MicroED structures of HIV-1 Gag CTD-SP1 reveal binding interactions with the maturation inhibitor bevirimat

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HIV-1 protease (PR) cleavage of the Gag polyprotein triggers the assembly of mature, infectious particles. Final cleavage of Gag occurs at the junction helix between the caspase protein CA and the SP1 spacer peptide. Here we used MicroED to delineate the binding interactions of the maturation inhibitor bevirimat (BVM) using very thin frozen-hydrated, 3D microcrystals of a CTD-SP1 Gag construct with and without bound BVM. The 2.9-Å MicroED structure revealed that a single BVM molecule stabilizes the six-helix bundle via both electrostatic interactions with the dimethylsucinyl moiety and hydrophobic interactions with the pentacyclic triterpenoid ring. These results provide insight into the mechanism of action of BVM and related maturation inhibitors that will inform further drug discovery efforts. This study also demonstrates the capabilities of MicroED for structure-based drug design.

HIV-1 | Gag | maturation inhibitors | drug discovery | cryoEM

In 2016, compelling evidence for the predicted six-helix bundle structure of the junction helices was provided by electron cryotomography and subtomogram averaging of ΔMA-Gag, non-encapsulated viruslike particles (VLPs) (19), X-ray crystallography of a Gag construct composed of the C-terminal domain (CTD) of CA and SP1 (20), and solid-state NMR spectroscopy of ΔMA-Gag VLPs (21). The cryoEM and X-ray structures revealed that the protease cleavage sites are sequestered on the interior of the six-helix bundle. The cryoEM map also showed that a single molecule of the maturation inhibitor bevirimat binds at the center of each six-helix bundle. To achieve a resolution of 3.9 Å, sixfold averaging was applied to the cryoEM map, which prevented characterization of the interactions between BVM and the CTD-SP1 junction helices.

To examine the molecular basis for BVM binding, we used electron diffraction from frozen-hydrated, 3D microcrystals, and solid-state NMR spectroscopy of HIV-1 Gag CTD-SP1 to reveal the detailed mechanism for binding of bevirimat, which will inform further drug discovery efforts.

Significance

During HIV maturation, protease cleaves the Gag polyprotein in immature particles, which culminates in dramatic morphological changes and assembly of mature, infectious particles. The final cleavage step occurs between the C-terminal domain (CTD) of the CA capsid protein and the SP1 spacer peptide. A potential therapeutic strategy is blockage of the CTD-SP1 protease binding, and the first-in-class maturation inhibitor was bevirimat. Unfortunately, therapeutic development was abrogated by resistance mutants. To date, the detailed mechanism of action of bevirimat has not been determined. Therefore, we performed electron cryomicroscopy and electron diffraction of thin 3D microcrystals of CTD-SP1 with and without bound bevirimat. The atomic resolution structures revealed the detailed mechanism for binding of bevirimat, which will inform further drug discovery efforts.

Author contributions: M.D.P., T.G., and M.Y. designed research; M.D.P., D.S., J.C., J.H., T.G., and M.Y. performed research; M.D.P., J.H., T.G., and M.Y. analyzed data; and M.D.P., T.G., and M.Y. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The atomic coordinates were deposited in the Protein Data Bank (drug-free CTD-SP1: PDB ID 6N3J; CTD-SP1 with bound bevirimat: PDB ID 6N3U). Because a unique bevirimat binding pose could not be determined, the drug is not included in the PDB coordinate file. MicroED maps were deposited in the EMDataBank (drug-free CTD-SP1: EMDB-0333; CTD-SP1 with bound bevirimat: EMDB-0337).

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so-called MicroED (microcrystal electron diffraction) (22–26), to solve the structures of drug-free and BVM-bound CTD-SP1 to resolutions of 3.0 and 2.9 Å, respectively, without applying sixfold symmetry (Table 1).

