Murzycki JE, Rosi C, Kurt Yilmaz N, Shafer RW, Schiffer CA (2013) Cooperative effects of drug-resistance mutations in the flap region of HIV-1 protease. *ACS Chem Biol* 8:513–518
- Fox JM, Zhao M, Fink MJ, Kang K, Whitesides GM (2018) The molecular origin of enthalpy/entropy compensation in biomolecular recognition. *Annu Rev Biophys* 47:223–250
- Freedberg DI, Ishima R, Jacob J, Wang YX, Kustanovich I, Louis JM, Torchia DA (2002) Rapid structural fluctuations of the free HIV protease flaps in solution: relationship to crystal structures and comparison with predictions of dynamics calculations. *Protein Sci* 11:221–232
- Galiano L, Bonora M, Fanucci GE (2007) Interflap distances in HIV-1 protease determined by pulsed EPR measurements. *J Am Chem Soc* 129:11004–11005
- Galiano L, Ding F, Vellozo AM, Blackburn ME, Simmerling C, Fanucci GE (2009) Drug pressure selected mutations in HIV-1 protease alter flap conformations. *J Am Chem Soc* 131:430–431
- Ghosh AK, Chapsal BD, Weber IT, Mitsuya H (2008) Design of HIV protease inhibitors targeting protein backbone: an effective strategy for combating drug resistance. *ACC Chem Res* 41:78–86
- Ghosh AK, Yu X, Osswald HL, Agniswamy J, Wang YF, Amano M, Weber IT, Mitsuya H (2015) Structure-based design of potent HIV-1 protease inhibitors with modified P1-biphenyl ligands: synthesis, biological evaluation, and enzyme-inhibitor X-ray structural studies. *J Med Chem* 58:5334–5343
- Harrison RW, Weber IT (1994) Molecular dynamics simulations of HIV-1 protease with peptide substrate. *Protein Eng Des Sel* 7:1353–1363
- Harte WE Jr, Swaminathan S, Beveridge DL (1992) Molecular dynamics of HIV-1 protease. *Proteins* 13:175–194
- Ishima R, Louis JM (2008) A diverse view of protein dynamics from NMR studies of HIV-1 protease flaps. *Proteins* 70:1408–1415
- Ishima R, Freedberg DI, Wang YX, Louis JM, Torchia DA (1999) Flap opening and dimer-interface flexibility in the free and inhibitor-bound HIV protease, and their implications for function. *Structure* 7:1047–1055
- Khan SN, Persons JD, Paulsen JL, Guerrero M, Schiffer CA, Kurt Yilmaz N, Ishima R (2018) Probing structural changes among analogous inhibitor-bound forms of HIV-1 protease and a drug-resistant mutant in solution by nuclear magnetic resonance. *Biochemistry* 57:1652–1662
- King NM, Prabu-Jeyabalan M, Nalivaika EA, Schiffer CA (2004) Combating susceptibility to drug resistance: lessons from HIV-1 protease. *Chem Biol* 11:1333–1338
- King NM, Prabu-Jeyabalan M, Bandaranayake RM, Nalam MN, Nalivaika EA, Ozen A, Haliloglu T, Yilmaz NK, Schiffer CA (2012) Extreme entropy-enthalpy compensation in a drug-resistant variant of HIV-1 protease. *ACS Chem Biol* 7:1536–1546
- Kloiber K, Konrat R (2000) Measurement of the protein backbone dihedral angle phi based on quantification of remote CSA/DD interference in inter-residue $^{13}\text{C}^{\prime}(i-1)-^{13}\text{C}^{\alpha}(i)$ multiple-quantum coherences. *J Biomol NMR* 17:265–268
- Koh Y, Amano M, Towata T, Danish M, Leshchenko-Yashchuk S, Das D, Nakayama M, Tojo Y, Ghosh AK, Mitsuya H (2010) In vitro selection of highly darunavir-resistant and replication-competent HIV-1 variants by using a mixture of clinical HIV-1 isolates resistant to multiple conventional protease inhibitors. *J Virol* 84:11961–11969
- Kohl NE, Emimi EA, Schleif WA, Davis LJ, Heimbach JC, Dixon RA, Scolnick EM, Sigal IS (1988) Active human immunodeficiency virus protease is required for viral infectivity. *Proc Natl Acad Sci USA* 85:4686–4690
- Korzhev DM, Kloiber K, Kanelis V, Tugarinov V, Kay LE (2004) Probing slow dynamics in high molecular weight proteins by methyl-TROSY NMR spectroscopy: application to a 723-residue enzyme. *J Am Chem Soc* 126:3964–3973

