DANA-FARBER CANCER INSTITUTE

Poster #531

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

HARVARD MEDICAL SCHOOL

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ABSTRACT

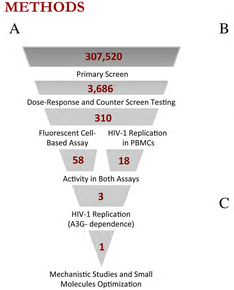
Background: APOBEC3G (A3G) is a cellular cytidine deaminase that restricts HIV replication by inducing G-to-A hypermutation in viral DNA and by deamination-independent mechanisms. Vif overcomes this innate antiviral activity by binding to A3G and targeting it for proteasomal degradation and by inhibiting its incorporation into virions. The interaction of Vif with A3G is essential for viral replication; as such, it represents a potential therapeutic target. Currently, there are no antivirals targeting Vif or its interaction with A3G.

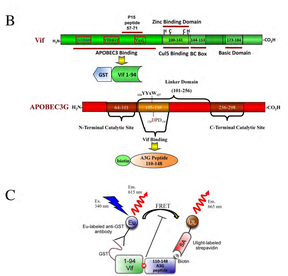
Methods: To identify compounds that inhibit interaction between A3G and HIV Vif in a high-throughput format, we developed a homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) assay. This assay detects the interaction between GST-Vif residues 1-94, which contains the A3G binding site, and a peptide consisting of A3G residues 110-148, a surrogate for the Vif binding site. A 307,520 compound library from the NIH Molecular Libraries Small Molecule Repository (MLSMR) was screened and 3686 hits were identified. To validate hits and eliminate false-positives, we employed a second TR-FRET-based assay and counter screen assays to evaluate dose response performance and off-target effects.

Results: In these primary and secondary screens, 310 hits were identified and then tested for antiviral activity against HIV-1_{Bx-L} virus replication in human PBMC and in a cell-based screen for their ability to inhibit Vif-dependent degradation of A3G. These studies identified 3 compounds that demonstrated both antiviral activity against HIV replication in PBMC and attenuation of Vif-dependent degradation of A3G. One compound, N.41, showed antiviral activity in H9 T cells (A3G+) but not in SupT1 T cells (A3G-) and dose-dependently reduced HIV-1 replication in PBMC. N.41 increased levels of A3G and its incorporation into virions in virus-producing cells, attenuated infectivity of single-round viruses in a Vif-dependent manner, and inhibited Vif-A3G interaction in co-immunoprecipitation assays. N.41 increased human but not African green monkey A3G levels in virus-producing cells in a Vif-dependent manner, and increased endogenous A3G levels in CEM T cells. Preliminary structure-activity relationship (SAR) studies suggested that a hydroxyl moiety located at the phenylamino group is critical for N.41 activity. Importantly, we identified several N.41 analogs with improved antiviral activities.

Conclusions: In this study, we identified a new lead compound that attenuates HIV replication by increasing A3G innate antiviral activity via liberating it from Vif regulation. SAR studies suggest that N.41 is a potential lead for further development as an antiviral.

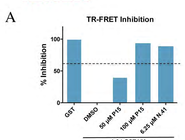
METHODS





High-throughput screening for HIV-1 Vif-APOBEC3G interaction inhibitors. A. Screening pipe-line. B. Development of high-throughput screen (HTS) to identify small molecules that inhibit binding of 1-94 GST-Vif to APOBEC3G peptide (AA 110-139, a surrogate for Vif binding site). C. Scheme of TR-FRET based assay used for screening inhibitors of HIV-1 Vif-APOBEC3G interaction. The interaction between purified 1-94 GST-Vif and biotinylated-A3G peptide, is detected by Europium (Eu- donor fluorophore)-labeled anti-GST antibodies and Streptavidin-Ulight (acceptor fluorophore). The median Z-value calculated for the primary screen was 0.84.

