Structural Basis of Potent and broad HIV-1 Fusion Inhibitor CP32M

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CAPSULE
Background: CP32M is a newly-designed HIV-1 fusion inhibitor.
Results: Crystal structure of CP32M and its potent activity against diverse HIV-1 variants were determined.
Conclusion: The crystal structure reveals the mechanistic insights of CP32M.
Significance: Our data provide important information for designing potent HIV-1 fusion inhibitors.

SUMMARY
CP32M is a newly designed peptide fusion inhibitor possessing potent anti-HIV activity, especially against T20-resistant HIV-1 strains. In this study, we show that CP32M can efficiently inhibit a large panel of diverse HIV-1 variants, including subtype B', CRF07_BC and CRF01_AE recombinants and naturally-occurring or induced T20-resistant viruses. To elucidate its mechanism of action, we determined the crystal structures of CP32M complexed by its target sequence. Different from its parental peptide CP621-652, the 621VEWNEMT627 motif of CP32M folds into two turns of α-helix at the N-terminus of the pocket-binding domain, forming a novel layer in the 6-helix bundle structure. Prominently, the residue Asn624 of the 621VEWNEMT627 motif is engaged in the polar interaction with a hydrophilic ridge that borders the hydrophobic pocket on the N-terminal coiled coil. The original inhibitor design of CP32M provides several intra- and inter-helical salt bridge / hydrogen bond interactions favoring the stability of helical conformation of CP32M and its interactions with NHR targets. We identified a novel salt bridge between Arg557 on NHR and Glu648 of CP32M, which is critical for the binding of CP32M and resistance against the inhibitor. Therefore, our data present important information for developing novel HIV-1 fusion inhibitors for clinical use.

INTRODUCTION
Infection of human immunodeficiency virus type 1 (HIV-1) requires a fusion reaction between the viral and cellular membranes. This process is mediated by the viral envelope glycoprotein (Env), a trimeric complex consisting of three transmembrane gp41 subunits and three non-covalently attached gp120 surface subunits (1-4). The gp120 subunit is responsible for attachment to the target cells that initiates the entry process by binding to the CD4 receptor and a co-receptor (CCR5 or CXCR4), while the gp41 subunit mediates the membrane fusion. In the current fusion model, the gp41 undergoes a dramatic transition from a native state into a pre-hairpin intermediate that releases its hydrophobic ectodomain in an extended conformation thus allowing insertion of the fusion peptide into the targeting cell membrane (4-5). This state may persist over 15 min and then collapses into a low energy trimeric hairpin structure (six-helix bundle, 6-HB) that juxtaposes the viral and host membranes. The crystal structure of 6-HB revealed that three N-terminal heptad repeat (NHR) regions of gp41 form a central trimeric coiled coil, whereas three C-terminal heptad repeats (CHR) segments pack as
antiparallel helices into the hydrophobic grooves on the NHR trimer (6-8). Prominently, a deep hydrophobic pocket presents in the C-terminal portion of NHR helices, while three hydrophobic residues (Trp628, Trp631, and Ile635) from the pocket-binding domain (PBD) of CHR helix penetrate into the pocket causing an extensive interaction. It is believed that the deep pocket can serve as an ideal target site for anti-HIV agents that block the 6-HB formation (5,9). It is becoming clear that the 6-HB structure is involved in the fusion mechanism of many enveloped viruses (1,3,10-12). During the extended pre-hairpin conformation, peptide or small molecule-based fusion inhibitors act by competitive binding to the exposed NHR or CHR thus blocking the 6-HB formation dominant-negatively (5,13-15).

T20 (Enfuvirtide, Fuzeon) is the first and only clinically approved HIV-1 fusion inhibitor that being used for treatment of HIV/AIDS patients failed to respond to current antiretroviral drugs (16-18). However, T20 requires frequent administration and resistance develops rapidly. To overcome these challenges, a series of more potent and stable peptide fusion inhibitors have been developed (13,15,19) and some of them have been evaluated by clinical trials, such as Sifuvirtide and T1249 (20-23). Notably, these peptide inhibitors were primarily derived from the gp41 CHR region not including the upstream sequence of the pocket-binding domain (PBD). In particular, the peptide C34 (aa 628-661) was widely used as a design template (Fig. 1). This limitation might arise from the lack of structural and functional information to this region. Indeed, all three pioneering structures of HIV-1 gp41 core were determined by using biosynthetic or synthesized peptide fragments that lack the loop region between the putative NHR and CHR (6-8). However, our recent studies may be challenging this belief (24-26). We found that the $^{621}$QWNNMT$^{627}$ motif, located immediately adjacent to the PBD, plays critical roles for 6-HB formation and the peptide CP621-652 containing this motif is a potent fusion inhibitor against the wild-type and T20-resistant HIV-1 strains (24). Based on this finding, we engineered the CP621-652 into CP32M (Fig. 1), which shows very strong inhibition on HIV-1 with different genotypes and phenotypes, especially T20-resistant viruses (25).

In this study, we further show that CP32M possesses potent and broad inhibitory activity against a large panel of diverse HIV-1 variants. We determined the crystal structures of CP32M in complex with the NHR target. In stark contrast to the largely disordered N-terminal motif of the parental peptide CP621-652, the engineered $^{621}$VEWNEMT$^{627}$ motif of CP32M adopts two turns $\alpha$-helix at the N-terminus of the PBD thereby forming a novel layer in the 6-HB structure that stabilize the pocket region. Several intra- and inter-helical salt bridges and hydrogen bonds synthetically favor the helical conformation of CP32M and its interactions with NHR targets. Therefore, our data present the mechanistic insights into CP32M and provide important information for designing HIV-1 fusion inhibitors.

**EXPERIMENTAL PROCEDURES**

**Peptide synthesis** — Peptides CP32M
and T20 were synthesized by a standard solid-phase FMOC method as described previously (25). All peptides were acetylated at the N-terminus and amidated at the C-terminus. They were purified by reversed-phase high-performance liquid chromatography (HPLC) and verified for purity >95% and correct amino acid composition by mass spectrometry. Concentrations of the peptides were determined by UV absorbance and a theoretically calculated molar-extinction coefficient (280 nm) of 5500 M⁻¹·cm⁻¹ and 1490 M⁻¹·cm⁻¹ based on the number of tryptophan and tyrosine residues (all the peptides tested contain Trp and/or Tyr), respectively.