- Lafont V, Armstrong AA, Ohtaka H, Kiso Y, Mario Amzel L, Freire E (2007) Compensating enthalpic and entropic changes hinder binding affinity optimization. *Chem Biol Drug Des* 69:413–422
- Layten M, Hornak V, Simmerling C (2006) The open structure of a multi-drug-resistant HIV-1 protease is stabilized by crystal packing contacts. *J Am Chem Soc* 128:13360–13361
- Lee CA, Kessler CM, Varon D, Martinowitz U, Heim M, Condra JH (1998) Resistance to HIV protease inhibitors. *Haemophilia* 4:610–615
- Leidner F, Kurt Yilmaz N, Paulsen J, Muller YA, Schiffer CA (2018) Hydration structure and dynamics of inhibitor-bound HIV-1 protease. *J Chem Theory Comput* 14:2784–2796
- LeMaster DM, Kushlan DM (1996) Dynamical mapping of *E. coli* Thioredoxin via ^{13}C NMR relaxation analysis. *J Am Chem Soc* 118:9255–9264
- Lienin SF, Bruschiweiler R (2000) Characterization of collective and anisotropic reorientational protein dynamics. *Phys Rev Lett* 84:5439–5442
- Lundstrom P, Mulder FA, Akke M (2005) Correlated dynamics of consecutive residues reveal transient and cooperative unfolding of secondary structure in proteins. *Proc Natl Acad Sci USA* 102:16984–16989
- Lundstrom P, Vallurupalli P, Religa TL, Dahlquist FW, Kay LE (2007) A single-quantum methyl ^{13}C -relaxation dispersion experiment with improved sensitivity. *J Biomol NMR* 38:79–88
- Luque I, Freire E (2002) Structural parameterization of the binding enthalpy of small ligands. *Proteins* 49:181–190
- Luque I, Todd MJ, Gomez J, Semo N, Freire E (1998) Molecular basis of resistance to HIV-1 protease inhibition: a plausible hypothesis. *Biochemistry* 37:5791–5797
- Mandel AM, Akke M, Palmer IIIAG (1995) Backbone dynamics of *Escherichia coli* ribonuclease HI: correlations with structure and function in an active enzyme. *J Mol Biol* 246:144–163
- Muhandiram DR, Yamazaki T, Sykes BD, Kay LE (1995) Measurement of H-2 T-1 and T-1p relaxation-times in uniformly C-13-labeled and fractionally H-2-labeled proteins in solution. *J Am Chem Soc* 117:11536–11544
- Murthy KH, Winborne EL, Minnich MD, Culp JS, Debouck C (1992) The crystal structures at 2.2-Å resolution of hydroxyethylene-based inhibitors bound to human immunodeficiency virus type 1 protease show that the inhibitors are present in two distinct orientations. *J Biol Chem* 267:22770–22778
- Nalam MN, Schiffer CA (2008) New approaches to HIV protease inhibitor drug design II: testing the substrate envelope hypothesis to avoid drug resistance and discover robust inhibitors. *Curr Opin HIV AIDS* 3:642–646
- Nalam MN, Ali A, Altman MD, Reddy GS, Chellappan S, Kairys V, Ozen A, Cao H, Gilson MK, Tidor B, Rana TM, Schiffer CA (2010) Evaluating the substrate-envelope hypothesis: structural analysis of novel HIV-1 protease inhibitors designed to be robust against drug resistance. *J Virol* 84:5368–5378
- Nalam MN, Ali A, Reddy GS, Cao H, Anjum SG, Altman MD, Yilmaz NK, Tidor B, Rana TM, Schiffer CA (2013) Substrate envelope-designed potent HIV-1 protease inhibitors to avoid drug resistance. *Chem Biol* 20:1116–1124
- Navia MA, Fitzgerald PM, McKeever BM, Leu CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP (1989) Three-dimensional structure of aspartyl protease from human immunodeficiency virus HIV-1. *Nature* 337:615–620
- Nicholson LK, Yamazaki T, Torchia DA, Grzesiek S, Bax A, Stahl SJ, Kaufman JD, Wingfield PT, Lam PY, Jadhav PK et al (1995) Flexibility and function in HIV-1 protease. *Nat Struct Biol* 2:274–280
- Ohtaka H, Freire E (2005) Adaptive inhibitors of the HIV-1 protease. *Prog Biophys Mol Biol* 88:193–208
- Oroszlan S, Luftig RB (1990) Retroviral proteinases. *Curr Top Microbiol Immunol* 157:153–185
- Paulsen JL, Leidner F, Ragland DA, Kurt Yilmaz N, Schiffer CA (2017) Interdependence of inhibitor recognition in HIV-1 protease. *J Chem Theory Comput* 13:2300–2309
