Small Molecular Compounds Inhibit HIV-1 Replication through Specifically Stabilizing APOBEC3G*  

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APOBEC3G (hA3G) is a host inhibitor for human immunodeficiency virus, type 1 (HIV-1). However, HIV-1 Vif binds hA3G and induces its degradation. We have established a screening system to discover inhibitors that protect hA3G from Vif-mediated degradation. Through screening, compounds IMB-26 and IMB-35 were identified to be specific inhibitors for the degradation of hA3G by Vif. The inhibitors suppressed HIV-1 replication in hA3G-containing cells but not in those without hA3G. The anti-HIV effect correlated with the endogenous hA3G level. HIV-1 particles from hA3G(-) cells treated with IMB-26/35 contained a hA3G level higher than that from those without IMB-26/35 treatment and showed decreased infectivity. IMB-26/35 bound directly to the hA3G protein, suppressed Vif/hA3G interaction, and therefore protected hA3G from Vif-mediated degradation. The compounds were safe with an anti-HIV therapeutic index > 200 in vitro. LD50 of IMB-26 in mice was > 1000 mg/kg (intraperitoneally). Therefore, IMB-26 and IMB-35 are novel anti-HIV leads working through specific stabilization of hA3G.

Host cells respond with defensive strategies in human immunodeficiency virus type 1 (HIV-1) infection. To overcome the host defensive responses, the virus develops offensive tactics, among which the HIV-1 accessory protein Vif (virion infectivity factor) plays an important role in the host/pathogen interaction. Vif is a 190–240-amino acid protein that is encoded by all of the lentiviruses except equine infectious anemia virus (1–6). It is required for HIV-1 to replicate in certain “nonpermissive” cell types, which include major HIV-1 host cells of primary T-lymphocytes, macrophages, as well as T-cell lines (for instance H9 cells). Vif is not required for viral replication in the “permissive” cell types, such as SupT1, Jurkat, 293T, HeLa, and CEM-SS cell lines (3, 5, 7). Recent studies demonstrate that nonpermissive cells contain a protein called human APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G, hereafter referred to as hA3G), which inhibits HIV-1 replication in the absence of Vif (8). The hA3G belongs to an APOBEC superfamily, which covers at least 10 members sharing a cytidine deaminase motif (a conserved His-X-Glu and Cys-X-X-Cys Zn2+ coordination motif) (9). Accumulating evidence demonstrates that hA3G induces G-to-A hypermutation in newly synthesized viral DNA and is capable of inhibiting the replication of a variety of retroviruses and nonretroviruses, suggesting that hA3G and its analogs are novel components of innate immunity against viral infection.

HIV-1 Vif binds to hA3G in the cytoplasm as part of the Vif-Cul5-SCF complex, that facilitates the hA3G ubiquitination and subsequent degradation by proteasomes (10). It prevents hA3G from its incorporation into HIV-1 and thereby abolishes the anti-HIV activity of hA3G (11). As hA3G expresses in human cells hosting HIV-1, blockage of Vif-mediated hA3G degradation represents a new anti-HIV-1 strategy for drug discovery. Due to the essential role of the ubiquitin proteasome pathway system in cell survival, interruption of proteasomal function is not considered an option in this study. We focused our attention in finding small molecule inhibitors that selectively block the Vif-hA3G interaction.