**Plasmids and cells** — Six CRF07_BC Env clones (CH064.20, CH070.1, CH091.9, CH110.2, CH119.10 and CH120.6) were generous gifts by Dr. Yiming Shao in the Chinese Center for Disease Control and Prevention, Beijing, China. A panel of Env clones derived from subtype B’ (B01, B02, B04, and 43-22), CRF01_AE (SHX335.24, YN192.31, AE01, AE03, GX2010.36, GX11.13, GX2010.36H, BJ5.11), and CRF07_BC (BC02, BC03, BC05, BC07, BC14, SC19-15, BJ22-5, YN148R-9, XJ50-6, HB5-3) were kindly provided by Dr. Youchun Wang from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. A panel of Env clones derived from subtype B’ (B01, B02, B04, and 43-22), CRF01_AE (SHX335.24, YN192.31, AE01, AE03, GX2010.36, GX11.13, GX2010.36H, BJ5.11), and CRF07_BC (BC02, BC03, BC05, BC07, BC14, SC19-15, BJ22-5, YN148R-9, XJ50-6, HB5-3) were kindly provided by Dr. Youchun Wang from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. TZM-bl cells were contributed by J.C. Kappes and X. Wu through the ARRRP, Division of AIDS, NIAID, NIH. This reporter cell line stably expresses high levels of CD4, CCR5, and CXCR4 and contains Tat-responsive reporter genes for firefly luciferase and β-galactosidase under the control of an HIV-1 long terminal repeat promoter (47). The cells were maintained in Dulbecco’s modified Eagle’s medium (D-MEM) growth medium (Gibco/Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS). Cells were harvested using trypsin/EDTA solution (Gibco/Invitrogen). All cell lines were maintained at 37°C in humidified air containing 5% CO2.

**Inhibition of HIV-1 single-cycle infection** — HIV-1 pseudoviruses were generated as described previously (32-33). Briefly, 293T cells (5 x 10⁶ cells in 15 ml of growth medium in a T-75 culture flask) were cotransfected with 10 μg of an Env-expressing plasmid and 20μg of a backbone plasmid pSG3Δenv that encodes Env-defective, luciferase-expressing HIV-1 genome using Lipofectamine 2000 reagent (Invitrogen). Pseudovirus-containing culture supernatants were harvested 48 hours after transfection and filtered by 0.45-mm pore size, and stored at -80°C in 1-ml aliquots until use. The 50% tissue culture infectious dose (TCID₅₀) of a single thawed aliquot of each pseudovirus batch was determined in TZM-bl cells. The antiviral activity of CP32M or T20 was determined using TZM-bl cells as described previously. Briefly, the peptides were prepared with ten series dilutions in a 3-fold stepwise manner and mixed with 100 TCID₅₀ viruses and incubated 1 hour at room temperature. The mixture was added to TZM-bl cells (10⁴/well) and incubation at 37°C for 48 hours. Luciferase activity was measured using luciferase assay regents (Promega, Medison, WI) and a Luminescence Counter (Promega) according to the manufacture’s instructions.
Inhibition of HIV-1 NL4-3 variants —
HIV-1 molecular clone NL4-3 wild-type (wt) or variants carrying naturally-occurring mutations (L33V, L34M, S35F, Q39R, L54M or Q56K) and T20-resistant mutations (L33S, I37Q/V38Q, I37Q/V38M, I37V/V38T) (29-30) were kindly provided by Dr. Frank Kirchhoff from the Institute of Virology, the University of Ulm, Ulm, Germany. The mutant viruses were generated by transient transfection of NL4-3 plasmids into 293T cells. The virus stocks were harvested 48 hours posttransfection and quantified for TCID<sub>50</sub>. Inhibition of the peptides (CP32M and T20) on the NL4-3 mutants was performed as described for pseudoviruses. In brief, 100 TCID<sub>50</sub> viruses were used to infect TZM-bl cells in the absence or presence of serially diluted peptides. Two days post-infection, the cells were harvested and lysed in reporter lysis buffer, and the luciferase activity was measured.

Protein expression and purification —
The 6-HB containing CP32M was prepared by expressing a NHR546-588/CP32M chimera. The cDNA encoding the chimera containing the N-terminal 6xHis tag, HIV-1 gp41 NHR546-588, a linker “SGGRGG” (with a trypsin cleavage site) and CP32M (MHHHHHHSSGLVPRGSAMADIGSGFSGIVQQQNLLRAIEAQQHLLQL TVWGIKQLQRICALAVERYLKSQGRGGVWENMTWMEWERIEINTKLIYKILESSEQQ) was synthesized and inserted into an expression vector pETH (derived from pET32a). The chimera was overexpressed in E.coli strain B834(DE3). Bacteria were grown in LeMaster Medium to an OD<sub>600</sub> nm of 1.0 and induced with IPTG (1mM) at 18 °C overnight. The bacteria were harvested by centrifugation, suspended in lysis buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl) and disrupted by ultrasonication. The inclusion body containing the fusion protein was collected by centrifugation and was solubilized in denaturing buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris HCl pH 8.0, 8 M urea) at 65°C for 20 minutes. The protein refolding was conducted by the dialysis against the buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl at 4°C overnight. The dialyzed sample was clarified by centrifugation and was purified with Ni-NTA resin (Qiagen). The N-terminal 6xHis-tag was eventually removed by thrombin (Sigma-Aldrich) digestion prior to the ion exchange chromatography (HighTrap Q, GE healthcare). The 6-HB was finally purified by size-exclusion chromatography (superdex75 10/300 GL, GE healthcare). The peak corresponding to the size of a 6-HB (~39kDa) was used for crystallization trials.

Circular dichroism spectroscopy —
The circular dichroism (CD) spectroscopy was performed as previously described (24). The final concentration of NHR546-588/CP32M was 10 μM in PBS buffer (pH 7.2). The CD spectra were acquired on Jasco spectropolarimeter (model J-815) using a 1 nm bandwidth with a 1 nm step resolution from 195 to 260 nm at room temperature. The spectra were corrected by subtraction of a blank corresponding to the solvent. Data were averaged over three accumulations. The α-helical content was calculated from the CD signal by dividing the mean residue ellipticity [θ] at 222 nm by the value expected for 100% helix formation.
(−33,000 degrees.cm².dmol⁻¹). The thermal denaturation experiment was performed by monitoring the change in ellipticity [θ] at 222 nm at the increasing temperature (20–98 °C) using temperature controller. The temperature was increased at a rate of 1.2°C per min; data were acquired at a 1 nm bandwidth at 222 nm at a frequency of 0.25 Hz. The melting curve was smoothened, and the midpoint of the thermal unfolding transition (Tm) values were taken as the maximum of the derivative d[θ]222/dT.

