Small-Molecule Inhibition of Human Immunodeficiency Virus Type 1 Replication by Targeting the Interaction between Vif and ElonginC

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The HIV-1 viral infectivity factor (Vif) protein is essential for viral replication. Vif recruits cellular ElonginB/C-Cullin5 E3 ubiquitin ligase to target the host antiviral protein APOBEC3G (A3G) for proteasomal degradation. In the absence of Vif, A3G is packaged into budding HIV-1 virions and introduces multiple mutations in the newly synthesized minus-strand viral DNA to restrict virus replication. Thus, the A3G-Vif-E3 complex represents an attractive target for development of novel anti-HIV drugs. In this study, we identified a potent small molecular compound (VEC-5) by virtual screening and validated its anti-Vif activity through biochemical analysis. We show that VEC-5 inhibits virus replication only in A3G-positive cells. Treatment with VEC-5 increased cellular A3G levels when Vif was coexpressed and enhanced A3G incorporation into HIV-1 virions to reduce viral infectivity. Coimmunoprecipitation and computational analysis further attributed the anti-Vif activity of VEC-5 to the inhibition of Vif from direct binding to the ElonginC protein. These findings support the notion that suppressing Vif function can liberate A3G to carry out its antiviral activity and demonstrate that regulation of the Vif-ElonginC interaction is a novel target for small-molecule inhibitors of HIV-1.

Since the human immunodeficiency virus type 1 (HIV-1) was first isolated 30 years ago, tremendous progress has been made in the prevention and treatment of HIV/AIDS. The introduction of highly active antiretroviral therapies (HAART) has proven to be exceedingly effective at reducing viral load and improving the clinical status of many patients. However, drug-resistant viruses continually emerge, which highlights the urgent need to discover effective inhibitors with novel targets and mechanisms (2, 8, 19).

A relatively new antiviral strategy lies in pursuing “host restriction factors” (6, 38), which are intrinsic cellular proteins that provide defenses by restricting HIV via different approaches. APOBEC3G (A3G), an archetype of the APOBEC3 (A3) subfamily of single-stranded DNA (ssDNA) cytidine deaminases, is such a protein with a remarkable ability to restrict HIV-1 replication. In the absence of HIV-1 Vif, A3G is packaged into HIV-1 virions and introduces G-to-A hypermutations in viral minus-strand DNA during reverse transcription, which leads to the production of nonfunctional proviruses (20, 26, 39, 69). Other APOBEC3 proteins also exhibit similar antiviral functions to various degrees (9, 12, 14, 29). However, the APOBEC3-imposed replication block is primarily overcome by the HIV-1 viral infectivity factor (Vif) protein that triggers the degradation of APOBEC3 through polyubiquitination and proteasomal degradation. Vif accomplishes this by interacting with and adapting APOBEC3s to an E3 ubiquitin complex that consists mainly of ElonginB, ElonginC, and Cullin5. In this complex, Vif uses diverse residues in its N terminus to recognize different APOBEC3s, as well as highly conserved ¹⁴⁴SLQ¹⁴⁶ and HCCH (residues 108 to 139) motifs for ElonginC and Cullin5 binding (16, 44, 47, 58, 63). Therefore, disrupting protein-protein interactions within the APOBEC3-Vif-E3 complex may effectively restore APOBEC3 protein levels and unleash the body's own natural defenses. This complex has stimulated much interest, for it offers an attractive target in development of novel anti-HIV therapies. Recently, two groups identified small-molecule Vif inhibitors,

RN-18 and IMB-26/35, by cell-based screening from chemical libraries. These compounds were shown to reduce the capacity of Vif to downregulate A3G (10, 46).

Compared with cell-based screening, structure-based virtual screening is a more rational and efficient approach to exploring novel pharmaceutical agents. However, little structural data are available on the HIV-1 Vif protein, which presents a major roadblock in the path to designing potent Vif inhibitors. To partially overcome this barrier, we previously constructed a three-dimensional (3D) Vif-ElonginB/C homology model (36), revealing structural information on the Vif protein at the molecular level to explore potential Vif antagonists.

In order to activate different APOBEC3 proteins and maximize antiviral activity, our research in the present study was focused on identifying Vif inhibitors targeting the Vif-ElonginC interface. We performed a virtual screening based on the Vif-ElonginB/C homology model mentioned above to find possible Vif antagonists. Subsequent biochemical investigations led to the identification of a small-molecule Vif inhibitor, designated VEC-5, which could restrict HIV-1 in Vif-nonpermissive cells. VEC-5 was shown to protect A3G, APOBEC3C (A3C), and APOBEC3F (A3F) from Vif-mediated degradation and drastically inhibit Vif function through blocking the interaction between Vif and ElonginC. Furthermore, VEC-5 could enhance A3G incorporation into HIV-1 virions to reduce viral infectivity. Thus, the identification of this

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Vif inhibitor initially reveals the potential for the Vif-ElonginC interaction as a novel target for anti-HIV-1 therapy.

MATERIALS AND METHODS

Structure-based virtual screening and preparation of compounds. The three-dimensional model of HIV-1 Vif that we described previously (36) was used to identify potential inhibitors of HIV-1 Vif in the virtual screening of a database of compounds, the Available Chemicals Directory (ACD). To improve efficiency, the ACD database was prescreened by using a filter designed to identify potential drug compounds with suitable physicochemical properties (molecular weight; ≤ 600 , number of hydrogen bond donors, ≤ 5 ; sum of nitrogen and oxygen atoms, ≤ 10 ; number of rings, ≤ 6) such that only 600,000 molecules were retained. In this study, the binding of Vif with ElonginC was the target of interest, and the binding site was defined as all residues within a radius of 9 Å of Vif Leu145. DOCK, version 4.0, was used as the screening tool. Before the screen was run, the structure of Vif was removed, and all missing hydrogens were added by Discovery Studio (version 2.1; Accelrys, Inc. San Diego, CA). The binding energies for all the docked compounds were evaluated by two scoring functions, Dock score and X-cscore. During soft docking simulations, only those molecules that were among the top 1,000 in both scoring functions were kept. We then manually checked the binding mode for these top-ranked molecules, and a cluster analysis was carried out by using Pipeline Pilot to minimize structural redundancy. Hits returned from the screening were provided by Sigma, Inc. (St. Louis, MO) and diluted in dimethyl sulfoxide (DMSO) for biological testing.

