Interaction Between HIV-1 Nef and Calnexin
From Modeling to Small Molecule Inhibitors Reversing HIV-Induced Lipid Accumulation

Ruth Hunegnaw, Marina Vassylyeva, Larisa Dubrovsky, Tatiana Pushkarsky, Dmitri Sviridov, Anastasia A. Anashkina, Aykut Üren, Beda Brichacek, Dmitry G. Vassylyev, Alexei A. Adzhubei, Michael Bukrinsky

Objective—HIV-infected patients are at an increased risk of developing atherosclerosis, in part because of downmodulation and functional impairment of ATP-binding cassette A1 (ABCA1) cholesterol transporter by the HIV-1 protein Nef. The mechanism of this effect involves Nef interacting with an ER chaperone calnexin and disrupting calnexin binding to ABCA1, leading to ABCA1 retention in ER, its degradation and resulting suppression of cholesterol efflux. However, molecular details of Nef–calnexin interaction remained unknown, limiting the translational impact of this finding.

Approach and Results—Here, we used molecular modeling and mutagenesis to characterize Nef–calnexin interaction and to identify small molecule compounds that could block it. We demonstrated that the interaction between Nef and calnexin is direct and can be reconstituted using recombinant proteins in vitro with a binding affinity of 89.1 nmol/L measured by surface plasmon resonance. The cytoplasmic tail of calnexin is essential and sufficient for interaction with Nef, and binds Nef with an affinity of 9.4 nmol/L. Replacing lysine residues in positions 4 and 7 of Nef with alanines abrogates Nef–calnexin interaction, prevents ABCA1 downregulation by Nef, and preserves cholesterol efflux from HIV-infected cells. Through virtual screening of the National Cancer Institute library of compounds, we identified a compound, 1[(7-oxo-7H-benz[de]anthracene-3-yl)amino]anthraquinone, which blocked Nef–calnexin interaction, partially restored ABCA1 activity in HIV-infected cells, and reduced foam cell formation in a culture of HIV-infected macrophages.

Conclusion—This study identifies potential targets that can be exploited to block the pathogenic effect of HIV infection on cholesterol metabolism and prevent atherosclerosis in HIV-infected subjects. (Arterioscler Thromb Vasc Biol. 2016;36:1758-1771. DOI: 10.1161/ATVBAHA.116.307997.)

Key Words: calnexin ■ cholesterol efflux ■ foam cell ■ HIV-1 ■ molecular modeling ■ Nef ■ virtual screening

HIV-1 infection, via activity of viral protein Nef, impairs cholesterol efflux mediated by the cholesterol transporter ATP-binding cassette A1 (ABCA1).1 ABCA1 is the main cellular cholesterol transporter regulating delivery of cellular cholesterol to extracellular acceptor, apolipoprotein A-I. Studies in animal models demonstrated that this activity of Nef may be responsible for hypoalphalipoproteinemia and high risk of atherosclerosis observed in HIV-infected subjects.2,4 Our recent study identified calnexin, an integral endoplasmic reticulum (ER) membrane lectin-like chaperone, as a key player in the mechanism of Nef-mediated inhibition of ABCA1 and cholesterol efflux.5 Calnexin and its homologue calreticulin regulate folding and maturation of newly synthesized glycoproteins by engaging them in a calnexin/calreticulin cycle.6

See cover image

ABCA1 is a highly glycosylated protein.7 Although no evidence for the role of calnexin in ABCA1 biogenesis is available, 2 well-studied ABC transporters, ABCB7 (also known as cystic fibrosis transmembrane conductance regulator, CFTR) and ABCB1 (also known as multidrug resistance protein 1 or P-glycoprotein 1), interact with calnexin, and folding mutants of these transporters are retained within the ER by calnexin and eventually degraded.8,9 Importantly, ABCB7 and ABCB1 mutants that escape calnexin binding do not achieve mature glycosylation and these mutations result in reduced transporter function.9,9 Our recently published study demonstrated that ABCA1 interacts with calnexin, and the reduction of
calnexin expression by RNAi resulted in a significant decrease in functional activity of ABCA1, evidenced by reduced cholesterol efflux to ABCA1-specific acceptor apoA-I. We also showed that Nef impairs the interaction between ABCA1 and calnexin, and this effect of Nef is essential for the inactivation and downregulation of ABCA1. Importantly, the inhibition of ABCA1–calnexin interaction by Nef is specific, as interaction between ABCA1 and 2 other proteins, dystrophin and serine palmitoyltransferase, shown previously to bind ABCA1, was not affected. Also not affected was the interaction between calnexin and HIV-1 envelope glycoprotein, gp160; in fact this interaction was even enhanced by Nef. These findings suggested that Nef modulates the activity of calnexin, but the mechanism of this effect and molecular details of Nef/calnexin interaction remained unknown. Moreover, it was unclear whether the interaction between Nef and calnexin is direct, making screen for inhibitory compounds difficult.

Calnexin is a 592-amino acid type I transmembrane protein composed of 3 parts: a lumenal fragment consisting of a globular β-sandwich domain responsible for the interaction with carbohydrates and a proline-rich tandem sequence repeat domain (the P domain) involved in protein–protein interactions, a transmembrane domain, and a cytoplasmic domain of 90 residues. The cytoplasmic tail of calnexin can undergo phosphorylation and palmitoylation which regulate calnexin association with several proteins and protein complexes that influence functional activity of this chaperone. For example, phosphorylation at Ser563 has been shown to play an essential role in quality control functions of the Nef/calnexin interaction and identify a small molecule compound that blocks this interaction and reverses negative effects of HIV infection on cellular cholesterol metabolism.

Materials and Methods

Results

Cytoplasmic Domain of Calnexin Is Necessary for Interaction With Nef

In our previous study, we have shown that HIV-1 Nef interacts with the ER chaperone calnexin. To test which region of calnexin is necessary for binding to Nef, we used calnexin constructs that had deletion of the luminal repeat segment (aa 276–409) or truncation of the C-terminal cytoplasmic domain (aa 504–586; Figure 1A). We cotransfected human embryonic kidney (HEK) 293T cells with Nef-expressing vector and HA-tagged variants of wild-type (WT) calnexin or the deletion mutants and performed coimmunoprecipitation. Figure 1B shows that WT calnexin interacted strongly with Nef, whereas calnexin construct with internal repeat motif deletion (CNXΔ276–409) exhibited partially reduced binding (40% reduction). However, binding of Nef to calnexin construct carrying the truncation of the C-terminal cytoplasmic tail (CNXΔ504–586) was reduced dramatically (70% reduction). This finding highlights the importance of the cytoplasmic region of calnexin in interaction with Nef. The role of calnexin cytoplasmic tail in the interaction with Nef is consistent with Nef’s predominant localization to the cytoplasm and with the charges of the interacting domains: the cytoplasmic domain of calnexin is composed mainly of negatively charged amino acids, which may promote interaction with the N-terminal region of Nef (see below) enriched in positively charged residues. The modest effect that deletions in the luminal repeat motif of calnexin had on Nef binding (40% reduction of binding), and residual binding (30%) of Nef to calnexin construct with deleted cytoplasmic domain, may be because of artefacts caused by overexpression. In addition, mutations in the luminal domain may affect binding properties of the cytoplasmic domain via reverse signal transduction mechanism (Discussion section of this article). These results provided an initial lead, which was followed in subsequent experiments.

Computational Model of Nef–Calnexin Interaction

Experimentally solved molecular structure of calnexin is available only for the luminal domain, and to obtain 3-dimensional structure of calnexin cytoplasmic domain we performed its modeling with several modeling servers implementing different methods, which produced several models ranging from the fully folded structures to structures that included natively disordered regions. The models have been assessed for accuracy and the final round of modeling was performed with the server QA-RecombineIt. The final model had a loosely folded structure (Figure 2A, a). Computational prediction of Nef–calnexin complexes showed Nef N-terminal α-helix forming the interaction interface with calnexin cytoplasmic domain (Figure 2A, b and c).
In comparison with the calnexin cytoplasmic domain model, the model of Nef was based on several experimental structures and thus had better accuracy. Nef–calnexin interaction has been modeled by global docking using four different docking servers: Cluspro, HEX, SwarmDock, and Zdock. A combined set of the best Nef–calnexin docking models produced with these servers contained 80 models. The advantage of this approach is that the resulting models represented Nef–calnexin interaction modeled by four different, unrelated methods and, therefore, it was more reliable than using a single server. From these, 49 models have been filtered out as possibly interfering with interaction of Nef with ER membrane. Intermolecular interactions in the remaining subset of 31 models have been identified. There are several distinct clusters of interactions, with sharp maxima for Lys7 and Arg in positions 8, 19, 22, 75, and 109 (Figure 2B).

