Published in final edited form as:

Chem Biol Drug Des. 2017 April ; 89(4): 608–618. doi:10.1111/cbdd.12886.

The Small Molecule 3G11 inhibits HIV-1 reverse transcription

Silvana Opp¹, Thomas Fricke¹, Caitlin Shepard², Dmytro Kovalsky³, Akash Bhattacharya³, Frank Herkules³, Dmitri N. Ivanov³, Baek Kim², Jose Valle-Casuso¹, and Felipe Diaz-Griffero^{1,*}

¹Department of Microbiology and Immunology, Albert Einstein College of Medicine Bronx, NY 10461

²Department of Pediatrics, Emory University, Atlanta, GA 30322

³Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78229

Abstract

The small molecule 6-(tert-butyl)-4-phenyl-4-(trifluoromethyl)-1H,3H-1,3,5-triazin-2-one (3G11) inhibits HIV-1 replication in the human T cell line MT-2. Here we showed that 3G11 specifically and potently blocks HIV-1 infection. By contrast, 3G11 did not block other retroviruses such as HIV-2, simian immunodeficiency virus (SIV_{mac}), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), N-tropic murine leukemia virus (N-MLV), B-tropic murine leukemia virus (B-MLV) and Moloney murine leukemia virus (Mo-MLV). Analysis of DNA metabolism by real-time PCR revealed that 3G11 blocks the formation of HIV-1 late reverse transcripts during infection prior to the first-strand transfer step. In agreement, an in vitro assay revealed that 3G11 blocks the enzymatic activity of HIV-1 reverse transcriptase as strong as Nevirapine. Docking of 3G11 to the HIV-1 reverse transcriptase enzyme suggested a direct interaction between residue L100 and 3G11. In agreement, an HIV-1 virus bearing the reverse transcriptase change L100I renders HIV-1 resistant to 3G11, which suggested that the reverse transcriptase enzyme is the viral determinant for HIV-1 sensitivity to 3G11. Although NMR experiments revealed that 3G11 binds to the HIV-1 capsid, functional experiments suggested that capsid is not the viral determinant for sensitivity to 3G11. Overall, we described a novel non-nucleoside reverse transcription inhibitor that blocks HIV-1 infection.

Graphical Abstract

Key Findings: 3G11 is a NNRTI that binds to capsid and blocks HIV-1 reverse transcription during infection.

*Corresponding author: Felipe Diaz-Griffero Ph.D., Albert Einstein College of Medicine, 1301 Morris Park – Price Center 501, New York, NY 10461, Phone: (718) 678-1191, Fax: (718) 632-4338, Felipe.Diaz-Griffero@einstein.yu.edu.

Keywords

HIV-1; reverse transcription; NNRTI; antiviral drug; capsid; L100

Introduction

HIV-1 uncoating is the shedding of monomeric capsids from the retroviral core, which is composed of ~1800 monomers of capsid, in the cytosol of the cell. Capsid plays a fundamental role in the early steps of HIV-1 replication, such as reverse transcription and nuclear import. Over the years several proteins, drugs and peptides that target capsid have been discovered: rhesus TRIM5 α ^{1,2}, owl monkey TRIMCyp^{3,4}, PF74⁵⁻⁹, BI-2^{9,10}, CAP-1^{3,4,6-12}, Ebselen^{1,2}, the peptide CAI^{9,13,14} and others. From studies using these proteins, drugs and peptides, we have learned much about the early steps of HIV-1 replication: 1) reverse transcription occurs before or during uncoating^{3,4,6-12,15}, 2) The decreased stability of the HIV-1 core, acceleration of uncoating, caused by rhesus TRIM5 α , owl monkey TRIMCyp, the small molecule PF-74 or the small molecule Bi-2, prevents HIV-1 infection^{3,8,9,13,14,16}, 3) The increased stability of the HIV-1 core, inhibition of uncoating, caused by the cytoplasmic expression of human CPSF6 or human MxB, similarly prevents HIV-1 infection¹⁷⁻¹⁹, and 4) Inhibition of reverse transcription increases stability of the HIV-1 core⁵. Altogether this evidence indicates that the HIV-1 uncoating process is fundamentally linked to reverse transcription.

Our previous studies suggested that genetic or pharmacological inhibition of HIV-1 reverse transcription a) stabilizes the HIV-1 core, and b) makes the HIV-1 core resistant to the destabilizing effects of rhesus TRIM5 α ⁵. These findings are in agreement with the hypothesis that the surface of the core is dynamic and could expose and hide protein domain---viral breathing--- depending upon changes within or outside the core, as shown for other viruses^{20,21}. The process of exposing and hiding domains or epitopes on the surface of the core creates a communication system between the core and its environment. This communication could be important for the core to sense the appropriate place and time to undergo uncoating and reverse transcription. Although progress has been made, several questions remain: 1) what triggers initiation of uncoating?, 2) what triggers initiation of reverse transcription, and 3) How are these processes connected? The understanding of

mechanisms used by novel inhibitors of early steps of HIV-1 replication will certainly help answering some of these questions.

Because finding additional small molecule inhibitors that target early steps of HIV-1 infection will help the better understanding of these processes, this work characterizes the HIV-1 steps that are affected by the novel HIV-1 inhibitor 6-(tert-butyl)-4-phenyl-4-(trifluoromethyl)-1H,3H-1,3,5-triazin-2-one (3G11).

Materials and Methods

Cell lines

Human HeLa (ATCC®#CCL-2), dog Cf2Th cells (ATCC®#CRL-1430) and CHME5 were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin.