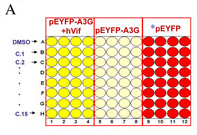
RESULTS

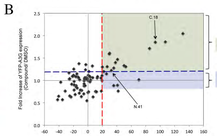


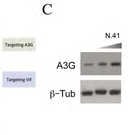
B

Hit	N.41
TR-FRET IC50 (μM)	2.18
TR-FRET Counter Screen Assay IC50 (μM)	>100
TR-FRET Counter Screen/TR-FRET Fold-Difference	>45.8
Tat-TAR Biochemical Assay IC50 (μM)	26.184
Tat-TAR/TR-FRET Fold-Difference	12.011
Vero Cell Cytotoxicity Assay TC50 (μM)	>40
Vero Cell Cytotoxicity/TR-FRET Fold-Difference	>18.3

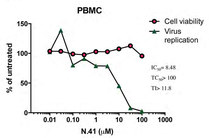
Compound N.41 inhibits HIV-1 Vif-APOBEC3G interaction in a TR-FRET based assay . A. TR-FRET inhibition graph. 100% TR-FRET assay inhibition is determined by using GST instead of 1-94 GST-Vif protein (positive control). DMSO (negative control). P15, 15-mer Vif peptide (positive control). Inhibition cutoff- 0.63% (broken line). B. Dose response TR-FRET assay and counter screen testing of compound N.41 for specificity and activity validation.

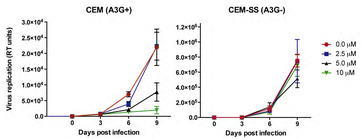




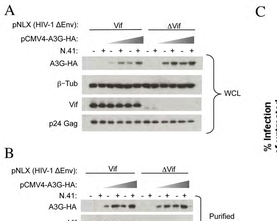


N.41 attenuates APOBEC3G degradation by Vif. A. Fluorescent cell-based screen to identify molecules that attenuate Vif-mediated degradation of YFP-A3G. 293T cells expressing YFP and YFP-A3G with or without Vif were treated with DMSO or 40 μM of tested compounds for 20 h, then fluorescense intensity (FI) of the treated cell lysate was measured. *This assay provides read-outs for cytotoxity, off-target effects, and nonspecific autofluorescence. B. On the X-axis of the scatter plot, is plotted compound/ DMSO percentage increase of FI tested in cells that express YFP-A3G and Vif. Compounds for which the % FI increase is above a certain cutoff (red broken line) are identified as actives. To determine if compounds potentially target Vif, compound value 'fold increase of YFP-A3G expression' was calculated as the change in FI measured from compound-treated cells that express YFP-A3G relative to DMSO, and was plotted on the Y-axis. Shown is cutoff (blue broken line) to resolve hit compounds into two groups corresponding to those targeting Vif (below cutoff) or A3G (above cutoff). C. N.41 stabilizes endogenous A3G expression in CEM T cells. CEM T cells were treated with 0, 10, and 20 μM N.41 for 48 h. Endogenous A3G and β-tubulin expression in CEM cell lysates were tested by Western.

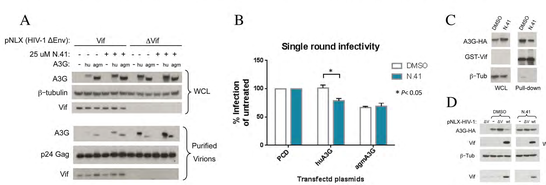




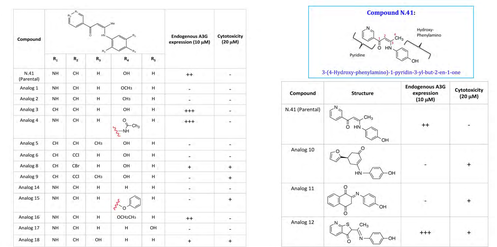
N.41 inhibits viral replication in PBMC and T cells in an A3G-dependent manner. A. Antiviral activity on HIV-1 replication in PBMC. N.41 was evaluated in dose-response using a 100 μM high-test concentration. Treated PBMC were infected by HIV-1_{BX-L} and 7 days post infection, both virus replication and cell viability were measured by RT and MTS assays. Percentage of virus replication and cell viability in N.41 treated cells are relative to virus replication and cell viability measured in DMSO-treated cells. B. HIV-1 spreading infection in T cells. CEM and CEM-SS T cells were infected by HIV-1_{NL4-3} and 3 h post infection cells were treated with 0, 2.5, 5 and 10 μM of N.41. Virus production from treated cells was tested 3, 6, and 9 days post infection by RT assay.