Notably, similar analysis of interactions performed on the full initial data set of 80 docking models showed similar clustering and maxima (not shown). We can therefore hypothesize that the identified residues represent the overall favorable Nef–calnexin interaction modeled by four different, unrelated methods and, therefore, it was more reliable than using a single server. From these, 49 models have been filtered out as possibly interfering with interaction of Nef with ER membrane. Intermolecular interactions in the remaining subset of 31 models have been identified. There are several distinct clusters of interactions, with sharp maxima for Lys7 and Arg in positions 8, 19, 22, 75, and 109 (Figure 2B). Notably, similar analysis of interactions performed on the full initial data set of 80 docking models showed similar clustering and maxima (not shown). We can therefore hypothesize that the identified residues represent the overall favorable Nef–calnexin interaction sites. All these residues, except Lys7 and Arg8, have been also identified as participating in interactions in the experimental structures of complexes which included Nef (Table). A representative model of Nef–calnexin binding is shown in Figure 2A, b and c. Analysis of the conserved residues in Nef performed with ConSurf revealed several conserved positions in the N-terminal region, including Lys4, Ser6, Lys7, and Arg19. Multiple sequence alignment of the human HIV Nef sequences from Uniprot showed that Lys7 is highly conserved across the spectrum of HIV-1 and HIV-2 sequences. Conserved residues indicate structurally and functionally important positions, including interaction sites. Therefore, Lys7 represents a new interaction site predicted by us, which was not previously identified in Nef interactions with other proteins (Table).

### Lysine Residues of Nef in Positions 4 and 7 Are Critical for Nef–Calnexin Interaction

From the perspective of drug design, targeting viral proteins has lower potential for side effects than targeting cellular partners. We thus focused our efforts on Nef. According to docking modeling and sequence conservation results, Lys7 possibly represents a new binding site in Nef, and accordingly it has been selected for mutagenesis experiments. Lys4 has been also selected because it is a Lys7 near-neighbor and, as demonstrated in the Nef model, it plays a key structural role for the N terminus (Figure 2A, d). Therefore, mutation of both Lys4 and Lys7 was predicted to invoke structural rearrangement in the Nef N-terminal region, thus disrupting the interaction between Nef and calnexin. Alanine substitution of basic residues at the N terminus of Nef has previously been shown to preserve membrane association and CD4 downregulation by Nef, and intracellular localization of the mutant Nef was indistinguishable from that of Nef WT.
To verify the role of these residues in Nef interaction with calnexin, we used the mutant HIV-1 NL4-3 clone carrying Nef with Lys4 and Lys7 changed to valine and alanine, respectively (NefK). Calnexin was immunoprecipitated from HEK293T cells transfected with WT or mutant HIV-1 clones, and the precipitate was immunoblotted for Nef. As shown in Figure 3A, interaction with calnexin was evident for Nef WT, but not for NefK. Interaction with the double mutant was reduced by 95%, indicating that the lysine residues in positions 4 and 7 are essential for Nef interaction with calnexin.

To look at the individual contribution of the 2 lysine residues to the interaction with calnexin, we mutated the NefK plasmid to create single and double lysine mutant constructs. To minimize variables introduced by cotransfection, we used HeLa cells that stably express ABCA1-GFP, transfected them with WT or mutant Nef constructs, and analyzed the amount of Nef that communoprecipitates with calnexin. Based on densitometric analysis, interaction of calnexin with NefK4A was reduced by 80%, whereas interaction with NefK7A was reduced by 70% when compared with interaction with WT Nef (Figure 3B). Interaction of calnexin with double mutant NefK4,7A was undetectable. Reduced interaction observed with the Nef single mutants was sufficient to downregulate ABCA1, as shown in Figure 3C. NefK4A and NefK7A mutants reduced ABCA1 abundance as much as the WT Nef, whereas near-control level of ABCA1 was observed when both lysine residues were mutated (Figure 3C). This result highlights the importance of both residues in ABCA1 downregulation and suggests that under Nef overexpression conditions even reduced interaction with calnexin observed
for NefK4A and NefK7A mutants is sufficient for ABCA1 downregulation. It also supports conclusions of the previous study that demonstrated that Nef–calnexin interaction is essential for Nef-mediated retention of ABCA1 in ER and subsequent degradation.5

To rule out the possibility that mutation of these residues grossly affected the behavior of the N-terminal domain of Nef, we tested the interaction of the mutant Nef with ABCA1. Previous studies demonstrated that interaction between Nef and ABCA1 also involves the N-terminal domain,1 although the specific residues involved have not been identified. Co-precipitation analysis revealed ≈30% reduction in NefK4,7A interaction with ABCA1 as compared with ABCA1 interaction with WT Nef (Figure 3D). The reduction, however, remains in stark contrast to the >95% loss of interaction observed in the Nef–calnexin interaction studies (Figure 3A).

To visualize the effect of mutations on Nef–calnexin interaction, HEK293T cells were transfected with vectors expressing WT or mutant (NefK4,7A) Nef, stained with a combination of anti-Nef rabbit polyclonal and anticalnexin mouse monoclonal antibody followed by a combination of Alexa Fluor647 anti-rabbit IgG (green) and DyLight 550 antimeouse IgG (red), counterstained with DAPI (blue), and analyzed by confocal fluorescent microscopy. Images were processed using Velocity software to identify colocalizing pixels (magenta staining). Consistent with our previous report,5 WT Nef was colocalized with calnexin (Figure 3E, left panels). However, the colocalization between calnexin and NefK4,7A was dramatically reduced (Figure 3E, right panels), confirming the key role of these residues in Nef–calnexin binding.

Table. Comparison of Nef Predicted Interacting Residues with Interactions Identified in Experimentally Determined Structures

<table>
<thead>
<tr>
<th>Predicted Interaction Clusters</th>
<th>Matching Residues</th>
<th>PDB Structures</th>
<th>UniProt Code</th>
<th>HIV Virus Subtype (Isolate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys7</td>
<td>No hits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg8</td>
<td>No hits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg19</td>
<td>Arg19</td>
<td>4emzC</td>
<td>AP-1 COMPLEX SUBUNIT MU-1</td>
<td>Q00VU7_9H1V1 Human immunodeficiency virus 1</td>
</tr>
<tr>
<td>Arg22</td>
<td>Arg22</td>
<td>4emzC</td>
<td>AP-1 COMPLEX SUBUNIT MU-1</td>
<td>Q00VU7_9H1V1 Human immunodeficiency virus 1</td>
</tr>
<tr>
<td>Arg75</td>
<td>Thr71, 4emzC, 4en2B</td>
<td>AP-1 COMPLEX SUBUNIT MU-1</td>
<td>Q00VU7_9H1V1 Human immunodeficiency virus 1</td>
<td></td>
</tr>
<tr>
<td>Arg71</td>
<td>1efnB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg71</td>
<td>4d8dB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg75</td>
<td>3rebA, 3rbbA, 3reaA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg109</td>
<td>Arg105</td>
<td>4neeC</td>
<td>AP-2 COMPLEX SUBUNIT ALPHA-2</td>
<td>P04601 (NEF_HV1H2) Human immunodeficiency virus type 1 group M subtype B (isolate HXB2)</td>
</tr>
</tbody>
</table>

Nef amino acid residues predicted in this study to form interactions with calnexin are compared with the interactions identified in experimental structures of complexes which include Nef. Comparison is done on the basis of structure/sequence alignments of the Nef model used to predict interactions with calnexin, and Protein Data Bank (PDB) experimental structures. Nef-interacting partners in the experimental structures are listed as they are given in the corresponding PDB files.

