Identification of a Novel HIV-1 Inhibitor Targeting Vif-dependent Degradation of Human APOBEC3G Protein*§

Erez Perry†§, Ann Sheehy†, N. Miranda Nebane§, Andrew Jay Brazier†, Vikas Misra†, Kottampatty S. Rajendran*§, Sara J. Buhrlage**, Marie K. Mankowski†§, Lynn Rasmussen†, E. Lucile White†, Roger G. Ptak†§, and Dana Gabuzda†§§

From the Departments of †Cancer Immunology and AIDS and **Cancer Biology, Dana Farber Cancer Institute and Departments of §Pathology and ¶Neurology (Microbiology), Harvard Medical School, Boston, Massachusetts 02115, Department of Biology, College of the Holy Cross, Worcester, Massachusetts 01610, Southern Research Institute High Throughput Screening Center, Birmingham, Alabama 35205, and ‡‡Southern Research Institute, Department of Infectious Disease Research, Frederick, Maryland 21701

Background: The interaction between HIV Vif protein and innate antiviral factor APOBEC3G represents a potential therapeutic target.

Results: Screening for inhibitors of Vif-APOBEC3G interaction identified a small molecule, N.41, that protects APOBEC3G from Vif-mediated degradation and exhibits antiviral activity.

Conclusion: N.41 is a lead for further development as an antiviral.

Significance: These findings suggest new strategies for developing anti-HIV therapeutics.

APOBEC3G (A3G) is a cellular cytidine deaminase that restricts HIV-1 replication by inducing G-to-A hypermutation in viral DNA and by deamination-independent mechanisms. HIV-1 Vif binds to A3G, resulting in its degradation via the 26 S proteasome. Therefore, this interaction represents a potential therapeutic target. To identify compounds that inhibit interaction between A3G and HIV-1 Vif in a high throughput format, we developed a homogeneous time-resolved fluorescence resonance energy transfer assay. A 307,520 compound library from the NIH Molecular Libraries Small Molecule Repository was screened. Secondary screens to evaluate dose-response performance and off-target effects, cell-based assays to identify compounds that attenuate Vif-dependent degradation of A3G, and assays testing antiviral activity in peripheral blood mononuclear cells and T cells were employed. One compound, N.41, showed potent antiviral activity in A3G(+) but not in A3G(−) T cells and had an IC50 as low as 8.4 μM and a TC50 of >100 μM when tested against HIV-1 Ba.L replication in peripheral blood mononuclear cells. N.41 inhibited the Vif-A3G interaction and increased cellular A3G levels and incorporation of A3G into virions, thereby attenuating virus infectivity in a Vif-dependent manner. N.41 activity was also species- and Vif-dependent. Preliminary structure-activity relationship studies suggest that a hydroxyl moiety located at a phenylamino group is critical for N.41 anti-HIV activity and identified N.41 analogs with better potency (IC50 as low as 4.2 μM). These findings identify a new lead compound that attenuates HIV replication by liberating A3G from Vif regulation and increasing its innate antiviral activity.

Retroviruses interact with cellular factors that can support or suppress viral replication; host cell factors that suppress viral replication are termed host restriction factors. The first restriction factors identified against human immunodeficiency virus type 1 (HIV-1) were members of the human cytidine deaminase apolipoprotein B mRNA-editing catalytic polypeptide-like 3 (APOBEC3) family. These proteins inhibit not only HIV-1 but also a broad range of other viruses and endogenous retroelements (1–4). Among the three members of the APOBEC family that exhibit the most potent anti-HIV-1 activity in vivo, APOBEC3D (A3D), APOBEC3F (A3F), and APOBEC3G (A3G), A3G is the most well characterized and potent HIV-1 inhibitor (5).

The HIV-1 virion infectivity factor (Vif) is a 23-kDa viral accessory protein that counteracts the innate anti-HIV activity of A3G. In the absence of Vif, A3G is actively packaged into HIV-1 virions and deaminates cytidines in viral minus-strand DNA during reverse transcription, resulting in a G-to-A hyper-
Small Molecule Inhibitor of HIV Vif-APOBEC3G Interaction

HIV-1 Vif binds to and inactivates human A3G (huA3G) but not A3G expressed in African green monkeys (AGM) or rhesus macaques (34–38). Conversely, AGM simian immunodeficiency virus (SIVagm) Vif inactivates AGM and rhesus macaque A3G but not huA3G. This species specificity was demonstrated by altering a single amino acid in the Vif-binding site, 128DPD130 of huA3G. The D128K mutation controls species specificity (34, 35, 37, 38). The Vif-binding site (128DPD130) is adjacent to residues 124YYWX127, which have been implicated in A3G packaging into HIV-1 virions (39).

Regions of Vif important for binding and neutralization of APOBEC proteins and species-specific recognition have been mapped to its N terminus (18, 40–45). Residues 14DRMR17 play a role in the species specificity of Vif, whereas distinct regions in the N-terminal half of HIV-1 Vif were shown to be important for its interaction with A3G: residues 21WXSLVK26, 40YRHGY44, and 55VXIPX4L64, 60YXX72, 81LGXXJLX89, and 96QX6ADX1107 (40, 41, 46–49).

Small molecules that inhibit HIV-1 Vif function in vitro have recently been identified, but these compounds do not inhibit the Vif-A3G interaction (50–53). Another study identified two compounds, IMB-26 and IMB-35, as specific inhibitors of Vif-dependent degradation of huA3G via stabilization of A3G (54). Although this study demonstrated a Vif-dependent effect on inhibition, a mechanistic explanation for the specific inhibition was unknown, and compound activity was not characterized in physiologically relevant target cells. Here, we used a high throughput screen for inhibitors of Vif-A3G binding to identify a novel lead compound that specifically protects A3G from Vif-mediated degradation, thereby increasing A3G antiviral activity against HIV-1 replication.

