A novel HIV-1 inhibitor that blocks viral replication and rescues APOBEC3s by interrupting vif/CBFβ interaction

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HIV remains a health challenge worldwide, partly because of the continued development of resistance to drugs. Therefore, it is urgent to find new HIV inhibitors and targets. Apolipoprotein B mRNA-editing catalytic polypeptide-like 3 family members (APOBEC3) are important host restriction factors that inhibit HIV-1 replication by their cytidine deaminase activity. HIV-1 viral infectivity factor (Vif) promotes proteasomal degradation of APOBEC3 proteins by recruiting the E3 ubiquitin ligase complex, in which core-binding factor β (CBFβ) is a necessary molecular chaperone. Interrupting the interaction between Vif and CBFβ can release APOBEC3 proteins to inhibit HIV-1 replication and may be useful for developing new drug targets for HIV-1. In this study, we identified a potent small molecule inhibitor CBFβ/Vif-3 (CV-3) of HIV-1 replication by employing structure-based virtual screening using the crystal structure of Vif and CBFβ (PDB: 4N9F) and validated CV-3’s antiviral activity. We found that CV-3 specifically inhibited HIV-1 replication (IC50 = 8.16 μM; 50% cytotoxic concentration >100 μM) in nonpermissive lymphocytes. Furthermore, CV-3 treatment rescued APOBEC3 family members (human APOBEC3G (hA3G), hA3C, and hA3F) in the presence of Vif and enabled hA3G packaging into HIV-1 virions, which resulted in Gly-to-Ala hypermutations in viral genomes. Finally, we used FRET to demonstrate that CV-3 inhibited the interaction between Vif and CBFβ by simultaneously forming hydrogen bonds with residues Gln-67, Ile-102, and Arg-131 of CBFβ. These findings demonstrate that CV-3 can effectively inhibit HIV-1 by blocking the interaction between Vif and CBFβ and that this interaction can serve as a new target for developing HIV-1 inhibitors.

Host restriction factors play an important role in innate immune responses that protect cells from viral pathogens such as HIV (1, 2). Apolipoprotein B mRNA-editing catalytic polypeptide-like 3 family members (APOBEC3), an intracellular family of cytidine deaminases, are important host restriction factors (3–5). The APOBEC3 family has seven members: A3A, A3B, A3C, A3DE, A3F, A3G, and A3H. A3G was first demonstrated to inhibit HIV-1 replication in the absence of viral infectivity factor (Vif) (3). A3G can be packaged into virions by binding to HIV-1 Gag and host RNA (6–8). A3G packaged into the virions can then interfere with reverse transcription (9, 10) and prevent the integration of the viral genome (11, 12). Most importantly, A3G can catalyze the hypermutation of the viral genome from Gly to Ala, thus leading to highly efficient interruption of HIV-1 replication (12–15). Other members of the A3 family, such as A3F, A3H, and A3D, also inhibit HIV-1 replication by Gly-to-Ala hypermutations (15–18).

The Vif protein expressed by HIV-1 can antagonize the antiviral activity of APOBEC3s by proteasome degradation (3, 19–22). The Vif-hijacked E3 ubiquitin ligase is composed of the scaffold protein Cul5n (Cul5) and adaptor proteins Elongin B (EloB) and Elongin C (Eloc) (23, 24); the specific cofactor core-binding factor beta (CBFβ) plays a critical role in stabilizing Vif and its assembly with the ligase (25, 26). It has been reported that Vif cannot degrade A3G and other Vif-sensitive A3s without CBFβ (27–29). The crystal structure of the Vif-EloBC-Cul5-CBFβ complex (PDB: 4N9F) was resolved, and the protein-protein interactions in the complex were elucidated. The interaction interface between CBFβ and Vif is extensive, and the Vif N terminus forms an antiparallel β-sheet with the β-strand S3 of CBFβ (30). This is consistent with previous reports that the N terminus of Vif is an important region for the interaction of CBFβ (29, 31, 32). A subsequent report showed that a tripartite interaction of Ile-55 and Phe-68 of CBFβ and Trp-5 of Vif is critical for Vif-CBFβ binding (33).

Various inhibitors targeting Vif function have been identified, but no small molecule compounds targeting Vif-CBFβ have been reported (34). O2-16 inhibits Vif oligomerization by targeting the Vif PPLP region (35). Some inhibitors block the binding of Vif-A3G, such as IMB-26/35 (36), N4.1 analogs (37), and analogs 14/26 (38). There are also some small molecule inhibitors that target the Vif-EloC interaction, including VEC-5 (39), ZBMA-1 (40), analogs 12c/13a (41), and Zif-15 (42). Here, we aimed to identify small molecule inhibitors that interrupt the Vif-CBFβ interaction, which can release not only APOBEC3s but also Vif-hijacking proteins in the E3-ubiquitin ligase complex, such as Cul5, EloB, and EloC.

By employing virtual screening based on the crystal structure of the Vif-CBFβ complex (PDB: 4N9F), we identified a small molecule compound called CV-3 that inhibited the interaction between Vif and CBFβ. CV-3 was found to inhibit HIV-1 replication (IC50 = 8.16 μM) and have a low cytotoxicity (50% cytotoxic concentration (CC50) > 100 μM). CV-3 can rescue A3G, promote A3G packaging into virions, and induce Gly-to-Ala hypermutation of the viral genome. FRET, yeast surface display,
and co-immunoprecipitation (Co-IP) experiments confirmed that CV-3 specifically blocked the binding of Vif-CBFβ. The results indicate that small molecule compound CV-3 can release APOBEC3 proteins by blocking the Vif-CBFβ interaction to achieve an antiviral effect; therefore, it could be used as a potential novel anti-HIV-1 drug candidate.