Functional Analysis of Nef Mutants

In our previous study, we reported that Nef plays a central role in the downmodulation of ABCA1 expression and function.1 This phenotype was associated with Nef’s ability to interact with calnexin and disrupt calnexin interaction with ABCA1. Identification of Nef residues required for interaction with calnexin provided an opportunity to verify the critical role of this interaction for the effects of Nef on cellular cholesterol metabolism. To assess the functional consequence of losing the Nef/calnexin interaction for ABCA1 functionality, we cotransfected HEK293T cells with ABCA1 and HIV-1 NL4-3 infectious clones that express either Nef WT or Nef K4VK7A (NefK).27 Lysates were immunoblotted for ABCA1
Consistent with the results obtained with Nef-expressing vector (Figure 3C), total ABCA1 abundance was significantly reduced in the presence of Nef WT; however, the expression of ABCA1 in the presence of NefK was comparable to that of the control sample, which was transfected with an empty vector. This result is consistent with conclusions of the previous study that identified Nef as the key viral factor responsible for ABCA1 downregulation.¹

Figure 3. Mutation of Nef lysine residues 4 and 7 blocks interaction with calnexin. A, HEK293T cells transfected with HIV-1 molecular clones encoding for Nef WT or Nef K4V7A (NefK), or B, HeLa ABCA1 cells transfected with pcDNA plasmids expressing Nef WT or mutants Nef K4A, Nef K7A, or NefK4,7A were lysed 48 h post transfection. Endogenous calnexin was immunoprecipitated using monoclonal anti-calnexin antibody and immunoprecipitates were blotted for Nef and calnexin (top). Whole-cell lysates were analyzed for expression of calnexin, Nef, and GAPDH (bottom). Numbers under the Nef panels show relative amounts of coprecipitated Nef obtained by gel densitometry. C, HeLa-ABCA1-GFP cells were transfected with Nef WT or mutants Nef K4A, Nef K7A, or Nef K4,7A. Cells were lysed 48 h post transfection and lysates were analyzed for expression of ABCA1, Nef, and GAPDH. Numbers under the ABCA1 panel show relative amounts of ABCA1 obtained by gel densitometry. D, HEK293T cells were cotransfected with ABCA1-FLAG and Nef WT or NefK4,7A and were lysed 48 h post transfection. ABCA1 was immunoprecipitated using anti-FLAG beads and precipitates were blotted for ABCA1 and Nef (top). Whole cell lysates were analyzed for expression of ABCA1-FLAG, Nef, and GAPDH (bottom). Numbers under the Nef panel show relative amounts of coprecipitated Nef obtained by gel densitometry. E, HEK293T cells were transfected with Nef WT (left) or NefK4,7A (right), stained with Alexa Fluor647 conjugated antibody for Nef (green), DyLight 550 conjugated antibody for calnexin (red), and DAPI for nuclei (blue), and analyzed by confocal fluorescent microscopy. Large panels show overlay of all 3 channels, whereas small panels show red channel (calnexin, top), green channel (Nef, middle), and magenta staining for colocalization obtained using Volocity software (bottom). Scale bar, 26.7 µm.
with HIV-1 expressing either WT Nef or NefK. Given that the virus used in this experiment was the X4-tropic strain NL4-3, we pseudotyped it with vesicular stomatitis virus-G to ensure 1-cycle replication. Both Nef WT and NefK viruses successfully infected the cells, establishing similar levels of Nef expression (Figure 4B, a). Seven days after infection, cholesterol efflux assay was performed. In agreement with previous reports,1,5,28 cells infected with the WT virus had significantly reduced cholesterol efflux relative to mock-infected cells (Figure 4Bb). However, infection with the virus carrying NefK did not lead to efflux decrease.

Interaction Between Nef and Calnexin Is Direct
To test whether Nef and calnexin interact directly with each other, we expressed calnexin and the cytoplasmic tail of calnexin (Calnexin-CT) in Escherichia coli and purified recombinant proteins by column chromatography. For the purification of full-length calnexin, we have developed and implemented a novel purification system based on the ultrahigh affinity (\(K_d \approx 10^{-14} - 10^{-17}\) M) small protein complex of genetically inactivated colicin 7 DNAse (CL7) and its inhibitor, immunity protein 7 (Im7).29–32 We have attached a CL7 variant, which possesses no DNAse activity but retains full Im7 affinity, as a C-terminal tag on His-tagged calnexin construct (Figure 5A, left). A cleavage site for the prescission protease (PSC) inserted between calnexin and CL7 allowed for the elution of

Figure 4. HIV-1 clones expressing mutant Nef K4VK7A do not downregulate ABCA1 or cholesterol efflux. A, HEK293T cells were cotransfected with ABCA1 and HIV-1 molecular clones encoding Nef WT or Nef K4VK7A (NefK). HIV-1 clone with a Nef deletion (DNef) was used as control. Cells were lysed 48 h post transfection and immunoblotted for ABCA1, Nef and GAPDH. B, THP-1 cells were infected with vesicular stomatitis virus-G (VSV) pseudotyped HIV-1 molecular clones used in A. Western blot shows expression of Nef in cell lysates (a). Cholesterol efflux was measured 7 days after infection (b). Results show apoA-I specific cholesterol efflux as mean±SEM of quadruplicates.

Figure 5. Nef directly binds to Calnexin and its cytoplasmic tail. A, Purification of calnexin and calnexin-CT. Steps of purification of full-length CL7-tagged calnexin (CNX-CL7) are shown in detail. Whole cell lysate (WCL) was centrifuged to remove cell debris, the supernatant (SN) was treated with 0.07% polyethylene-amine (PE) to precipitate DNA, the pellet (PL), which contained most of calnexin protein, was washed with detergent-containing buffer to release calnexin into solution, centrifuged, and the resulting supernatant was loaded on Immunity protein 7 (Im7) column. Bound proteins were eluted by treating the column with prescission protease (PSC; EL lane), whereas flow-through (FT) lane shows unbound proteins. ΔCNX-truncated calnexin fragment; small ubiquitin-like modifier (SUMO)—SUMO domain; P(PSC), P(SUMOP)—cleavage sites for the PSC and SUMO proteases, respectively; H8 – 8-Histidine tag. B, Surface plasmon resonance experiments were done in a Biacore T-200 by using a CM5 chip. Calnexin (left) and calnexin-CT (right) were captured by amine coupling, and myristoylated NefSF2 protein was injected over the chip surface at 6 different concentrations (6.25–200 nmol/L range) in triplicates. Colored lines represent actual data, and black lines represent curve fit to a monovalent analyte binding model in BiaEvaluation software.
Figure 6. Characterization of compounds targeting Nef–Calnexin interaction. A, a, Ten compounds with the best score (grey) from virtual screening performed on the Zinc NCI Plated 2007 data set. Their overall location in the structural alignment with the model of Nef (magenta)–calnexin (green) complex is shown as translucent molecular surface (cyan). The model shows that these compounds can block the Nef/calnexin interaction at calnexin residues Glu529, Glu532, and Glu533. The set of compounds includes NSC 1758, NSC 13987, and NSC 92938 selected for experimental testing. b, Compound NSC 13987 docked to Nef-binding site, which is centered on Lys4 and Lys7. Interactions of the compound with Nef include 2 hydrogen bonds with Nef amino acid residues Ser6 and Tyr124. B, Chemical structures and names of compounds NSC 1758, NSC 13987, and NSC 92938. C, Dose–response effect of NSC 1758, NSC 13987, and NSC 92938 on viability of THP-1 cells. THP-1 cells were treated with indicated compounds for 5 days and cytotoxicity was measured using MTT assay. D, HEK293T cells were transfected with HA-tagged Nef, treated with compounds NSC 1758, NSC 13987, or...
calnexin from the Im7 column through cleavage by PSC. A single purification step provided an excellent yield of ~90% pure protein (Figure 5A), in which major contamination represented calnexin molecules (confirmed by mass spectroscopy), most likely, truncated from the N terminus. The Calnexin-CT construct was designed with a single N-terminal His-tag and was purified using the standard procedure (Figure 5A, right).