Crystallization and structure determination — The 6-HB assembled by NHR546-588/CP32M chimera was crystallized by mixing equal volumes (0.8 μl) of purified protein (~10 mg/ml) and reservoir solution in a hanging drop vapor diffusion system at 22 °C. Two crystal forms were found. Crystal form 1 crystallized using reservoir buffer containing 0.2 M MgSO₄ and 20% PEG3350; Crystal form 2 crystallized using the reservoir buffer containing 0.1 M MgCl₂, 0.1 M sodium acetate pH4.6 and 25% PEG400). Trypsin was added to sample (protease:sample = 1:100 in molar ratio) prior to the crystallization trials. The in situ trypsinization was essential for obtaining the measurable crystals. Cryocooling treatments for crystal form 1 and crystal form 2 were achieved by soaking the crystal 0.5-1 minute in the reservoir solution containing 30% ethylene glycol and 15% glycerol. Subsequently, the crystals were flash-frozen in liquid nitrogen. Complete datasets were collected for crystal form 1 and 2 at beamline PX III SLS (Villigen, Switzerland) using X ray of wavelength 0.9787 Å and 1.000Å. Crystal form 1 belonged to the space group of P321, contained one third of a 6-HB (one NHR546-588/CP32M chimera) per asymmetry unit (ASU), diffracted the X ray to the resolution limit of 2.0 Å. Crystal form 2 belonged to the space group of P2₁, contained one complete 6-helical bundle (three NHR546-588/CP32M chimera) per asymmetry unit (ASU), diffracted the X ray to the resolution limit of 2.0 Å. The structures of both crystal forms were solved by molecule replacement (Phaser CCP4 suite) using HIV-1 gp41 core structure (PDB ID 3F4Y) as searching model. The initial electron density map was improved by manual model building (coot). The structures were refined using PHENIX (48). The final atomic models have excellent refinement statistics and stereochemistry qualities (Table 3). To identify a hydrogen bond: the distance from donor hydrogen to the electronegative acceptor is ≤3.0Å and the angle “donor-H-acceptor” is ≥120°. To identify a salt bridge, the distance between the positively charged atom of basic residue (K or R) and negatively charged atom on acidic residues (E or D) is ≤4 Å.

RESULTS

Potent inhibition of CP32M on diverse HIV-1 variants — Our previous studies demonstrated that CP32M had potent inhibitory activity against several representative primary HIV-1 isolates from multiple genotypes (subtypes A-G and group O) and phenotypes (R5, X4 and R5X4) (25). To advance CP32M for clinical development, we are interested to know whether CP32M acts effectively against HIV-1 variants that are currently predominating AIDS epidemics in China, including CRF07_BC (B/C) and CRF01_AE (A/E) recombinants and B’
(also known as Tai B) (27-28). A panel of 27 HIV-1 pseudoviruses with their EnvS showing high polymorphisms were constructed and used in single-cycle infection assays. As shown in Table 1, CP32M can inhibit CRF07_BC (B/C), CRF01_AE and B' with mean IC\textsubscript{50} values at 12.03 nM, 16.94 nM and 10.64 nM respectively. In comparison, T20 has much lower inhibitory activity against these HIV-1 variants.

Some naturally-occurring variations in the NHR region of gp41 have been observed in treatment-naïve patients infected with various HIV-1 subtypes and shown resistance to HIV-1 fusion inhibitors (29). Here, we characterized the susceptibility of several naturally-occurring variants on CP32M. Table 2 shows that L33V, L34M and L54M mutations result in significant resistance to T20 (fold-change > 15), but have no or only mild effects on CP32M. Compared to the wild-type, the susceptibility of S35M mutant decreased 5.86-folds for T20 while decreased 2.51-folds for CP32M. Furthermore, we tested CP32M by several well-characterized T20-resistant viruses that carry L33S point-mutation or I37Q/V38Q, I37Q/V38M and I37V/V38T double-mutations (29-30). The results demonstrate that CP32M can efficiently inhibit infection by these HIV-1 variants; in a sharp contrast T20 has no inhibitory activity at a concentration as high as 750 nM. These results indicate that CP32M has potent and broad anti-HIV activity.

**Biophysical characterization of a 6-HB formed by NHR-CP32M chimera** — Our previous studies demonstrated that CP23M possesses the outstanding antiviral activity and forms the 6-HB structure with improved thermostability and helical content (25). In order to elucidate the molecular determinants, we utilized the crystallographic approach. We assembled the 6-HB by expressing a chimera comprising a gp41 NHR target sequence (NHR546-588), a linker and CP32M. The molecular weight of the purified NHR546-588/CP32M chimera determined by SDS-PAGE was ~12 kDa, whereas the molecular weight measured by size-exclusion chromatography was ~39 kDa (Fig. 2A and the insert) corresponding to the size of a trimeric chimera. The dynamic light scattering analysis measured the hydrodynamic radius of NHR546-588/CP32M chimera as 2.99 ± 0.44 nm corresponding to an apparent molecular weight of 43.9 ± 19 kDa, which indicates not only the size for a trimeric chimera but also the size for other possible oligomers of the chimera. The resolution of this experimental method cannot provide the defined oligomerization state of the chimera. The circular dichroism (CD) spectroscopy demonstrated that the chimera maintains highly helical conformation with ultra high thermostability in solution (helical content ~80%, T\textsubscript{m} ~100\degree C) (Fig. 2B and the insert). The above biophysical studies confirm that the recombinant chimera assemble into a 6-HB structure in solution.

**Crystallization and structure determination** — The purified 6-HB consisting of NHR546-588/CP32M chimera was subjected to crystallization trials. We discovered two different crystal forms. The crystal form 1 belonged to the space group of P3\textsubscript{2}1, contained one NHR546-588/CP32M chimera (1/3 6-HB) per asymmetrical
The crystal form 2 belonged to the space group of P2₁, contained three NHR546-588/CP32M chimeras assembling into a complete 6-HB per ASU. The redundancy of the diffraction data collected for crystal form 2 is significantly lower than that for crystal form 1, which coincides with the lower symmetry in crystal form 2 comparing to the crystal form 1. The three-fold symmetry found in crystal form 1 is not present in crystal form 2. This observation suggests the possible conformational differences between the two crystal structures. We solved the both structures by molecular replacement. The final refined model has excellent refinement statistics and stereochemistry qualities (Table 3). The structures of crystal form 1 and 2 were validated by MolProbity analysis (31). The MolProbity score for the crystal form 1 is 1.82, rating 86th percentile among structures of comparable resolution. The Ramachandran plot finds all residues in the favored area. The MolProbity score for the crystal form 2 is 1.64, rating 93rd percentile among structures of comparable resolution. The Ramachandran plot finds all residues in the favored area.