**EXPERIMENTAL PROCEDURES**

**Cells**—HEK293T cells (from ATCC, Manassas, VA) and HEK293-APOBEC3G-HA cells (293/A3G, stably expressing HA-tagged A3G) were grown in DMEM supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories). HeLa-derived indicator TZM-bl cells (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl was from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc. (55)) were grown in DMEM supplemented with 10% FBS. T cell lines H9, CEM, CEM-SS, and SupT1 (obtained through the NIH AIDS Reagent Program) were grown in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin (Corning Cellgro). Fresh human PBMCs were isolated as previously described (56) from screened donors seronegative for HIV and hepatitis B virus (Biological Specialty Corp., Colmar, PA) and grown in RPMI 1640 supplemented with 15% FBS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin; cells were cultured with 4 µg/ml phytohemagglutinin (Sigma) for 48–72 h and cultured in RPMI 1640 supplemented with 15% FBS, 1-glutamine, penicillin, streptomycin, nonessential amino acids (MEM/NEAA; Hyclone), and 20 units/ml recombinant human IL-2 (R&D Systems Inc.) for 48 h before infection.

**Antibodies and Plasmids**—The following antibodies were used: rabbit anti-Vif (57), rat 3F10 anti-HA (Roche Applied Science), mouse anti-V5 (NOVEX), mouse anti-tubulin (Sigma), and rabbit anti-APOBEC3G (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: anti-APOBEC3G C-terminus from Dr. Jaisri Lingappa). The HIV-1 NL4–3 proviral plasmid pNLX (pNL4–3/Xmal) has been described previously (58). pNLXΔVif was created by cloning the ApaI-EcoRI fragment from NL4–3ΔVif. pAPOBEC3G-HA, pc-AGM-Apo3G-HA, and pEYFP-APOBEC3G were gifts of M. Malim (59), Nathaniel Landau, and T. Rana, respectively. pEYFP-C1 was from Clontech. pcDNA-HVif and pcDNA3.1-APOBEC3F-V5-His6 were obtained through the NIH AIDS Reagent Program: pcDNA-HVif was from Dr. Stephan Bour and Dr. Klaus Strebel (60), and pcDNA3.1-APOBEC3F-V5-His6 and pcDNA3.1-APOBEC3C-V5-His6 were from Drs. B. Matija Peterlin and Yong-Hui Zheng (61). Vif residues 1–94 and full-length Vif were cloned into pGEX-6P-1 expression vector (Novagen).

**Cell Transfection, Western Blot Analysis, and Co-immunoprecipitation**—HEK293T cells were cultured in DMEM with 10% FBS and transfected by Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. At 40–48 h post transfection, lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.5% Nonidet P-40, and 1% protease inhibitor mixture). Twenty-five µg of protein normalized by Bradford protein assay (Bio-Rad) were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore), and detected by standard Western blotting. For co-immunoprecipitation experiments, identical amounts of lysate were subjected to immunoprecipitation followed by Western blotting. HA-tagged proteins were immunoprecipitated by EZview Red anti-HA affinity gel (Sigma). For GST pulldown, 2.5 µg of recombinant protein was incubated with 10 µl of glutathione-Sepharose 4B beads and 250 µl of 293/A3G cells lysate for 1 h at 4 °C, the beads were washed, and isolated proteins were subjected to SDS-PAGE and Western blotting.

**Time-resolved Fluorescence Resonance Energy Transfer (TR-FRET) Assay**—The interaction between GST-Vif residues 1–94, which contains the A3G-biding site (mapped to residues 40–72), and a biotinylated peptide consisting of A3G residues 110–148 (bio-A3G) was detected using TR-FRET.
Small Molecule Inhibitor of HIV Vif-APOBEC3G Interaction

(40, 46). europium (EU-W1024-labeled anti-GST antibody, PerkinElmer Life Sciences) and Ulight (LANCE Ultra Ulighstreptavidin, PerkinElmer Life Sciences) served as the donor-acceptor pair. Briefly, 15 nM GST-Vif 1–94 protein was added to assay plates containing test compounds (1536 well format). After 30 min of incubation, 500 nM bio-A3G peptide was added. 2 nM europium-labeled anti-GST and 50 nM streptavidin-Ulight detection reagents were added 30 min later and incubated for 1 h. Samples were analyzed using Envision multiplate reader (PerkinElmer Life Sciences) with excitation at 340 nm and emission at 615 and 665 nm. The emission at 615 nm from europium-labeled anti-GST induces emission at 665 nm from Uligh conjugated to Streptavidin when the two molecules are in close proximity, resulting in a FRET signal (see Fig. 1A). A 307,520 compound library from the NIH Molecular Libraries Small Molecule Repository (MLSMR) was screened. The MLMSR collection of >300,000 compounds generically grouped into one of the following five categories: (a) specialty sets, comprising bioactive compounds such as known drugs and toxins, (b) non-commercial compounds, mainly from academic laboratories, (c) targeted libraries, (d) natural products, and (e) diversity compounds. A description of the library can be found at nih.gov. Negative control wells included all assay components in the absence of an inhibitor, and positive control wells included all assay components with GST-Vif 1–94 protein replaced by GST protein. Percent inhibition was determined as:

$\text{Percent inhibition} = \frac{100 \times \text{(test compound signal} - \text{median negative control signal)}}{\text{(median positive control signal} - \text{median negative control signal)}}$

Statistical analysis for calculating the % inhibition cutoff for selecting active hits was performed; FRET signal mean and standard deviation (S.D.) were calculated for all tested compounds, and the cutoff was set as 3 S.D. above the mean. All of the screening data has been published on PubChem under AID 1117320.

Dose-response and Counter Screen Assays—Compounds identified as hits in the screen were evaluated for dose-response in the TR-FRET assay, TR-FRET counter screen assay, and HIV-1 Tat-TAR fluorescence polarization (FP) assay. Compounds identified as hits in the screen were also evaluated in a Vero cell or THP-1 cell toxicity assay. The TR-FRET counter screen assay used 7.5 nM biotinylated GST protein in place of the unbound FAMTAR. Therefore, inhibition of Tat binding to FAMTAR leads to a decreased level of anisotropy. This assay was used to prioritize compounds, and the % increase in the y axis of a scatter plot.