Binding of myristoylated Nef(SF2)3 to calnexin and its cytoplasmic domain was analyzed using surface plasmon resonance (Figure 5B). Calnexin and calnexin-CT were immobilized on microchip surfaces and myristoylated Nef was injected over the surface. Nef(SF2) directly bound to calnexin with an affinity (K_d) of 89.1 nM (k_a=1.338E5 M^-1 s^-1, k_d=0.01192 s^-1, χ^2=2.77 RU; Figure 5B, left panel). Binding to calnexin-CT was observed to have higher affinity of K_d=9.4 nM (k_a=9.083E5 M^-1 s^-1, k_d=0.008569 s^-1, χ^2=0.474 RU; Figure 5B, right panel). Taken together, these experiments demonstrate that Nef/calnexin interaction is direct and involves the cytoplasmic domain of calnexin.

Virtual Screening for Compounds Interfering With Nef–Calnexin Interaction

Docking-based virtual screening has been performed on compounds from the Zinc NCI Plated 2007 data set with docking program Vina.34 Nef model described in Figure 2 has been used with the interaction site for ligand docking selected to cover amino acid residues Lys4 and Lys7. The data set consisted of 139735 compounds. Ten putative ligands were identified and prioritized according to the Vina ranking, and structural alignment of these compounds to the Nef–calnexin complex is shown in Figure 6A (a). The model shows that these compounds can block Nef/calnexin interaction at the calnexin residues Glu529, Glu532, and Glu533. Docking of NSC 13987, which turned out in the later studies to be the most effective inhibitor of the Nef–calnexin interaction, is shown in panel b (Figure 6A). Interactions of the compound with Nef include 2 hydrogen bonds with Nef amino acid residues Ser6 and Tyr124. Three of the 10 compounds, NSC 1758, NSC 13987, and NSC 92938, have been submitted for experimental testing. The chemical names and molecular structures of these compounds are shown in Figure 6B.

Testing the Compounds’ Activity

To test whether the compounds identified in our virtual screen can interfere with Nef–calnexin interaction, we first performed coimmunoprecipitation assay. HEK293T cells were transfected with plasmid encoding for Nef(SF2) and 6 hours post transfection were treated with NSC 1758 (4 μmol/L), NSC 13987 (5 μmol/L), or NSC 92938 (5 μmol/L). These concentrations of the compounds were determined by the MTT assay to reduce cell metabolism by <10% during 5-day incubation (Figure 6C). Among the 3 compounds tested, 1 compound, NSC 13987, inhibited coimmunoprecipitation of Nef and calnexin by over 50%, whereas the effect of NSC 1758 and NSC 92938 showed a partial inhibition of Nef/calnexin binding, which did not reach statistical significance (Figure 6D). We have previously shown that membrane localization of Nef is important for the interaction of Nef with calnexin.5 To rule out the possibility that the compound interferes with membrane localization of Nef, we tested whether NSC 13987 affects the interaction between Nef and ABCA1, as ABCA1–Nef interaction also requires membrane localization of Nef.1 As shown in Figure 6E, ABCA1–Nef interaction remained unaffected in the presence of compound indicating that the inhibition was specific for the molecular interaction of Nef and calnexin.

We next tested whether the 3 compounds could prevent the impairment of cholesterol efflux by Nef. THP-1 cells were transfected with a Nef-encoding plasmid, and drug treatment was started 6 hours after transfection. The following day, cells were activated with PMA after which cholesterol efflux assay was performed. Drug treatment was continued throughout the duration of the experiment. Figure 7Aa shows cholesterol efflux measured in untreated cells or cells treated with DMSO or each of the 3 compounds. Cholesterol efflux in Nef-transfected untreated or DMSO-treated cells was reduced by over 2-fold relative to mock-transfected cells. NSC 13987, which showed the inhibition of Nef–calnexin interaction (Figure 6D), significantly increased cholesterol efflux as compared with DMSO-treated Nef-expressing cells, although the rescue was partial and did not completely reverse the inhibition. Two other compounds did not significantly rescue Nef-suppressed cholesterol efflux. Treating untransfected THP-1 cells with the compounds did not lead to any changes in cholesterol efflux (Figure 7Ab). This result implies that the impact in efflux capacity observed in the presence of NSC 13987 was specific to the compound’s activity in Nef-expressing cells. It also demonstrates that the compounds were not toxic to cells.

To test the effect of NSC 13987 in the context of natural infection, we infected monocyte-derived macrophages (MDM) with HIV-1 ADA, treated them with NSC 13987 and measured cholesterol efflux. Viral replication in the presence of compound was reduced (Figure 7Ba), and fold change analysis showed on average a 2-fold reduction in reverse transcriptase (RT) activity measured in 3 independent experiments with cells from different donors (Figure 7Bb), consistent with demonstrated rescue by the compound of Nef-inhibited cholesterol efflux (Figure 7A) and previous studies demonstrating anti-HIV activity of ABCA1 and ABCA1-stimulated cholesterol efflux.28,35,36 Consistent with previous studies,1,5,36 cholesterol efflux from HIV-infected cells was decreased by 60%, whereas HIV-infected cells treated with NSC 13987 showed cholesterol efflux not significantly different from that of...
Figure 7. Compound NSC 13987 prevents impairment of cholesterol efflux by HIV-1 and Nef. A, THP-1 cells were transfected with Nef (or mock-transfected, control) and incubated with compounds NSC 1758, NSC 13987 and NSC 92938 or DMSO, and cholesterol efflux was measured 5 days post transfection (a). Cholesterol efflux was measured in THP-1 cells treated only with compounds NSC 1758, NSC 13987 and NSC 92938 or DMSO (b). Results are presented as mean±SEM (n=4; one-way ANOVA with post hoc Tukey HSD test). B, Primary MDM were infected in triplicates with HIV-1 ADA. Compound NSC 13987 (5 µmol/L) or DMSO was added 3 d after infection and maintained thereafter, and virus replication was monitored by measuring RT activity in the supernatant over a 14-day period. Results of 3 measurements are shown as mean±SEM, (n=3, *P<0.05; **P<0.07 by Student unpaired t test; a). Fold change in RT activity was calculated from 3 independent experiments performed using macrophages from 3 donors. Error bars show SD (n=3, P value was calculated using Student's unpaired t test; b). C, MDMs were infected with HIV-1 ADA or mock-infected and treated with DMSO or NSC 13987 as in B. Cholesterol efflux was measured 14 days postinfection in quadruplicate wells. Results are presented as mean±SEM (n=4; 1-way ANOVA with Tukey’s HSD test; a). Cholesterol efflux in MDM obtained from 2 donors was measured independently and fold change in efflux activity in the presence of drug is shown (b). Results are presented as mean±SEM (n=2, Student unpaired t test). D, a, MDMs were infected with VSV-G-pseudotyped HIV-1 NL4-3 (upper row), mock-infected (middle row), or infected with VSV-G-pseudotyped Nef-deficient HIV-1 ΔNef NL4-3 (bottom row). NSC 13987 (5 µmol/L) was added on day 5 postinfection and maintained thereafter. Ten days postinfection, cells were stained with ORO and analyzed by bright field microscopy at 100x magnification. b, Analysis of images in a was performed using Image J program. Histograms in the left represent pixel distribution along the 256 RGB shades, averaged from 55 cells in each condition. Pie charts in the right show percentage of cells in each condition with ORO-stained area over or under an arbitrarily selected value of 20%. c, MDMs were infected with HIV-1 ADA or mock-infected. NSC 13987 was added on day 5 and maintained thereafter. On day 14 post infection, cells were stained with ORO, counterstained with DAPI and analyzed by fluorescent microscopy at ×100 magnification.
mock-infected cells (Figure 7Ca). Fold change in cholesterol efflux from 2 independent experiments with cells from different donors showed consistent efflux rescue by the compound (Figure 7Cb). A more potent reversal by NSC 13987 of cholesterol efflux inhibited by HIV-1 infection (Figure 7C) than by Nef transfection (Figure 7Aa) is likely because of higher levels of Nef expression in transfected cells, and a combined effect of reduced virus replication and inhibition of the Nef–calnexin interaction in HIV-infected cells.

