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Crystallographic Fragment Based Drug Discovery: Use of a Brominated Fragment Library Targeting HIV Protease

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Abstract

A library of 68 brominated fragments was screened against a new crystal form of inhibited HIV-1 protease in order to probe surface sites in soaking experiments. Often fragments are weak binders with partial occupancy, resulting in weak, difficult-to-fit electron density. The use of a brominated fragment library addresses this challenge, as bromine can be located unequivocally via anomalous scattering. Data collection was carried out in an automated fashion using AutoDrug at SSRL. Novel hits were identified in the known surface sites: 3-bromo-2,6-dimethoxybenzoic acid (Br6) in the flap site, and 1-bromo-2-naphthoic acid (Br27) in the exosite, expanding the chemistry of known fragments for development of higher affinity potential allosteric inhibitors. At the same time, mapping the binding sites of a number of weaker binding Br-fragments provides further insight into the nature of these surface pockets.

Introduction

Fragment-based drug discovery is increasingly replacing large high-throughput screens as a technique to design novel therapeutics, but optimal library design is an ongoing challenge in the field. The potential utility of brominated fragment libraries for crystallization-based screening has been previously described(1, 2), but few successes have been reported. The fragment library used by SGX pharmaceuticals (now part of Lilly) includes a large number of brominated compounds selected for the ease of detection of bromine using anomalous scattering(2). Bauman et. al. observed a hit rate of 23.5% for brominated compounds and a 24.1% hit rate for fluorinated compounds in a crystallography-based screen against HIV-1 reverse transcriptase, compared to an overall hit rate of 4.4% for the 742 compounds screened(1).

In addition to its utility as a label for compound detection via anomalous scattering, bromine may contribute to the binding efficiency of a ligand via the recently-described halogen bonding interaction. The halogen atoms chlorine, bromine, and iodine bind in a noncovalent Lewis acid-base type interaction with carbonyl oxygen atoms of the protein backbone or π systems, and can increase protein-ligand affinity(3). A halogen-enriched fragment library (HEFLib) was used by Boeckler and colleagues to identify small molecules that stabilize the p53 mutant Y220C using NMR, ITC and DSF as detection methods(4).

Prior work has focused on the application of fragment screening to target surface binding pockets on HIV protease (PR)(5, 6). In an initial cocrystallization effort, three hits were observed from a library of 384 compounds that bound at two sites, giving an overall hit rate of 0.8%(5). Further work identified a third fragment hit that bound in the flap site and suggests that flap site binding favors a closed flap conformation of PR(6). Through crystal engineering, a new crystal form of PR complexed with the active site inhibitor TL-3(Lee, 1998) was developed. In these crystals, both of the previously-described surface-exposed binding sites, the flap site and the exosite, are solvent accessible. In the present work, a small library of 68 commercially-available brominated molecules was selected for soaking, and data sets were collected for each of the 68 compounds. Of these 68 data sets, 23 had at least one bromine anomalous peak. Most of these peaks correspond to a hit that is uninteresting: isolated electron density for a bromine atom, binding in an interstitial site, or binding at a site formed by TL-3 in addition to side-chains of HIV-PR.. At the same time, two well-defined novel hits were obtained – one compound binding in the flap site and one binding in the exosite, for a hit rate of ~3%. If the TL-3 site is taken into account as a “binding site”, although dependent on the presence of a specific active site ligand, the hit rate for well-ordered compounds binding in a single orientation is increased to ~6%. While this is not as high as the hit rate reported for brominated fragments against other targets, it is four to eight times that observed in the initial ActiveSite screen. Importantly, the hits confirm the two surface pockets in PR and bromine binding further delineates the extent of the two sites. Results presented here suggest this methodology can be used as an efficient probing tool for identifying potential binding pockets on unknown targets.

Materials and Methods

Protein Expression

The W6A mutation was introduced into wild type NL4-3 HIV-1 PR using site-directed mutagenesis as previously described.(7) The mutant PR was expressed in *E. coli* cells and induced with 1mM IPTG. Inclusion bodies were isolated by centrifugation, solubilized, and purified by ion-exchange chromatography on a Pharmacia FPLC. PR was dialyzed against 10 mM sodium acetate buffer, pH 5.2, 0.1% 2-mercaptoethanol prior to filtration and concentration to 3–5mg/mL. PR purity was verified by SDS-PAGE and protein identity was verified by Western blot using rabbit antiserum against HIV PR.