**Results**

**CV-3 protects APOBEC3G from Vif-mediated degradation and decreases HIV-1 infectivity**

Because the N terminus of Vif is a key region for the interaction between CBFβ and Vif (30) and the tripartite interaction of Ile-55 and Phe-68 of CBFβ and Trp-5 of Vif is critical for Vif-CBFβ binding (33), it is desirable to screen for small molecules capable of blocking this region through structure-based virtual screening. The N-terminal of Vif is a linear structure that binds to a shallow pocket on the N-terminal of CBFβ. Based on this structure feature, CBFβ (full sequence) was defined as the receptor. The virtual screening process is described in detail under “Experimental procedures”. In brief, the binding of Vif to CBFβ (PDB: 4N9F) was the target of our study. The shallow surface pocket on CBFβ coincides with the position of Vif residues 5–11 in the complex. Thus, the “binding site” was defined as the pocket on CBFβ that form hydrogen bonds with the compounds. The LigandFit program was run by Discovery studio 2.5. The predicted binding free energy that was calculated by the “calculate binding energy” protocol and residues on CBFβ that form hydrogen bonds with the compounds are marked. In the “calculate binding energy” protocol, “in situ ligand minimization” and “ligand conformational entropy” were selected as “true.” “Implicit solvent model” was selected as “distance-dependent dielectrics.”

![Figure 1. Structure-based virtual screening of small molecule inhibitors of Vif-CBFβ.](image)

**A**, virtual screening process. The small molecule compound database was docked to the binding site defined on CBFβ. First, the LigandFit program was performed with three partitions, and then the small molecule compounds successfully docked were scored by the consensus score. Small molecules with a score of 6 or more were further subjected to the CDOCKER procedure. The small molecule compounds with higher scores were selected for visual screening, and 7 small molecule hits were selected. The blue, red, and green meshed regions are the binding sites on CBFβ (purple) in the LigandFit program, and the translucent red sphere is the docking site in the CDOCKER program. **B**, the structures of candidate molecular compounds. The virtual screen was run by Discovery studio 2.5. The predicted binding free energy that was calculated by the “calculate binding energy” protocol and residues on CBFβ that form hydrogen bonds with the compounds are marked. In the “calculate binding energy” protocol, “in situ ligand minimization” and “ligand conformational entropy” were selected as “true.” “Implicit solvent model” was selected as “distance-dependent dielectrics.”
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**A**

|       | NC   | Blank | CV-1 |
|-------|------|-------|------|
| CV-2  | 3.56%| 0.01% | 1.97% |
| CV-3  | 1.45%| 0.84% | 0.86% |
| CV-4  | 1.27%| 0.52% | 1.51% |

**B**

|       | Blank | NC | CV-1 |
|-------|-------|----|------|
| CV-2  | 40 µm | 40 µm | 40 µm |
| CV-3  | 40 µm | 40 µm | 40 µm |
| CV-4  | 40 µm | 40 µm | 40 µm |

**C**

|        | Blank | NC | CV-1 | CV-2 | CV-3 | CV-4 | CV-5 | CV-6 | CV-7 |
|--------|-------|----|------|------|------|------|------|------|------|
| A3G    |       |    |      |      |      |      |      |      |      |
| kDa    | 75    | 50 |      |      |      |      |      |      |      |
| Vif    | 1.0   | 0.1| 0.9  | 0.7  | 0.9  | 0.9  | 0.9  | 0.2  |
| Tubulin|       |    |      |      |      |      |      |      |      |
| Vif    |       |    |      |      |      |      |      |      |      |
| VR1012 |       |    |      |      |      |      |      |      |      |

**D**

|       | Blank | NC | CV-1 | CV-2 | CV-3 | CV-4 | CV-5 | CV-6 | CV-7 |
|-------|-------|----|------|------|------|------|------|------|------|
| Virus infectivity (%) | 100 | 75 | 50  | 25  | 75  | 50  | 75  | 50  | 75  |

**E**

- **Cell viability (%)**
  - CV-1:
    - 0: 100%
    - 25: 75%
    - 50: 50%
    - 75: 25%
    - 100: 0%

- **Virus infectivity (%)**
  - CV-3:
    - 0: 100%
    - 25: 75%
    - 50: 50%
    - 75: 25%
    - 100: 0%

**F**

- **Cell viability (%)**
  - CV-1:
    - 1: 100%
    - 10: 75%
    - 100: 50%
    - 1000: 25%
    - 10000: 0%

- **Virus infectivity (%)**
  - CV-3:
    - 1: 100%
    - 10: 75%
    - 100: 50%
    - 1000: 25%
    - 10000: 0%

**Addendum**

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Compounds are shown in Fig. 1B. All of the candidates have very low predicted biotoxicity (LD_{50} 3.6 g/kg), and most of them form H bonds with residues Gln-67, Phe-69, Arg-83, and Arg-131 of CBFb.