Lack of a small animal model of HIV-associated atherosclerosis prevented us from testing the antiatherogenic potential of NSC 13987 in vivo. We therefore used the best available in vitro approximation of HIV-associated proatherogenic changes, conversion of HIV-infected macrophages into the foam cells.1 In this experiment, we used the vesicular stomatitis virus G protein-pseudotyped HIV-1 NL4-3 and its Nef-deficient mutant (HIV-1ΔNef), which can go through only 1 cycle of replication in MDM cultures. This approach allowed us to eliminate the drug’s effect on viral replication, leaving changes in cholesterol metabolism as the only cause of potential effects on lipid droplets accumulation. Indeed, virus replication, as measured by RT activity in culture supernatants, was 400 to 500 cpm/μL for both WT and ΔNef viruses treated or untreated with NSC 13987. As revealed by bright field microscopy, MDM cultures infected with WT HIV-1 (Figure 7Da, top left panel) were enriched in Oil-Red-O (ORO)–stained lipid droplets compared with mock-infected cells (middle left panel), and treatment with NSC 13987 (top right panel) reduced the number of stained cells and decreased the intensity of staining to the level observed in mock-infected cells. Importantly, infection with the Nef-deficient HIV-1 did not increase the number of lipid droplets (Figure 7Da, bottom left panel), indicating that the observed effect was mediated by Nef expression. Of note, treatment with NSC 13987 of mock-infected MDM or MDM infected with the ΔNef virus did not decrease lipid droplets, indicating the compound’s effect was dependent on Nef. Quantitative analysis of the images performed on 55 cells from each condition is presented in Figure 7Db. Histograms in the left panel demonstrate a shift of RGB pixel distribution to low-intensity area (indicative of reduction of ORO-stained lipid droplets) in MDMs infected with WT HIV-1 and treated with NSC 13987 relative to HIV-infected untreated culture, whereas treatment of mock-infected cultures or cultures infected with the ΔNef virus did not significantly affect pixel distribution. Importantly, histograms obtained with HIV-infected cells treated with NSC 13987 were similar to those with mock–infected cells, indicating that the drug fully reversed HIV-induced accumulation of lipid droplets. The pie-chart graphs on the right show the distribution of cells according to ORO-stained area; 65% of HIV-infected untreated cells had over 20% of cell area stained with ORO, whereas such cells constituted only 42% and 44% in mock- and HIV-1ΔNef–infected MDMs, respectively. NSC 13987 reduced the percentage of such cells in HIV-infected MDM to 22%, which was even smaller in drug–treated uninfected cells.

To better visualize lipid droplets, we used fluorescent microscopy. We also used macrophage-tropic HIV-1 isolate ADA to better mimic natural conditions. The diameter of ORO-stained lipid droplets in HIV-infected cells varied from 0.1 to 3 μm (Figure 7Dc, middle panel). The size of lipid droplets accumulated in HIV-infected MDMs treated with NSC 13987 was visibly reduced and did not exceed 0.5 μm (right panel); in fact, it was similar to the size of the droplets in mock-infected cells (left panel).

Taken together, these results provide a proof of concept for the idea that HIV-induced impairment of cholesterol efflux can be reversed pharmacologically by blocking the Nef/calnexin interaction.

Discussion

Highly active anti-retroviral therapy (HAART) has transformed the treatment of the HIV disease changing the prognosis from acutely lethal to chronic illness, and lifespan of HIV-infected subjects approximates that of uninfected individuals. However, highly active anti-retroviral therapy does not cure HIV, and chronic HIV infection is associated with several comorbidities, such as premature atherosclerosis and cardiovascular disease.37 An essential component in pathogenesis of cardiovascular disease in HIV-infected subjects is HIV-associated dyslipidemia, which is caused both by drugs used to treat HIV infection and by the effects of HIV itself on cholesterol metabolism.38 In this report, we identify a small-molecule compound that blocks HIV-mediated impairment of cellular cholesterol metabolism. Excitingly, this compound also inhibited the replication of HIV, suggesting that, if developed into a drug, it can target both HIV infection and virus-induced metabolic comorbidities.

Our previous studies demonstrated that HIV critically depends on the interaction with host cholesterol metabolism and modifies it for the optimization of viral replication.1,2,28,35,36 Specifically, HIV, through viral protein Nef, reduces abundance and impairs functional activity of ABCA1, a key transporter in cholesterol efflux pathway.1 As a result, host cells accumulate excessive cholesterol promoting the formation of plasma membrane lipid rafts, which are sites of HIV entry, assembly, and budding.39 Recently, we demonstrated that an important mechanism of downregulation and functional impairment of ABCA1 by HIV is Nef-mediated inhibition of the interaction between ABCA1 and the ER chaperone, calnexin.5 This study provides the first characterization of the exact molecular structures involved in Nef–calnexin interaction.

First, we established that the interaction between Nef and calnexin involves the cytoplasmic domain of calnexin. Although this finding is consistent with demonstrated localization of Nef to the cytoplasmic side of membranes27 and lack of evidence for Nef localization to ER, it is surprising given that the C-tail of calnexin is not involved in the interaction between calnexin and ABCA1, which is disrupted by Nef.5 Indeed, calnexin interactions with glycosylated proteins are mediated by its luminal domains.8 Therefore, Nef interaction with the C-tail alters the activity of the luminal domains of calnexin. How Nef is doing it is unknown and several possibilities can be considered. Binding of Nef may prevent post-translational modifications of the C-tail of calnexin, such as phosphorylation on Ser563 that has been shown to regulate calnexin interaction with α1-antitrypsin and several other
glycoproteins.\textsuperscript{15} However, docking analysis did not reveal Ser563 as a likely site for interaction with Nef (Figure 5B). The same argument can be applied to SUMOylation (small ubiquitin-like modifier) at Lys506, which has been shown to regulate calnexin interaction with another ER protein, protein tyrosine phosphatase 1B\textsuperscript{40}: Lys506 is not among the preferred sites for Nef binding. It is likely that Nef binding to the cytoplasmic domain of calnexin results in signal transduction from the cytoplasmic to the luminal domain, eg, via a conformational change in calnexin. This explanation is consistent with the partial reduction of Nef binding to calnexin carrying deletion in the luminal domain (Figure 1B), which may be because of reverse signaling from luminal to cytoplasmic domain. Mechanistic details of such an effect await careful structural analysis. Regardless of the mechanism, this finding provides the first example of a pathogen utilizing the calnexin C-tail to regulate functional activity of this chaperone.

Second, we identified the Nef residues critical for interaction with calnexin: mutation of lysine residues in positions 4 and 7 of Nef abrogated Nef–calnexin binding, prevented ABCA1 downregulation, and restored cholesterol efflux in cells infected with HIV-1. Our finding that Nef–calnexin interaction involves the flexible N-terminal region of Nef was surprising, as this region has not been implicated before in protein–protein interactions, but its basic and hydrophobic residues were shown to be essential for membrane association of Nef.\textsuperscript{41} Our finding that Nef–calnexin interaction involves the flexible N-terminal region of Nef was surprising, as this region has not been implicated before in protein–protein interactions (Table). However, molecular modeling (Figure 2) suggests that Lys4 or Nef forms a hydrogen bond with Asp90 located in an α-helix, thus contributing to stabilization of the structure of the N-terminal region, and therefore acts as a structural anchor for the Nef Lys7 interaction with calnexin. Nef Lys7 is predicted to form a strong interaction with Glu533 in calnexin through the hydrogen and ionic bonds. Thus, mutation of both lysine residues destabilizes the structure of Nef, and cancels the strong interaction with calnexin provided by Lys7, which explains the dramatic effect of these mutations on Nef–calnexin interaction. The N-terminal region of Nef has not been involved in protein–protein interactions, but its basic and hydrophobic residues were shown to be essential for membrane association of Nef.\textsuperscript{41} Interestingly, lysine residues at positions 4 and 7, which participate in interaction with calnexin, were not essential for the membrane association of Nef.\textsuperscript{42} Therefore, our study identified a novel epitope on Nef involved in the interaction with the cytoplasmic tail of calnexin.