Crystallization

4.3 mg/mL W6A PR was mixed with 10% 200 mM TL-3 in DMSO. Crystals were grown via the sitting drop method with a 1:1 ratio of protein:reservoir in drops with a total volume of 0.4 μ L in a 24-well plate. Reservoir solutions (1mL) were 2–3 M NaNO₃, 0.1 M NaOAc pH 5.5.

Soaking

First, crystals were transferred to a cryo solution by serial mixing with the crystallization drop. The cryo solution contained 2.5% DMSO, 20% glycerol, 0.1 M NaOAc pH 5.0 and 4.2M NH₄NO₃. It was observed that crystal stability was higher with this cryo solution than with one prepared with NaNO₃. 4 μ L of cryo solution was added to the 4 μ L drop, mixed, and then 4 μ L was removed from the drop, being careful not to remove any crystals. This process was repeated 3–4 times. One to three crystals were then transferred to the cover of a Qiagen hanging-drop reservoir in 4 μ L total solution. Crystals were placed over a reservoir of 3M NaNO₃. After a full soaking tray (15 wells) of crystals were harvested, compounds were added. A solution of each compound in DMSO was diluted 1:1 with a solution containing 7M NH₄NO₃ and 30% glycerol, and 1 μ L of this solution was added to the 4 μ L drop containing the crystals. This leads to final soaking conditions of ~10% DMSO, 4M

NH₄NO₃, and 19% glycerol. The initial concentration of the compounds in DMSO ranged from 50–200 mM, leading to final concentrations in the soaks of 5–20 mM. However, many compounds were at saturation under soaking conditions (SI Table 1). Soaks were carried out for 5 days at 25 °C, and crystals were frozen in LN₂ directly from the soaking conditions without further cryoprotection.

Data Collection and Processing

Data were collected on BL11-1 at SSRL with the wavelength tuned near the absorption edge of bromine at 13,500 eV ($\lambda = 0.9184$) to maximize anomalous signal. A trial version of Autodrug was used to automate crystal orientation and data collection. (8) Data integration and scaling were completed using Autoxds. (9–11) For each compound, two crystals were mounted. One data set was collected; a second data set was collected if a second crystal had significantly better criteria as scored by Blu-ice. PDB_id: 4K4P was used as a model for molecular replacement with Phaser. (11) Bromine anomalous maps were generated for each compound using FFT. (Read, 1988) After one round of Refmac5, datasets were visually inspected for anomalous difference peaks, and data were further analyzed when a bromine anomalous peak was observed. Bromine occupancy was further refined when an anomalous peak was present. Refinements were completed using Coot, MiFit, and Refmac5. (11–14)

Results and Discussion

Crystal Engineering

The most common crystal form of HIV-PR observed with the active site inhibitor TL-3 (7) is the hexagonal P6₁22 form (i.e. PDB_id: 3KFP). In the cocrystallization experiments previously described, (5) deviations from this crystal form were observed only upon compound binding. The binding of 4D9 in the exosite caused the crystal packing to change to the P2₁2₁2 form (i.e. PDB_id: 3KF0), which is frequently observed for pepstatin-inhibited PR, and the binding of 2F4 and 1F1 in the flap site caused the crystals to grow in a P2₁2₁2₁ form (i.e. PDB_id: 3KFR), only observed for PR in the presence of flap site binders. (5, 6) Soaking of the P6₁22 crystals with the flap site binder 1F1 led to crystal destruction, and electron density for exosite-binding compound 4D9 was not observed in soaking with this crystal form. (5, 6)

The packing of the P2₁2₁2 crystal form relies on interactions of Trp6 with proline residues on symmetry related molecules, while packing of P6₁22 crystals is stabilized by interaction of Trp6 with Gln18 and Thr91. Although Trp6 is a conserved residue in HIV protease, it is surface-exposed and not conserved in related viruses, suggesting that it may be important in protein-protein interactions but likely not important for the stability of the protease. To facilitate the formation of novel crystal forms, Trp6 was mutated to alanine, and this mutation was found to be compatible with stable protein folding.