HIV-1 Replication in Peripheral Blood Mononuclear Cells—Compounds were evaluated in dose-response assays using a 100 μM high test concentration (9 total concentrations using half-log dilutions) as described (56). Test drug dilutions were prepared at a 2× concentration in microtiter tubes, and 100 μl of each concentration was placed in designated wells. Activated

YFP-APOBEC3G Degradation Assay—2.5 × 10^6 HEK293T cells seeded in 96-well plates were transfected by Lipofectamine 2000 (Invitrogen) with pEYFP (counter screen), pEYFP-A3G, or pEYFP-A3G and HIV Vif plasmids. 24 h post-transfection, media were replaced with fresh media supplemented with DMSO (0.4%) or 40 μM concentrations of tested compound for 20 h. At 44 h post transfection, cells were lysed by M-PER Mammalian protein extraction reagent (Thermo Scientific) supplemented with protease inhibitor mixture (Roche Applied Science). Cleared supernatants of cell lysates were transferred to BD OptiLux 96-well plates (BD Biosciences), and YFP fluorescence intensity (FI) was measured by SpectraMax M5e (Molecular Devices) Multi-Mode Microplate Reader (excitation at 510 nm and emission at 530 nm). Small molecules increasing or decreasing YFP expression from pEYFP were excluded due to their nonspecific effects. The mean FI was calculated for each tested compound (2 replicates) and control (DMSO). To exclude molecules that increase YFP expression nonspecifically, a cutoff of 3 S.D. above the mean FI(YFP) measured for the DMSO control was established. A cutoff of 2 S.D. below the mean FI(YFP) measured for the DMSO control was established to exclude cytotoxic molecules. The remaining molecules were tested in cells expressing YFP-A3G alone or YFP-A3G and Vif. FI means were calculated for each tested compound (2 replicates) and control (DMSO) for treated cells expressing YFP-A3G + Vif or YFP-A3G alone. The following formula was used to calculate the percentage increase in YFP-A3G levels in compound-treated cells relative to non-treated cells (DMSO):

$\text{100} \times \frac{\text{FI(YFP-A3G)} \text{compound} - \text{FI(YFP-A3G)} \text{DMSO}}{\text{FI(YFP-A3G)} \text{DMSO}}$

100% represents the FI of DMSO-treated cells expressing YFP-A3G, whereas 0% represents the FI from DMSO-treated cells expressing YFP-A3G + HIV. A cutoff of 2 S.D. above the % increase in YFP-A3G increase calculated for the DMSO control was used to prioritize compounds, and % increase in FI relative to DMSO-treated control cells was calculated for each compound (see Fig. 2A). Molecules stabilizing YFP-A3G levels independently of Vif were expected to increase YFP-A3G levels (measured as FI), whereas compounds inhibiting YFP-A3G degradation through targeting Vif were expected not to increase YFP-A3G levels in the absence of Vif. To classify compounds as predicted to target either Vif or A3G proteins, we calculated -fold increase of YFP-A3G levels for each tested compound using the formula: compound-mediated -fold increase of YFP-A3G levels = FI(YFP-A3G)/FI(YFP-A3G)DMSO. We set a cutoff of 1 S.D. above the -fold increase of YFP-A3G levels calculated for the DMSO control (4 replicates). The -fold increase of YFP-A3G FI calculated for each compound is represented in the y axis of a scatter plot.

HIV-1 Replication in Peripheral Blood Mononuclear Cells—Compounds were evaluated in dose-response assays using a 100 μM high test concentration (9 total concentrations using half-log dilutions) as described (56). Test drug dilutions were prepared at a 2× concentration in microtiter tubes, and 100 μl of each concentration was placed in designated wells. Activated
PBMCs were plated at 50 µl/well (5 × 10^4 cells/well) in 96-well plates. A multiplicity of infection of ~0.1 of HIV-1_BaL (laboratory-adapted, Group M, Subtype B) was used to infect the reporter cell line TZM-bl. The titer of viruses collected 3, 6, and 9 days post infection were measured by RT assay as described (65). Virus stocks were prepared and monitored for cell viability using an MTS assay in freshly obtained PBMCs. Parallel plates lacking virus were prepared and monitored for cell viability using an MTS assay (Promega). PBMC cultures were maintained for 7 days at 37 °C, 5% CO₂. Cell-free supernatant samples were then collected and analyzed for reverse transcriptase (RT) activity (64), whereas cytotoxicity was measured by MTS assay.

**HIV-1 Replication in T Cells—**

HIV-1 NL4-3 (CXCR4-tropic) and NL4-3ΔVif viruses were produced by co-transfection of HEK293T cells, and viral titers were measured by RT assay as described (65). Virus stock (1 × 10^8 cpd) was used to infect 1 × 10^4 H9 and CEM T cells, and 2 × 10^4 cpd were used to infect SupT1 and CEM-SS T cells. 3 h post incubation at 37 °C, 5% CO₂, infected cells were washed 3 × to remove free viruses, and 100 µl of infected cells were added to wells in 96-well plates. Compounds were evaluated in dose-response assays using a 40 µM high test concentration (3–4 total concentrations using 1:2 dilutions). Test drug dilutions were prepared at a 2 × concentration in microtiter tubes, and 100 µl of each concentration was placed in appropriate wells. Ritonavir (protease inhibitor) was included as a positive control antiviral compound. Separate plates without virus were prepared in parallel for drug cytotoxicity studies. Plates were incubated for 6 days, and virus production was measured using p24 ELISA (PerkinElmer Life Sciences) according to the manufacturer’s instructions; compound cytotoxicity was measured by MTS assay. For testing antiviral activity against HIV-1 spreading infection, infected CEM and CEM-SS T cells were treated by increasing concentrations of 0, 2.5, 5, and 10 µM N.41. Viral production from infected cells was collected every 3 days, and infected cells were washed and incubated with fresh N.41 supplemented media. The titer of viruses collected 3, 6, and 9 days post infection was evaluated by RT assay.

**Single-round Infectivity Assay, Virus Purification, and A3G Virion Packaging Levels—** Viruses were produced by co-transfecting HEK293T cells with VSVG (vesicular stomatitis virus-G envelope protein) envelope plasmid, AGM or human APOBEC3G, human APOBEC3F, or human APOBEC3C and Vif-deficient proviral plasmid pNLXΔEnvA3Vif or pNLXΔEnvVif. Viruses were quantitated by RT assays, and normalized amounts were used to infect the reporter cell line TZM-bl. Infectivity was measured 48 h after infection by performing luciferase (Promega) or β-galactosidase assays (Applied Biosystems). To purify viruses, 150 × 10^3 cpd virus-containing supernatants (8.5 ml) were concentrated by ultracentrifugation through 1.7 ml of 20% sucrose in phosphate-buffered saline (PBS). Purified viruses were resuspended in Laemmli sample buffer (Bio-Rad) supplemented with 5% 2-mercaptoethanol, and p24Gag and A3G protein levels in the purified viruses were detected by Western blot. Statistical significance was evaluated by using Student’s t test (p < 0.05).