Using this information, we performed virtual screening for compounds that can potentially disrupt Nef–calnexin interaction, and identified several candidates. One of these compounds, 1[(7-Oxo-7H-benz[de]anthracene-3-yl)amin]-anthraquione (NSC 13987), prevented co precipitation of calnexin with Nef, reversed Nef-mediated effect on ABCA1 abundance, and restored cholesterol efflux impaired by Nef, thus effectively reversing the effects of Nef on host cholesterol metabolism. In addition, the compound resulted in a near 2-fold inhibition of viral replication (Figure 7B). This latter effect may have 2 main explanations. First, the compound prevents ABCA1 downregulation by Nef, and ABCA1 has been shown to inhibit HIV-1 replication by reducing lipid rafts’ abundance on the plasma membrane and affecting the production and infectivity of nascent virions.\textsuperscript{3,28,35,36} Second, previous reports presented evidence that anthraquinone derivatives inhibit the ribonuclease H function of HIV-1 reverse transcriptase.\textsuperscript{43,44} Therefore, the action of compound NSC 13987 in HIV-1-infected cells may be a combination of inhibiting Nef–calnexin interaction and a separate antiviral activity. This, together with differences in Nef expression, could explain why the rescue of the cholesterol efflux by the compound was only partial in Nef-transfected cells (Figure 7Aa), but almost complete in HIV-infected macrophages (Figure 7C). Our findings provide the basis for using NSC 13987 as a foundation for the development of novel treatment approaches for HIV-associated atherosclerosis and other Nef-dependent metabolic comorbidities. Indeed, the effects of Nef secreted from HIV-infected cells may be responsible for many lipid-related complications of HIV disease, such as atherosclerosis, diabetes mellitus, lipodystrophy, and neurodegeneration, so the compounds similar to the one identified in this study may reverse HIV-induced impairment of cholesterol metabolism in uninfected cells mitigating lipid-related complications of HIV infection.

Unfortunately, no small animal model is available to test in vivo whether NSC 13987 reverses the development of atherosclerosis associated with HIV infection. Indeed, mice do not develop atherosclerosis unless certain genes (apoE or ldlr) are knocked out, and humanization of such mutant mice to make them susceptible to HIV infection has not been attempted. Injection of Nef into mice reproduces only some features of the disease and it remains uncertain whether these effects of Nef involve calnexin.\textsuperscript{4} Here, we demonstrated that NSC 13987 inhibits the accumulation of lipid droplets in HIV-infected macrophage cultures (Figure 7D), which is a characteristic feature of foam cells, and foam macrophages are a hallmark of the development of atherosclerosis.\textsuperscript{45} Our findings indicate that the effect of NSC 13987 is specific for Nef-expressing cells, as the compound did not affect lipid droplets in mock-infected macrophages or cells infected with Nef-deficient HIV-1 (Figure 7D). This result supports our conclusion that the protective effect of NSC 13987 is because of the inhibition of Nef–calnexin interaction, rather than to an off-target effect on cholesterol metabolism.

Calnexin is an ER-integral membrane protein and is responsible for the folding of several glycoproteins. Depletion of calnexin has been shown to result in the elevation of several other ER-folding factors minimizing aberrant protein folding and expression.\textsuperscript{46} This is mainly true for glycoproteins which are common substrates of other soluble ER chaperones like calreticulin. However, solubility and oligosaccharide variability impose a limit on this commonality, making calnexin vital for expression and function of proteins like ABCA1 and several others.\textsuperscript{5,47,48} Nef’s ability to target several host factors, such as CD4, MHC I, and CXCR4, may in part be because of the limitation Nef imposes on the access of these proteins to calnexin. Therefore, the protective effect of compound NSC 13987 may well extend to restoring the expression and function of other proteins targeted by Nef.

In conclusion, in this study we identified the molecular mechanisms and structural epitopes involved in interaction between HIV-1 Nef and host calnexin and characterized a...
compound capable of reversing the effects of Nef, thus presenting potential utility in treatment of HIV-1 infection and its metabolic side effects.

**Sources of Funding**

This study was supported by Russian Foundation for Basic Research grant 13-04-91458; by National Institutes of Health (NIH) grants HL093818, HL101274, and AI108533; by the District of Columbia Center for AIDS Research (DC CFAR), an NIH-funded program (5F30 AI055019); and by the Molecular and Cellular Biology Program of the Russian Academy of Sciences. We thank the Biacore Molecular Interaction shared resources at the Lombardi Comprehensive Cancer Center (Georgetown University), which is supported by a grant P30 CA51708 (PI Louis Weiner) from the National Cancer Institute. We thank Dr. Geyer from University of Bonn Institute of Innate Immunity for myristoylated recombiant Nef protein. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH: pT7consnefhis6 from Dr. Chang; and p83-10 and p210-8 from Dr. Desrosiers. Ruth Hunegnaw is a predoctoral student in the Microbiology and Immunology Program of the Institute for Biomedical Sciences at the George Washington University. This work is from a dissertation to be presented to the above program in partial fulfillment of the requirements for the Ph.D. degree.

**Disclosures**

None.

**References**


---

**Highlights**

- HIV-1 Nef interacts directly with the cytoplasmic tail of the endoplasmic reticulum chaperone calnexin.
- Mutation of lysine residues in positions 4 and 7 of Nef disrupts Nef-calnexin interaction and prevents Nef-mediated inhibition of ABCA1 and impairment of cholesterol efflux.
- Anthraquinone derivative NSC 13987 blocks Nef-calnexin interaction, reverses impairment of cholesterol efflux and reduces accumulation of lipid droplets in HIV-infected macrophages.
Interaction Between HIV-1 Nef and Calnexin: From Modeling to Small Molecule Inhibitors Reversing HIV-Induced Lipid Accumulation
Ruth Hunegnaw, Marina Vassylyeva, Larisa Dubrovsky, Tatiana Pushkarsky, Dmitri Sviridov, Anastasia A. Anashkina, Aykut Üren, Beda Brichacek, Dmitry G. Vassylyev, Alexei A. Adzhubei and Michael Bukrinsky

Arterioscler Thromb Vasc Biol. 2016;36:1758-1771; originally published online July 28, 2016; doi: 10.1161/ATVBAHA.116.307997
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/36/9/1758

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2016/07/28/ATVBAHA.116.307997.DC1
http://atvb.ahajournals.org/content/suppl/2016/07/28/ATVBAHA.116.307997.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and Methods

**Reagents** - The following reagents were purchased from the indicated suppliers: mouse monoclonal anti-calnexin (ab31290, Abcam); anti-HA Epitope tag Antibody (NB600-363, Novus Biologicals); anti-Nef serum (NIH AIDS Reagent Program); anti-ABCA1 (NB400-105, Novus Biologicals); polyclonal rabbit anti-calnexin (H-70, Santa Cruz Biotech); anti-GAPDH (G9545, Sigma Aldrich); HRP conjugated donkey anti-rabbit and Goat anti-mouse (Jackson Immuno Research); anti-HA Agarose (26181, Thermo Scientific); EZview Red Protein A Affinity Gel (P6486, Sigma-Aldrich). Metafectene® (Biontex; IGEPA (CA-630, SigmaAldrich); Triton™ (X-100, Sigma Aldrich); 10% SDS (Corning).

**Nef and Calnexin Mutagenesis** – Nefcons expression plasmid pT7consnefhis6 was obtained from the NIH AIDS Reagent Program. Nef was recloned into the pHCMV3 vector for expression and mutagenesis. Nef mutants K4A, K7A and K4,7A were generated using site-directed mutagenesis with Pfu Ultra High-Fidelity DNA Polymerase (Agilent Technologies). Forward and reverse primer sequences used were as follows:

- Nef K4A Fwd, 5'-TTTGCTATAAGATGGGTGGCGCGTGGTCAAAAAGTAGTGTGG-3'; Nef K4A Rev, 5'-CCACACTACTTTTGGACCGCCACCCCATCTTTATAGCAA-3';
- Nef K7A Fwd, 5'GATGGGTGGCAAGTGGTCAGCAAGTAGTGTGGTTGATGG-3'; Nef K7A Rev, 5'-CCATCCAACCACACTACTTGCTGACCACCCCATC-3;
- NefK4,7A Fwd, 5'-GGGTGGCGCGTGGTCAGCAAGTAGTGTGGTTGGA-3';
- NefK4,7A Rev, 5'-TCCAACCACACTACTTGCTGACCACCCACC-3'.