**Overall structure** — In both crystal form 1 and crystal form 2, CP32M and its NHR counterpart form a typical 6-HB structure as anticipated (Fig. 3 A,B). Three CP32M helices wrap around the outside of the NHR coiled coil trimer in an antiparallel orientation. The N-terminal “QIWNMT” motif of the parental peptide CP621-652 was engineered to “VEWNEMT” in CP32M (25). Our previous crystallographic study revealed that the “QIWNMT” motif is largely disordered but its residues Met626 and Thr627 folds into a unique M-T hook structure that stabilizes the hydrophobic pocket on NHR trimer (26). Surprisingly, the crystal structure of CP32M shows that the peptide is fully α-helical, in which the N-terminal “VEWNEMT” motif folds into two turns of α-helix targeting a novel region on NHR trimer (see details in the results below). However, the conformation of the additional α-helix is not as stable as the rest of 6-HB structure. The “VEWNEMT” motif is associated with high temperature factor in both crystal forms. The average temperature factor of this motif in both crystal forms ranges 60-61Å²; whereas the average temperature factor of the 6-HBs ranges 41-42Å². Interestingly, in the crystal form 2, the “VEWNEMT” motif of one particular CP32M peptide is largely disordered. The N-terminus of this peptide is directed away from central NHR core (Fig. 3B), thus, the internal three-fold symmetry of the 6-HB structure is lost (space group for crystal form 1 is P32₁, whereas the space group for crystal form 2 is P2₁). As shown in Fig. 4A, the unusual conformation of the particular CP23M molecule is caused by the collision with a nearby 6-HB in crystal. Although most of the residues on the “VEWNEMT” motif are disordered in the electron density, the conserved residues Met626 and Thr627 fold into a M-T hook structure similar to that observed in CP621-652 (26) (Fig. 4B). This exceptional structure suggests that the M-T hook could be another possible conformation for the “VEWNEMT” motif of CP32M if the preferred α-helical conformation is disrupted. In such case, the conserved residues...
Met626 and Thr627 could still contribute to the stability of 6-HB structure.

**The molecular determinants underlying the stability of the 6-HB structure** —

To stabilize the helical conformation of CP32M, charged residues were introduced to promote ion pairing between residues at i and i+4 positions during the inhibitor design (25). The crystal structure of CP32M confirms that the substitution of Asn636 by a glutamic acid, resulting the pairing between Glu636 and Lys640. The distance from Oε2 atom of Glu636 to the hydrogen donated by NZ atom of Lys640 is 2.14Å (angle, 147.1°), indicating a hydrogen bond interaction (Fig. 5A). A lysine substitution of Ser644 leads to the formation of a salt-bridge between Lys644 and Glu648 (3.8 Å). Intriguingly, we observed a novel intermolecular salt bridge between Glu648 and Arg557 on NHR helix (2.7Å), indicating a stronger salt bridging interaction. However, this salt bridge is not present in the crystal structure of CP621-652 regardless of that the Glu648 remained unchanged in the inhibitor design (see details in the results below). We previously identified a conserved salt bridge between Asp632 on CHR and Lys574 on NHR, which is critical for the HIV-1 entry and 6-HB stability (32-33). In CP32M design, Asp632 was changed to Glu632 to improve this salt bridge interaction. The crystal structure reveals that the longer side chain of Glu632 allows its carboxylate group to orient in a favored syn orientation to accept a hydrogen bond from the side chain of Lys574 (2.04 Å, 147.5°), which contribute to the inter-helical interaction between CP32M and its NHR target.

To summarize, our crystallographic study reveals the novel hydrogen bonds and salt bridges that contribute to the stability of the helical conformation of CP32M and its binding affinity to the NHR target. Especially, a single CP32M helix is able to salt bridging two NHR helices simultaneously, enhancing the overall stability of the 6-HB structure (Fig. 4B).

**The “VEWNEMT” motif targets a novel region on NHR trimer** —

Except one particular CP32M molecule in crystal form 2 clashing with a nearby molecule, resulted in the significant conformational rearrangement of the “VEWNEMT” motif, all other CP32M molecules in both crystal forms are fully α-helical. As shown in Fig. 6A, the N-terminal “VEWNEMT” motif extends the α-helical conformation by two turns, in which the side chains of Glu622, Trp623, Glu625 and Met626 face the exterior of the 6-HB; the side chains of Val621, Asn624 and Thr627 face the NHR target. The targeting region on NHR trimer involves a part of the hydrophobic pocket and a novel region adjacent to the pocket. The β-branched side chain of Thr627 (at “g” position of CP32M helix) is positioned on top of the bulky side chain of Trp571, stabilizing the hydrophobic pocket below (Fig. 6A, B). Residue Asn624 (at “d” position of CP32M helix) projects its side chain toward a remarkable hydrophilic region comprising of Gln575, Gln577, Arg579 and Glu584 on NHR trimer. This hydrophilic region is located next to the hydrophobic pocket, forming a high ridge that borders the deep pocket (Fig. 6A). The side chains of Arg579 and Glu584 (at “g” and “e” positions on NHR helix) on the ridge are tightly
connected to each other by two ideal hydrogen bonds (H-bond 1: 2.1 Å, 144.3°; H-bond 2: 2.1 Å, 168.1°), stabilizing the interaction between two NHR helices. Based on our current crystal structures, we cannot identify the well-defined intermolecular forces between the Asn624 and the residues on the hydrophilic ridge. However, the side chain of Asn624 is close to the side chain of Gln575 (at “c” positions on NHR helix), but their distance (from Nδ of Asn642 to Oε of Glu575) ranges 3.5-4.5 Å in both crystal forms, indicating a possible hydrogen bonding interaction. The molecular dynamic approach may be employed to further investigate the interaction between Asn624 and the residues on the hydrophilic ridge (Fig. 6A,B). Residue Val621 (at “a” position of CP32M helix) orients the side chain toward NHR trimer; however, the distance to the NHR counterpart (7-8 Å) is out of the range of any possible intermolecular forces.

Collectively, the “VEWNEMT” domain of CP32M and its NHR counterparts form an additional layer of the 6-HB structure (Fig. 5C). Three Leu576 residues form the hydrophobic core of NHR trimer that is locked tightly by three Arg584-Glu579 hasps (Glu579 belongs to the adjacent front layer) (Fig. 6A,C). The side chain of Thr627 stabilizes the hydrophobic pocket on NHR, and the side chain of Asn624 is in close contact with a hydrophilic ridge bordering the hydrophobic pocket on NHR trimer, which contributes to the stabilization of the 6-HB structure.