EXPERIMENTAL PROCEDURES

Plasmids—SVC21BH10 is a simian virus 40-based vector that contains full-length wild type HIV-1 proviral DNA. The sequence coding for full-length Vif was amplified with PCR from SVC21.BH10 and inserted into pcDNA3.1 (Invitrogen). The cDNA fragment coding for hA3G was also cloned into pcDNA3.1, which expresses wild type hA3G with a fused hemagglutinin (HA) tag at the C terminus, as described previously (12). To generate Vif-induced degradation reporter, hA3G coding sequence was PCR-amplified and cloned into pEYFP-C1 (Clontech), and resultant plasmid pH3A3G-YFP expressed a fused YFP at the C terminus of hA3G. The adenovirus E4orf6 and human p53 expression vectors (pCMV6.9 and pC53SN3, respectively) were gifts from Gary Ketner (13). For BRET2 assay, the sequence coding for full-length hA3G or the N-terminal fragment of Vif (amino acids 1–69) were cloned...
into pHGF2 or pRLuc (Perkin-Elmer), respectively, generating RLuc-hA3G and GFP2-Vif.deltaC. All of the cDNA clones were verified by sequencing.

**Cells, Transfections, and Virus Purification**—The 293T, H9, MT-4, and SupT1 cells were obtained from the American Type Culture Collection. TZM-BL indicator cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (8129). Procedure for cell culture, cell transfection, and virus purification were described previously (12). To estimate viable HIV-1 virions in culture supernatants, TCID_{50} (50% tissue culture infectious dose) was determined using a streamlined end point dilution assay that was analyzed with the Spearman-Karber statistical method. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) was used to evaluate cytotoxicity.

**Compounds**—IMB-26 [(3-(α-bromopropionyl)aminoo-4-methoxybenzene][formyl-3′,4′,5′-trimethoxybenzene] amide, C_{20}H_{23}N_{2}O_{6}Br, M, 467.31] and IMB-35 [(3-(α-bromopropionyl)aminoo-4-methoxybenzene][formyl-3′,4′,5′-trimethoxybenzene] ester, C_{20}H_{22}NO_{7}Br, M, 468.30] were synthesized in a medicinal chemistry laboratory at the Institute of Medicinal Biotechnology, with a purity of >98.5%. The proteasome inhibitor MG132 was purchased from Sigma.

**Protein Analysis**—Cellular and viral proteins were extracted with radiomimic precipitation assay buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 2 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin A, 100 mg/ml phenylmethylsulfonyl fluoride) and analyzed in SDS-PAGE (10% acrylamide), followed by blotting onto nitrocellulose membranes (Amersham Biosciences Pharmacia). Western blots were probed with monoclonal antibodies that are specifically reactive with HIV-1 p24 (ZeptoMetrix, Inc.), YFP, HA (Santa Cruz Biotechnology), β-actin (Sigma), or with hA3G, Vif-specific polyclonal antisera (National Institutes of Health AIDS Research and Reference Reagent Program). Protein detection was performed with enhanced chemiluminescence (NEN Life Sciences Products), using anti-mouse (for p24 and β-actin) and anti-rabbit (for HA, Myc, and Vif) secondary antibodies (Amersham Biosciences). Co-immunoprecipitation assay was done as described previously (10). Briefly, 293T cells were lysed in TNT buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1% Triton X-100); the supernatant containing an equal amount of protein were incubated with Vif-specific antibody, followed by addition of protein A-Sepharose. The immunoprecipitate was washed three times with TNT buffer and twice with phosphate-buffered saline and then boiled in the loading buffer. The resulting supernatant was analyzed using Western blots.

**Real-time PCR**—Total cellular RNA was extracted with TrizolTM (Invitrogen), and β-actin mRNA was quantitated using real-time RT-PCR. Total cellular RNA samples containing equal amounts of β-actin mRNA were used to generate cDNA, using SuperScriptTM II reverse transcriptase (Invitrogen) and with dT-oligomer. The amount of hA3G cDNA produced from an equal amount of total cellular RNA was determined using real-time fluorescence-monitored PCR with pairs of primers specific for hA3G, as described previously (12).