**Expression and Purification of GST and GST-Vif Fusion Proteins in Escherichia coli—** Briefly, E. coli Rosetta (DE3)pLysS competent cells (Novagen) were transformed with pGEX-6P-1 vectors expressing 1–94 GST-Vif and full-length GST-Vif constructs, and expression of recombinant proteins was induced by isopropyl 1-thio-β-D-galactopyranoside. Next, the bacterial culture was lysed and sonicated, and soluble GST recombinant proteins were purified through glutathione-Sepharose 4B fast Flow beads (GE Healthcare).

**RESULTS**

To identify compounds that inhibit the interaction between HIV-1 Vif and A3G in a high throughput format, we developed a homogeneous TR-FRET assay using LANCE (Lanthanide Chelate Excite) reagents (Fig. 1A) (40, 46). In this assay interaction between purified GST-Vif residues 1–94 (1–94 GST-Vif), which includes A3G binding sites, and a synthetic biotinylated peptide containing A3G residues 110–148 (bio-A3G), which includes the Vif-binding site, is detected by europium (europium-donor fluorophore)-labeled anti-GST antibodies and Streptavidin-Ulight (acceptor fluorophore). Interaction between GST-Vif and bio-A3G brings europium and Ulight into close proximity (~9 nm or less), supporting energy transfer between these molecules measured as a FRET signal. The attenuation of GST-Vif-bio-A3G interaction is expected to result in FRET signal reduction.

The screening pipeline to identify inhibitors of the HIV-1 Vif-A3G interaction is outlined in Fig. 1B. A 307,520 compound library from the NIH MLSMR was screened at a concentration of 6.25 µM. The compounds were screened in three different sets of ~100,000 compounds each. The median z-values calculated for each of the sets were 0.84, 0.70, and 0.77. Statistical analysis identified 63.0%, 65.1%, and 33.7% inhibition as cutoffs between inactive and hit compounds (see % inhibition formula under “Experimental Procedures”). Based on these statistical criteria, 3686 hits representing 3650 unique compounds were identified (36 hits overlapped between the three screens), and the overall hit rate for the screen was 1.2%. For initial follow-up testing of the compounds, hits were evaluated for dose response in the TR-FRET biochemical assay as well as in a PerkinElmer Life Sciences TR-FRET Counter Screen assay to identify compounds that quench the fluorescence signal or inhibit the GST anti-GST antibody interaction instead of Vif-A3G binding (i.e. to eliminate nonspecific inhibitors). The compounds were also tested in an HIV-1 Tat-TAR FP assay, which was selected as an additional fluorescence-based counter screen to determine specificity for Vif-A3G interaction. Lastly, a cytotoxicity assay using Vero (hits from first 100,000 compound set) or THP-1 cells (hits from second and third 100,000 compound sets) was used to identify cytotoxic compounds. Of 3686 hits identified in the screen, 195 hits achieved an IC_{50} value of <25 µM in the TR-FRET assay and were not active in any of the counter screen
secondary dose-response assays tested or were potent in the TR-FRET assay with an IC$_{50}$ of 0.195 μM (the low test concentration used in the experiments) with minimal activity observed in various counter screens. An additional 116 hits were flagged as potential Vif inhibitors because they exhibited at least a 10-fold difference in IC$_{50}$ values when secondary counter screen dose-response assays were compared with the TR-FRET biochemical assay. These combined 311 hits representing 303 unique compounds were identified for additional follow-up testing. Compound N.41 inhibited the Vif-A3G interaction by 89% when tested at 6.25 μM in the TR-FRET high throughput screening assay. For comparison, P15, a 15-mer Vif peptide (residues 57–71) used as a positive control (40, 46), exhibited an average inhibition of 40% at 50 μM and 93.5% at 100 μM. The secondary TR-FRET-based assay and counter screen testing of compound N.41 for specificity and activity validation. The z-values calculated for the counter screens and cytotoxicity assays ranged from 0.70 to 0.91.

We developed a cell-based assay to identify compounds that attenuate Vif-dependent degradation of YFP-A3G to screen the 311 hits selected for additional follow-up testing based on the primary screen and secondary dose-response experiments.

Compounds that significantly increased or decreased YFP levels in control experiments were excluded from additional follow-up testing. Cells expressing both YFP-A3G and Vif proteins were treated with DMSO (negative control) and the compounds of interest for 20 h. Cell lysates were assessed for YFP-A3G FI. The relative increase in FI in compound-treated cells that expressed YFP-A3G and Vif relative to untreated cells (DMSO control) was plotted. Fig. 2A shows a representative experiment. Fifty-eight of the tested compounds were identified as active molecules based on the percent increase of the FI being above a calculated cutoff (red dashed line in Fig. 2A).

Compounds targeting Vif were not expected to increase YFP-A3G levels in the absence of Vif. To identify such compounds, the fold increase of FI in compound-treated cells expressing YFP-A3G compared with DMSO-treated cells was determined, and a cutoff (blue dashed line in Fig. 2A) was set to classify hit compounds into two groups corresponding to those potentially targeting Vif (below the cutoff) or targeting A3G (above cutoff). Nineteen compounds that increased YFP-A3G levels in the presence of Vif also increased A3G levels in the absence of Vif (a subset of these compounds is shown in Fig. 2A, right upper quadrant). One example is compound C.18, a molecule identified in the cell-based screen as a false positive that
significantly increases YFP levels. Based on this phenotype, C.18 was included in subsequent experiments as a positive control for molecules targeting YFP-A3G. Compound N.41 fell just above the cutoff set to identify compounds potentially targeting A3G, suggesting it has an effect on A3G protein levels. To further investigate whether compound N.41 targeted Vif versus A3G, we examined the effect of N.41 treatment on endogenous A3G protein levels in CEM cells (A3G+). In these cells N.41 increased endogenous A3G protein in a dose-dependent manner (Fig. 2B).