The structural basis for the inhibitor efficacy and drug resistance — CP32M is highly effective against HIV-1 strains that are resistant to T20 (25). One major determinant for T20 resistance is the mutation at the $^{36}$GIV$^{38}$ motif on the NHR of gp41 (reference to the gp41 numbering of HIV-1HXB2 for comparison, corresponding to the positions 547-549 in the gp160 numbering) (34). Our data demonstrate that HIV-1 bearing mutation V38T, V38Q, V38M or V38A is highly resistant to T20, C34 and SFT, but barely resistant to CP32M (25,35). The crystal structure of CP32M confirms that inhibitor does not bind the “GIV” motif, and thus the mutations occurring at this motif cannot influence the binding affinity of CP32M. Intriguingly, a combined mutation V38A/N42D induced ~18-folds of resistance against CP32M (25), whereas other substitutions of Asn42 (equivalent to Asn553), like V38A/N42T or N42S, did not contribute to CP32M resistance. These data suggest that Asn42 is one of the key residues for CP32M resistance. The crystal structures of CP32M reveal the underlying mechanism. As shown in Fig. 7, Asn553 on NHR helix does not interact with CP32M, thus cannot contribute the binding affinity of CP32M directly. An upstream residue Arg557 forms a salt bridge with Glu648 on CP32M, favoring the electrostatic interaction between CP32M and the NHR target (Fig. 4A,B and Fig. 7A). When Asn553 was replaced with the aspartic acid (modeled by coot, Fig. 7B), the negatively charged side chain of Asp553 could then form a salt bridge with the positively charge Arg557, neutralizing the charging on Arg557. Note that Arg557 and N553D are situated at $i$ and $i+4$ positions on the same NHR helix, the ideal positions for ion pairing, therefore this salt bridge
may pre-form before the CP32M coming into play. In such case, Glu648 of CP32M will then have little chance to pair with Arg557 on NHR, thus disfavoring the binding of CP32M to NHR target. Although Glu648 remains unchanged in the original inhibitor design, the corresponding salt bridge between Arg557 and Glu648 (distance=4.8Å) in the crystal structure of CP621-652 cannot be identified (Fig. 7C). This is likely due to the presence of the uncharged Ser644 in the parental sequence, which is unable to form a salt-bridge with Glu648. By contrast, in CP32M, mutation S644K not only leads to the salt bridge formation between Lys644 and Glu648, but also brings the side chain of Glu648 to the proximity of the side chain of Arg557, leading to the formation of the salt bridge between the residues. Therefore, the substitution of Ser644 by lysine offers two advantages for the efficacy of CP32M. First, forms an intra-helical salt-bridge to stabilize the helical conformation; second, promotes the formation of an inter-helical salt bridge to favor the binding of CP32M to the NHR target.

Recent studies have identified mutations of Gln64, Glu66 and Asn113 (equivalent to Gln575, Gln577 and Asn624, respectively) of gp41 are related to drug resistance against a number of HIV-1 fusion inhibitors, such as T2635, C34 and CP32M (36-37). Interestingly, these residues are involved in the interactions between the ‘VEWNEMT’ motif of CP32M and its targeting sites on NHR. Eggink et al found that Asn113 on the C-peptide of gp41 is in close contact with Gln66 on the N-peptide based on a theoretical model of gp41 ectodomain and the mutagenesis study (37). They concluded that the mutations at these positions to oppositely charged amino acids, such as Q66R/N113E allowing salt bridging, could speed up the formation of the fusogenic core of gp41, thus decrease the time window for T2635 to act (37). By contrast, our crystal structures show that Asn624 of CP32M is closer to Gln575 (3.5-4.5Å) rather than to Gln577 (7-8Å) on different NHR helices (Fig. 6A,C), suggesting a possible hydrogen bond interaction between Asn624 and Gln575. This observation is supported by the mutagenesis study by Yu et al that the HIV-1 bearing mutation Q575A or Q575L that presumably disrupt the hydrogen bond interaction between Asn624 and Gln575 is highly resistance to CP32M (36). They suggested a model for the interaction between Asn624 and Gln575, which is similar to our crystal structures. Therefore, the interaction between the key residue Asn624 on “VEWNENT” motif of CP32M and the hydrophilic ridge on NHR is critical for binding and the antiviral activity of the inhibitor. Collectively, the structural data provide the molecular basis for the improved inhibitor efficacy of CP32M comparing to its parental peptide as well as the mechanisms underlying the drug resistance.

**DISCUSSION**

In the previous study, we designed CP32M as a peptide HIV-1 fusion inhibitor on the basis of the structural and functional information of gp41 and a recently identified anti-HIV peptide (CP621–652) containing a motif (623QIWNNMT627) that is critical for the 6-HB formation and stability (24-25). We showed that CP32M has great
potential to serve as a unique anti-HIV drug for treatment of HIV variants that are resistant to the first generation fusion inhibitor T20. To develop CP32M for clinical use, we are currently performing preclinical studies for its safety and pharmacokinetic properties in animals. In this parallel leading study, we have firstly characterized its anti-HIV spectrum with a particular attention for HIV-1 strains that are currently circulating in China, including CRF07_BC (B/C) and CRF01_AE (A/E) recombinants and subtype B’ viruses that are responsible for a total nationwide infection of about 95% (27-28). The results demonstrate that CP32M has potent inhibitory activity against diverse HIV-1 variants, highlighting its broad-spectrum.

Resistance to T20 usually maps to the amino acid 36–45 region of the peptide binding site in the NHR domain of the viral gp41, with the 36GIV38 motif being a hotspot for resistance (14,34,38-40). However, our previous studies demonstrated that CP32M could efficiently inhibit T20-resistant HIV-1 variants that carry single or double mutations in the amino acid 36–45 region (e.g. V38A, V38A/N42T, N42T/N43K) (20). In this study, we have also evaluated CP32M for its anti-HIV spectrum with a panel of HIV-1 variants that carry naturally-occurring or induced T20-resistant mutations. From the results we can conclude that CP32M possesses broad inhibitory activity on the diverse HIV-1 mutants. Among the tested variants, L33S mutant was initially induced by the peptide C34 and showed conferring cross-resistance to T20, C34 and T1249 (30,37,41).

Therefore, our results here indicate further that CP32M maintains a high genetic barrier for resistance. We previously explained that the CP32M-targeting site does not contain the T20 and C34-resistance hotspots, and the N-terminal 621VEWNEMT627 motif plays a critical role for the interactions between CP32M and its NHR target (25).