W6A protease was readily crystallized when complexed with TL-3, with multiple conditions yielding crystals in commercially available 96-well screens. Pepstatin-inhibited W6A was more challenging to crystallize. The majority of crystals of W6A:TL-3 were determined to be hexagonal P6₁ crystals, a crystal form closely related to the original P6₁22 crystals observed for NL-3:TL-3 and not compatible with soaking for the flap site. However, novel crystal forms were observed when W6A:TL-3 was crystallized under high nitrate conditions. Crystallization in 5–7M ammonium nitrate led to the formation of octahedral crystals with similar morphologies: I4₁22 crystals containing a dimer in the asymmetric unit, and P4₁22 crystals containing two dimers in the asymmetric unit. The I4₁22 crystal form meets criteria for soaking: the crystals grow rapidly, routinely diffract to 2.5 Å or better, and both the flap site and the exosite are solvent exposed in at least one monomer of the dimer. The related

P4₁22 crystal form is not compatible with soaking. Subsequently, substitution of 2–3 M sodium nitrate for the ammonium nitrate led to exclusive formation of I4₁22 crystals.

Library Selection, Soaking, and Data Collection

Compounds with a molecular weight of 160–300 kD and containing at least one bromine were selected using Maybridge Hit Finder, and of the 70 compounds identified, 68 were available. 58 of these fragments were soluble to 200mM in DMSO; the remainder were dissolved at 50–100mM. Soaking conditions were developed in which the crystals were stable for 5 days at 25°C in the context of ~10% DMSO and glycerol for cryoprotection(details in Methods). Compounds were individually soaked and were either at 20mM in the drop or at saturation (SI Table 1). After five days, crystals were mounted directly from the soaking drops and frozen with liquid nitrogen.

Data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) using BluIce(15) and in a completely automated mode using a customized AutoDrug(8) workflow. Data were collected on beamline 11-1 at SSRL with the wavelength near the bromine absorption edge (13,500 eV) to maximize the anomalous signal. At least one dataset with a resolution of 3.5 Å or better was collected for each compound, with datasets better than 3.0 Å for 53 compounds (SI Table 1). For all data sets, Autoxds was used for data processing through scaling, and a model I4₁22 structure (soaked with a DMSO control; PDB_ID: 4K4P) used for molecular replacement with Phaser.(11) The anomalous difference fourier map was also calculated using FFT.(16) After one round of refinement to add water molecules, a Fo-Fc difference map and the anomalous map were visually inspected for each compound.(11, 12, 14) Peaks in the anomalous maps allowed unambiguous identification of positive electron density difference peaks arising from bromine compound binding.

Soaking Results

23 out of 68 data sets contained at least one anomalous peak corresponding to a bromine atom (Table 1; SI Table 1). Several compounds were promiscuous, with bromine anomalous peaks at several sites on the protease. For many compounds, only the position of the bromine atom was well-defined, and the rest of the molecule was either completely disordered or had several different, but overlapping orientations, generating complex electron density maps. Compound binding was only observed in three different sites within the crystals: the ‘outside/top’ flap site,(5, 6) the exosite,(5) and an adventitious site formed by the interaction of a terminal phenyl ring of TL-3 with Phe53 and Pro81 (Figure 1; Table 1). This latter site is uninteresting for drug development because it relies on the presence of the active-site inhibitor TL-3, but it represents the most promiscuous binding site for this set of bromine-containing compounds in the I4₁22 crystal form. Bromine peaks were observed for nine compounds in both the exosite and flap site(Figures 2, 3), and for sixteen compounds in the TL-3 site, with a number of compounds demonstrating binding in two or more of these sites. Three compounds had electron density for the light atoms of the compound in the flap site, four in the exosite, and twelve in the TL-3 site; the remainder only showed a peak for bromine. In addition, some of the compounds bind in association with symmetry-related molecules, such as Br14 and Br15 in the flap site and Br22 and Br23 in the exosite(SI Figures 1, 2). For example, the bromine of Br15 binds between Pro44 and Met46 in the flap site, but binding is stabilized by a cation- π interaction between the phenyl ring of Br15 and Arg8 of a symmetry-related molecule(SI Figure 1). For both Br22 and Br23, compound binding is at the crystal packing interface(SI Figure 2), and for Br23, the compound binds in multiple orientations(not shown). In spite of this context-dependent binding behavior for the brominated fragments, two well-defined hits were obtained with Br6 in the flap site and Br27 in the exosite, and these structures were fully refined (Table 1,

Table 2, SI Table 2, Figures 4 and 5). Data are summarized in SI Table 1. These hits confirm the ability of fragments to bind specifically in the surface pockets of PR.