Plasmids. The infectious molecular clone pNL4-3 and the Vif mutant pNL4-3ΔVif construct were obtained from the National Institutes of Health AIDS Research and Reference Reagents Program (NIH-ARRRP), Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID). VR1012, A3G fused to hemagglutinin (HA) tag (A3G-HA), A3C-HA, A3F-V5, African green monkey (AGM) A3G-HA, feline A3Z3-HA ([fA3Z3-HA] where A3Z3 is the A3 protein with a Z3 domain), pHIV-1 Vif-cmyc, Vif-HA, SIVagmTan Vif-cmyc (where SIVagmTan is simian immunodeficiency virus isolated from tantalus African green monkeys), feline immunodeficiency virus (FIV) Vif-cmyc, Cullin5-HA, and ElonginC-HA have been previously described (33, 34, 61, 67, 68, 71, 72). A polycistronic expression vector, pST39 (57), was used to express the ElonginB/C complex in *Escherichia coli*. ElonginC with an N-terminal His₆ tag was inserted between XbaI and BamHI. ElonginB was cloned into pST39 using the SacI and KpnI sites.

Cells, antibodies, and proteins. HEK293T (CRL-11268) cells were purchased from the American Tissue Culture Collection (ATCC). MAGI-CCR5 cells (catalog number 3522) were obtained from NIH-ARRRP. HEK293T and MAGI-CCR5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. Human T cell lines H9, CEM, and CEM-SS were kind gifts from Xiao-Fang Yu (Department of Molecular Microbiology and Immunology, Johns Hopkins University, Baltimore, MD). SupT1 was a gift from Shan Cen (Department of Virology, Institute of Medicinal Biotechnology, Chinese Academy of Medical Science). The chronically infected cell line H9/HxB2Neo and A3G-expressing SupT1/A3G cells were described previously (27, 32). T cells were maintained in RPMI 1640 medium with 10% FBS. The antibodies used in this study have been previously described (35, 71): anti-HA antibody ([Ab] Covance, Emeryville, CA), anti-myc antibody (Millipore, Billerica, MA), anti-V5 antibody (Invitrogen, Carlsbad, CA) and anti-tubulin antibody (Covance). Pr55Gag and CAp24 were detected with a monoclonal anti-HIV capsid antibody generated by an HIV-1 p24 hybridoma (NIH-ARRRP). The anti-APOBEC3G antiserum was obtained from NIH-ARRRP (catalog number 9906).

The ElonginB/C protein complex was expressed in *E. coli* strain BL21. Cells were grown in LB medium with 100 mg/liter ampicillin at 37°C until an optical density (OD) of ≈ 0.8 , and protein expression was induced by adding 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 20°C for

20 h. Cells were harvested, resuspended in phosphate-buffered saline (PBS) buffer, sonicated, and clarified by centrifugation. The supernatant was then applied to a nickel affinity column, and the fraction containing the ElonginB/C complex was pooled and concentrated in PBS (pH 8.0) buffer. Purified HIV-1 Vif (catalog number 11096) was obtained through the NIH-ARRRP.

Transfection and virus purification. DNA transfections were carried out using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Virus in cell culture supernatants was precleared of cellular debris by centrifugation at 3,000 rpm for 10 min and filtration through a 0.22- μ m-pore-size membrane. Virus particles were then concentrated through a 20% sucrose cushion by ultracentrifugation at $100,000 \times g$ for 2 h at 4°C. For immunoblotting, viral pellets were resuspended in radio-immunoprecipitation assay (RIPA) buffer. For the viral infectivity assay, virus was resuspended in sterilized PBS.

Viral infectivity (MAGI) assay. Viral infection was determined by a multinuclear activation of a galactosidase indicator (MAGI) assay as previously described (67). In general, MAGI-CCR5 cells were prepared in 24-well plates in DMEM 1 day before infection, and the cells were at 30 to 40% confluence on the day of infection. Viral supernatants were normalized by the level of p24. Virus samples with equal p24 units were mixed with DEAE-dextran (Sigma) at a final concentration of 20 μ g/ml and then incubated with MAGI-CCR5 indicator cells for 2 h, followed by addition of fresh DMEM. After incubation for 48 h at 37°C under 5% CO₂, supernatants were removed, and the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), the substrate for β -galactosidase produced under the control of the HIV-1 long terminal repeat (LTR) promoter. Positive blue dots (indicative of β -galactosidase activity) were counted to determine viral infectivity.

Cytotoxicity assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used to assess cytotoxicity of VEC-5. Exponentially growing HEK293T, MAGI-CCR5, and H9 cells were plated (at 1,000, 1,000 and 2,000 per well, respectively) in 96-well plates and cultured for 24 h at 37°C under 5% CO₂. A 200- μ l aliquot of drug solution, diluted with DMEM plus 10% FBS and 0.5% DMSO (final concentration), was added to the cells and incubated for 48 h at 37°C under 5% CO₂. After the supernatants were removed, MTT solution was added to each well, and the plates were incubated for 4 h. The absorbance at 490 nm was measured using a multiwell plate reader. The DMSO-treated control cell group was set at 100%. Each experiment was performed in quintuplicate.