The discovery of virus-derived peptide fusion inhibitors did open a bright avenue for exploring the mechanism of HIV-1 entry and for developing antiviral therapeutics (14,42-45). Several pioneering crystal structures deciphered the fusion-active 6-HB core of gp41, which delivered series stories about the membrane fusion of many viruses. On the basis of gp41 core structure, a number of new generation peptide inhibitors with improved antiviral activity and pharmacokinetic profiles have been developed. However, there are limited structural data available for the engineered peptides. We have recently reported the crystal structures of two fusion inhibitors (21,26). The structure of Sifuvirtide (SFT) confirms its fully helical conformation stabilized by the multiple engineered salt bridges, providing direct evidence to support the rational inhibitor designing strategy (21). The structure of CP621-652, the parental peptide of CP32M, reveals the key motifs and residues responsible for the stability and anti-HIV activity (26). Importantly, our structures found that the N-terminal 621QIWNNMT627 motif of CP621-652 is highly flexible but the residues Met626 and Thr627 form a unique hook-like structure (termed M-T hook) that plays the important roles in
the 6-HB stabilization and the antiviral activity (26). In this study, we continued our crystallographic studies for CP32M to visualize its molecular details at the atomic resolution.

In the designing rationale of CP32M (25), the N-terminal $^{621}$QIWNNMT$^{627}$ motif of CP621-652 template was engineered by changing three residues. The first residue Gln621 at the “a” position in the heptad repeat was replaced by a hydrophobic valine to enhance its hydrophobic interaction with the NHR target. The residues Ile622 and Asn625, which are located at the “b” and “e” positions in the helical wheel, were replaced by negatively charged glutamic acid to increase the hydrophilicity of the peptide. Surprisingly, our crystal structures show that the engineered “VEWNEMT” motif of CP32M becomes fully helical in one crystal form and predominantly helical in another crystal form. By adopting the helical conformation, the “VEWNEMT” motif folds into two turns of $\alpha$-helix, forming the extensive interaction layer with the pocket region of NHR helix. Previous structural studies have revealed that several distinguished layers of residues that are highly conserved in HIV-1 and SIV gp41, such as Thr/Trp, Gln50 and Gln40/Asn140 layers, which are critical for the stability of the 6-HB structure. The novel layer involving the residues on the “VEWNEMT” motif of CP32M presents another unique layer. Residues Gln575, Gln577, Arg579 and Glu584 form the hydrophilic ridge, severing as the C-terminal wall of the hydrophobic pocket. The hydrophilic ridge stabilizes the NHR helices by multiple inter-helical hydrogen bonds and mediates the interaction with the Asn624 of the “VEWNEMT” motif of CP32M helix via a possible hydrogen bond. Therefore, we believe that the novel layer can benefit the NHR-binding stability of CP32M and thus confer the antiviral activity. The “VEWNEMT” motif of one CP32M molecule in the crystal form 2 is largely disordered, albeit the M-T hook structure is well conserved to be identical to that of CP621-652. It is obvious that this unusual conformation was caused by the crystallographic artifacts (Fig. 4), further suggesting the flexibility of the CHR region upstream of the pocket binding domain. However, this feature may provide valuable strategy to engineer the peptide-based fusion inhibitors.

Indeed, three pioneering gp41 core structures were determined by taking the advantages of the limited proteolysis treatments that eliminate flexible or unstructured peptide loop (6-8), thus the linking region between the putative NHR and CHR of HIV gp41 are largely invisible. In other words, the upstream sequence of the PBD or the boundary of a C-helix could not be observed due to their intrinsic flexibility. Although the “VEWNEMT” motif in the CP32M was engineered to the $\alpha$-helical conformation, however, it is apparently not as stable as the rest part of the peptide. This is evidenced by the finding that the relatively weak crystal packing force could induce such a dramatic change that the $\alpha$-helical conformation was completely disordered. The distance between the extreme N-terminus of CP32M and its NHR counterpart are out of range of any type of molecular force (7-8Å), suggesting that N-terminal residues Val621 and Glu622 may not contribute to the binding affinity to the
NHR target significantly. This idea has been tested by our mutagenesis experiments that CP32M peptide missing two N-terminal residues does not suffer the significant losses of the antiviral activity (data not shown).

Interestingly, the analogous "WNNET" motif in SIV gp41 core appear to be folded into a helical structure while the amino acid sequence difference in this region is a glutamic acid instead of a Met626. In case of an M-T hook structure, the position 626 demands a hydrophobic amino acid to be able to cap the hydrophobic pocket on HIV gp41 NHR trimer. Thus, adopting the M-T hook-like structure to stabilize the 6-HB is not an option for the "WNNET" motif of SIV gp41. In the structure of SIV gp41, the conformation of "PKWNNET" motif and its interaction mode with the NHR are highly similar to that of "VEWNEMT" motif of CP32M. The side chain of Thr111 is positioned above the side chain of Trp59 on NHR pocket; the side chain of Asn108 points down to a hydrophilic region comprising Arg67, Glu72, Asn63 and Gln65. These data suggest that the "VEWNEMT" motif of CP32M utilizes a similar mechanism for targeting NHR helices as that utilized by SIV gp41 CHR. As two residues in the N-terminus of CP32M were substituted by glutamic acid, it will be interesting to investigate whether these negatively charged residues are the molecular determinants to the local conformation and thus affects the 6-HB structure and HIV-1 entry.

The first and secondary generation HIV-1 fusion inhibitors can easily induce drug resistance by single or multiple mutations at the inhibitor binding site that obstruct the docking of the peptide (15,19,37,46). By contrast, the third generation inhibitor T2635 is more difficult to induce drug resistance. Combined mutations at various gp41 domains, especially the mutations outside the binding site, were found important to escape T2635. The novel inhibitor CP32M belongs to the third-generation inhibitor, which is highly effective against T20-resistant HIV-1 strains. The careful characterization of CP32M resistance is certainly essential for the further studies of the inhibitor. Nevertheless, the available data have revealed at least two important mutations triggering CP32M resistance. The mutation positions 575 and 553 are located around the N- and C-terminal edges of CP32M binding site on NHR helices, suggesting these residues may play the key roles during the docking of CP32M. The Asn624 of "VEWNEMT" motif is engaged in the hydrophilic interaction with the hydrophilic ridge on NHR trimer and possibly forms a hydrogen bond with Gln575 on the ridge. Therefore, substitution of Gln575 by nonpolar residues could abolish the interaction, disfavoring the docking of the inhibitor. On the other side, our crystal structure demonstrates that Asn553 is clearly out of the binding site for CP32M. Therefore, a number of Asn553 substitutions (N42T and N42S) do not induce resistance against CP32M. The only mutation N42D caused the dramatic resistance against CP32M is due to the negatively charged aspartic acid side chain that may form a salt bridge with Arg557, thereby abolish an important inter-helical salt-bridge between Arg557 and Glu648 on CP32M,
which contributes significantly to the binding affinity of CP32M. This observation provides a novel mechanism causing resistance against HIV-1 fusion inhibitor. Mutation outside of the inhibitor binding site can indirectly influence the binding of the inhibitor to its target on NHR trimer by rearranging the salt-bridge interactions between inhibitor and its target.