To determine the candidate compounds’ ability to block Vif-CBFβ, Vif was displayed on the surface of the yeast cell strain EBY100. The cells were incubated with CBFb protein for 4 h with a 100 µM concentration of candidate compounds or Figure 3. CV-3 specifically inhibits HIV-1 in Vif nonpermissive cells. A, CV-3 showed A3G-dependent inhibition of viral infectivity in HIV-1 chronically infected cells. 5 × 10^5 chronically infected Jurkat/HXB2 cells (left) and H9/HXB2 cells (right) in a 12-well plate were treated with 50 µM CV-3 for 48 h. Viral infectivity was tested by infection of TZM-bl cells (mean ± S.D. (error bars); triplicate experiments). Statistical significance was assessed by Student’s t test; *p < 0.05; **p < 0.001; ***p < 0.0001; NS, not significant. B, CV-3 dose-dependently inhibited HIV-1 replication. H9/HXB2 cells (5 × 10^5 in a 6-well plate) were cultured for 48 h with different concentrations of CV-3, and the infectivity of the virus was detected in TZM-bl cells (mean ± S.D. (error bars); triplicate experiments). C, CV-3 restricted HIV-1 replication in nonpermissive cells. WT or vif-defective HIV-1 NL4-3 was produced from 293T cells after transfection with pNL4-3 or pNL4-3Δvif. CEM, CEM-SS, SupT1-A3G, and SupT1 cells were then infected with these viruses, and viral production was monitored at the indicated time points using a p24 ELISA Kit for 12 days. D, IC_{50} of CV-3 in CEM cells. NL4-3 infected CEM cells were cultured with the indicated concentrations of CV-3 for 7 days, and the infectivity was detected by a p24 ELISA Kit.
DMSO. After binding to the CBFβ antibody, the cells were incubated with FITC-IgG antibody. The binding of Vif-CBFβ was analyzed by measuring FITC intensity via flow cytometry (Fig. 2A). The results showed that all of the candidates can inhibit Vif-CBFβ interaction significantly. Next, the protective effect of compounds toward A3G in the cytoplasm was determined. 293T cells were transfected with A3G-YFP and Vif-HA. After 4 h of transfection, 100 μM small molecule compounds or DMSO were added. The expression of A3G-YFP was observed under a fluorescence microscope (Fig. 2B), and protein levels in cell lysates were analyzed by Western blotting (Fig. 2C) 48 h later. For both of these methods, the expression levels of A3G with CV-5 or CV-7 were comparable with the DMSO negative control (Fig. S1). The antiviral activities of the candidates were then examined. After infecting CEM cells with the HIV-1 NL4-3, the cells were treated with 100 μM of candidate compounds or DMSO for 6 days. The viral infectivity was then tested using TZM-bl cells. During 6 days of culture incubation, no additional drugs were added to the culture medium so the inhibition ability detected would be weaker. Only CV-1 and CV-3 significantly decreased virus infectivity (Fig. 2D). To explore the cytotoxicity of the compounds, cell viability was monitored by cell counting after incubating HIV-1-infected CEM cells at different concentrations of CV-1 and CV-3 for 6 days, and the infectivity of the produced virus was determined (Fig. 2E). Treatment with CV-3 did not affect cell viability, and the infectivity was dose-dependently decreased. Virus infectivity only declined at 100 μM CV-1, which suggested that CV-1 reduced the infectivity of HIV-1 due to its cytotoxicity. In addition, the CC50 of CV-1 (87.5 μM) was much lower than that of CV-3 (264.9 μM) in CEM cells (Fig. 2F). Therefore, CV-3 was selected for subsequent investigations.

**APOBEC3 specificity of the Vif-mediated antiviral effect of CV-3**

Vif expression is essential for HIV-1 derived from nonpermissive cells, including cell lines H9 and CEM, whereas the virus produced from permissive cells, such as CEM-SS, Jurkat, and SupT1 cells, is fully infectious regardless of the presence of Vif (43). To further examine whether the antiviral activity of CV-3 is APOBEC3-dependent, H9 and Jurkat cells stably infected with HIV-1 HXB2 were incubated with 50 μM CV-3 or DMSO for 48 h. The infectivity was assayed using TZM-bl cells. Notably, only the infectivity of virus produced from nonpermissive cell line H9/HXB2 was inhibited by CV-3 (Fig. 3A). This indicated that the antiviral function of CV-3 was related to the expression of APOBEC3s. Moreover, as the concentration of CV-3 became higher in the H9/HXB2 culture system, the viral infectivity gradually weakened, which showed that the antiviral function of CV-3 was dose dependent (Fig. 3B).

We next evaluated the effect of CV-3 on viral replication in different human T lymphocyte lines. Equal amounts of NL4-3 or NL4-3ΔVif were utilized to infect permissive cell lines (CEM-SS and SupT1) and nonpermissive cell lines (CEM and SupT1-A3G). After infection, the cells were cultured in the medium with different concentrations of CV-3. In the following 12 days, p24 antigen in the supernatants was quantified to construct virus replication curves (Fig. 3C). Both HIV-1 and HIV-1ΔVif were able to replicate at the same level in permissive cell
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Figure 5. CV-3 recovers the antiviral activity of A3G. A, CV-3 increased the level of A3G packaged into virions. 293T cells were transfected with A3G-cmyc and pNL4-3 or pNL4-3ΔVif as indicated and then treated with 10 or 50 μM CV-3 or DMSO. After 48 h, the cells and pelleted virions were examined for A3G expression. Pr55 Gag was examined as a transfection control. GAPDH and p24 were detected as a loading control. A3G of the pNL4-3ΔVif group was set to 100%, and the expression of A3G in cell lysates or virions was normalized by the level of GAPDH or p24. Protein expression levels determined by Western blotting were quantified by Quantity One. B, infectivity of the virus in panel A was determined using TZM-bl cells (mean ± S.D. (error bars); triplicate experiments). C, CV-3 treatment recovered the Gly-to-Ala hypermutation induced by A3G. TZM-bl cells were infected with the viruses for 48 h, and then genomic DNA was extracted. An 885-bp fragment of pol was amplified by nested PCR, then ligated to the T-easy cloning vector for sequencing (mean ± S.D. (error bars); n = 10). Statistical significance was assessed by Student’s t test; *p < 0.05; **p < 0.001; ***p < 0.0001; NS, not significant.