Investigation of the bromine binding provides insight into the nature of these two surface sites of HIV PR. First, these binding sites are promiscuous, but the binding of most compounds is weak, and otherwise difficult to identify in crystal structures due to partial occupancy and multiple fragment orientations. Second, the observed bromine sites in each region of the protein surface delineate the pockets while providing insight into directions for fragment expansion. In the flap site, the bromines cluster into two preferred sites: one between Trp42 and Lys55, where 1F1 (indole-6-carboxylic acid) and 1F1-N (3-indolepropionic acid) were shown to bind, and a more highly populated second site showing one of the potential directions for expanding the flap site hits, towards Met46 (Figure 2).(6) In the exosite, the major cluster of Br sites extends beyond the original description of the exosite, based on the crystal structures with 4D9 (2-methylcyclohexanol) bound (PDB_id: 3KF0, 3KFN), where Lys14 defined the end of the cavity.(5) In these 14₁22 crystals, Lys14 shows significant mobility, opening up this ‘end’ of the exosite for the binding of brominated compounds. The second cluster of bromine sites in the exosite is between Leu63 and Glu65 (Figure 3).

Br6 (3-bromo-2,6-dimethoxybenzoic acid) binds specifically in the flap site, exploiting similar interactions to the previously described compounds 1F1 (indole-6-carboxylic acid), 2F4 (benzothiophene), and 1F1-N (3-indolepropionic acid).(5, 6) The phenyl ring packs between Pro44 and Lys55, while the bromine interacts with the backbone carbonyl of Val56 and the carboxylic acid interacts with the amino group of Lys55(Figure 4). Unlike 1F1 and 1F1-N, interaction with the backbone amide of Val56 is not observed, presumably replaced by the halogen-carbonyl interaction. Hence, the same interactions with protease are exploited by a very different chemical compound.

Br27 (1-bromo-2-naphthoic acid) binds at the base of the exosite, packing against the side chain of Lys70 (Figure 5). The bromine binds in a pocket formed by Leu63, Glu65, and Lys14. Lys14 moves to accommodate compound binding and forms a salt bridge with the carboxylic acid of Br27. The naphthalene ring interacts with Leu63, Glu65, and Lys70, and has weak van der Waals contacts with Phe53 from a symmetry-related molecule (SI Figure 3). Br27 is a much larger molecule than the previously reported hit in the exosite, 4D9,(5) and shows that stacking interactions with Leu63, Glu65, and Lys70 can be exploited in a similar manner. Importantly, a new interaction is revealed via induced-fit salt bridging with Lys14.

Conclusions and Future Directions

Screening of a 68-member brominated fragment library against HIV PR led to the identification of two new compounds that bind to two known surface sites of PR. The numerous ‘quasi-hits’ where anomalous density was observed for the bromine atom but the density for the rest of the compound was weak or disordered provide insight into the challenges of obtaining hits for these weak surface sites via crystal soaking or co-crystallization. The presence of a bromine anomalous signal provides unambiguous evidence of bromine binding, and improves interpretation of the map for compounds with lower occupancy or disordered binding modes, suggesting that the selection of brominated molecules is not only a good way to improve the hit rate for a crystallization-based screen, but is also beneficial for defining the orientation of hits from otherwise unclear electron density maps. While a bromine atom is anticipated to influence compound binding, the bromine does not dominate the binding for this set of compounds, as evidenced by the lack of consistent binding to ‘bromine sites’ for all compounds tested. Additionally, when the

fragment has specific alternative interactions with the protein, such as in the case of Br6 in the flap site and Br27 in the exosite, these interactions override the ‘preferred bromine binding’ and the compounds bind with bromine occupying a distinct site within these surface pockets (Figures 4 and 5). The soaking strategy and Autodrug data collection represent a generally applicable strategy for finding fragments that bind to solvent-accessible sites on any soakable crystal form. Even with a small library of 68 compounds, the hit rate with brominated fragments is much higher than that observed previously with the ActiveSite fragment library,⁽⁵⁾ 3–6% vs. 0.8%. Additionally, even weakly binding fragments or those that bind at the crystal interface provide insights into the nature of these surface binding pockets and can help to guide future fragment design. For example, the mobility of Lys14 observed in this experiment has widened our view of the extent of the exosite of HIV PR. This information, along with the specific interactions of Br27, is being used to inform ongoing computational studies to identify larger exosite-binding fragments. In the flap site, the bromine-rich site that extends from the edge of identified flap site binders (Br6, 1F1 and 1F1-N(6)) shows a clear direction for extension of these compounds in future work. The robustness of the methodology in confirming known sites, the relatively high “sensitivity” to low affinity binding pockets, and the higher hit rate when compared to non-brominated fragment libraries make this protocol a very useful tool to probe unknown targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic of binding sites for brominated fragments on TL-3:W6A-PR. Key interaction residues are shown in cyan for the flap site, yellow for the TL-3:site, and pink for the exosite.