In conclusion, our studies have further demonstrated that CP32M is a potent fusion inhibitor against diverse HIV-1 variants. We determined the high resolution crystal structure of CP32M, which identifies the key motifs and residues underlying the improved antiviral activity and stability of the inhibitor, and provides the structural basis for drug-resistance. Therefore, the present works have multiple implications for understanding the mechanism of HIV-1 fusion and for designing gp41 inhibitors.

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**FOOTNOTES**

**Accession codes**

Protein Data Bank: Coordinates and structure factors for the HIV-1 gp41 NHR546-588/CP32M crystal form 1 and 2 were deposited with the PDB ID: 3VGY and 3VH7

**Acknowledgements**

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FIGURE LEGENDS

Fig. 1. Schematic illustration of HIV-1 gp41 functional regions and NHR or CHR-derived peptide sequences. The residue numbers of each region corresponding to their positions in gp160 of HIV-1HXB2. FP, fusion peptide; NHR, N-terminal heptad repeat; CHR, C-terminal heptad repeat; PBD, pocket-binding domain; TM, transmembrane domain. The residues corresponding to the NHR pocket region are marked in magenta; the residues for the PBD are marked in yellow, the critical motif adjacent to the N-terminal of PBD are marked in red; the hotspot “GIV” motif for drug resistance on NHR are marked in green.

Fig. 2. Assembly and the biophysical characterizations of the 6-HB formed by gp41 NHR546-588/CP32M chimera. A. Size-exclusion chromatography and SDS-PAGE analyses of HIV gp41 NHR546-588/CP32M chimera. Chromatographic profile is UV absorbance at 280nm. Upper-right inset: the log (MW) values of the standard proteins for size-exclusion column calibration (Superdex75 10/300 GL) are plotted as the function of Ve (elution volume) (•). The data are fitted linearly to derive standard curve. The molecular mass of HIV gp41 NHR546-588/CP32M chimera is calculated as ~39 kDa (○). Upper-left inset: SDS-PAGE analysis of purified HIV gp41 NHR546-588/CP32M chimera. Lane 1, HIV gp41 NHR546-588/CP32M chimera (~12kDa); lane 2, molecular weight standards. B. The melting curve of NHR546-588/CP32M chimera showing the thermostability of NHR546-588/CP32M chimera. Insert, normalized circular dichroism spectrum showing the α-helical conformation of the 6-HB formed by the chimera.

Fig. 3. Overall structure of the 6-HB structures formed by HIV gp41 NHR546-588/CP32M chimera. Ribbon models of two 6-HB structures formed by NHR546-588/CP32M chimera. The NHR trimers are colored in gra, the CP32M peptides are colored in blue and the “VEWNEMT” motifs are colored in orange with the labels. The same color scheme of the 6-HB is used in all following figures. The CP32M molecule with the disordered N-terminal motif is marked with an asterisk. A. Crystal form 1 (space group P321). Left, side view; right, top view. B. Crystal form 2 (space group P21). Left, side view; right, top view.

Fig. 4. Crystal packing interactions caused the conformational rearrangement of the N-terminal motif of one particular CP32M in crystal form 2. A. Ribbon model of a 6-HB formed by NHR546-588/CP32M chimera (positioned horizontally) collided by a nearby 6-HB (colored in olive), causing the conformational rearrangement of the N-terminal “VEWNEMT” motif of one particular CP32M. The collision site is highlighted with a red box. B. The magnification of the red-boxed area in panel A. The “VEWNEMT” motif of the CP32M peptide is largely disorderd, whereas the conserved residues Met626 and Thr627 adopt the M-T hook like structure similar to that found in the crystal structure of CP621-625 (26). The M-T hook
residues and the residues interacting with the M-T hook are shown in stick model with the labels.

**Fig. 5. The molecular determinants underlying the stability of the helical conformation of CP32M and the 6-HB structure.**  
*A.* Ribbon model of the 6-HB formed by NHR546-588/CP32M chimera with the charged residues forming the intra- and inter-helical salt bridges / hydrogen bonds shown in stick model with the labels. The salt bridges / hydrogen bonds are indicated with the dashed lines.  
*B.* The sequence of CP32M with various features indicated. A single CP32M peptide is interacting with two NHR helices (white bars). The changed residues during the inhibitor design are colored in red; the unchanged residues are colored in black. The “VEWNEMT” motif is highlighted by orange background. The intra-helical salt bridges / hydrogen bonds observed in the crystal structures are indicated with the solid lines; the inter-helical salt bridges / hydrogen bonds observed in the crystal structures are indicated with the dashed lines.

**Fig. 6. The “VEWNEMT” motif of CP32M targets a hydrophilic region on NHR trimer and form a novel layer of the 6-HB structure.**  
*A.* A portion of the ribbon model of 6-HB structure form by NHR546-588/CP32M chimera (positioned horizontally). The residues on the N-terminal “VEWNEMT” motif of CP32M, the residues of the pocket-binding domain and the residues of NHR binding site of CP32M are shown in stick model with the labels. The hydrophilic ridge comprising Gln575, Gln577, Glu584 and Arg579 are colored in yellow. The hydrophobic pocket on NHR is indicated. The hydrogen bonds 1 and 2 between Glu584 and Arg579 are indicated with black dashed lines. The possible hydrogen bond between Asn624 and Gln575 is indicated with red dashed line.  
*B.* Helical wheel presentation showing the interaction between CP32M and NHR trimer. Three NHR helices and one CP32M helix are shown in helical wheel projections. The view is from the bottom of the 6-HB. Three NHR helices form the central coiled coil, and a CP32M helix is packed against the inter-helical groove between two NHR helices. At the top of the complex, the N-terminal of CP32M (gray shading) is slightly tilted toward the upper NHR helix, while at the bottom of the complex, the C-terminal of the CP32M is tilted toward the lower NHR helix. The color code for the residues is: black, hydrophobic; orange, uncharged; blue, positively charged; red, negatively charged. Residues on the “VEWNEMT” motif of CP32M are labeled with the residue numbers. Residues mutated from the parental sequence to generate CP32M are highlighted by yellow background. Red dash lines indicate the possible hydrogen bonds between Asn624 on CP32M and Gln575 on NHR helices. Black dashed lines indicates the hydrogen bond between Glu584 and Arg579.  
*C.* The “VEWNEMT” motif of CP32M forms the novel layer of the 6-HB. Three Leu576 form the hydrophobic core of NHR trimer that is locked by three Glu584-Arg579 “hasps” (the side chain of Glu584 comes from the front layer, thus not visible here, see panel A). Gln575, Gln577, Glu584 and Arg579 form a hydrophilic region on NHR trimer, which mediates the hydrophilic interaction with Asn624 on the “VEWNEMT” motif of CP32M. The possible hydrogen bond
between Asn624 and Gln575 are indicated with red dashed lines.