CV-3 protects APOBEC3s from Vif-induced degradation and enhances A3G incorporation into HIV-1 particles

Blocking the interaction of Vif-CBFβ results in the inability to form the E3 ubiquitin ligase complex; therefore, CV-3 should be able to protect not only A3G but also other proteins of the APOBEC3 family (26, 28, 29). To verify this, the protective effect of CV-3 on hA3C, hA3F, and hA3G was examined. hA3C-FLAG, hA3F-V5, or hA3G-cmyc was cotransfected with Vif-HA into 293T cells. After incubation with 10 or 50 μM CV-3 for 48 h, the expression of hA3C, hA3F, and hA3G in cells treated with CV-3 was rescued to comparable levels as the DMSO control group in the presence of Vif (Fig. 4, A–C). The feline immunodeficiency virus (FIV) Vif can degrade feline APOBEC3s without CBFβ as a molecular chaperone (47–49). We found that feline A3Z2 (fA3Z2)-cmyc (Fig. 4D) and fA3Z3-cmyc (Fig. 4F) could not be recovered by CV-3. The results suggested that CV-3 could release other APOBEC3 proteins by blocking Vif-CBFβ.

APOBEC3 proteins could be packaged into the HIV-1 budding virus. In the next round of infection, the cytidine deamination of APOBEC3s induces Gly-to-Ala hypermutation of the viral single-stranded cDNA, thereby inhibiting HIV-1 replication (6, 7, 13). To verify whether CV-3 can enhance the encapsidation of A3G, A3G was cotransfected into 293T cells with pNL4-3 or pNL4-3ΔVif. Then, 10 or 50 μM of CV-3 or DMSO was added into the cell culture and incubated for 48 h. After incubation, the cells were harvested and the virus particles in the supernatant were obtained by ultracentrifugation through a 20% sucrose cushion. The expression of A3G in cell lysates and virions was analyzed by Western blotting. The IC50 of CV-3 detected in CEM cells was ~8.16 μM (Fig. 3D). In addition, the CC50 detected by the MTT method was greater than 100 μM in all utilized lymphocyte lines (Fig. S3).
was amplified by nested PCR and was ligated into a T-easy vector for sequencing. The numbers of Gly-to-Ala mutations in HIV-1ΔVif and CV-3-treated WT HIV-1 group were significantly higher than in the HIV-1 control group. Moreover, there was no significant difference in hypermutation levels between HIV-1ΔVif and CV-3-treated HIV-1 (Fig. 5C). The results indicated that the addition of CV-3 enabled A3G to be packaged into the virions and produced Gly-to-Ala mutations in the HIV-1 NL4-3 genome.

**CV-3 inhibits the interaction between Vif and CBFβ**

After demonstrating the antiviral activity of CV-3, it was necessary to confirm whether CV-3 achieved antiviral activity by blocking Vif-CBFβ as envisaged. In a FRET assay, 293T cells were transfected with CBFβ-CFP and Vif-YFP, and VR1012 as a control. If CBFβ-CFP binds to Vif-YFP, the emitted light of CFP would excite YFP to emit light at 530 nm. Compared with the DMSO-treated control, the presence of CV-3 attenuated the emission of YFP at 530 nm, which indicated that CV-3 may block the interaction of CBFβ-CFP and Vif-YFP (Fig. 6A). According to the calculation formula described under “Experimental procedures,” the FRET efficiency of CBFβ-CFP combined with Vif-YFP was 18.4. When CV-3 was added, the FRET efficiency decreased to 11.

The effect of CV-3 on the Vif-CBFβ interaction was further verified by Co-IP. Vif-HA was cotransfected in 293T cells with CBFβ-cmyc. Western blotting showed that Vif could co-immunoprecipitate with CBFβ; however, in the presence of CV-3, the binding of Vif-CBFβ was blocked (Fig. 6B). It has been reported that CBFβ enhances the binding of Cul5 with Vif, so the interruption of the binding between Vif and CBFβ may affect the assembly of the E3 complex (29). Thus, we next decided to further validate the role of CV-3 in the formation of the Vif-CBFβ-Cul5-EloB-EloC complex. Vif-HA and Cul5-cmyc were transfected into 293T cells for Co-IP analysis. With the addition of CV-3, the binding of Vif-Cul5 was weakened compared with the DMSO-treated control (Fig. 6C). The result indicated that CV-3 blocked the binding of Vif-CBFβ and thus disrupted the assembly of the E3 ubiquitin ligase complex to inhibit HIV-1.

It has been reported that the combination of Vif and CBFβ will affect the gene expression regulated by runt-related transcription factor 1 (RUNX1)/CBFβ (28). In addition, there is an overlap between the areas where CBFβ binds to Vif and RUNX1 (30), so it is necessary to verify whether CV-3 will affect the binding of CBFβ to RUNX1. A macrophage colony-stimulating factor receptor (MCSFR) promoter-reporting system was used to test the effect of CV-3 on CBFβ/RUNX1. The 293T cells transfected with MCSFR-luciferase and RUNX1 were treated with different concentrations of CV-3 for 48 h. The results showed that high concentration (80 μM) of CV-3 inhibited the gene expression regulated by CBFβ/RUNX1, whereas lower doses (10, 20, and 40 μM) of CV-3 had no significant effect on that (Fig. S4). Then we tested whether CV-3 can interrupt the occupation of CBFβ by Vif at the concentration that did not affect the gene expression driven by CBFβ/RUNX1. 293T cells were transfected with MCSFR-luciferase and RUNX1 in the presence or absence of Vif and were treated with 20 μM CV-3 or DMSO. As in previous reports (28), the expression of Vif decreased the fluorescence intensity due to the occupation of CBFβ by Vif. However, when 20 μM CV-3 was added, the fluorescence intensity was restored; that is, CV-3 released the occupation of CBFβ by Vif without affecting the gene expression regulated by CBFβ/RUNX1 (Fig. 6D). These results indicated that CV-3 can preferentially relieve the occupation of CBFβ by Vif, whereas a high concentration of CV-3 can block the binding of RUNX1 and CBFβ (Fig. S4). Considering that PPP2R5 family proteins are likely to be degraded by Vif through Cul5-based E3 ligase, just like APOBEC3s (50), we detected the effect of CV-3 on Vif-mediated degradation of PPP2R5A. The results showed that CV-3 could not inhibit the degradation of PPP2R5A (Fig. 6E and F). This indicates that CV-3 can specifically rescue A3G but not PPP2R5A in the presence of Vif.