Cell viability was also measured by trypan blue exclusion analysis. Human T cells (0.6×10^6) were cultured with VEC-5 or VEC-6 as indicated in the figure legends. Equal volumes of the cell suspension and 0.4% (wt/vol) trypan blue in PBS were mixed, and the living cell number was scored under a microscope using a hemacytometer.

Coimmunoprecipitation. Transfected 293T cells were harvested, washed twice with cold PBS, and disrupted in lysis buffer (50 mM Tris, pH 7.5, with 150 mM NaCl, 1% Triton X-100, and complete protease inhibitor cocktail tablets) at 4°C for 1 h, and then centrifuged at $10,000 \times g$ for 30 min. The precleared cell lysates were then mixed with anti-hemagglutinin Ab-conjugated agarose beads (Roche, Mannheim, Germany) and incubated at 4°C for 3 h. Samples were then washed three times with washing buffer (20 mM Tris, pH 7.5, with 100 mM NaCl, 0.1 mM EDTA and 0.05% Tween 20). Elution buffer (0.1 M glycine-HCl, pH 2.0) was applied to the beads, and the eluted materials were then analyzed by SDS-PAGE and immunoblotting.

Immunoblot analysis. Cells and viruses were harvested 48 h after transfection and lysed with RIPA buffer. Samples were boiled for 10 min, subjected to standard SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred to nitrocellulose membranes for Western blotting. Secondary antibodies were alkaline phosphatase-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), and staining was carried out with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) solutions.

HIV-1 infection of human T cell lines. A total of 0.6×10^6 cells were incubated with 2 to 10 ng wild-type (WT) or Vif-defective HIV-1 virus at 37°C for 3 h. After removal of the inocula and three extensive washings, cells were cultured with VEC-5 for 12 days. p24 levels were monitored by using a p24 enzyme-linked immunosorbent assay (ELISA) kit (Perkin Elmer, Norwalk, CT, USA). The concentrations of VEC-5 and time points for p24 evaluation are shown in Fig. 2B.

BLI binding assay. A protein binding assay was performed by biolayer interferometry (BLI) using an Octet RED96 instrument (ForteBio, Inc., Menlo Park, CA). In this assay, the protein of interest is immobilized on the surface of a sensor tip and then exposed to potential binding partners in solution. The binding of analytes to the immobilized protein leads to a shift in the wavelength of light reflected off the binding surface. Thus, the protein-protein binding is measured as a wavelength shift (in nanometers). Data were analyzed and presented using the system software of Octet RED96.

In this experiment, ElonginB/C protein complex was biotinylated with *N*-hydroxysuccinimide (NHS)-LC-LC-biotin (Pierce/ThermoFisher, Rockford, IL) in PBS at 4°C for 1 h. Excess biotinylation reagent was removed using Pepclean C_{18} spin columns (Pierce/ThermoFisher). Biotinylated ElonginB/C complex was then diluted to 100 μ g/ml in kinetics buffer (PBS containing 0.02% Tween 20, 0.005% sodium azide, and 100 μ g/ml bovine serum albumin) and were immobilized onto streptavidin binding sensor (SA sensors). Vif protein was diluted to 80 μ g/ml in kinetics buffer plus DMSO or 50 μ M VEC-5. Sensors coated with ElonginB/C were then allowed to incubate with Vif protein. The shift in wavelength was monitored for 420 s.

Molecular docking and dynamics simulation. The structure of VEC-5 was constructed based upon the crystal coordinates of VEC-5 followed by energy minimization. It was then docked into the binding pocket of ElonginC using Discovery Studio, version 2.1.

After docking, one conformation with a relatively low energy was selected as the starting conformation for the subsequent molecular dynamics simulation. The molecular topology file for VEC-5 was generated by the PRODRG2 (50) server (<http://davapc1.bioch.dundee.ac.uk/prodrg/>). The partial atomic charges of the compound were calculated using the Gaussian 03 program at the level of HF/6-31G*. GROMACS, version 3.3.1 (30), software was used to perform the simulations with the force field GROMOS96 43a1 applied for the protein. The VEC-5/ElonginC complex was placed into a cubic periodic box with an edge approximately 10 Å from the periphery of the system in each dimension, and then 10,000 simple point charge (SPC) water molecules were added into the box. After that, the total charge in the computational box was neutralized by the addition of 2 Na^+ to the simulation. During the entire simulation, all bonds were constrained via the SHAKE algorithm. Long-range electrostatic interactions were calculated using the particle mesh Ewald (PME) method. The Berendsen thermostat was applied using a coupling time of 0.1 ps to maintain the system at a constant temperature of 300 K, and the pressure was also maintained by coupling to a reference pressure of 10^5 Pa by a Berendsen thermostat. The complex simulation began with 5,000-step energy minimization with conjugate gradient algorithm. The solvent equilibration was then performed in 40 ps with the protein and the ligand fixed. Following that, a second 100-ps simulation was carried out with the main chain and the ligand fixed. Another 100-ps simulation was used to relax the whole system except for the $\text{C}\alpha$ atoms and the ligand. Finally, the production simulation of 2 ns was performed on the whole system. The system was equilibrated after about 1 ns, and the final structure was obtained after 2 ns, which was considered the stable binding mode of VEC-5.