Resulting cDNA was transformed into XL10-Gold Ultracompetent Cells and final plasmid preps were sequenced to confirm mutations.

The plasmids pCG-NL4-3 expressing infectious provirus with wild-type Nef, and pCG-NL4-3NefK expressing provirus with K4V,K7A mutant Nef were kindly provided by Dr. Marc Harris 2. Recombinant myristoylated NefSF2 was provided by Dr. Matthias Geyer 3.

Human CNX cDNA construct with a C-terminal HA tag was prepared by standard PCR method from CNX cDNA clone (Open Biosystems) in the pHCMV3 vector (Gelantis). Truncated CNX construct was generated similarly by using primers described previously 4. The HA-tagged CNX∆504-586 construct lacks the 87 residues of the cytoplasmic tail. Deletion of the repeated sequence motifs referred to as CNX∆276-409 was made by restriction digest of full length HA-tagged pHCMV3-CN and re-ligation 5.

**Expression and Purification of Calnexin** – We have designed a CNX expression vector with a His-tag at the N-terminus and the CL7-tag, which can be cleaved by the PSC protease, at the CNX C-terminus (Fig. 5A). The CNX-CT construct was designed with a single N-terminal His-tag followed by the SUMO-domain (to allow His-tag cleavage by the SUMO-protease, SUMO-P). CNX and CNX-CT were expressed in E. coli BL21 DE. Cells were grown at 37°C to OD ~ 0.8-0.9, then temperature was decreased to 18-20°C and expression was induced with 0.1 mM IPTG overnight. Cells were frozen at -80°C until protein purification.

We have developed and implemented a novel purification system based on the natural ultra-high affinity complex (K_m ~ 10^{-14}-10^{-17} M) between the colicin E7 DNAase domain (CL7) and its inhibitor, immunity protein 7 (Im7) 6-9. The proteins have been modified to remove DNAse activity of CL7 and allow for efficient immobilization of the Im7 unit on the activated agarose beads (DGV, unpublished results). The CNX construct tagged at the C-terminus with CL7 was expressed in E. coli, the cells were lysed, centrifuged to remove cell debris, and the supernatant was treated with 0.07% polyethylene-ename (PE) to precipitate CNX (Fig. 5A).
pellet was washed with 20 mM Tris-HCl, pH 8.0, 600 mM NaCl, 1.5% dodecyl-maltopyranoside to release CNX into solution, centrifuged again, and the resulting supernatant was loaded on the Im7 column. CNX protein was eluted from the column upon treatment by PSC protease. This single purification step provided an excellent yield of ~90-95% pure protein, in which major contamination represents truncated CNX molecules (confirmed by mass-spectroscopy). Given that these molecules are retained on the column and that affinity tag is localized at the C-terminus of CNX, truncation occurred most likely from the N-terminus (proteolytic sensitivity of CNX at the N-end was reported previously 10). CNX-CT was purified in standard procedure using the commercial His-Trap column. All procedures were carried out at 4°C.

Surface Plasmon Resonance Experiments - Direct binding between purified recombinant proteins was evaluated by surface plasmon resonance technology utilizing a Biacore T-200 instrument at the Biacore Molecular Interaction Shared Resource of Georgetown University. Full length CNX and the C-terminal (cytoplasmic) domain of CNX (CNX-CT) were captured on CM5 chips by amine coupling. Three surfaces of CM5 chip were activated by NHS/CDC (N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) for 720 sec. Flow cell 1 was left empty as a reference surface. Full length CNX and CNX-CT were diluted in 10 mM sodium acetate (pH 4.0) buffer at 1.8 µM and 10.6 µM, respectively, and captured on flow cell 3 and flow cell 2, at 3200 RU and 16600 RU, respectively. After protein capture, all 3 flow cells were inactivated by 720 sec injection of 1 M ethanolamine. Myristoylated NefSF2 protein was injected over the chip surface at 6 different concentrations (6.25 nM, 12.5 nM, 25 nM, 50 nM, 100 nM, 200 nM) in triplicates. All binding studies were done at 25°C. Flow rate for protein capture was 10 µl/min, and kinetics experiment was at 50 µl/min. HPS-P (10 mM HEPES pH 7.4, 150 mM NaCl, 0.005% surfactant P-20) + 2 mM CaCl2 was used as the running buffer. The data was analyzed by BioEvaluation software using the bivalent analyte model.

Cells and Transfection - HEK293T and THP-1 cells were cultured in RPMI supplemented with 10% fetal bovine serum and antibiotics. For transfection, HEK 293T or HeLa-ABCA1-GFP cells were passaged and cultured overnight in 6-well plates and transfected with plasmid DNA using Metafectene according to the manufacturer’s (Biontex) instructions. THP-1 cells were transfected using the Lipofectamine™ LTX reagent according to manufacturer’s instructions (Thermo Fisher Scientific).

Compounds – Three out of 10 compounds obtained from NCI drug database were tested for blocking Nef/CNX interaction. Tested compounds were 1,3-DI-9-Phenanthrylguanidine (NSC 1758), 1[(7-Oxo-7H-benz[de]anthracene-3-yl)amino]anthraquinone (NSC 13987) and 5H-Naphtho(2,3-a)carbazole-5,13(12H)-dione (NSC 92938). All compounds were dissolved in DMSO and diluted in cell culture medium (RPMI 1640 with 10% fetal bovine serum and antibiotics) to ensure the final concentration of the solvent to be <1%.

MTT Assay – THP-1 cells were seeded in 96 well plates (30,000 cells/well) and incubated at 37°C, 5% CO2 in the presence of compounds for 5 days. The MTT assay for cytotoxicity was done in quadruplicates according to manufacturer’s instructions (Sigma-Aldrich). The concentrations selected for experimental testing - 4 µM for NSC 1758, and 5 µM for NSC 13987 and NSC 92938 – reduced MTT metabolism by less than 10% relative to untreated cultures.

Immunoprecipitation - For calnexin mutant/Nef interaction analysis, HEK293T cells were transfected with HA-tagged calnexin mutants and Nefcons expression plasmid. Cells were lysed
48 h post-transfection with 1% IGEPAL and 0.1% SDS lysis buffer on ice for 30 min. Lysates were incubated with anti-HA agarose beads for 2 h at 4°C with rotation. Respective immunoprecipitates were washed three times with TBS (150 mM NaCl, 30 mM Tris-HCl, 5 mM EDTA, pH 7.5). Bound complexes were eluted by boiling in sample buffer for 5 min. Supernatants were separated by SDS-PAGE for immunoblotting. Immunoprecipitation of calnexin from cells transfected with pCG-NL4-3-IRES-GFP or mutant variant was performed similarly with the following modifications. Cell lysates were incubated with mouse monoclonal anti-calnexin antibody (Abcam) for 2 h with rotation at 4°C. EZView protein A agarose beads (SigmaAldrich) were then added and the mix was further incubated for 1 h at 4°C with rotation. Bound complexes were recovered as described above. To analyze interaction between Nef single mutants and calnexin, HeLa-ABCA1-GFP cells were transfected with pcDNA3.1 empty vector, WT Nef, NefK4A, NefK7A or NefK4,7A and lysed 48 h post-transfection. Endogenous calnexin was immunoprecipitated by sequential incubations at 4°C first with mouse monoclonal anti-calnexin antibody (Abcam) for 2 h, and then with protein A/G agarose beads (SantaCruz) for an additional 1 h. Beads were collected and complexes were recovered as described above. For studies of Nef/CNX interactions in the presence of compounds, HEK293T cells were first transfected with HA-tagged Nef and compounds were added after 6 h. Cells were lysed 48 h post-transfection and immunoprecipitation was carried out using anti-HA agarose beads as described above. For analysis of ABCA1-FLAG/Nef interaction, HEK293T cells transfected with ABCA1-FLAG and empty pcDNA3.1 vector, Nef WT or NefK4,7A were lysed 48 h post-transfection with 1% IGEPAL. Lysates were incubated with anti-FLAG agarose beads (Sigma-Aldrich) for 2 h, beads were harvested and washed 3 times with TBS. Immunoprecipitates were recovered by boiling in sample buffer for 5 min and analyzed by Western blot.