**Results**

The CTD-SP1 crystals were of the same form that was used to solve the drug-free X-ray crystallographic structure to 3.27-Å resolution (20). The CTD-SP1 Gag construct was expressed and purified as described previously (20), and the crystallization conditions were adapted to grow microcrystals <1 μm thick (as described in Methods and SI Appendix, Fig. S1). The asymmetric unit contains one CTD-SP1 hexamer, and layers of the C2 crystals approximate the HIV-1 Gag immature lattice (Fig. 2). Molecular replacement phasing of the CTD-SP1-BVM MicroED data with the CTD X-ray structure (PDB 5I4T) resulted in a map that contains BVM density at the center of the six-helix bundle formed by the CTD-SP1 junction helices (Fig. 2 D–F). The density attributed to BVM is absent in our MicroED map of drug-free CTD-SP1 (Fig. 2 A–C). The location of the single BVM molecule within the six-helix bundle is consistent with a 3.9-Å map of HIV-1 Gag VLPs determined by electron cryotomography and subtomogram averaging (19). In the case of the cryoEM map of VLPs, the BVM density was a featureless ellipsoid due to sixfold averaging required to achieve a resolution of 3.9 Å for the protein density. However, sixfold averaging was not applied in the determination of the CTD-SP1-BVM MicroED map, and the BVM density is asymmetric with a dominant feature consistent with en face (Fig. 3C) and profile (Fig. 3D) views of the pentacyclic triterpenoid moiety of BVM. The presence of a unique mode of binding is presumably a consequence of the twofold symmetric crystallographic environment of the binding site (Fig. 2E).

We define the axial orientation of the bound drug according to the location of the dimethylsuccinyl group, either oriented toward (BVM-up, Fig. 4A) or away from (BVM-down, Fig. 4B) the CA domain. There is a distinct satellite density directed toward the CA domain, which we ascribe to the dimethylsuccinyl moiety (Fig. 3A–E). This BVM-up orientation places the carboxylic acid of the dimethylsuccinyl moiety in proximity to a ring of lysine residues (K359). Interestingly, the extent of the density of the K359 side chains varies between protomers in the BVM-bound map. In particular, the side chain density is better defined for the K359 residue that is closest to the density peak that we ascribe to the dimethylsuccinyl (Fig. 3E and G). In contrast, density for the K359 side chain is absent in five of six protomers in the drug-free CTD-SP1 map (Fig. 3H), likely due to conformational flexibility.

Refinement of the structure with BVM in the up orientation also supports our interpretation (Fig. 4A), whereas refinement with BVM in the down orientation positions the C-28 carboxyl more than 7 Å away from the nearest K359 Nε, and the dimethylsuccinyl extends out of the junction helix bundle (Fig. 4B and C).

The presence of carboxylic acid groups at each end of BVM (Fig. 1A) makes consideration of the physics of electron scattering relevant to our analysis of BVM binding to Gag because carboxylates frequently have diminished density in maps derived from cryoEM (27–30). Electron diffraction is a result of elastic scattering due to Coulomb forces between the incident electron beam and the crystal and therefore is dependent on the charge distribution in the crystal. Unlike atomic scattering factors for X-rays, electron scattering factors for some atoms are strongly dependent on scattering angle. In the case of negatively charged oxygen (O⁻), the atomic scattering factor is negative at low resolution (less than about 8 Å) and positive at higher resolution. To examine these effects on our maps, we examined the

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**Table 1. MicroED data collection and refinement statistics**

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Drug-free CTD-SP1*</th>
<th>CTD-SP1-BVM†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>C121</td>
<td>C121</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b, c, Å</td>
<td>70.6, 122.5, 78.7</td>
<td>71.4, 123.0, 81.8</td>
</tr>
<tr>
<td>R, β, γ, °</td>
<td>90.0, 90.0, 96.5</td>
<td>90.0, 90.0, 95.0</td>
</tr>
<tr>
<td>Resolution, Å</td>
<td>27.5–3.0 (3.2–3.0)</td>
<td>27.4–2.9 (3.1–2.9)</td>
</tr>
<tr>
<td>Rpim, &lt;film&gt;</td>
<td>0.255 (1.324)</td>
<td>0.180 (0.860)</td>
</tr>
<tr>
<td>CC1/2</td>
<td>0.90 (0.35)</td>
<td>0.96 (0.10)</td>
</tr>
<tr>
<td>Completeness, %</td>
<td>79.8 (78.7)</td>
<td>83.4 (82.3)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>5.7 (5.7)</td>
<td>7.5 (7.5)</td>
</tr>
</tbody>
</table>