RESULTS

VEC-5 decreases HIV-1 particle infectivity. To identify small molecules that can interfere with the Vif-ElonginC interaction, we carried out virtual screening on the basis of the Vif-ElonginB/C 3D homology model reported in our earlier study (36). In essence, the $^{144}\text{SLQ}^{146}$ motif in the BC box and related residues in Elong-

inC had been defined as a binding pocket for molecular docking. The compound database used for screening, the Available Chemicals Directory (ACD), contains structural information of over 1,160,000 unique chemicals. Fifteen compounds with top docking scores were selected as candidates and tested in bioactivity assays to evaluate their potential anti-Vif activities. Effects of these leading compounds on HIV-1 infectivity were tested using chronically infected H9/HxB2Neo cells, which continuously produce HIV-1 HxB2 particles (27). The H9/HxB2Neo cells were cultured with 100 μ M each compound or control DMSO for 48 h. Viral infectivity was tested in a standard MAGI assay as previously described (59, 70, 71). The relative infectivities of virus cultured with all candidate compounds were calculated based on the infectivity of DMSO-treated virus set to 100% (Fig. 1A). Among the 15 candidates, compounds VEC-5 and VEC-6 demonstrated the strongest inhibitory effects, up to 85% and 75%, respectively, on HIV-1 infectivity. Importantly, VEC-5 showed little adverse effects on cell viability (Fig. 1B). H9 cells treated with 5 to 50 μ M VEC-5 for 48 h consistently had viabilities greater than 95%. In the 100 μ M treatment group, viable cell rates remained higher than 85%. In contrast, VEC-6 strongly reduced cell viability, which dropped to 22% at 100 μ M, indicating that its ability to suppress virus was at least in part the consequence of injury to host cells. Because VEC-6 showed unacceptably high cell toxicity, it was excluded from further analysis, and our subsequent investigations focused on VEC-5. The purity of VEC-5 was >99%, which was confirmed by high-performance liquid chromatography (HPLC) (data not shown). The structure of VEC-5 is shown in Fig. 1C.

The presence of A3G is indispensable for the antiviral activity of VEC-5. The ability of Vif to enhance virus infectivity is producer cell-type dependent. That is, Vif expression is essential for virus derived from nonpermissive cells, such as human T cell lines H9 and CEM, whereas virus produced from permissive cells, including CEM-SS and SupT1 cells, is fully infectious regardless of the presence of Vif (17, 18, 24, 38, 60). Because VEC-5 was expected to counteract Vif function, it was important to determine its activity in cells devoid of A3G expression. We therefore compared the efficiency of VEC-5 on HIV-1 infectivity using viruses produced from 293T cells with or without A3G expression. 293T cells were cotransfected with pNL4-3 plus an A3G expression vector or a control vector. Cells were then cultured in 0 to 100 μ M VEC-5 as indicated in Fig. 2A. VEC-5 specifically reduced the infectivity of viruses produced from 293T cells expressing A3G in a dose-dependent manner but showed no effect on that of viruses from cells without A3G expression. From these data we determined VEC-5 to have a 50% inhibitory concentration (IC_{50}) of 24.48 μ M (95% confidence interval is 20.34 to 29.48 μ M). Western blot analysis was used to further validate the A3G abundance in the virus-producing 293T cells (Fig. 2A, bottom panel). The A3G protein level clearly negatively correlated with HIV-1 infectivity. When VEC-5 was used at the active concentration in this experiment, little cytotoxicity to cells was observed as evaluated by the MTT assay (Table 1). Therefore, the dose-dependent and A3G-dependent inhibition profile indicates that the antiviral activity observed with VEC-5 is likely based on interference of Vif function and enhancement of the A3G level.

In addition to its role in virus infectivity, Vif also plays an essential role in virus replication. In nonpermissive cells, Vif is essential for the virus to counteract the restriction imposed by A3G and normally replicate (2, 37, 53). In contrast, Vif-deficient

FIG 1 Effects of 15 candidate anti-Vif compounds on HIV-1 infectivity. (A) Chronically infected H9/HxB2Neo cells were treated with the indicated compounds at 100 μ M for 48 h. Viral infectivity was tested in MAGI assays, with virus infectivity in DMSO set to 100%. Error bars represent the standard deviation calculated from three independent infections. (B) Effects of VEC-5 and VEC-6 on cell viability. H9 cells were cultured in the indicated concentrations of VEC-5 or VEC-6 for 48 h. Cell survival rates were measured by trypan blue exclusion and are represented as percentages compared with DMSO-treated cells. Values are presented as the average of three independent experiments. (C) Structure of compound VEC-5.

virus can efficiently replicate in permissive cells (24, 38). To further investigate the inhibitory activity exerted by VEC-5, we analyzed its effect on virus replication in different types of human T cell lines in a spreading infection assay (Fig. 2B). Wild-type HIV-1 NL4-3 or Vif-deficient virus NL4-3 Δ Vif was used to infect non-permissive (CEM and SupT1/A3G) and permissive (CEM-SS and SupT1) cells separately. The SupT1/A3G cell line was generated by stably transfecting SupT1 cells with the A3G expression vector. Expression of transduced A3G converted this cell line from the permissive to the nonpermissive phenotype for HIV-1 Vif mutant viruses (32). After infection, the cells were treated with various concentrations of VEC-5. In the following 12 days, p24 antigen levels in the supernatants were quantified to determine virus replication curves. Consistent with previous findings, both wild-type and Vif-defective HIV-1 showed robust replication levels in SupT1 and CEM-SS cells. In contrast, the replication of HIV-1 Δ Vif was completely restricted in the nonpermissive CEM and SupT1/A3G cell lines. In the presence of VEC-5, p24 antigen levels in CEM and SupT1/A3G cells decreased substantially and in a dose-dependent manner. In particular, VEC-5 at 50 μ M impaired replication of wild-type virus to a level similar to that of Vif-deficient virus. In sharp contrast, VEC-5 did not affect p24 levels in CEM-SS or SupT1 permissive cells. The expression levels of A3G in these T-cell lines were confirmed by Western blotting using anti-A3G antiserum (Fig. 2C). A3G protein was detected only in SupT1/A3G and CEM cells. Moreover, VEC-5 had no effect on the

growth of the host cells (Fig. 2D). Because VEC-5 inhibited HIV-1 replication only in nonpermissive cells and not in permissive cells, the results suggest that the presence of A3G is indispensable for the antiviral activity of this compound. Furthermore, the protective effects of VEC-5 may be through targeting the Vif pathway and activating endogenously expressed A3G.