**Predicted binding mode of CV-3 with CBFβ**

To confirm the mechanism of the antiviral action of CV-3, the docking poses of CV-3 with CBFβ were analyzed. After docking small molecule compounds with CBFβ using CDOCKER, following the protocol from Discovery Studio 2.5, CV-3 was observed to have greater H bond formation with CBFβ (residues Gln-67, Phe-69, Ile-102, and Arg-131) than the other six candidates (Fig. 1B). Three binding postures were predominant. In the first case, CV-3 formed H bonds with residues Gln-67, Ile-102, and Arg-131 of CBFβ (Fig. 7), with Gln-67-NH, Ile-102-NH, and Arg-131-HH acting as the hydrogen bond donors. In the second case, CV-3 formed H bonds with CBFβ residues Gln-67 and Phe-69 (Fig. S5A), and Gln-67-NH and Phe-69-NH acted as the H-bond donors. Additionally, in the third case, CV-3 formed H bonds with residues Ile-102 and Arg-131 of CBFβ (Fig. S5B), with Ile-102-NH and Arg-131-HH serving as the H-bond donors. Among these interaction models, the binding postures of CV-3 were found to block the extension of Vif’s N terminus into the CBFβ structure and the tripartite hydrophobic interaction (CBFβ-Phe-68 and Ile-55 with Trp5 of Vif) (33). The Vif N terminus acts as the key region for binding to CBFβ (30, 31), and blocking the interaction between the N terminus of Vif with CBFβ can effectively block the binding of Vif-CBFβ.

To confirm the binding sites of CV-3, mutants containing key residues, including CBFβQ67A-CFP, CBFβR69A-CFP, CBFβI102A-CFP, and CBFβR131A-CFP, were constructed for FRET analysis. CBFβ-CFP or mutants were transfected with Vif-YFP into 293T cells, and the FRET efficiency was detected after 48 h (Table 1). The expression of CBFβ-CFP or the mutants and the co-expressed Vif-YFP were detected by Western blotting (Fig. S6). The results showed that F69A and R131A mutation greatly reduced the combination efficiency. CBFβ residue Phe-69 was reported to be a key site of Vif-CBFβ interaction (51), whereas R131 was a newly discovered one. Additionally, Q67A and I102A mutations slightly weakened the binding of Vif-CBFβ. Compared with DMSO, the addition of CV-3 impaired the interaction between CBFβ-Vif (FRET efficiency decreased from 19.2 to 13.3, p < 0.05). Although the CBFβI102A
mutant has a very weak binding affinity to Vif, the addition of CV-3 still significantly reduced FRET efficiency to 2.6 (p < 0.05). This result showed that Phe-69 was not the active site of CV-3. However, the FRET efficiency of Vif with CBFβQ67A, CBFβI102A, and CBFβR131A mutants did not change significantly when CV-3 was added (p > 0.05), indicating that Gln-67, Ile-102, and Arg-131 were involved in the action of CV-3, and the addition of CV-7 had no significant change in the interaction of CBFβ and its mutants with Vif (p > 0.05). Moreover, mutation of any of these residues caused CV-3 to lose its inhibitory activity, indicating that these three sites may form H bonds with CV-3 simultaneously, which is consistent with the action model shown in Fig. 7.

To further determine the CV-3 binding model, we made further mutations (Table 1) and found that the side chain changes of Phe-69 (to Ala, Trp, or Tyr) did not abolish the inhibitory effect of CV-3, which further suggests that Phe-69 is not the key site of CV-3. Interestingly, mutation of Phe-69 to Trp improved the function of CV-3 and even CV-7 showed an inhibitory effect (p < 0.05); that is, CV-3 and CV-7 may have stronger binding with CBFβF69W. When Gln-67 was mutated to Ile (hydrophobic) and Ile-102 to Gln (hydrophilic), CV-3 lost its inhibitory effect on the binding of CBFβ and Vif (p < 0.05), and the interaction of CBFβQ67N or CBFβI102V with Vif was still inhibited by CV-3 (p < 0.05). The results were consistent with the prediction model of CV-3 with CBFβ that CV-3 was...
predicted to be hydrogen-bonded to the backbone of Gln-67 and Ile-102. In the model, CV-3 formed a hydrogen bond with the side chain of Arg-131. The FRET efficiency of CBFβR131K showed that the shorter side chain weakened the inhibitory effect of CV-3 (p > 0.05) and the mutation of R131E (acidic) made CV-3 completely lose its inhibitory effect (p > 0.05). The experimental results are consistent with the predicted model with the highest score; that is, CV-3 may interact with Gln-67, Ile-102, and Arg-131, but not with Phe-69 on CBFβ, to block the binding of Vif to CBFβ.

Discussion

The detailed interactions of the HIV-1 Vif-E3 complex have been brought to light through the crystal structure of the full-length protein, which was recently published by the Huang group (PDB: 4N9F) (30). This structure provides information for the development of anti-HIV-1 inhibitors against Vif. In this complex, Vif likely forms at least five potentially druggable interfaces including Vif-CBFβ, Vif-EloC, Vif-Cul5, Vif-Vif (dimerization domain), and Vif-APOBEC3s (A3D, F, G, or H). Theoretically, disrupting any of these protein-protein interactions (PPI) would abrogate the degradation of A3s. In the past decade, three kinds of inhibitors targeting the PPI of the Vif-E3 complex were reported, which target Vif-A3G (36–38), Vif-EC (39–42), or Vif dimerization (35). Vif-Cul5 is considered to be a lower-priority target given the higher risk of off-target interactions (52).