**BRET2 Assay**—The BRET assay used for determination of the protein/protein interaction has been described previously in detail (15). Briefly, 293T cells were co-transfected with hGFP2-Vif.deltaC and hRLuc-hA3G and harvested 40 h after transfection. Then, 293T cells were distributed into 96-well microplates (white Optiplate; Perkin-Elmer) with a density of 2 × 10^3 cells/well; substrate DeepBlueC (Perkin-Elmer) was injected at a final concentration of 5 μM. The signals detected at 395 and 510 nm were measured sequentially on a Mithras LB 940 plate reader (Berthold Technologies). BRET ratio was determined using the equation below: ((emission at 510 nm/emission at 395 nm) in cells expressing hRLuc and hGFP2 fusion proteins) − (emission at 510 nm/emission at 410 in cells expressing hRLuc alone).

**BIAcore**—Surface plasmon resonance experiments were performed on a BIAcore biosensor system (Biacore AB). In brief, the carboxymethylated surface of the sensor chip CM5 was first activated with a mixture of N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Biacore AB). Subsequently, 35 μl of purified Vif or hA3G at a concentration of 60 μg/ml was injected into the flow cell 2 (FC2) for immobilization on the sensor surface. No protein was injected into the FC1. Experiments were performed at 25 °C in HBS buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% Nonidet P-40, pH 7.4) at a flow rate of 10 μl/min. To analyze the binding of the compounds with Vif or hA3G, 30 μl of IMB-26/35 (10 μM in HBS buffer) were injected, followed by flowing the buffer over the chip and an elution step with 2 mM dithiothreitol in HBS buffer. Difference resonance spectra (FC2–FC1) were recorded. Data were evaluated using the software BIAevaluation 3.0 (Biacore AB). Purified HIV-1 Vif (>99% purity, catalog no. 11096) and hA3G (>95% purity, catalog no. 10067) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health.

**HIV-1 Infectivity Analysis**—HIV-containing supernatant from the 293T cells transfected with proviral DNA was used for infection. Viruses normalized by p24 level were added to the H9, MT4, or SupT1 cell lines, and HIV-1 replication was determined by measuring the amount of p24 in the culture supernatant with an HIV-1 p24 enzyme-linked immunosorbent assay kit. Single cycle infectivity was determined by challenging 10^5 TZM-BL indicator cells with viruses corresponding to 5 ng of p24 for 28 h and then measuring the induced expression of luciferase activity in the cell lysates. The TZM-BL indicator cells were generated from stably transfected CD4+ HeLa cell line, JC53, by introducing separate integrated copies of the luciferase and β-galactosidase genes under control of the HIV-1 promoter. Infection was measured by the induction of luciferase activity, which was measured using luminometer Lumat LB9507 (Berthold).

**Acute Toxicity**—Male Balb/c mice, weighing 19 ± 1 g, were purchased from the Institute of Laboratory Animal Science, Beijing, China. They were fed with regular rodent chow and housed in an air-conditioned room with five mice per cage. The mice were randomly divided into five groups with five mice each and received saline (as control) or IMB-26 at 250, 500, 750, or 1000 mg/kg, respectively. IMB-26 was given once intraperi-
toneally. After treatment, animals were monitored for body weight change as well as death. 14 days later, animals were sacrificed; their livers, kidneys, hearts, lungs, and spleen were fixed in 10% formaldehyde at room temperature for hematoxylin and eosin staining.