VEC-5 protects APOBECs from Vif-induced degradation and enhances A3G incorporation into HIV-1 particles. It is well established that Vif impedes the antiretroviral activity of A3G (40, 43, 52, 56) by hijacking the cellular ubiquitin system and targeting A3G for proteasomal degradation (25, 31, 41, 43, 56, 67). Since VEC-5 was designed to conceal Vif and reestablish A3G expression, we then determined the effect of VEC-5 on A3G degradation. For this purpose, 293T cells were cotransfected with an HA-tagged APOBEC3G expression vector and cmc-tagged Vif as indicated in Fig. 3A. Transfected cells were then cultured with control DMSO (lanes 1, 3, and 5), proteasome inhibitor MG132 (lane 6), or the VEC-5 compound (lanes 2, 4, and 7 to 10), and protein levels in cell lysates were analyzed by Western blotting at 48 h posttransfection. As expected, Vif induced the degradation of A3G, which was blocked by MG132 (lanes 5 and 6). In the absence of Vif, the level of A3G was not changed by VEC-5 (lanes 1 and 2). When Vif was coexpressed, however, the A3G abundance was increased by treatment with VEC-5 in a dose-dependent manner, implying that VEC-5 is potentially capable of protecting A3G expression from Vif-mediated degradation.

FIG 2 VEC-5 specifically inhibits HIV-1 in Vif-nonpermissive cells. (A) VEC-5 showed dose-dependent inhibition of viral infectivity in HEK293T cells in the presence of A3G. HEK293T cells were transfected with pNL4-3 and control plasmid pcDNA3.1 or an expression vector for A3G. Cells were then cultured with the indicated concentration of VEC-5 or control DMSO. Cells and virus-containing supernatants were harvested at 48 h posttransfection. Viral infectivity was tested in a MAGI assay, with virus infectivity without A3G and treated with DMSO set to 100%. Results are the average of five independent experiments. A3G expression in cell lysates was analyzed by immunoblotting. Tubulin was detected as a loading control. The VEC-5 concentrations and transfected plasmid indicated in the histogram also correspond to the Western blot results. (B) VEC-5 restricted HIV-1 replication in nonpermissive cells. Wild-type or Vif-defective HIV-1 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 indicated times using a p24 ELISA for 12 days. (C) Expression level of A3G in cells. Cell lysates were analyzed by Western blotting using A3G antiserum. (D) Effect of VEC-5 on cell growth. Cells (0.6×10^6) were cultured in 0 to 50 μ M VEC-5 as indicated. Living cell numbers were determined by trypan blue exclusion.

TABLE 1 Effect of VEC-5 on cell viability^a

Cell line	VEC-5 concn (μ M)	% Viability (avg $\pm$ SD)
HEK293T	5	99 $\pm$ 0.6
	50	91 $\pm$ 0.3
	100	80 $\pm$ 1.6
MAGI-CCR5	5	100 $\pm$ 2.3
	50	86 $\pm$ 1.0
	100	79 $\pm$ 1.6
H9	5	98 $\pm$ 3.8
	50	93 $\pm$ 1.2
	100	85 $\pm$ 2.3

^a Cell toxicity associated with VEC-5 treatment was assessed by determining cell viability after 48 h of treatment with different concentrations of VEC-5. Cell viability was determined using an MTT assay.

APOBEC3F (A3F) and APOBEC3C (A3C) are two host factors belonging to the same family of cytidine deaminases with A3G (7, 21, 23, 29, 71). These two proteins are degraded by Vif through a similar mechanism to that of A3G (48, 58, 71). In 293T cells, we found that VEC-5 also enhanced A3C and A3F levels when coexpressed with Vif (Fig. 3B and C, lanes 3). Interestingly, Vif expression was also improved by VEC-5. A likely explanation for the enhancement of Vif could be that VEC-5 collapses the Vif-E3 complex and thereby protects both APOBEC3 and Vif proteins, which are self-ubiquitinated (16, 38, 42), from proteasomal degradation.

Vif protein of SIVagmTan (simian immunodeficiency virus from a tamarin African green monkey [AGM]) has been shown to be capable of overcoming AGM A3G in 293T cells through the ElonginB-ElonginC-Cullin5 complex, which is a conserved path-

FIG 3 VEC-5 protects APOBECs from Vif-induced degradation. (A) VEC-5 stabilized A3G levels in a dose-dependent manner. HEK293T cells were cotransfected with A3G-HA and Vif-cmyc as indicated and then treated with control DMSO (lanes 1, 3, and 5), proteasome inhibitor MG132 (lane 6), or VEC-5 (lanes 2 and 4, 50 μ M; lanes 7 to 10, 5, 10, 25, and 50 μ M, respectively) for 48 h. (B and C) Similarly, VEC-5 enhanced A3C (B) and A3F (C) when Vif was coexpressed. HEK293T cells were cotransfected with an expression plasmid A3C-HA (B) or A3F-V5 (C) and Vif-cmyc as indicated. Transfected cells were then cultured with control DMSO or 50 μ M VEC-5 for 48 h. (D) VEC-5 prevented AGM A3G from SIVagmTan Vif-mediated degradation. HEK293T cells transfected with AGM A3G-HA and SIVagmTan Vif-cmyc were cultured with DMSO or 50 μ M VEC-5 for 48 h. (E) VEC-5 showed no effect on FIV Vif. HEK293T cells transfected with feline A3Z3-HA (fA3Z3-HA) and FIV Vif-cmyc were cultured with DMSO or 50 μ M VEC-5 for 48 h. For all panels, Western blot analysis was performed on cells after the treatments described above.