| Refinement          |                   |             |
| Resolution, Å       | 19.9–3.0 (3.2–3.0) | 19.9–2.9 (3.0–2.9) |
| No. reflections     | 10,480 (1,006)    | 11,635 (1,137) |
| Rpim/Refe           | 0.254/0.292       | 0.236/0.280  |
| No. protein atoms   | 7,410             | 7,814        |
| ADP, Å2             |                   |             |
| Mean                | 41.1              | 52.6         |
| Minimum/Maximum     | 6.8/177.9         | 19.2/160.5   |
| rms deviations      | Bond lengths, Å   | 0.003        | 0.002 |
| Bond angles, °      | 0.561             | 0.474        |
| MolProbity clashscore | 1.48             | 0.38         |

*Data from six crystals.
†Data from five crystals.
resolution dependence of our Coulomb potential maps (Fig. 5A and D). Including low-resolution intensities (20–2.9 Å) resulted in MicroED maps with a negative contribution to acidic side chains (Fig. 5B and E) (30). In contrast, maps calculated only with data in the higher-resolution range (8–2.9 Å), in which scattering factors for O\(^-\) are positive, were unaffected (Fig. 5C and F). The electron scattering factor for uncharged oxygen is fairly constant; therefore, neutralization of acidic side chains by direct interaction with, or proximity to, basic residues eliminates or minimizes the resolution dependence of the density of these side chains (e.g., E291 in a salt bridge with R299) (Fig. 5B and C). Similarly, such Coulomb potential neutralization of the BVM dimethylsuccinyl carboxylic acid by the nearby ring of K359 lysine residues (Fig. 3E) likely accounts for the presence of density for this moiety.

The absence of acidic side chain density can also be due to mobility and/or radiation damage. O\(^-\) is more susceptible to radiation damage by electrons than neutral and positively charged atoms, as shown in the analysis of radiation sensitivity of acidic
side chains in cryoEM experiments (31, 32). These studies also demonstrated that radiation damage to acidic side chains is dose-dependent, and its manifestation in the density varies with resolution. At the average electron doses (~3 e⁻/Å²) and resolutions observed in this study, we expect radiation damage to have only minor effects on the density.

**Discussion**

BVM is known to act by stabilizing the immature Gag lattice (14). The location of the pentacyclic triterpenoid adjacent to the junction helices clearly indicates that hydrophobic interactions are important for the mechanism of BVM binding (Figs. 3 A–D and 6 E and F). Specifically, L363 and M367 from each of the six protomers contribute the majority of the BVM binding interactions (Fig. 3 C, D, and F). The BVM dimethylsuccinyl ester is also thought to contribute to the activity (18), and we propose that BVM stabilizes Gag hexamers by electrostatically anchoring K359 of the CA/CTD domain via the dimethylsuccinyl carboxylate (Fig. 6 C and D) and binding together the junction helices via hydrophobic interactions between L363 and M367 from each protomer and the BVM pentacyclic triterpenoid moiety (Fig. 6 E and F). This BVM binding mechanism could accommodate lattice and hexamer distortions due to the nonspecific nature of the hydrophobic interactions and the electrostatic attraction with the six K359 residues, which is amplified by a second coaxial ring of lysine residues (K290) (Fig. 3 C and D). This mechanism of BVM binding and Gag stabilization is also consistent with the structures of second-generation HIV-1 maturation inhibitors in which the dimethylsuccinyl ester is replaced with benzoic acid (Fig. 1B). C-3 benzoic acid substitution places a carboxyl in proximity to K359 while preserving the hydrophobic interactions between the junction helices and the pentacyclic triterpenoid. The fact that the C-3 benzoic acid substituted compound exhibited antiviral activity comparable to BVM and had no effect on the potency against the BVM Gag escape mutant V370A provides additional support for BVM binding with the succinic acid positioned in the vicinity of K359 and away from V370. In addition, the BVM-up orientation positions C-28 of BVM near M367, and C-28 derivatives that extend BVM analogs toward the C-terminal end of SP-1 have the highest potency against HIV-1 with the Gag V370A polymorphism (Fig. 7) (16–18). BVM analogs with C-28 extensions may provide additional, compensatory interactions at the C-terminal end of the six-helix bundle that are sufficient to prevent protease access to the junction helix cleavage site.