- Pearl LH, Taylor WR (1987) A structural model for the retroviral proteases. *Nature* 329:351–354
- Perryman AL, Lin JH, McCammon JA (2004) HIV-1 protease molecular dynamics of a wild-type and of the V82F/I84V mutant: possible contributions to drug resistance and a potential new target site for drugs. *Protein Sci* 13:1108–1123
- Persons JD, Khan SN, Ishima R (2018) An NMR strategy to detect conformational differences in a protein complexed with highly analogous inhibitors in solution. *Methods* 148:9–18
- Prabu-Jeyabalan M, Nalivaika E, Schiffer CA (2002) Substrate shape determines specificity of recognition for HIV-1 protease: analysis of crystal structures of six substrate complexes. *Structure* 10:369–381
- Quinones-Mateu ME, Moore-Dudley DM, Jegede O, Weber J, Arts JE (2008) Viral drug resistance and fitness. *Adv Pharmacol* 56:257–296
- Ragland DA, Nalivaika EA, Nalam MN, Prachanonarong KL, Cao H, Bandaranayake RM, Cai Y, Kurt-Yilmaz N, Schiffer CA (2014) Drug resistance conferred by mutations outside the active site through alterations in the dynamic and structural ensemble of HIV-1 protease. *J Am Chem Soc* 136:11956–11963
- Richarz R, Nagayama K, Wuethrich K (2002) Carbon-13 nuclear magnetic resonance relaxation studies of internal mobility of the polypeptide chain in basic pancreatic trypsin inhibitor and a selectively reduced analog. *Biochemistry* 19:5189–5196
- Rick SW, Erickson JW, Burt SK (1998) Reaction path and free energy calculations of the transition between alternate conformations of HIV-1 protease. *Proteins Struct Funct Genet* 32:7–16
- Ridky T, Leis J (1995) Development of drug resistance to HIV-1 protease inhibitors. *J Biol Chem* 270:29621–29623
- Robustelli P, Stafford KA, Palmer AG 3rd (2012) Interpreting protein structural dynamics from NMR chemical shifts. *J Am Chem Soc* 134:6365–6374
- Roche J, Louis JM, Bax A (2015) Conformation of inhibitor-free HIV-1 protease derived from NMR spectroscopy in a weakly oriented solution. *ChemBioChem* 16:214–218
- Ryde U (2014) A fundamental view of enthalpy–entropy compensation. *Med Chem Commun* 5:1324–1336
- Shafer RW, Jung DR, Betts BJ, Xi Y, Gonzales MJ (2000) Human immunodeficiency virus reverse transcriptase and protease sequence database. *Nucleic Acids Res* 28:346–348
- Showalter SA, Bruschiweiler R (2007) Validation of molecular dynamics simulations of biomolecules using NMR spin relaxation as benchmarks: application to the AMBER99SB Force Field. *J Chem Theory Comput* 3:961–975
- Silva AM, Cachau RE, Sham HL, Erickson JW (1996) Inhibition and catalytic mechanism of HIV-1 aspartic protease. *J Mol Biol* 255:321–346
- Skrynnikov NR, Mulder FAA, Hon B, Dahlquist FW, Kay LE (2001) Probing slow time scale dynamics at methyl-containing side chains in proteins by relaxation dispersion NMR measurements: application to methionine residues in a cavity mutant of T4 lysozyme. *J Am Chem Soc* 123:4556–4566
- Spinelli S, Liu Q, Alzari P, Hirel P, Poljak R (1991) The three-dimensional structure of the aspartyl protease from the HIV-1 isolate BRU. *Biochimie* 73:1391–1396
- Sun H, Long D, Bruschiweiler R, Tugarinov V (2013) Carbon relaxation in ^{13}C alpha-Halpa and ^{13}C alpha-Dalpa spin pairs as a probe of backbone dynamics in proteins. *J Phys Chem B* 117:1308–1320
- Surleraux DL, de Kock HA, Verschueren WG, Pille GM, Maes LJ, Peeters A, Vendeville S, De Meyer S, Azijn H, Pauwels R, de Bethune MP, King NM, Prabu-Jeyabalan M, Schiffer CA, Wigerinck

- PB (2005a) Design of HIV-1 protease inhibitors active on multi-drug-resistant virus. *J Med Chem* 48:1965–1973
- Surleraux DL, Tahri A, Verschuereen WG, Pille GM, de Kock HA, Jonckers TH, Peeters A, De Meyer S, Azijn H, Pauwels R, de Bethune MP, King NM, Prabu-Jeyabalan M, Schiffer CA, Wigerinck PB (2005b) Discovery and selection of TMC114, a next generation HIV-1 protease inhibitor. *J Med Chem* 48:1813–1822