We next determined the antiviral activity of hit compounds against HIV-1Ba-L replication in PBMCs. Only 255 of the 311 hits (250 of 303 unique compounds) identified were commercially available and obtainable in quantities necessary for followup studies. This assay identified 18 compounds in which the % increase in FI is above a specified cutoff (% increase in FIComp % increase in FI_DMSO + 2 S.D., red dashed line) used to classify hit compounds into two groups corresponding to those predicted to target Vif (below the cutoff) or A3G (above the cutoff). Compound C.18, identified in the cell-based assay as a false positive that significantly increases YFP levels, was included as a positive control for molecules that enhance YFP-A3G levels. N.41 likely stabilizes endogenous A3G protein levels in CEM cells. CEM T cells were treated with 0, 10, and 20 μM N.41 for 48 h. Endogenous A3G and β-tubulin protein levels in CEM cell lysates were detected by Western blotting. Results are representative of two independent experiments.

FIGURE 2. N.41 attenuates APOBEC3G degradation by Vif. A, fluorescent cell-based screen to identify molecules that attenuate Vif-mediated degradation of YFP-A3G. 293T cells expressing YFP-A3G with or without Vif were treated with DMSO or 40 μM concentrations of tested compounds for 20 h, then FI of treated cell lysates was measured. The change in FI (percentage change relative to untreated (DMSO) controls) in compound-treated cells expressing YFP-A3G and Vif is plotted on the x axis. Compounds for which the % increase in FI is above a specified cutoff (% increase in FIComp % increase in FI_DMSO + 2 S.D., red dashed line) are identified as active hits. To identify compounds that potentially target Vif, -fold increase in YFP-A3G levels was calculated as the change in FI in compound-treated cells expressing YFP-A3G relative to untreated (DMSO) control cells (y axis). Shown is a representative experiment and cutoff (-fold increase FI(YFP-A3G)_DMSO + 1 S.D., blue dashed line) used to classify hit compounds into two groups corresponding to those predicted to target Vif (below the cutoff) or A3G (above the cutoff). Compound C.18, identified in the cell-based screen as a false positive that significantly increases YFP levels, was included as a positive control for molecules targeting YFP-A3G. 293T cells expressing YFP-A3G with or without Vif were treated with DMSO or 40 μM concentrations of tested compounds for 20 h, then FI of treated cell lysates was measured. The change in FI (percentage change relative to untreated (DMSO) controls) in compound-treated cells expressing YFP-A3G and Vif is plotted on the x axis. Compounds for which the % increase in FI is above a specified cutoff (% increase in FIComp % increase in FI_DMSO + 2 S.D., red dashed line) are identified as active hits. To identify compounds that potentially target Vif, -fold increase in YFP-A3G levels was calculated as the change in FI in compound-treated cells expressing YFP-A3G relative to untreated (DMSO) control cells (y axis). Shown is a representative experiment and cutoff (-fold increase FI(YFP-A3G)_DMSO + 1 S.D., blue dashed line) used to classify hit compounds into two groups corresponding to those predicted to target Vif (below the cutoff) or A3G (above the cutoff).
left upper panel). A3F levels were increased by N.41 treatment only in cells co-expressing HIV-1 Vif, and A3C levels were unaffected by N.41 (Fig. 4A, right upper panels). No change in Vif protein levels was detected in N.41-treated cells. A3G levels were increased in virus particles produced from N.41-treated cells regardless of Vif expression (Fig. 4A, left lower panel), whereas A3F and A3C levels in virus particles were increased by N.41 treatment only in Vif-expressing cells (Fig. 4A, right lower panels). Next, we infected TZM-bl reporter cells with equivalent amounts of virus and found that N.41-treated cells expressing A3G produced virus with lower infectivity compared with untreated cells (Fig. 4B, left panel). In contrast, N.41 treatment had minor effects on virus infectivity that were not statistically significant in virus-producing cells expressing A3C and enhanced virus infectivity in cells expressing A3F (Fig. 4B, right panel).

Although cellular and virion-packaged A3G protein levels were increased in N.41-treated cells in the absence of Vif (Fig. 4A), the effect of N.41 on A3G protein levels was greater when Vif was present. HuA3G but not African green monkey A3G (agmA3G) is subject to HIV-1 Vif regulation. To examine if N.41 antiviral activity targeted the Vif-huA3G interaction, we tested whether N.41 could attenuate the production of infectious viruses from cells expressing agmA3G. VSV-G pseudotyped Vif(H11001) HIV-1 viruses and corresponding viruses that lack Vif (H9004Vif) were produced in 293T cells ectopically expressing HA-tagged huA3G or agmA3G proteins (Fig. 5A). Notably, in this experiment lower levels of A3G protein were expressed compared with the levels used in the infectivity assay (75 ng of transfected plasmid DNA instead of 200 and 400 ng used in Fig. 4) in an effort to achieve A3G expression at levels closer to physiological levels. Producer cells were treated for 40 h with 25 μM N.41 or DMSO (untreated). N.41 treatment significantly increased huA3G protein levels in the presence of HIV-1 Vif, whereas only a modest change was seen in its absence (Fig. 5A). N.41 had no effect on agmA3G protein levels, suggesting spe-
N.41 increases HIV-1 virion incorporation of A3G and decreases virus infectivity. A, 293T cells were co-transfected with 0, 200, or 400 ng of A3G-3xHA (left panel) or 250 ng of A3F-V5 or 250 ng A3C-V5 (right panels) and either pNLX HIV-1 ΔEnv or pNLX HIV-1 ΔEnv ΔVif plasmids. VSV-G was used for pseudotyping in single round infections. At 5 h post transfection, the media was replaced with fresh media supplemented with DMSO or 40 μM N.41. At 40 h post transfection, supernatants containing virus were collected, and producer cells were lysed. A3G, A3F, A3C, Vif, p24 Gag, and β-tubulin protein levels in producer cell lysates were analyzed by Western blotting. WCL, whole cell lysates (upper panels). Compound N.41 increases A3G incorporation into HIV-1 virions. Virions normalized for equivalent RT units were purified through 20% sucrose. A3G, A3F, A3C, Vif, and p24 Gag protein levels in virion lysates were detected by Western blotting (lower panels). Results are representative of two independent experiments. B, N.41 reduces infectivity of viruses produced from cells expressing A3G but not A3C or A3F proteins. TZM-bl reporter cells were infected with viruses corresponding to 4000 RT units. Luminescence values from infected cells were measured 48 h post infection. Shown is the percentage of infection relative to infection of untreated producer cells. *, p value is based on Student’s t test. Results are representative of two independent experiments each done in duplicate.
DISCUSSION