way used by diverse primate lentiviral Vif proteins (34). To characterize the breadth of inhibitory activities of VEC-5 against lentiviral Vif, we then asked whether VEC-5 could affect SIVagmTan Vif-induced AGM A3G degradation. As shown in Fig. 3D, expression of SIVagmTan Vif drastically reduced the level of AGM A3G (Fig. 3D, lane 2), which was partially rescued by VEC-5 (lane 3). To further evaluate the specificity of VEC-5, we used a recently characterized ElonginB/C-Cullin5 system. Feline immunodeficiency virus (FIV) Vif (fVif) was shown to specifically assemble with Cullin5 and ElonginB/C to degrade feline APOBECs in 293T cells (61), a mechanism quite similar to that of HIV/SIV Vif. Interestingly, the expression level of fA3Z3 (a feline APOBEC protein) was not changed by VEC-5 (Fig. 3E). We propose that this is due to differences between the human Vif-ElonginC (hVif-ElonginC) and fVif-ElonginC interfaces. It is possible that VEC-5 specifically acts on primate lentiviral Vif molecules, such as HIV Vif and SIV Vif, which possess a more conserved BC box. Because little structural data on these Vif proteins are available, we could not characterize the precise functional specificity of VEC-5.

Incorporation into the budding HIV virions allows A3G to exert its antiviral function. When these virions enter new host cells, A3G introduces multiple cytidine deaminations on the HIV-1 minus-strand cDNA and acts as a postentry block to viral infection (20, 39–41, 69). To provide direct evidence that the anti-HIV activity observed with VEC-5 is the consequence of enhanc-

ing A3G encapsidation, we next determined whether treatment of VEC-5 would lead to greater recovery of A3G levels in viral particles. The A3G expression plasmid was cotransfected into 293T cells with either a control vector (Fig. 4A, lane 1), HIV-1 proviral DNA pNL4-3 wild-type (lanes 2 and 4), or NL4-3 Δ Vif (lane 3). Transfected cells were then cultured with or without 50 μ M VEC-5 for 48 h before cell lysates and virions were collected for Western blot analysis. A3G proteins were detected in both cell lysates and virus particles. Tubulin, p55, and p24 were also detected as loading, transfection, and virus release controls. As expected, NL4-3 (WT) effectively blocked A3G packaging into virions, whereas NL4-3 Δ Vif failed to induce A3G degradation in cells and subsequently failed to exclude the virion packaging of A3G (Fig. 4A, lanes 2 and 3). Remarkably, addition of VEC-5 significantly restored A3G encapsidation in wild-type virions (Fig. 4A, lane 4) without blocking virus particle release. Therefore, VEC-5 increased the abundance of A3G in both HIV-1 producer cells and virions, indicating that this compound has the ability to improve both the metabolic stability and viral packaging of A3G.

A potential disadvantage for designing inhibitors to target the Vif-ElonginC interface is that Vif would still be bound to A3G, which may hinder A3G catalytic activity. Therefore, the next step was to confirm whether the A3G protected by VEC-5 still retained its antiviral activity. Virus produced by 293T cells described above was subsequently titrated in a MAGI assay. As

FIG 4 VEC-5 enhances viral incorporation of A3G and reduces viral infectivity. (A) HEK293T cells were cotransfected with A3G-HA and pNL4-3 or pNL4-3ΔVif as indicated and then treated with 50 μ M VEC-5 or control DMSO. After 48 h, cells and pelleted virions were examined for A3G expression. Pr55Gag was examined as a transfection control. Tubulin was detected as a loading control. (B) Relative infectivity of viruses produced in the experiment shown in panel A was assayed by infecting MAGI-CCR5 cells, with wild-type NL4-3 treated with DMSO set to 100%. Results shown are the average of three independent infections.

shown in Fig. 4B, the level of relative infectivity agreed with that of A3G in virions. VEC-5 treatment severely reduced the infectivity of wild-type NL4-3 by 70%. Our findings demonstrated that A3G molecules protected by VEC-5 retained their intrinsic antiviral activity.

VEC-5 inhibits the interaction between Vif and ElonginC. Vif recruits the ElonginB/C-Cullin5 complex and directly binds A3G for degradation (67). The major objective in our study was to design a small-molecule inhibitor specifically for disrupting Vif binding to ElonginC, thereby shielding A3G from Vif-mediated proteasomal degradation. After validating the antiviral activity of the VEC-5 compound, we then set out to locate its target within the A3G-Vif-E3 complex and determine whether it functioned as designed through the pathway described above. The biological relevance of VEC-5 in formation of the A3G-Vif-E3 complex was examined by a coimmunoprecipitation assay, in which the Vif-expressing vector was cotransfected with A3G, ElonginC, or Cullin5. As expected, in the absence of VEC-5, Vif exhibited strong binding affinities for A3G, ElonginC, and Cullin5 (Fig. 5A to C). Treatment with VEC-5 showed no effect on Vif binding to A3G (Fig. 5A), while the compound almost completely abolished binding of ElonginC (Fig. 5B) and Cullin5 (Fig. 5C).