We realize that our interpretation of the binding mechanism of BVM to the six-helix bundle is based on a comparison of MicroED

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**Fig. 4.** MicroED map suggests the orientation of the bound bevirimat. (A and B) CTD-SP1-BVM 2Fo–Fc (gray, contoured at 1.0σ) and Fo–Fc (green and red, contoured at ±3.0σ) maps with BVM modeled in different axial orientations. (A) In the up orientation, the dimethylsuccinyl group points toward the CTD. (B) In the down orientation it points away from the CTD. (A) In the up orientation, the pentacyclic triterpenoid moiety occupies the large map feature near the CTD-protease cleavage site, and the carboxyl of BVM is proximal to the ring of K359 lysines. (B) In the down orientation, when the pentacyclic triterpenoid is positioned in the primary drug density, there is no density for the dimethylsuccinyl group, and the C-28 carboxyl is positioned far from K359. During refinement, without BVM, some maps include weak extensions of the primary drug density peak (>0.6σ) out of the helical bundle, that is, away from the CTD. However, in mostly maps, the drug density terminates abruptly. Following refinement with BVM in the down orientation, no density is present for the dimethylsuccinyl, which could be attributable to high mobility. (C) Maps are the same as in B with an additional 2Fo–Fc contour level at 0.6σ above the mean.

**Fig. 5.** Coulomb potential map of CTD-SP1-BVM at 2.9-Å resolution. (A and D) The CTD-SP1-BVM Coulomb potential surface [−8 kcal/mol e⁻ (red) to 8 kcal/mol e⁻ (blue)] calculated from the model (49) shows significant negative charge for solvent-exposed acidic residues (D295, E307, and E319) and neutralization in the case of an acidic residue involved in a salt bridge (E291), (B, C, E, and F) CTD-SP1-BVM MicroED maps showing acidic and basic residues in a variety of environments. Electronic atomic scattering factors are strongly dependent on charge at low resolution, and excluding these reflections results in additional side chain density for residues containing O⁻; e.g., note the reduced density of D295, E307, and E319 (red ellipses) in 8 and E (map calculated from 20–2.9 Å, gray mesh) compared with C and F (map calculated from 8–2.9 Å, pink mesh). Scattering factors for neutral oxygen are much less dependent on resolution and are positive at all scattering angles; therefore, densities are nearly identical for acidic side chains involved in salt bridges (e.g., E291 in B and C) or neutral oxygen-containing side chains (e.g., N315 and T318 in E and F) whether the full or high-resolution range is used in map calculations.
structures with and without the drug. For instance, photoaffinity labeling of detergent-stripped VLPs suggested that BVM may bind in the down orientation (33). This would be consistent with more distal mutants such as S373P and I376V conferring low-level BVM resistance (34–36). Likewise, the orientation may be influenced by the chemical properties of R-group substitutions. In the context of assembly of the immature hexagonal Gag lattice at the plasma membrane, it may be possible that other factors such as binding of viral RNA may influence the up–down distribution of BVM orientations as well as susceptibility to resistance. For instance, V20I confers strong resistance to bevirimat, even though valine and isoleucine are both hydrophobic, β-branched amino acids. The main difference is that the extra methylene increases the volume of isoleucine by ~20% (165 versus 139 Å²) (37). Notably, the MicroED structure shows that this residue resides on the exterior of the helical bundle, where it would appear to have minimal effect on the stability of α-helical packing within the bundle. Regardless of uncertainties in the mechanisms of resistance, our results provide atomic resolution results that provide insight into the molecular basis for BVM binding.