In this study the HIV-1 Vif-A3G interaction served as the primary target in a high throughput screen to detect inhibitors of this protein-protein interaction (Fig. 1B). We successfully identified a novel lead compound N.41 that disrupts this critical interaction. N.41 had a calculated IC$_{50}$ of 2.18 μM for inhibiting the interaction between GST-Vif (amino acids 1–94) and bio-A3G (amino acids 110–148) peptide in a TR-FRET-based assay (Fig. 1C). N.41 also attenuated Vif-dependent degradation of A3G in a cell-based assay (Fig. 2A) and inhibited viral replication in PBMCs (IC$_{50}$ as low as 8.4 μM and TC$_{50}$ > 100 μM). N.41 also inhibited virus replication in H9 (A3G+ T) cells and attenuated HIV-1 replication in a spreading infection assay in CEM (A3G+ T) cells. The difference in N.41 antiviral activity in H9 compared with CEM cells (Fig. 3, B and C) is likely attributable to differences in experimental design; the H9 experiment is a dose response at one time point, whereas the CEM experiment is a virus replication study over time. When the CEM experiment is plotted as a dose response for the same time point (day 6), N.41 shows similar antiviral activity in H9 and CEM cells (data not shown). N.41 exhibited no significant antiviral activity in the absence of A3G expression (SupT1 and CEM-SS T cells), suggesting an A3G-dependent mechanism. Further characterization of the mechanism of action of N.41 supported this conclusion. The cell-based assay showed that N.41 treatment reduced the infectivity of viruses produced from A3G-expressing cells (Fig. 4B), and Western blotting revealed that N.41 increased A3G protein levels in both producer cells and newly produced virions (Fig. 4A). Furthermore, N.41 treatment of CEM T cell lines increased endogenous A3G protein levels (Fig. 2B). Although N.41 treatment increased both cellular and virion A3G protein levels in the absence of Vif, effects on A3G protein levels were even more significant when Vif was present (Fig. 4A, left panel). Together, these results support the idea that compound N.41 specifically targets the A3G protein.

Vif preferentially suppresses APOBEC3 proteins of its host species (34–38). A single amino acid in A3G, aspartic acid at position 128 in huA3G versus lysine in agmA3G, controls this specificity by direct effects on Vif-A3G binding. As expected from the design of our screen, N.41 attenuated huA3G degradation by Vif and inhibited the production of infectious virions from cells expressing huA3G, but not agmA3G (Fig. 5, A and B). Given these findings, we predict that N.41 binds huA3G in a region close to this essential aspartic acid, thereby hindering the Vif-A3G interaction. A pulldown assay (Fig. 5C) and co-immunoprecipitation analysis (Fig. 5D) further supported this prediction. Interestingly, the packaging of both huA3G and agmA3G proteins was increased in Vif-deficient virions pro-
duced from N.41-treated cells (Fig. 5A, lower panel). The huA3G and agmA3G protein packaging motif (\(124\)YY\(X\)W\(127\)) is adjacent to the aspartic acid and lysine at position 128 (huA3G and agmA3G) that controls interaction with Vif. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.

In this study we found that N.41 treatment attenuated A3F but not A3C degradation in virus producing cells in a Vif-dependent manner (Fig. 4A, right panels). A recent study (67) revealed new determinants of Vif binding in the A3F-CTD: Glu-316, Ser-320, and Glu-324. These residues were shown to be part of a negatively charged interface that is directly involved in Vif binding. We speculate that N.41 binds both huA3G and agmA3G in this region and thereby increases packaging of these A3G proteins into virions.
that the hydroxyl moiety (analogs 3 and 12) or the amide hydrogen of the N-methyl-amide moiety (analogue 4) located at the para position of the phenylamino group are essential for activity, potentially through donation of a hydrogen bond to its target. Furthermore, conformational stabilization of the pyridine ring (as in analogue 12) appeared important for optimal potency, although the nitrogen itself was not required (as in analogue 3). These three active analogs also increased A3G levels in virus producer cells, but only analogs 3 and 12 increased A3G levels in newly produced virions (Fig. 7A). Unlike treatment with analogue 4, treatment with analogues 3 and 12 resulted in a less infectious virus produced in cells expressing A3G (Fig. 7B). Analogue 3 and 12 treatment also inhibited HIV-1 replication in PBMCs more potently than the parental compound N.41, with analogue 12 demonstrating an IC50 as low as 4.2 μM (Fig. 7C). The structure-activity relationship results imply that N.41 interacts with A3G and obstructs its recruitment and degradation by