The interaction between Vif and ElonginB/C complex has been detected *in vitro* (5). Here, we used label-free biolayer interferometry (BLI) technology to test the effect of VEC-5 on Vif-ElonginB/C binding (Fig. 5D). BLI is an optical technique that analyzes the interference pattern of light reflected from a layer of immobilized protein on the tip of a biosensor. Macromolecules binding to the biosensor produce an increase in optical thickness at the bio-

FIG 5 VEC-5 blocks Vif binding to ElonginB/C-Cullin5 ligase. (A) VEC-5 showed no effect on the A3G-Vif interaction. HEK293T cells were cotransfected with A3G-HA and Vif-cmyc. Cells were cultured in control DMSO or 50 μ M VEC-5. DMSO in the control group was replaced by 5 μ M MG132 24 h after transfection. At 48 h posttransfection, cell lysates were prepared and immunoprecipitated (IP) with anti-HA Ab-conjugated agarose beads. Expression of A3G in the cells and its interaction with Vif were detected by Western blotting. (B and C) VEC-5 prevented Vif binding to ElonginC (B) and Cullin5 (C). HEK293T cells expressing Vif and ElonginC (B) or Cullin5 (C) were treated with control DMSO or 50 μ M VEC-5. Protein interactions were detected by coimmunoprecipitation and Western blotting. (D) VEC-5 disrupts the interaction between recombinant Vif and ElonginB/C protein. Biosensors immobilized with the ElonginB/C protein complex were incubated with Vif protein in the presence of control DMSO or VEC-5. Binding to the biosensor, which was indicated by a wavelength shift detected, was monitored for 420 s.

FIG 6 Proposed model for the mechanism of action of VEC-5. (A) The top-scoring docking pose for VEC-5 to ElonginC and superposition onto the crystal structure of the Vif BC box in complex with ElonginC (PDB code 3DCG). Shown are the Vif BC box (pink) and ElonginC (gray). VEC-5 (yellow) largely mimics and interferes with three key residues in Vif, rendered by a stick model: Gly 143, Ser 144, and Leu 145. (B) Geometries of key residues in ElonginC (gray) that produce favorable interactions with VEC-5 (yellow) were modeled in the complexes according to the stable structure from the molecular dynamics simulation.

sensor tip, which results in a wavelength shift that can be followed in real time (1, 28). In our study, sensor tips were immobilized with the recombinant ElonginB/C complex followed by incubation with the Vif protein in the absence (Fig. 5D, blue line) or presence (red line) of 50 μ M VEC-5. The shift in wavelength was monitored for 420 s. As shown in Fig. 5D, addition of VEC-5 inhibited Vif binding to the ElonginB/C complex, thus reducing the signal detected by the sensor tip. Taken together, these results indicate that the ability of VEC-5 to prevent Vif-induced A3G degradation is through disruption of the Vif-ElonginC interaction, thereby preventing Vif from functioning as an adaptor to the E3 complex.

Predicted binding mode of VEC-5. Although tremendous work has been devoted to solving the structure of the HIV-1 Vif protein, the full-length Vif structure is not yet available, and computational analysis is still the best approach for designing and analyzing the binding mode of Vif inhibitors. To understand the structural basis of the binding of VEC-5, we performed computational docking studies using Discovery Studio. We docked VEC-5 into the ElonginC protein from the crystal structure of the Vif BC box in complex with ElonginB/C (Protein Data Bank [PDB] code 3DCG) (55) and compared the binding model of VEC-5 to that of the Vif BC box (Fig. 6A). The crystal structure showed that the Vif BC box forms a loop-helix structure, which protrudes into a hydrophobic pocket of ElonginC and forms a tight connection between Vif and ElonginC. Vif L145, which is located in the tip of the α -helix, fits into a hydrophobic pocket of ElonginC and is required for binding with ElonginC. The docking results showed that VEC-5 mimics the conformation of the Vif BC box and largely excludes the loop region. The ethoxycarbonyl in the side chain of VEC-5 lies in the same region as Vif G143. More importantly, the naphthalene ring overlaps with Vif L145, which may result in steric hindrance for insertion of L145.

To further elucidate the binding mode of VEC-5 with ElonginC, molecular dynamics simulation was performed. The position of VEC-5 with respect to the key residues in the binding site is shown in Fig. 6B. The naphthalene ring of VEC-5 stretches into

the large hydrophobic cavity formed by Pro95, Ile96, Ala97, Val73, Val74, Leu104, Leu105, Ala108, and Tyr76. The most important interaction here is that the naphthalene ring is almost parallel to Tyr76 and forms π - π stacking and hydrophobic interactions. The terminal phenyl is located in another small groove formed by Glu93, Pro92, and Ile91. These two aromatic terminals are linked by the indolizine ring in the center. Hydrogen bonds between the ethoxycarbonyl group and Lys80 and between benzoyl and Lys80 could also be observed. Such information can help us improve the efficacy of this compound through structure-guided optimization. However, to clearly understand the binding of VEC-5 with ElonginC, more detailed investigation is needed.

Since the crystal structure of full-length Vif has not been reported, it is difficult to identify the accurate binding site of VEC-5 and the conformational change in Vif-ElonginB/C-Cullin5 caused by this compound. Nevertheless, our data confirmed that the small-molecule compound VEC-5 is capable of disrupting Vif binding to ElonginB/C-Cullin5, thus allowing A3G to exhibit its antiviral function. VEC-5 may represent a prototype of a new class of HIV-1 Vif inhibitors that can break into the Vif-ElonginC interface and liberate the antiviral activity of APOBECs, effectively restricting viral replication.

DISCUSSION

In this study, we identified a potent Vif inhibitor, VEC-5, and showed that it could restrict virus replication in Vif-nonpermissive cells with low cytotoxic effects. The anti-HIV property of this compound was associated with its capacity to impair Vif-induced degradation of A3G. Treatment with VEC-5 resulted in a marked increase in A3G recovery from viral particles, which displayed reduced infectivity. VEC-5 could also stabilize levels of A3C and A3F when Vif was coexpressed. Thus, VEC-5 is a novel anti-HIV compound that can disrupt the formation of the A3G-Vif-E3 complex by blocking the interaction between Vif and ElonginC.