CBFβ is a transcriptional cofactor of RUNX, and the binding of CBFβ to RUNX1 and Vif is mutually exclusive because the binding regions of these two proteins on CBFβ overlap (30). The combination of Vif and CBFβ can reduce the expression of RUNX1-driven genes (28), so blocking the interaction between Vif and CBFβ may also affect the function of CBFβ/RUNX1. A high concentration of CV-3 (80 μM) reduced the expression of PPP2R5 family proteins after knockdown of Cul5, CBFβ, or Elongin B/C; that is, Vif mediated degradation of PPP2R5 family proteins through Cul5-based E3 ligase, just like degradation of APOBEC3s (50). Our results showed that CV-3 could not inhibit the degradation of PPP2RSA mediated by Vif (Fig. 6, E and F).
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reason may be that we used a Cul5/EloBC/CFBβ/Vif complex to screen the inhibitors and CV-3 was screened out by first detecting if the inhibitors can rescue APOBEC3s. When PPP2R5s bind to the complex, the conformation of the complex may be different from the structure containing A3G. It has been reported that the key residues required for Vif-induced degradation of A3G and PPP2R5s were not the same, indicating that the binding mode of E3-complex with PPP2R5s may be different from that with APOBEC3s (53, 54). Therefore, the CV-3 that rescues APOBEC3s may not have the same ability to rescue PPP2R5α in the presence of Vif.

However, Vif-CBFβ is a good target for the development of drug candidates because blocking this PPI can release all Vif-sensitive APOBEC3s and can prevent Vif from hijacking components of the E3 complex, which can reduce nonspecific binding of drugs. A recent report revealed the mechanism of some Vif mutants with a dominant-negative phenotype, which rescue A3G by competitively binding CBFβ (55). This suggests that Vif-CBFβ can be used as a therapeutic target to develop anti-HIV-1 drugs. However, it seems difficult to find a drug-like molecule that can interrupt this interaction for the large interface between Vif and CBFβ (~4800 Å² of surface area) (30).

The docking site of CV-3 in our study was based on a tripartite hydrophobic interaction (CBFβ Phe-68 and Ile-55 with Trp5 of Vif) in the N-terminal interaction region of Vif-CBFβ (33). The screening of CV-3 shows that, even in a large interface, occupying key sites can block the Vif-CBFβ interaction. The molecular weight of CV-3 is 501.536 Da, which is the largest of the seven candidates, close to the suggested upper size limit (500 Da). Additionally, the experimental results indicated that CV-3 may interact with CBFβ residues Gln-67, Ile-102, and Arg-131 simultaneously so that it can effectively block the Vif-CBFβ interaction (Table 1). This suggests that to interrupt Vif-CBFβ, molecules with higher molecular weight and tighter binding may be required.

A reported inhibitor targeting Vif dimerization O2-16 can rescue A3G and A3F (35), and a Vif-EC inhibitor VEC-5 can also save them (39). This protection is consistent with the theoretical expectation. Compared with inhibitors targeting Vif-A3G, CV-3 can recover A3G, A3F, and A3C as an inhibitor targeting Vif-CBFβ (Fig. 3). This shows that Vif oligomerization, Vif-EC, and Vif-CBFβ are broad-spectrum targets for protecting APOBEC3s against Vif and should be selected preferentially. A previous study has shown that hijacking CBFβ by Vif mutants with a dominant-negative phenotype can make it lose the function of stabilizing Vif and thus decrease the expression of Vif (55). Furthermore, the interaction of Vif and CBFβ will reduce the expression of RUNX1-driven genes (28). This is notable because one of the target genes of RUNX1 is APOBEC3G (56). We have demonstrated that 20 μM CV-3 can relieve Vif from occupying CBFβ and restore the expression of RUNX1-driven genes. These data indicate that blocking Vif-CBFβ interaction with the appropriate dose of CV-3 cannot only counteract the degradation of A3G by Vif but may also affect the expression of Vif and increase the transcription level of A3G. CV-3 can promote A3G packaged into the virus and induce Gly-to-Ala hypermutation (Fig. 4), indicating that CV-3 can restore the antiviral activity of A3s and have a stronger inhibitory effect on HIV-1 compared with other known Vif inhibitors. These results indicate that Vif-CBFβ is an effective anti-HIV-1 target and can achieve antiviral effects by blocking its N-terminal interactions.

Most of the drugs currently being developed are targeted at viral proteins, although antiviral therapy is very effective but produces resistance. The antiviral effect of host-restricted factors and the characteristic of less susceptible to mutate make it a novel target for the development of HIV drugs. In this work, CV-3, which was obtained through virtual screening based on structural insights, can effectively block the interaction between Vif-CBFβ and lead to inhibition of viral replication. CV-3 has a low cytotoxicity, although the IC₅₀ of CV-3 is about 8.16 μM in CEM cells; further structural optimization may lead to a more effective HIV-1 inhibitor.

Experimental procedures

Structure-based virtual screening and preparation of compounds

The three-dimensional model of CBFβ-Vif was obtained from a crystal structure of the Vif-CBFβ-Cul5-EloB-EloC complex (PDB: 4N9F). A database of compounds obtained from the Alfa Aesar entry in the ZINC database (http://zinc.docking.org/catalogs/alfa) can be directly used for docking (57). Discovery Studio (version 2.5; Accelrys, Inc. San Diego, CA) was used as a screening tool. The binding of Vif (Gly) to CBFβ (Phe) was the target of our study. For the LigandFit docking protocol, the binding site was defined as the volume of residues 5–11 of Vif by the tool “find sites as volume of selected ligand” with three partition sites. The docking was run with variable numbers of Monte Carlo steps and scored using default scoring functions (LigScore1, LigScore2, PLP1, PLP2, Jain, and PMF). The consensus score was calculated using these six scores and the LigandFit docking score. For the CDOCKER protocol, the binding site sphere was also defined from these residues with a 14.4-Å radius. Before the screen was run, the structure of Vif was removed, and the structure of CBFβ was prepared using the Prepare Protein tool. After running the LigandFit protocol, molecules having a consensus score >6 went through the CDOCKER program with duplicate structures removed. CDOCKER was also run on default parameters. (Briefly, the random conformations and orientations were both defined as “true.” “CHARMM force-field” and “grid-based potential” were selected.) Only those molecules whose -CDOCKER energy was more than 40 were kept for visual inspection. Moreover, compounds that could form H bonds with CBFβ were chosen for further biological testing. Hit compounds were provided by Thermo Fisher Scientific and dissolved in DMSO.