---

**FIGURE 7.** Effect of compound N.41 and its most potent analogs on A3G virion incorporation, viral infectivity, and viral replication in PBMCs. A, compound N.41 and its analogs 3 and 12 increase A3G virion incorporation. 293T cells were co-transfected with or without 100 ng of huA3G-3xHA and pNLX HIV-1ΔEnv plasmids. VSV-G was used for pseudotyping HIV-1 envelope for single-round infections. At 5 h post transfection, media were replaced with fresh media supplemented with DMSO (untreated controls) or 40 μM N.41 or its analogs 3 and 4 or 20 μM concentration of analogue 12. At 40 h post-transfection, supernatants containing virus were collected, and producer cells were lysed. A3G, Vif, and β-tubulin protein levels in producer cell lysates were analyzed by Western blotting (upper panel; WCL, whole cell lysates). To assess A3G incorporation into virions, virions normalized for equivalent RT units were purified through 20% sucrose. A3G, Vif, and p24 Gag protein levels in virus lysates were detected by Western blotting (bottom panel). Results are representative of two independent experiments. B, effect of N.41 and analogs 3, 4, and 12 on infectivity of viruses produced from cells expressing A3G. TZM-bl reporter cells were infected with viruses corresponding to 4000 RT units. Luminescence from infected cells was measured 48 h post infection. Percentage of infection is relative to virus infection of untreated producer cells. *, p value based on Student’s t test. Results are representative of two independent infection experiments each carried out in duplicate. C, antiviral activity of the most potent N.41 analogs against HIV-1 replication in PBMCs. N.41 and its analogs were evaluated in dose-response assays using a 100 μM high test concentration and half-log dilutions. Compound-treated PBMCs were infected by the indicated HIV1 isolates (HIV-1Ba-L or NL4-3), and 7 days post infection virus replication and cell viability were measured by RT and MTS assays. Shown is the percentage virus replication and cell viability in compound-treated cells relative to levels measured in untreated cells. The table shows the virus isolates used for infections, and IC50, IC90, TC50, and Therapeutic Index of the tested compounds.
Vif, but due to the proximity of the Vif-binding site and A3G packaging motif, some molecules, such as analog 4, may also negatively affect A3G packaging into virions and weaken its antiviral capacity. N41 and its analogs such as 3 and 4 are enaminoketones (enaminones); unfortunately, such enaminones can have problems with stability. Therefore, goals for future work are to design lead compounds with better potency along with modified structures designed to increase their stability.

In summary, a primary screen for inhibitors of HIV-1 Vif-A3G binding together with cell-based screens assays for antiviral activity in relevant primary cells, and preliminary structure-activity relationship studies identified a parental N41 molecule as well as two analogs that can suppress HIV replication via A3G-mediated restriction. Further structure optimization studies may lead to the identification of additional potent HIV-1 inhibitors that could specifically shield A3G from degradation by Vif and unleash A3G innate antiviral activity.

Acknowledgments—We thank Dr. Caroline Shamu and staff at the Institute of Chemistry and Cell Biology Longwood Screening Facility, Harvard Medical School, Boston, MA, for helpful discussions and advice during performance of the high throughput screening and Dr. Marintha Heil, Southern Research Institute Department of Infectious Disease Research, for helpful discussions and advice. Core facilities received support from the Harvard Center for AIDS Research Grant P30 AI060354 and Dana-Farber Cancer Institute/Harvard Cancer Center Research Grant P30 CA06516.

REFERENCES

1. Esnault, C., Heidmann, O., Delebecque, F., Dewannieux, M., Ribet, D., Hance, A. J., Heidmann, T., and Schwartz, O. (2005) APOBEC3G cytidine deaminase inhibits retrotransposition of endogenous retroviruses. Nature 433, 420–423
2. Esnault, C., Millet, J., Schwartz, O., and Heidmann, T. (2006) Dual inhibitory effects of APOBEC family proteins on retrotransposition of mammalian endogenous retroviruses. Nucleic Acids Res. 34, 1522–1531
3. Harris, R. S., Bishop, K. N., Holmes, R. K., Craig, H. M., Klein, K. C., Lingappa, J. R., Albrecht, A. L., Cimermancic, P., Burlingame, A., Sali, A., Craik, C. S., Harris, R. S., Anderson, B. D., Yen, L., Stanley, D., Mahon, C., Kane, J., Franks-Skiba, N., and Malim, M. H. (2003) DNA deaminase inhibits retrotransposition. J. Virol. 7792–7798
4. Mehle, A., Strack, B., Ancuta, P., Zhang, C., McPike, M., and Gabuzda, D. (2004) Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway. J. Biol. Chem. 279, 7792–7798
5. Mehle, A., Thomas, E. R., Rajendran, K. S., and Gabuzda, D. (2006) A zinc-binding region in Vif binds Cul5 and determines cullin selection. J. Biol. Chem. 281, 17259–17265
6. Sheehy, A. M., Siddiqui, T., and Malim, M. H. (2007) The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1. Nat. Med. 9, 7238–7248
7. Stopak, K., de Noronha, C., Yonomoto, W., and Greene, W. C. (2003) HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. Mol. Cell 12, 591–601
8. Yu, X., Xu, Y., Liu, B., Luo, K., Kong, W., Mao, P., and Yu, X. F. (2003) Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. Science 302, 1056–1060
9. Yu, X., Xio, Z., Ehrlich, E. S., Xu, Y., and Yu, X. F. (2004) Selective assembly of HIV-1 Vif-Cul5-ElonginB-ElonginC E3 ubiquitin ligase complex through a novel SOCS box and upstream cysteines. Genes Dev. 18, 2867–2872
10. Mehle, A., Goncalves, J., Santa-Marta, M., McPike, M., and Gabuzda, D. (2004) Phosphorylation of a novel SOCS-box regulates assembly of the HIV-1 Vif-Cul5 complex that promotes APOBEC3G degradation. Genes Dev. 18, 2861–2866
11. Paul, I., Cui, J., and Maynard, E. L. (2006) Zinc binding to the HCC motif of HIV-1 virion infectivity factor induces a conformational change that mediates protein-protein interactions. Proc. Natl. Acad. Sci. USA. 103, 18475–18480
12. Stanley, B. J., Ehrlich, E. S., Short, L., Yu, Y., Xiao, Z., Yu, X. F., and Xiong, Y. (2008) Structural insight into the human immunodeficiency virus Vif SOCS box and its role in human E3 ubiquitin ligase assembly. J. Virol. 82, 8656–8663
13. Wang, X., Wang, X., Zhang, H., Lv, M., Zuo, T., Wu, H., Wang, J., Liu, D., Wang, C., Zhang, J., Li, X., Wu, J., Yu, B., Kong, W., and Yu, X. (2013) Interactions between HIV-1 Vif and human ElonginB-ElonginC are important for CBF-beta binding to Vif. Retrovirology 10, 94
14. Bisel, K., Dietz, D., Hultquist, J. F., Shindo, K., LaRue, R. S., Kwon, E., Li, M., Anderson, B. D., Yen, L., Stanley, D., Mahon, C., Kane, J., Franks-Skiba, K., Cimermancic, P., Burlingame, A., Sali, A., Craik, C. S., Harris, R. S.,
small molecule inhibitor of HIV Vif-APOBEC3G interaction

Gross, J. D., and Krogan, N. J. (2012) Vif hijacks CBF-B to degrade APOBEC3G and promote HIV-1 infection. *Nature* 481, 371–375