RESULTS AND DISCUSSION

We first established an assay in the 293T cells to measure Vif-mediated degradation of hA3G using an YFP-fused hA3G as a reporter. In this assay, co-expression of hA3G-YFP and Vif causes degradation of YFP that is fused with hA3G. The amount of YFP in the cells reflects the Vif-mediated hA3G degradation and could be measured by quantification of the YFP fluorescent emission using a fluorescence reader. The assay model is illustrated in Fig. 1A, and it has been adapted to a cell-based high throughput screening in our laboratory to identify inhibitors for Vif-mediated hA3G degradation. To ensure the quality and reproducibility of the assay, the test condition was optimized after evaluation, modification, and validation of the prototype of high throughput screening assay based on the Z'-factor calculation, signal/noise ratio, and well-to-well variability (coefficient of variability) (16). Next, using this assay model, we performed a screening test against compounds in the chemical library and natural product collection at the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Beijing, China. Among 8634 samples screened, two compounds
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with a code of IMB-26 (C_{20}H_{23}N_{2}O_{7}Br, MW, 467.31) and IMB-35 (C_{20}H_{22}NO_{7}Br, MW, 468.30) were discovered to be effective in inhibiting hA3G degradation in this model. Interestingly, the two compounds have a similar structure (Fig. 1B), confirming the activity of the compounds with this chemical backbone. As the two compounds are analogs, we expressed them as IMB-26/35 hereafter. Like MG132, a well known proteasome inhibitor, IMB-26/35 significantly restored the fluorescent emission and stabilized expression of hA3G-YFP in the presence of Vif; in contrast, the two compounds were inactive on hA3G in the absence of Vif (Fig. 1, C and D). It suggests that the stabilization of hA3G-YFP by IMB-26/35 resulted from the inhibition of Vif-mediated hA3G degradation.

To learn whether IMB-26/35 inhibits Vif-mediated degradation of hA3G specifically or interrupt the proteasomal-dependent degradation pathway as MG132 does, we examined the effect of IMB-26/35 on the well characterized Cul5 E3 ligase systems. The adenovirus protein E4orf6 is known to act as a Cul5 substrate receptor, with a function of degrading cellular p53 (13). Similar to the screening assay described above, a plasmid expressing YFP-tagged p53 was constructed. It was used to transfect 293T cells alone, or co-transfect the cells with an E4orf6-containing plasmid. The expression level of p53 was altered by the treatment (Fig. 2A, lower panel). In a one-round HIV-1 infectivity assay using TZM-BL cells, IMB-26/35 treatment reduced the infectivity by 90% for the HIV-1 particles generated from the hA3G-positive 293T cells, whereas they appeared to be inactive to the infectivity of the HIV-1 from the 293T cells free of hA3G (Fig. 2B), indicating that IMB-26/35 inhibits HIV infectivity through hA3G. We next assessed the inhibitory effect of IMB-26/35 upon HIV-1 replication in human T-cell lines H9, MT-4, and SupT1 cells. After HIV-1 infection, the T-cells were treated with 2 μM IMB-26/35. As a viral replication indicator HIV-1 p24 antigen was determined in culture supernatant at day 4 post-infection. IMB-26/35 displayed an anti-HIV effect at different degrees in the three cell lines, with 97% p24 reduction in H9, 85% in MT-4, and ~20% in SupT1 cells (Fig. 2C). To further evaluate the antiviral effectiveness of the compounds, we measured HIV-1 replication curves over a 2-week period in the presence of IMB-35 (Fig. 2D). Consistent with the results presented in Fig. 2C, IMB-35 showed a significant inhibition on HIV-1 replication in H9 but not in SupT1 cells. We then determined the expression level of the endogenous hA3G in the three cell lines by quantification of its mRNA using real-time RT-PCR. We found that the hA3G mRNA level was in the order of H9 > MT-4 > SupT1 (data not determined by quantification of the emission of YFP fused at the C-terminus of p53 and by Western blotting of cell lysates. As shown in Fig. 1, E and F, introduction of E4orf6 reduced the p53 level by >70%, whereas addition of MG132 restored the emission to 80% of that absent of the viral protein. However, addition of IMB-26/35 to the culture medium of the co-transfected cells had almost no protective effect on the cellular level of p53 in the presence of E4orf6, suggesting that IMB-26/35 are specific inhibitors for Vif-mediated hA3G degradation and have no inhibitory effect on the proteasomal pathway.