The HIV-1 Vif protein is indispensable for viral evasion of APOBEC3G-imposed restriction, and the mechanism of A3G degradation induced by Vif has been extensively studied. In gen-

eral, Vif functions as an adaptor, bridging A3G with cellular ElonginB/C-Cullin5 E3 ubiquitin ligase for downstream proteasomal degradation (11, 21, 31, 42, 52, 67, 73). Thus, there is great potential for inhibitors, such as small molecules and peptides, to prevent the assembly of the A3G-Vif-E3 complex and release A3G to perform its natural antiviral activity. This complex offers several protein-protein interactions as promising therapeutic targets.

First, disrupting the Vif-A3G interface is the most straightforward strategy to preserve A3G from Vif-induced degradation. The IMB-26/35 analog compounds are such Vif-A3G-targeted inhibitors with potent activity against HIV-1. In addition to A3G, however, other members of the APOBEC3 family (APOBEC3A to -H) also display various degrees of antiviral activity (22). Vif uses different N-terminal residues to bind and neutralize these proteins (48, 49, 54), which may narrow the breadth of inhibitory activities of the agents. It is conceivable that an inhibitor that can interrupt the Vif-A3G interaction and perhaps successfully enhance A3G activity yet may fail to rescue other APOBEC3s from Vif-mediated degradation.

A second plausible target is the multimerization domain in Vif, which allows it to form dimers, trimers, and even tetramers (3, 45). The homodimerization of Vif involving the PPLP domain has been shown to be critical for HIV infectivity (65, 66). It has been reported that peptides that can disrupt the Vif dimerization domain expectedly produce virions with a higher content of A3G and lower infectivity (45, 65).

Another potential therapeutic target is the Vif-E3 interaction. That is, Vif recruitment of cellular E3 ligase may be blocked by interfering with the binding of Cullin5 or ElonginC to Vif. Vif utilizes its BC box to interact with ElonginC and the HCCH motif to interact with Cullin5 (34, 42, 63, 64). One recent study demonstrated that a membrane-permeable zinc chelator, *N,N,N',N'*-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) can prevent Vif-Cullin5 binding and increase the stability of A3G (62). Until now, the Vif-ElonginC interaction was the last Vif function to be explored as a target for anti-HIV therapeutics. In the present study, we showed that the small-molecule compound VEC-5 was able to prevent Vif function through blocking the interaction between Vif and ElonginC. The highly conserved ¹⁴⁴SLQ¹⁴⁶ motif in Vif is responsible for its direct binding to ElonginC (4, 47, 68). It is further suggested that the SLQ motif is also involved in the recruitment of Cullin5. Introduction of an SLQ Vif mutant has been shown to significantly reduce the level of Cullin5 coprecipitated with Vif, indicating that the SLQ motif is required for Cullin5 association (42) although the molecular mechanism has not been fully explained. Interestingly, in our study the addition of VEC-5 drastically weakened the affinity of Vif to Cullin5 (Fig. 5C). It is reasonable that VEC-5 may block the key SLQ motif interacting with ElonginC, which, in turn, prevents the interaction with Cullin5.

A major concern of using Vif-ElonginC as a drug target is the possibility that Vif may still bind and coencapsidate with A3G, thus impairing A3G antiviral activity (19). Here, we showed that A3G protected by VEC-5 was capable of packaging into virions (Fig. 4A) and suppressing virus replication (Fig. 2B). Moreover, VEC-5 could also protect A3C and A3F from Vif-mediated destruction (Fig. 3B and C). It is reasonable that inhibitors that target the Vif-E3 interaction may display a broader antiviral activity than Vif-A3G inhibitors. On the other hand, an important principle in designing a Vif-E3 inhibitor is to avoid inhibition of the

cellular proteasomal pathway and minimize toxic side effects. Our results showed that VEC-5 also inhibited SIVagmTan Vif function (Fig. 3D), while it had no effect on FIV Vif (Fig. 3E), initially indicating that the antagonistic activity of VEC-5 is selective for lentiviral Vif. Additional studies are necessary to identify the precise binding site of VEC-5 and further delineate its molecular mechanism. VEC-5 may also serve as an initial candidate that can be further optimized by analyzing structure-function relationships to improve its inhibitory activity and target selectivity.

As mentioned above, several small molecules have been demonstrated to be capable of targeting the A3G-Vif-E3 axis and inhibiting Vif function (10, 15, 46). Among these compounds, IMB-26/35 acts through blocking the Vif-A3G interaction (10), while the precise working mechanisms of RN-18 and the more recently identified SN-2 remain unclear (15, 46). Although all of these compounds can stabilize the level of A3G, we found that they have different effects on Vif protein. Vif abundance is not significantly changed by IMB-26/35 or SN-2, but it is downregulated by RN-18. In contrast, we found that both A3G and Vif expression levels were increased when treated with our inhibitor VEC-5 (Fig. 3A). To date, there is no consensus on the necessity for polyubiquitination of Vif for A3G degradation (13, 51). However, the notion that both hA3G and HIV Vif are ubiquitinated and degraded by the same E3, ElonginB/C-Cullin5, is generally accepted (13, 16, 31, 43). The positive relationship between A3G and Vif level indicates that treatment with VEC-5 may result in collapse of the whole A3G-Vif-E3 complex, allowing both A3G and Vif to escape from degradation. The fact that RN-18 can downregulate Vif supports a hypothesis that this drug might block the polyubiquitination of A3G and therefore transfer more ubiquitin to Vif.

Regardless of whether VEC-5 itself will eventually be developed as a novel therapeutic, our *in silico* method for discovery of small-molecule inhibitors for HIV and evaluation of the resulting candidate compound provides valuable insights into the virus-host interaction, which may contribute to future anti-HIV interventions.

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