- Tjandra N, Wingfield P, Stahl S, Bax A (1996) Anisotropic rotational diffusion of perdeuterated HIV protease from 15 N NMR relaxation measurements at two magnetic fields. *J Biomol NMR* 8:273–284
- Todd MJ, Semo N, Freire E (1998) The structural stability of the HIV-1 protease. *J Mol Biol* 283:475–488
- Van Marck H, Dierynck I, Kraus G, Hallenberger S, Pattery T, Muyldermans G, Geeraert L, Borozdina L, Bonesteel R, Aston C, Shaw E, Chen Q, Martinez C, Koka V, Lee J, Chi E, de Bethune MP, Hertogs K (2009) The impact of individual human immunodeficiency virus type 1 protease mutations on drug susceptibility is highly influenced by complex interactions with the background protease sequence. *J Virol* 83:9512–9520
- Vega S, Kang LW, Velazquez-Campoy A, Kiso Y, Amzel LM, Freire E (2004) A structural and thermodynamic escape mechanism from a drug resistant mutation of the HIV-1 protease. *Proteins* 55:594–602
- Velazquez-Campoy A, Todd MJ, Freire E (2000) HIV-1 protease inhibitors: enthalpic versus entropic optimization of the binding affinity. *Biochemistry* 39:2201–2207
- Velazquez-Campoy A, Todd MJ, Vega S, Freire E (2001) Catalytic efficiency and vitality of HIV-1 proteases from African viral subtypes. *Proc Natl Acad Sci USA* 98:6062–6067
- Velazquez-Campoy A, Muzammil S, Ohtaka H, Schon A, Vega S, Freire E (2003) Structural and thermodynamic basis of resistance to HIV-1 protease inhibition: implications for inhibitor design. *Curr Drug Targets Infect Disord* 3:311–328
- Walch JC, Jones CD, Barnes EA, Gazzard BG, Mitchell SM (1998) Increasing survival in AIDS patients with cytomegalovirus retinitis treated with combination antiretroviral therapy including HIV protease inhibitors. *AIDS* 12:613–618
- Wang YX, Freedberg DI, Grzesiek S, Torchia DA, Wingfield PT, Kaufman JD, Stahl SJ, Chang CH, Hodge CN (1996) Mapping hydration water molecules in the HIV-1 protease/DMP323 complex in solution by NMR spectroscopy. *Biochemistry* 35:12694–12704
- Wang T, Cai S, Zuiderweg ER (2003) Temperature dependence of anisotropic protein backbone dynamics. *J Am Chem Soc* 125:8639–8643
- Weber IT, Agniswamy J (2009) HIV-1 protease: structural perspectives on drug resistance. *Viruses* 1:1110–1136
- Wlodawer A, Erickson JW (1993) Structure-based inhibitors of HIV-1 protease. *Annu Rev Biochem* 62:543–585
- Wlodawer A, Miller M, Jaskolski M, Sathyanarayana BK, Baldwin E, Weber IT, Selk LM, Clawson L, Schneider J, Kent SB (1989) Conserved folding in retroviral proteases: crystal structure of a synthetic HIV-1 protease. *Science* 245:616–621
- Wong V, Case DA (2008) Evaluating rotational diffusion from protein MD simulations. *J Phys Chem B* 112:6013–6024
- Yamazaki T, Hinck AP, Wang YX, Nicholson LK, Torchia DA, Wingfield P, Stahl SJ, Kaufman JD, Chang CH, Domaille PJ, Lam PY (1996) Three-dimensional solution structure of the HIV-1 protease complexed with DMP323, a novel cyclic urea-type inhibitor, determined by nuclear magnetic resonance spectroscopy. *Protein Sci* 5:495–506
- Yang D, Gardner KH, Kay LE (1998) A sensitive pulse scheme for measuring the backbone dihedral angle ψ based on cross-correlation between $(^{13}\text{C}(\alpha)-^1\text{H})$ dipolar and carbonyl chemical shift anisotropy relaxation interactions. *J Biomol NMR* 11:213–220
- York DM, Darden TA, Pedersen LG, Anderson MW (2002) Molecular dynamics simulation of HIV-1 protease in a crystalline environment and in solution. *Biochemistry* 32:1443–1453
- Yu Y, Wang J, Shao Q, Shi J, Zhu W (2015) Effects of drug-resistant mutations on the dynamic properties of HIV-1 protease and inhibition by Amprenavir and Darunavir. *Sci Rep* 5:10517

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