We next tested IMB-26/35 for their effect on the infectivity of HIV-1 particles produced from 293T cells. The 293T cells were co-transfected with HIV-1 proviral DNA and hA3G-expressing plasmid and then cultured for 48 h with or without IMB-26/35 treatment. The resultant viruses were collected from the culture supernatant. Western blot of HIV viral lysates showed that IMB-26/35 significantly restored virion encapsidation of hA3G to a level similar to that detected in the Vif-deficient HIV-1 (Fig. 2A, upper panel). The HIV-1 p24 level in the lysates was not...
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The viruses were used to infect TZM-BL indicator cells; the viral infectivity was measured as the induction of luciferase activity. Values presented are the normalized infectivity relative to HIV-1.Vif control. As shown). This result was consistent with previous measurement of the endogenous hA3G in these cells (17) and positively correlated with the anti-HIV effect of the compounds. It further validates the hA3G-mediated anti-HIV mechanism of the two compounds. It appears that the anti-HIV activity of IMB-26/35 results from their ability of blocking Vif-mediated hA3G degradation in host cells. It is noteworthy that IMB-26/35 has no activity on HIV-1 reverse transcriptase, protease, and integrase (data not shown). IMB-35 displayed a potant activity against HIV-1 in the permissive H9 cells with an EC_{50} of 17 nM; no cytotoxicity was detected in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay even when the concentration of IMB-35 was up to 3638 nM, resulting in a therapeutic safety index of >200. IMB-26 showed an anti-HIV effect similar to that of IMB-35.

As IMB-26/35 specifically inhibits the degradation of hA3G by Vif but not the degradation of p53 by E4orf6, the compounds may target Vif recruiting of the Cul5 complex or Vif binding to hA3G. Co-immunoprecipitation assay showed that IMB-26/35 treatment significantly reduced the amount of Vif bound to hA3G, with no effect upon the interaction between Vif and Cul5 (Fig. 3A), hinting that the Vif/hA3G interaction may be the target of IMB-26/35. To further confirm the co-immunoprecipitation result, we investigated the effect of IMB-26/35 upon the Vif/hA3G interaction using a BRET2 assay. In this assay, GFP and luciferase (RLuc) were fused with the N-terminal part of Vif (a truncated form of Vif able to bind to hA3G but not to degrade hA3G) and hA3G, respectively; a BRET value quantitatively reflects the interaction between the two proteins (Vif and hA3G that were fused with GFP and RLuc, respectively). As shown in Fig. 3B, addition of IMB-26/35 to the cultured 293T cells co-expressing the fusing Vif and hA3G reduced the BRET value in a dose-dependent manner. These results reveal that the Vif/hA3G interaction was inhibited in IMB-26/35 treated cells. Furthermore, addition of more IMB-26/35 in the concentration range as indicated above accompanied with an increase of cellular hA3G (Fig. 3C) as well as a decrease of the infectivity of HIV-1 particles from the hA3G-positive 293T cells (Fig. 3D). These data show a striking correlation among the effect of the compounds on viral infectivity, hA3G degradation, and Vif/hA3G interaction and indicate that the Vif/hA3G interaction may be the target of IMB-26/35. In fact, no hA3G polyubiquitination by Vif was detected in the IMB-26/35 (2 μM) treated samples (data not shown), agreeing with the conclusion that hA3G polyubiquitination occurs only after hA3G/Vif interaction.

Vif has been reported to bind to hA3G through a direct protein/protein interaction (18, 19); thus, we hypothesized that IMB-26/35 might bind to hA3G or Vif, resulting in a blockage of Vif/hA3G interaction. To test this hypothesis, we used a BIAcore system to measure the binding affinity of IMB-26/35 in its interaction with either hA3G or Vif in vitro and searched for the primary binding target of IMB-26/35 by comparing the binding affinity of these two interactions. The result in Fig. 3E showed a direct interaction of IMB-26/35 with hA3G but not Vif. The fact that IMB-26/35 were able to inhibit the interaction of Vif/hA3G but not Vif/Cullin5 provides further evidence to
support that these compounds appear to bind at hA3G directly, inhibit the interaction between Vif and hA3G, and thereby stabilize hA3G. The mode of action of IMB-26/35 seems to be very different from that of RN-18, which inhibits Vif/hA3G interaction through reduction of Vif in the host cells (14).