Molecular Modeling and Docking – Structure modeling of the calnexin cytoplasmic domain was performed using servers Hhpred 11, iTasser 12, ModWeb 13, Phyre2 14, and RaptorX 15, with subsequent quality assessment of the obtained models and building of the final model by the QA-RecombineIt server 16. The Modbase 13 GI 66933005 model based on PDB 1JHN (96% sequence identity) was used for the luminal domain structure. Nef structure has been modeled using as templates the crystallographic and solution NMR experimental structures covering different parts of Nef sequence, available from the PDB 17: 4EN2, 3TB8, 4EMZ, 3REB, 3RBB, 3REA, 1EFN. These structures are based on the HIV-1 sequences P03404, P03406, P03407, Q90VU7 (Uniprot 18). Modeling was performed for the target sequence P03407 (HIV-1 group M subtype B isolate ARV2/BRU) with servers Hhpred, iTasser, M4T 19, ModWeb, Phyre2, Swiss-Model 20. The resulting models have been then submitted to the QA-RecombineIt server, producing the final model used for interactions prediction.

In order to obtain indications as to which regions of Nef can represent interactions interfaces, location of possible interactions sites has been estimated with a sequence-based method ConSurf 21. Sequence conservation for Nef has been assessed by constructing multiple alignments using T-Coffee 22. We have subsequently carried out docking of the structure models of calnexin cytoplasmic domain and Nef to identify the sites in Nef interacting with calnexin. Docking was performed using servers Cluspro 23, HEX 24, SwarmDock 25, Zdock 26, each run producing 10 best models. To obtain a representative array of docking models, docking has been carried out for calnexin and Nef submitted to docking runs alternatively as receptor and ligand. Since both calnexin cytoplasmic domain and Nef bind to ER membrane, the resulting docking models that were able to disrupt this binding have been filtered out from the final data.
To assess average number of interactions for each residue position in Nef and CNX sequences in the set of docking models of binding between CNX cytoplasmic domain and Nef, the overall number of Nef-CNX interactions for all models, for each residue in Nef and CNX involved in intermolecular interaction has been calculated. Number of interactions for each amino acid residue in Nef and CNX is the total number of interactions for this residue in docking models where such interaction was identified.

Virtual screening – structure-based virtual screening (docking-based) was carried out on the NCI Plated 2007 dataset (http://zinc.docking.org/catalogs/ncip) from Zinc database (http://zinc.docking.org/). Locally installed docking program AutoDock Vina has been used for screening.

HIV Infection – HIV particles pseudotyped with VSV-G were produced from HEK293T cells co-transfected with pHEF-VSVG and pCG-NL4-3 or pCG-NL4-3NefK mutant derivative. Viruses were normalized by RT activity prior to infection, and infection was allowed to proceed for 10 days. For infection of human monocyte derived macrophages (MDM) with HIV-1 ADA, virus was amplified on primary PBLs. Infection was monitored by RT assay for up to 15 days.

Cholesterol Efflux - Infected cells seeded in a 24-well plate were labeled with $[^{3}H]$ cholesterol (PerkinElmer) for 48 h. Cells were washed and ABCA1 expression was stimulated by adding 1µM T0901317 (Sigma-Aldrich) and incubating overnight. Following this step, cells were washed with PBS to remove any free cholesterol and efflux was initiated by adding 20 µg/mL apoA-I (Calbiochem) and incubating for 3 h in serum free medium. The media from the wells was then carefully collected and any cell debris was removed by centrifuging at 5,000 rpm for 5 min. Cell monolayers were lysed with 1% Triton X-100. Level of radioactivity in the media as well as in the cells was determined by scintillation counting. Cholesterol efflux was calculated as the percentage of radioactivity in the media divided by the total amount measured in the cells and media. Cholesterol efflux in the presence of compounds was performed similarly with the following modifications. THP-1 cells were first transfected with Nef using Lipofectamine™ LTX reagent (Thermo Fisher Scientific). Compounds were added to cells 6 h post transfection and incubated overnight. The following day cells were washed with PBS and treatment with compounds was continued for 48 h with the addition of $[^{3}H]$ cholesterol and Phorbol 12-myristate 13-acetate (PMA). Efflux measurements were then performed as described above.

Oil Red O Staining - Human monocyte derived macrophages grown on coverslips were infected with HIV-1 ADA, VSV-G-pseudotyped HIV-1 NL4-3 or HIV-1 NL4-3∆Nef, or mock-infected. Five days post infection, cells were treated with DMSO or NSC 13987. Ten days post-infection (for VSV-G-pseudotype NL4-3 virus) or 14 days post-infection (for HIV-1 ADA), cells were washed and fixed with 3.7% formaldehyde for 30 min. A working solution of Oil Red O was prepared according to Koopman et al. The staining solution was applied on top of the coverslips and incubated for 7 min. Cover slips were washed 3 times with deionized water and dipped in a solution containing DAPI according to manufacturer’s recommendations (Invitrogen). Cells were observed on Leica DMi8 microscope under a 100x magnification, using bright field or fluorescent modes. Bright field images were analyzed in ImageJ program. Histograms of RGB pixels distribution along the axis of 256 shades were created for each cell in an image, and values for each shade were averaged for all cells (about 50-70 cells). Left side of the histogram represents pixels with high color intensity, characteristic for ORO stained droplets,
right side – pixels with low intensity. These histograms represent shade distribution in an average cell for each condition. To quantify the number of cells displaying increased accumulation of ORO staining, the shade profile was defined in each condition for ORO-stained areas of cells with high accumulation of lipid droplets and for ORO-stained areas of cells with no visible lipid droplets (negative cells). Pixels corresponding to shades left of the negative cell shade profile were considered positive. Area of positive pixels in each cell was determined as percentage of total cell area. Pie charts for each condition were plotted comparing the number of cells with positive pixels forming less and more than 20% of total cell area.

Confocal Fluorescent Microscopy - Cultures of HEK 293T cells, grown on coverslips, were transfected with wild-type HIV-1 Nefcons or K4,7A Nefcons. Forty eight hours post transfection, cultures were fixed with 3.5% formaldehyde, permeabilized with Triton X-100, blocked with 1% BSA and subsequently immunostained with a combination of anti-HIV Nefcons rabbit serum (NIH AIDS Reagents Program) (1:750) and anti-Calnexin/ER membrane marker antibody (mouse monoclonal AF18, Abcam) (1:750) followed by combination of Alexa Fluor® 647 Goat Anti-Rabbit IgG (Molecular Probes, Invitrogen) (1:500) and DyLight 550 Goat Anti-Mouse IgG (MyBiosource) (1:500). All antibody dilutions were done in PBS supplemented with 1% BSA and 5% Normal Goat Serum. Immunostained cultures were counterstained with DAPI (Molecular Probes, Invitrogen) and coverslips were mounted with Fluoromount G (SouthernBiotech).

Images were captured with an Axio Cell Observer Spinning Disk fluorescent microscope (Carl Zeiss) equipped with Yokogawa CSU X1 spinning disk and Evolve Delta EM CCD cameras (512 x 512, Photometrics), using a Plan Apochromat 100x/1.46 oil immersion lens. The image was processed using Volocity software (PerkinElmer Life Sciences).

Statistical Analysis – Statistical analyses were performed with the use of GraphPad PRISM 6.0 (Software MacKiev). A significant difference was accepted at $p < 0.05$. 
References


27. Irwin JJ, Shoichet BK. Zinc--a free database of commercially available compounds for virtual screening. *Journal of chemical information and modeling.* 2005;45:177-182