**Fig. 7. Molecular basis of the a key residue that are critical for CP32M resistance.**  
**A.** A portion of a ribbon model of the 6-HB structure formed by NHR546-588/CP32M chimera. Residues that are important to CP32M resistance and the interaction with NHR helix are shown in stick model. Glu648 and Lys644 form an intra-helical salt bridge on CP32M and Glu648 forms another inter-helical salt bridge with Arg557 on NHR helix. The salt bridges are indicated with the dashed line.  
**B.** The model (generated by coot and Pymol) of the 6-HB structure formed by NHR546-588/CP32M chimera with mutation N553D (equivalent to N42D). The assumed salt bridge between Asp553 and Arg557 (indicated with red dashed line) will neutralize the positive charging of Arg557, thus disrupt the inter-helical salt bridge between Arg557 and Glu648 on CP32M, disfavoring the electrostatic interaction between CP32M and is NHR target.  
**C.** A portion of a ribbon model of the 6-HB structure formed by T21/CP621-652 peptide (PDB ID: 3VGX). The NHR helices are colored in gray and CP621-652 peptide are colored in green. The corresponding residues in panel A, B are shown in stick model with the labels. The corresponding salt bridge between Arg557 and Glu648 (distance 4.8Å) cannot be identified in this structure.
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Table. 3 Data collection and refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>NHR546-588/CP32M complex Crystal form 1</th>
<th>NHR546-588/CP32M complex Crystal form 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P32_1</td>
<td>P2_1</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>45.09, 45.09, 73.03</td>
<td>50.51, 45.50, 55.51</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90.0, 90.0, 120.0</td>
<td>90.0, 107.65, 90.0</td>
</tr>
<tr>
<td>X ray source</td>
<td>PSI-SLS BEAMLINE PX III</td>
<td>PSI-SLS BEAMLINE PX III</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.9787</td>
<td>1.0000</td>
</tr>
<tr>
<td>Data range (Å)</td>
<td>39.05-2.03</td>
<td>42.61-2.02</td>
</tr>
<tr>
<td>R&lt;sub&gt;sym&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (last shell)</td>
<td>0.079 (0.63)</td>
<td>0.084 (0.75)</td>
</tr>
<tr>
<td>I / σI</td>
<td>16.42 (2.89)</td>
<td>7.8 (1.2)</td>
</tr>
<tr>
<td>Completeness (%) (last shell)</td>
<td>99.8 (98.8)</td>
<td>90.8 (63.3)</td>
</tr>
<tr>
<td>Redundancy (last shell)</td>
<td>5.77 (5.61)</td>
<td>1.97 (1.24)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>39.05-2.03</td>
<td>42.61-2.02</td>
</tr>
<tr>
<td>Reflections, cutoff, cross validation</td>
<td>5846, F&gt;1.99, 529</td>
<td>14944, F&gt;1.45, 748</td>
</tr>
<tr>
<td>R&lt;sub&gt;work&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; / R&lt;sub&gt;free&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt; (last shell)</td>
<td>0.1969 / 0.2590</td>
<td>0.2216 / 0.2741</td>
</tr>
<tr>
<td>Non hydrogen protein atoms</td>
<td>708</td>
<td>2181</td>
</tr>
<tr>
<td>Protein</td>
<td>686</td>
<td>2113</td>
</tr>
<tr>
<td>Water</td>
<td>22</td>
<td>68</td>
</tr>
<tr>
<td>B-factors average</td>
<td>42.17</td>
<td>41.19</td>
</tr>
<tr>
<td>Protein (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>42.25</td>
<td>41.16</td>
</tr>
<tr>
<td>Water (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>37.04</td>
<td>38.77</td>
</tr>
<tr>
<td>r.m.s.d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.014</td>
<td>0.002</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>1.359</td>
<td>0.545</td>
</tr>
<tr>
<td><strong>Validation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MolProbity score</td>
<td>1.82, rating 86&lt;sup&gt;th&lt;/sup&gt; percentile among structures of comparable resolution</td>
<td>1.64, rating 93&lt;sup&gt;nd&lt;/sup&gt; percentile among structures of comparable resolution</td>
</tr>
<tr>
<td>% residues in favored regions, allowed regions, outliers in Ramachandran plot</td>
<td>100.0, 0, 0</td>
<td>100.0, 0, 0</td>
</tr>
</tbody>
</table>

<sup>a</sup> R<sub>sym</sub> = Σ<sub>hkl</sub>Σ<sub>j</sub> |I<sub>hlk,i</sub> - I<sub>hlk</sub>| / Σ<sub>hkl</sub>Σ<sub>j</sub>I<sub>hlk,i</sub>, where I<sub>hlk</sub> is the average of symmetry-related observations of a unique reflection.

<sup>b</sup> R<sub>work</sub> = Σ<sub>hkl</sub> |F<sub>obs</sub>(hkl)| - |F<sub>calc</sub>(hkl)| / Σ<sub>hkl</sub>|F<sub>obs</sub>(hkl)|.

<sup>c</sup> R<sub>free</sub> is the cross-validation R factor for 5% of reflections against which the model was not refined.
Fig. 2

A

Retention Volume (ml)

[1 2 66 45 35 25 18.4 14.4]

~39kDa

B

[θ 22] (10^3 deg cm^2 dmol^-1 res^-1)

25 50 75 100

-25 -20 -15 -10

Temperature °C

-20 -15 -10
Fig. 4

A

Nearby 6-HB

“VEWNEMT” motif

NHR trimer

B

“VEWNEMT” motif

NHR trimer
Fig. 5

A

NHR trimer

VEWNEMT motif

B

C'  K574  NHR  i, i+4

N'  621  VEWNEMTWMEWEREIENYTKIYLEEQ  652  C'

N'  R557  C'

C'  NHR  K574
Structural basis of potent and broad HIV-1 fusion inhibitor CP32M
Xue Yao, Huihui Chong, Chao Zhang, Zongliu Qiu, Bo Qin, Ruiyun Han, Sandro Waltersperger, Meitian Wang, Yuxian He and Sheng Cui

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