Interestingly, IMB-26/35 did not alter the editing activity of hA3G in vivo, whereas a direct interaction between the compounds and hA3G was observed. We infected the SupT1 cells with Vif-deficient HIV-1, which was produced from hA3G-expressing 293T cells with or without IMB-26 treatment. DNA was extracted from the cells 24 h post-infection, and PCR products representing the BH10 DNA sequence 492–764 were sequenced and examined for mutations, using the method described in our previous work (20). As shown in Table 1, we did not find significant difference in the G-to-A mutation rate between the two group samples, i.e. a total of 58 mutations in 2720 nucleotides sequenced was found in the untreated sample and 55 mutations in the compound-treated sample, suggesting that IMB-26 did not affect the deaminating activity of hA3G.

Furthermore, the infectivity of these viruses was measured, using TZM-BL cells as described above, and normalized to that obtained for HIV-1.Vif- without hA3G (Table 1). In agreement with the study on deaminating activity of hA3G, the result indicated that IMB-26 significantly did not affect the antiviral activity of hA3G.

One explanation could be that the compound binds to the N-terminal portion of hA3G, which involves in binding to Vif, and does not interrupt the catalytic site of the deaminase located in the C terminus. Nevertheless, other possibilities cannot be excluded thus far, i.e. manipulating localization or trafficking of hA3G and Vif to prevent the hA3G/Vif interaction. Further investigations are needed to define the detailed action.

One of our concerns was the possible host toxicity after stabilization of host hA3G. Therefore, IMB-26 was selected for in vivo study and injected intraperitoneally into normal mice, followed by body weight monitoring and organ function examination. After 2 weeks of follow-up, we found that IMB-26 at doses between 250–1000 mg/kg intraperitoneally did not cause sig-

![Graph](image-url)

**FIGURE 4. Acute toxicological study of IMB-26.** Male Balb/c mice were treated with IMB-26 (intraperitoneally) at the doses indicated in the figure. The measurements done 14 days after IMB-26 treatment includes body weight (A), liver (B, upper panel), and kidney (B, lower panel) function as well as organ histological examination (C). Presented are mean ± S.E. (n = 5). GPT, glutamate-pyruvate transaminase; GOP, glutamate-oxaloacetate transaminase; BUN, blood urea nitrogen; CRE, creatine; U/L, unit per liter. Error bars, S.E.
nificant change of body weight (Fig. 4A). Blood samples taken at the end of the 14-day acute toxicity experiment were examined for liver and kidney functions. As shown in Fig. 4B, significant abnormality was not found in blood glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, blood urea nitrogen, and creatine after IMB-26 injection. Histological changes in liver, kidney, heart, and lung (Fig. 4C) was not detectable. Together with the results in vitro, we conclude that stabilization of hA3G by IMB-26 is relatively safe and might not alter the physiological function of major organs.

Anti-HIV therapeutics used today primarily target viral proteins. Although the combination drug therapies have been successful in prolonging the life span of HIV-infected individuals, they often lose their effectiveness due to the selection of drug-resistant mutants. It drives the discovery and development of novel anti-HIV agents directed at novel targets. Drugs acting through host cellular factors that are involved in HIV replication might have reduced the chance of causing drug-resistant mutation because the chemotherapeutic pressure is not on the virus. In this work, we have identified two active compounds, IMB-26 and IMB-35, both of which directly bind at host hA3G, specifically inhibit the Vif/hA3G interaction, and thereby abolish the Vif-mediated degradation of hA3G. The compounds efficiently inhibit HIV-1 infectivity/replication in hA3G expressing cells and are relatively safe in vivo. Therefore, they could be lead inhibitors for future anti-HIV intervention.

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