Plasmids

The infectious molecular clone pNL4-3 and the vif deficiency mutant pNL4-3ΔVif construct, as well as VR1012, A3G-HA, A3G-YFP, A3C-HA, A3F-V5, fA3Z2-HA, fA3Z3-HA, HIV-1 Vif-cmyc, Vif-HA, fIV Vif-cmyc, and Cul5-cmyc have been previously described (39). CBFβ was amplified through RT-PCR with mRNA from Hela cells serving as the template. Cmyc
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and His-epitope tag sequences were added to the C terminus of CBFβ by PCR. Then the tagged CBFβ was inserted into VR1012 and PET-28a vectors, respectively. N-terminally CFP-fused CBFβ and N-terminally YFP-fused Vif were constructed by overlap PCR and then cloned into the EcoR I and Xba I sites of VR1012. Vif was inserted into the Nhe I and BamH I sites of pCTcon2 (Addgene, 41843). pMCSF- luciferase and RUNX1-myc were gifts from Dr. Wenyan Zhang (Institute of Virology and AIDS Research, First Hospital of Jilin University). PPP2R5A was amplified from genomic cDNA that had been reverse-transcribed from the total mRNA isolated from 293T cells by PCR. HA tag and enhanced GFP (EGFP) sequences were added to the C terminus of PPP2R5A. Then the HA-tagged and EGFP-fused PPP2R5A fragment was inserted into the VR1012 vector.

Cells, antibodies, and protein

HEK293T cells (CRL-11268) were purchased from ATCC. HeLa-derived indicator TZM-bl cells and human T cell lines H9, CEM, CEM-SS were obtained through the National Institutes of Health AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases. HEK293T and TZM-bl cells were cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO₂. SupT1 was a gift from Dr. Shan Cen (Department of Virology, Institute of Medicinal Biotechnology, Chinese Academy of Medical Science). The chronically infected cell lines H9/HXB2, Jurkat/HXB2, and A3G-expressing SupT1 cells were described previously (58, 59). T cells were maintained in RPMI 1640 medium with 10% FBS.

The antibodies used in this study have been previously described (39): anti-HA antibody (Covance, Emeryville, CA), anti-myc antibody (Millipore), anti-V5 antibody (Invitrogen), and anti-tubulin antibody (Covance). Pr55 Gag and caspid p24 reverse-transcripted from the total mRNA isolated from 293T cells were added to the C terminus of PPP2R5A. Then the HA-tagged and EGFP-fused PPP2R5A fragment was inserted into the VR1012 vector.

Yeast surface display

Vif-pcTCON2 was transformed into Saccharomyces cerevisiae strain EBY100 (Invitrogen), and transformants were selected on SDCAA medium (2% dextrose, 0.67% yeast nitrogen base, and 0.5% casamino acids), then confirmed by PCR. Selected transformants were inoculated into 1-ml SDCAA medium and incubated overnight at 30°C; protein expression was induced in SGCAA (SDCAA with 2% galactose in place of dextrose) at A600 = 1 and 20°C for 36 h. Induction was verified via immunolabelling of induced cells with a mAb that targets the internal HA tag located in the linker region bridging the Aga2a domain and Vif of the surface display protein. Approximately 1 × 10⁶ cells were incubated in 200 μl of PBS with 20 μg of CBFβ protein for 4 h at 4°C. The cells were washed twice with PBS and incubated with 200 μl of 1:500 diluted CBFβ antibody for 40 min at 4°C. Then the cells were washed twice with PBS and incubated with 200 μl of 1:100 diluted goat anti-rabbit IgG conjugated to FITC antibody for 40 min at 4°C. After washing twice with PBS, the cells were analyzed by flow cytometry C6 (BD Biosciences).

Transfection and virus purification

DNA transfections were performed using jetPRIME (Polyplus-transfection, Illkirch, France) as recommended by the manufacturer’s protocol. Virus in cell culture supernatants was precleared of cellular debris by centrifugation at 3,000 rpm for 10 min. Virus particles were then concentrated through a 20% sucrose cushion by ultracentrifugation at 100,000 × g for 2 h, then viral pellets were resuspended in radioimmune precipitation assay buffer.

Viral infectivity assay

Viral supernatants were normalized by the level of p24. Virus samples with equal p24 units were mixed with DEAE-dextran (Sigma-Aldrich) at a final concentration of 20 μg/ml and then incubated with 1 × 10⁶ TZM-bl indicator cells/well in 96-well plates. Infectivity was measured at 48 h after infection by performing luciferase assay (Promega). Typically, infections were done in triplicate. Statistical significance was evaluated using Student’s t test.

Cytotoxicity assay

MTT was used to assess the cytotoxicity of CV-3. Exponentially growing CEM, CEM-SS, SupT1, and SupT1-A3G cells were plated (1 × 10⁵) in 96-well plates and cultured for 24 h. A 200-μl aliquot of drug solution, diluted with RPMI 1640 medium plus 10% FBS and 0.5% DMSO, was added to the cells and incubated for 7 days. After removing the supernatants, the MTT solution was added, and the plates were incubated for 4 h at 37°C. After the supernatant was removed, DMSO was added, and the plates were incubated at 25°C for 20 min. The absorbance at 490 nm was measured using a multi-well plate reader. The DMSO-treated control cell group was set at 100%. Each experiment was performed in quadruplicate.