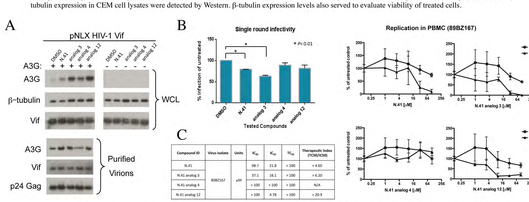
N.41 increases HIV-1 virion incorporation of A3G and decreases virus infectivity. A. 293T cells were co- transfected with 0, 200, and 400 ng A3G-3xHA plasmids and either pNLX HIV-1 ΔEnv or pNLX HIV-1 ΔEnv ΔVif plasmids. VSVG plasmid was used for pseudotyping in single round infections. 5 h post transfection, cell media was replaced with 10% FBS DMEM media supplemented with DMSO or 40 μM N.41. 40 h post transfection, supernatants containing viruses were collected, and producer cells were lysed. Producer cell A3G, Vif, p24 Gag, and β-tubulin protein expression levels were analyzed by Western. **B.** N.41 increases A3G virion incorporation. Virions normalized for the same RT units were purified through 20% sucrose. A3G, Vif and p24 Gag protein expression levels in virion lysates were tested by Western. **C.** N.41 treatment reduces infectivity of viruses produced from cells expressing A3G 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. Percentage of infection is relative to virus infection of untreated producer cells.



N.41 targets Vif-A3G interaction. A. N.41 attenuates Vif-dependent degradation of huA3G but not agmA3G. 293T cells were co transfected with 75 ng of pCDNA3 (empty vector), huA3G-3xHA or agmA3G-HA plasmids and with pNLX HIV-1 ΔEnv or pNLX HIV-1 ΔEnv ΔVif plasmids. VSVG plasmid used for pseudotyping in single round infection. 5 h post transfection, cells media was replaced with 10% FBS DMEM media supplemented with DMSO or 25 μM N.41. 40 h post transfection, supernatants containing viruses were collected, and producer cells were lysed. Producer cell protein A3G, Vif and β-tubulin expression levels were analyzed by Western (Upper panel). A3G, Vif and p24 Gag proteins expression levels from virus lysates were tested by Western (Bottom panel). **B.** N.41 treatment reduces infectivity of viruses produced from cells expressing huA3G but not agmA3G proteins. Percentage of infection is relative to virus infection of untreated producer cells. **C.** N.41 attenuates Vif-A3G interaction in in-vitro binding assay: Recombinant GST-Vif proteins were incubated with 293-ago lysate (293 cells that stably express A3G-HA). GST-Vif pull-down was performed in the presence of DMSO or 40 μM N.41. GST-Vif-A3G complexes were detected by Western using anti-GST and anti-HA antibodies. **D.** N.41 attenuates HIV-1 Vif-A3G interaction in 293T cells in a co-immunoprecipitation assay. Cells expressing A3G-HA and HIV-1 Vif were treated with 40 μM N.41 or DMSO for 40 h. Western blot of cell lysate or anti-HA co-immunoprecipitated proteins were probed using anti-Vif and anti-HA antibodies.



N.41 molecule optimization. A. 16 commercially available molecules were identified as potential N.41 analogs for follow-up studies. **B.** Effect of N.41 analogs on endogenous A3G expression in CEM cells. CEM T cells were treated with 0, 10, and 20 μM N.41 and its analogs for 48 h. Endogenous A3G and β-tubulin expression in CEM cell lysates were detected by Western. β-tubulin expression levels also served to evaluate viability of treated cells.



Effect of N.41 and its most potent analogs on A3G virion incorporation, viral infectivity and viral replication in PBMC. A. Compound N.41 and analogs 3 and 12 increase A3G virion incorporation. 293T cells were co-transfected with or without 100 ng huA3G-3xHA plasmids and with pNLX HIV-1 ΔEnv plasmid. VSVG plasmid used for pseudotyping HIV-1 envelope for single round infection. 5 h post transfection, cells media were replaced with 10% FBS DMEM media supplemented with DMSO or one of 40 μM N.41, its analogs 3 and 4 or 20 μM of analog 12. 40 h post transfection, supernatants containing viruses were collected, and producer cells were lysed. Producer cells proteins A3G, Vif and β-tubulin expression levels were analyzed by Western (Upper panel). A3G, Vif and p24 Gag proteins expression levels from virus lysates were tested by Western (Bottom panel). **B.** Effect of N.41 and analogs 3, 4, and 12 on infectivity of viruses produced from cells expressing A3G proteins. TZM-BL reporter cells were infected with viruses corresponding to 4000 RT units . Luminescence from infected cells were measured 48 h post infection. Percentage of infection is relative to virus infection of untreated producer cells. **C.** Antiviral activity of N.41 most potent analogs on HIV-1 replication in PBMC. N.41 and the analogs were evaluated in dose-response using a 100 μM high-test concentration (using half-log dilutions). Compound treated PBMC were infected by HIV-isolate 89BZ167 and 7 days post infection, both virus replication and cell viability were measured by RT and MTS assays.