Binding a single BVM molecule to the Gag hexamer presents challenges for structure-based drug design, but this mode of binding is likely responsible for the efficacy of BVM and related maturation inhibitors that bind to Gag, despite structural heterogeneity inherent in the quasi-hexagonal immature lattice. The CTD-SP1-BVM structure provides additional insights into BVM binding and activity that will inform further development of HIV-1 maturation inhibitors.

The CTD-SP1-BVM structure also demonstrates the capability of MicroED for structure-based drug design and discovery even in challenging cases (i.e., low crystallographic symmetry and diffraction to moderate resolution). The increasing availability of electron microscopes and the ability to obtain microcrystals, either by screening under low dose in an electron microscope or generated by mechanical disruption of larger crystals (38), make MicroED an attractive option for high-resolution macromolecular structure determination, even for proteins that are far too small for analysis by single-particle cryoEM methods.

**Methods**

**Purification and Crystallization of CTD-SP1 and CTD-SP1-BVM.** The protein was expressed and purified as described previously (20). The conditions for growing microcrystals were adapted from the conditions to grow larger crystals for X-ray crystallography (20). The crystallization solution contained 0.1 M bis-Tris propane (pH: 7.0), 1.1–1.2 M LiSO₄, and 0.8–1.0 mg/mL Gag CTD-SP1. The drug molar excess was 48x, assuming 1 BVM molecule/Gag hexamer; 600 μL of the crystallization solution was added to the well of a hanging drop plate. The protein solution was mixed with BVM (e.g., for a 1:8 protein: drug ratio, 10 μL of 1 mg/mL protein were mixed with 10 μL of a solution of 0.64 mM BVM in DMSO and water). An aliquot (1 μL) of the protein–BVM solution was mixed with 2 μL of the well solution. The crystallization plates were incubated at 17 °C, and plate-like microcrystals appeared after 1–2 d. The same conditions were used to grow drug-free crystals. Initial screening of the crystals was performed by applying a 3-mm continuous carbon-coated EM grid to the crystallization drop for 90 s, blotting the grid with filter paper, and allowing the grid to dry at room temperature. Successful transfer of the crystals to the grid was first confirmed by light microscopy at 40x magnification (Supplementary Appendix, Fig. S1). The grid was then examined in an FEI T20 electron microscope operating at 200 kV. The unstained crystals were sufficiently thick to be readily observed from 10,000–40,000x magnification, and successful crystallization was confirmed by electron diffraction.

**MicroED Sample Preparation, Data Collection, and Processing.** Quantifoil EM grids were glow-discharged for 30 s on both sides, and the grid was placed onto the crystallization droplet with the carbon film side facing the droplet. After a 5- to 10-min incubation, tweezers were used to separate the grid from the droplet, and an additional 1.5 μL of solution from the same droplet was pipetted onto the back side of the grid. The grid was then mounted in an FEI Vitrobot, which was used to plunge-freeze the grid into an ethane slush, with the following settings: blotting time of 7–15 s, force of 24, and 40% humidity. The grid was transferred to a Gatan 626 cryostage, and continuous-rotation...
MicroED data were recorded using an FEI TF20 microscope equipped with a field emission electron source and a bottom-mounted TVIPS TemCam-F416 cooled chargecoupled device camera. The 1D X-ray projection of the CTD and the backbone model of the CTD hexamer from the Gag CA structure were determined by a combination of tomography and helical reconstruction. We aligned a copy of the 4COP monomer to each protomer of the 4D1K (46) CTD hexamer and performed molecular replacement with Phaser using the all-atom hexameric CTD as the search model. The top Phaser solution (TF2 = 9.4) showed well-defined density for the CTD and clear a-helical density for SPF. We generated an ideal helix in Coot within the best SPF density and then generated the initial six-helix bundle by application of sifxod NCS. We then performed rounds of building and refinement in Coot and Phenix. The resultant refined BVM-bound CTD-SP1 model and associated maps recapitulated those resulting from molecular replacement with the CTD-SP1 X-ray structure.

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