Cell viability was measured by trypan blue exclusion analysis. In brief, CEM cells (5 × 10⁵) were cultured with CV-1 or CV-3 as indicated in the figure legends. Equal volumes of the cell suspension and 0.4% (w/v) trypan blue in PBS were mixed, and the number of living cells was scored under a microscope using a hemocytometer.

Co-immunoprecipitation

Transfected 293T cells were washed with cold PBS and disrupted in lysis buffer (50 mm Tris, pH 7.5, with 150 mm NaCl,
products were cloned into the pGEM-T-Easy Vector (Promega).

ond round of PCR was amplified using inner primers Fwd TGCAAAGCTAGATGAATTGCTTGTAAC-3’ and Rev TGCAAAGCTAGATGAATTGCTTGTAAC-3’.

The cell supernatant was centrifuged to remove debris and lysed with radioimmune precipitation assay buffer. The samples were boiled for 10 min, subjected to standard SDS-PAGE, and then transferred to nitrocellulose membranes for Western blotting. Secondary antibodies were alkaline phosphatase-conjugated anti-mouse IgG (Jackson ImmunoResearch), and staining was carried out with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium solutions.

IC50 assay

The cells and viruses were harvested at 48 h after transfection and lysed with radioimmune precipitation assay buffer. The samples were boiled for 10 min, subjected to standard SDS-PAGE, and then transferred to nitrocellulose membranes for Western blotting. Secondary antibodies were alkaline phosphatase-conjugated anti-mouse IgG (Jackson ImmunoResearch), and staining was carried out with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium solutions.

HIV-1 replication in human T cells

A total of 5 × 10^6 cells were incubated with 2 to 10 ng WT or Vif-defective HIV-1 p24 antigen at 37 °C for 3 h. The virus-containing supernatants were then removed by washing three times with PBS. The cells were cultured with CV-3 for 12 days. p24 levels were monitored using a p24 ELISA kit (PerkinElmer).

IC50 assay

CEM cells (5 × 10^6) were infected with the equivalent of 4 ng of HIV-1 p24 antigen for 3 h at 37 °C. The virus-containing supernatants were then removed by washing three times with PBS. The cells were treated with compound CV-3, and HIV-1 replication was monitored using a p24 ELISA kit on day 7 post-infection. The IC50 was calculated using an IC50 calculator (AAT Bioquest, Inc.).

Hypermutation analysis

A3G-HA, pNL4-3ΔVif, or pNL4-3 was transfected into 293T cells, and 50 μM CV-3 was added into cell cultures 4 h later. Next, the cells were cultured in a 0.5% DMSO system for 48 h. The cell supernatant was centrifuged to remove debris and treated with Dpn I (20 units/ml) at 37 °C for 1 h. Next, 1 × 10^6 TZM-bl cells were infected with equal loads of virion (100 ng of p24 antigen), and genomic DNA was isolated from the cells at 48 h post-infection using a TIANamp Genomic DNA kit (Tiangen Biotech) according to the manufacturer’s protocol. An 885-bp DNAfragment of pol was amplified with Taq DNA polymerase (Invitrogen) using the outer primers Fwd (5’-GCACCTTTAAAAAATTTCATTAGTCC-3’) and Rev (5’-TGCAAAAGCTAGATGAATTGCTTGTAAC-3’). The second round of PCR was amplified using inner primers Fwd (5’-GTATAGTGTGTACATTACCACCTAGATGAACTAC-3’) and Rev (5’-GGAGGCTTGATGAAACTAC-3’) (35). The PCR products were cloned into the pGEM-T-Easy Vector (Promega).

The clones were sequenced, and the sequencing results were analyzed on Hypermut 2.0 (RRID:SCR_014933). Statistical analysis was evaluated by Student’s t test.

FRET

FRET experiments for detecting protein-protein interaction were performed with methods adapted from the reference publication (60). Briefly, Vif-YFP and CBFβ-CFP were transfected into 293T cells and cultured for 48 h. The cells were harvested and resuspended with PBS for detection. Fluorescence signals were detected in a 1.5-ml quartz microcuvette with a magnetic stir bar using an LS-55 Spectrofluorometer (PerkinElmer). In each FRET experiment, fluorescence emission spectra were recorded from four separate samples: 1) a buffer-only blank, 2) a sample containing only CBFβ-CFP, 3) a sample containing only Vif-YFP, and 4) a sample containing both CBFβ-CFP and Vif-YFP. FRET interaction between CFP-tagged and YFP-tagged receptors was detected by following a procedure to remove contributions from the background signal, CFP emission (“bleed-through”), and direct YFP emission (“cross-talk”) from the CFP-tagged and YFP-tagged sample emission spectrum. These were achieved following a previously described method (60).

A parameter termed the “apparent FRET efficiency” was calculated as follows (60):

\[ E^{\text{app}} = \frac{F^{\text{DA}}}{F^{\text{DA}}_{\lambda}} \times 100 \]

Where \( E^{\text{app}} \) is the apparent FRET efficiency, \( F^{\text{DA}} \) is the fluorescence intensity attributable to FRET, and \( F^{\text{DA}}_{\lambda} \) is the fluorescence intensity of the acceptor when excited at \( \lambda_{\text{max}} \).

Data availability

All data are contained within this article and in the supporting information.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: APOBEC3, apolipoprotein B mRNA-editing catalytic polypeptide-like 3 family members; Vif, viral infectivity factor; CBFβ, core binding factor β; CDC53, 50% cytotoxic concentration; A3, APOBEC3; hA3, human A3; Cul5, Cullin5; Elo, Elongin; Co-IP, co-immunoprecipitation; H bonds, hydrogen bonds; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); FIV, feline immunodeficiency virus; RUNX, runt-related transcription factor; MCSFR, macrophage colony-stimulating factor receptor; PPI, protein-protein interactions; fA3, feline A3.

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