Zhang, W., Du, J., Evans, S. L., Yu, Y., and Yu, X. F. (2012) T-cell differentiation factor CBF-B regulates HIV-1 Vif-mediated evasion of host restriction. *Nature* 481, 376–379

Kao, S., Khan, M. A., Miyagi, E., Plishka, R., Buckler-White, A., and Strebel, K. (2003) The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15), a cellular inhibitor of virus infectivity. *J. Virol.* 77, 11398–11407

Kao, S., Miyagi, E., Khan, M. A., Takeuchi, H., Opi, S., Goila-Gaur, R., and Strebel, K. (2004) Production of infectious human immunodeficiency virus type 1 does not require depletion of APOBEC3G from virus-producing cells. *Retrovirology* 1, 27

Santa-Mart, M., da Silva, F. A., Fonseca, A. M., and Gonçalves, J. (2005) HIV-1 Vif can directly inhibit apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G-mediated cytidine deamination by using a single amino acid interaction and without protein degradation. *J. Biol. Chem.* 280, 8765–8775

Boger, H. P., Doehle, B. P., Wiegand, H. L., and Cullen, B. R. (2004) A single amino acid difference in the host APOBEC3G protein controls the primate species specificity of HIV type 1 virion infectivity factor. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3770–3774

Mangeat, B., Turelli, P., Liao, S., and Trono, D. (2004) A single amino acid determinant governs the species-specific sensitivity of APOBEC3G to Vif action. *J. Biol. Chem.* 279, 14481–14483

Mariani, R., Chen, D., Schröfelbauer, B., Navarro, F., König, R., Bollman, B., Münk, C., Nymark-Mcmahon, H., and Landau, N. R. (2003) Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* 114, 21–31

Schröfelbauer, B., Chen, D., and Landau, N. R. (2004) A single amino acid of APOBEC3G controls its species-specific interaction with virion infectivity factor (Vif). *Proc. Natl. Acad. Sci. U.S.A.* 101, 3927–3932

Xu, H., Svarovskia, E. S., Barr, R., Zhang, Y., Khan, M. A., Strebel, K., and Pathak, V. K. (2004) A single amino acid substitution in human immunodeficiency virus type 1 Vif protein confers resistance to HIV-1 virion infectivity factor-induced depletion. *Proc. Natl. Acad. Sci. U.S.A.* 101, 5652–5657

Huthoff, H., and Malim, M. H. (2007) Identification of amino acid residues in APOBEC3G required for regulation by human immunodeficiency virus type 1 Vif and Virion encapsidation. *J. Virol.* 81, 3807–3815

Mehle, A., Wilson, H., Zhang, C., Brazier, A. J., McPike, M., Perry, E., and Gabuzda, D. (2007) Identification of an APOBEC3G binding site in human immunodeficiency virus type 1 Vif and inhibitors of Vif-APOBEC3G binding. *J. Virol.* 81, 12325–12341

Russell, R. A., and Pathak, V. K. (2007) Identification of two distinct human immunodeficiency virus type 1 Vif determinants critical for interactions with human APOBEC3G and APOBEC3F. *J. Virol.* 81, 8201–8210

Schröfelbauer, B., Senger, T., Manning, G., and Landau, N. R. (2006) Mutational alteration of human immunodeficiency virus type 1 Vif allows for functional interaction with nonhuman primate APOBEC3G. *J. Virol.* 80, 5984–5991

Simon, V., Zennou, V., Murray, D., Huang, Y., Ho, D. D., and Bieniasz, P. D. (2005) Natural variation in Vif: differential impact on APOBEC3G/3F and APOBEC3F neutralizing activity. *Nature* 430, 8701–8709

Gonzalez, J., Afouda, B., Cardona, F., Lozada, L., and Popovic, M. (1986) The role of mononuclear phagocytes in HTLV-I/HIV-1 infection. *Science* 233, 215–219

Louwagie, J., Delwart, E. L., Mullins, J. I., McCutchan, F. E., Eddy, G., and Burke, D. S. (1994) Genetic analysis of HIV-1 isolates from Brazil reveals presence of two distinct genetic subtypes. *AIDS Res. Hum. Retroviruses* 10, 561–567

Buckheit, R. W., Jr., and Swanstrom, R. (1991) Characterization of an HIV-1 isolate displaying an apparent absence of virion-associated reverse transcriptase activity. *AIDS Res. Hum. Retroviruses* 7, 295–302

Gonzalez, J., Korin, Y., Zack, J., and Gabuzda, D. (1996) Role of Vif in human immunodeficiency virus type 1 reverse transcriptase. *J. Virol.* 70, 8701–8709

Yu, Q., Chen, D., König, R., Mariani, R., Unutmaz, D., and Landau, N. R.
APOBEC3B and APOBEC3C are potent inhibitors of simian immunodeficiency virus replication. *J. Biol. Chem.* **279**, 53379–53386

67. Siu, K. K., Sultana, A., Azimi, F. C., and Lee, J. E. (2013) Structural determinants of HIV-1 Vif susceptibility and DNA binding in APOBEC3F. *Nat. Commun.* **4**, 2593

68. Kitamura, S., Ode, H., Nakashima, M., Imahashi, M., Naganawa, Y., Kurosawa, T., Yokomaku, Y., Yamane, T., Watanabe, N., Suzuki, A., Sugiura, W., and Iwatani, Y. (2012) The APOBEC3C crystal structure and the interface for HIV-1 Vif binding. *Nat. Struct. Mol. Biol.* **19**, 1005–1010

69. Aydin, H., Taylor, M. W., and Lee, J. E. (2014) Structure-guided analysis of the human APOBEC3-HIV restrictome. *Structure* **22**, 668-684

70. Bohn, M. F., Shandilya, S. M., Albin, J. S., Kouno, T., Anderson, B. D., McDougle, R. M., Carpenter, M. A., Rathore, A., Evans, L., Davis, A. N., Zhang, J., Lu, Y., Somasundaran, M., Matsuo, H., Harris, R. S., and Schiffer, C. A. (2013) Crystal structure of the DNA cytosine deaminase APOBEC3F: the catalytically active and HIV-1 Vif-binding domain. *Structure* **21**, 1042–1050