Figure 2. Bromine binding sites in the flap site of TL-3:W6A-PR in the I4₁22 crystal form. Colors correspond to compounds in Table 1. Red, Br6; Orange, Br14; Green, Br15; Blue, Br22; Purple, Br27; Magenta, Br33; Teal, Br50.

Figure 3. Bromine binding sites in the exosite of TL-3:W6A-PR in the $I4_122$ crystal form. Colors correspond to the compounds in Table 1. Orange, Br14; Blue, Br22; Burgundy, Br23; Purple, Br27; Green, Br28; Dark magenta, Br58.

Figure 4.

A) Electron density for Br6. Unbiased electron density for Br6 is shown in dark blue; biased density after 1 round of refinement with compound included is shown in gray-blue, both contoured at 1 σ . Bromine anomalous signal is shown in green; contoured at σ .

B) Interactions of Br6 in the flap site of HIV PR.

Figure 5.

A) Unbiased electron density for Br27 is shown in blue, contoured at 1 σ ; bromine anomalous signal is shown in green, contoured at 4 σ .

B) Interactions of Br27 in the exosite.

Table 1

Compounds with >25% bromine occupancy in the flap site or exosite, as represented in Figures 2 and 3.

ID	Structure	Br Site	Res (Å)	R	R _{free}
6		Flap site	1.8	19.89	23.94
14		Both	2.2	22.43	27.45
15		Flap site	2.0	19.28	22.87
22		Both	2.0	21.17	25.84
23		Exosite	1.9	21.70	24.25
27		Both	1.8	20.10	23.97
28		Exosite	2.5	20.72	28.04

ID	Structure	Br Site	Res (Å)	R	R _{free}
33		Flap site	2.5	21.40	26.71
50		Flap site	2.5	18.92	23.81
58		Exosite	2.5	20.47	25.90

Table 2

Data Collection and Refinement Statistics

Crystals			
Protein Complex	W6A_PR	W6A_PR + Br6	W6A_PR + Br27
PDB code	4K4P	4K4Q	4K4R
Space group	I4 ₁ 22	I4 ₁ 22	I4 ₁ 22
Unit cell dimensions (Å)	98.5 98.5 96.4 90 90 90	97.8 97.8 98.8 90 90 90	98.5 98.5 96.6 90 90 90
Solvent content	53.75	54.22	53.81
Data			
Total obs. > 0σ _F	68,114	142,814	139,560
Unique reflections > 0σ _F	10, 686	22,302	22,354
Redundancy (last shell)	6.4 (6.6)	6.4 (6.4)	6.2 (5.3)
Completeness	99.5 (97.3)	99.6 (98.2)	99.4 (97.0)
Resolution (Å)	2.31	1.80	1.79
<I/ σ _I > all data	16.4(2.1)	13.8 (1.5)	12.8 (1.9)
Rmerge all data	0.029 (0.38)	0.036 (0.49)	0.030 (0.41)
Refinement			
R-factor	18.87	20.37	20.60
Rfree	21.87	23.65	23.78
Reflections used	10,151	21,148	21,194
Test set	4.8 %	5.1 %	5.1 %
Bond lengths RMSD (Å)	0.0106	0.0069	0.0076
Bond angles RMSD (°)	1.5795	1.3190	1.4341
Ramachandran Favored regions	100 %	100 %	98.97 %
Ramachandran Allowed regions	0 %	0 %	1.03 %
Model			
	Residues / Avg. B (3 ²) ¹	Residues / Avg. B (3 ²)	Residues / Avg. B (3 ²)
Chain A	56.95	29.67	37.12
Chain B	55.69	31.12	39.19
Ligand	n/a	34.2	44.06
NO ₃	82.0	41.8	44.10
DMSO/glyc./BME	n/a	56.9	59.9
TL-3	79.7	33.6	45.33
H ₂ O molecules	57.3	38.5	42.8