Purification and assembly of HIV-1 CA-NC complexes

The HIV-1-CA-NC protein was expressed and purified, as previously described²². HIV-1 CA-NC particles were assembled in vitro by diluting the CA-NC protein to a concentration of 0.3 mM in 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl and 2 mg/ml DNA oligo-(TG)50. The mixture was incubated at 4°C overnight and centrifuged at 8,600×g for 5 minutes. The pellet was resuspended in assembly buffer (50 mM Tris-HCl (pH 8.0), 0.5 M NaCl) at a final protein concentration of 0.15 mM, and stored at 4°C until needed.

CA-NC stability assay

CA-NC stability assay was performed as described previously^{9,23}. Briefly, CA-NC tubes [assembled in 50 mM Tris-HCl pH8.0, 0.5 M NaCl and 2 mg/mL DNA oligo(Tg)25 (50 bp long)], were incubated with either stabilization buffer (10 mM Tris-HCl pH8.0, 10 mM KCl, 2 mM MgCl₂, 0.5 mM DTT) or destabilization buffer (20 mM Tris-HCl pH8.0, 10 mM KCl, 2 mM MgCl₂, 0.5 mM DTT, 1% glycerol, 0.1% NP40) for 1 hr at room temperature, in presence of increasing concentrations of 3G11. An aliquot of this mixture was referred to as “input”. The mixture was spun through a 70% sucrose cushion at 100,000 × g in a SW55 rotor (Beckman) for 1hr at 4°C. After centrifugation, the pellet was resuspended in 1x SDS-PAGE loading buffer. The levels of HIV-1 CA-NC protein in the input and the pellet were assessed by western blotting using anti-p24 antibodies.

Infection with viruses expressing GFP

Recombinant HIV-1-GFP, HIV-2-GFP, SIV_{mac}-GFP, BIV-GFP, FIV-GFP, EIAV-GFP, N-MLV-GFP, B-MLV-GFP, and MO-MLV-GFP viruses were prepared as described previously²⁴. Recombinant viruses were pseudotyped with the VSV-G glycoprotein. For infections, 24-well plates were seeded with 25 000 Cf2Th or HeLa cells and incubated at 37°C with virus for 24 h. 250 µl of medium was added and cells were returned to culture for another 24 h. GFP-positive cells were analyzed using a flow cytometer (Becton Dickinson).

Real-time PCR to detect HIV-1 late reverse transcripts (LRTs) and 2-LTR circles, and formation of the HIV-1 minus-strand DNA

Total cellular DNA from infected cells was isolated using the QIAamp DNA microkit (Qiagen) at 7 h (LRT) and 24 h (2-LTR) post-infection. Before infection, viruses were treated for 30 min at 37°C with 1,000 U of DNase I (Roche). As a control of the experiment, we performed infection in the presence of 10 µM nevirapine and heat inactivated viruses for 30 min at 100°C. We measured late reverse transcripts using the following primers and probe: forward primer (GACGTAAACGGCCACAAG); reverse primer, (GGTCTTGTAGTTGCCGTCGT); and probe 5'/56-FAM/CCTACGGCAAGCTGACCC/36-TAMSp/-3. A standard curve was created using the GFP sequence of the HIV-1 reporter virus. Similarly, for the detection of 2-LTR circles, we used the following primers and probe: forward primer (ACCTAGGGAACCCACTGCTTAAG), reverse primer (TCCACAGATCAAGGATATCTTGTC) and probe 5-/56-FAM/ACACTACTTGAAGCA CTCAAGGCAAGCTTT/36-TAMSp/-3. A standard curve was created using the pUC2LTR plasmid, which contains the HIV-1 2-LTR junction. β-Actin amplification was used for normalization using the following primers and probes for actin: DNA samples for real-time PCR from canine Cf2Th cells were normalized by amplification of canine actin housekeeping gene using the following primers by SYBR Green: forward primer (GCATCCTGACCCTCAAGTAG) and reverse primer (ACATACATGGCTGCTGGGGTGTT) and probe SYBR green Reaction mixtures contained 1× Fast SYBR Green Master Mix (Applied Biosystems), 300 nM forward primer, 300 nM reverse primer, 100 nM probe primer, and template DNA. Denaturation steps (95°C for 17 min) with 40 cycles of amplification were carried out (95°C for 15 s, 58°C for 30 s, and 72°C for 30s).

Similar experiments at 7 hours post-infection were performed to detect formation of the minus-strand DNA by real-time PCR (see Figure 6C):

Primer1: (GGCTAACTAGGGAACCCACTG)

Primer2: (GCTAGAGATTTTCCACACTGACTAA)

Fate of the capsid assay

The fate of the capsid assay was performed as previously described²⁵. Cf2Th cells were infected with HIV-1-GFP and 3 µM 3G11, 5 µM PF74, or 50 µM BI-2 was added. After an incubation time of 8 hours, cells were detached with Pronase for 5 min on ice and washed 3 times with ice-cold PBS. Cell pellets were resuspended in hypotonic buffer [10 mM tris-HCl pH8; 10 mM KCl; 1 mM EDTA] and incubated for 15 min on ice. Cells were lysed in a 7.0 ml Dounce homogenizer with pestle B. Cellular debris were cleared by centrifugation for 7 minutes at 3000 rpm. The cleared lysate was layered onto a 50% sucrose (weight: volume) cushion in 1× PBS and centrifuged at 125,000 × g for 2 hours at 4°C in a Beckman SW41 rotor. Input, soluble and pellet fractions were analyzed by Western blotting using anti-HIV-1 p24 antibody.

RT inhibition assay

We examined the effect of two drugs on RNA-dependent DNA polymerization activity of RT enzyme using a 5' ³²P-labeled 17-mer primer annealed to 40-mer RNA template. Nevirapine and 3G11 were diluted so that final concentrations in the assay were 100 μ M, 10 μ M and 1 μ M. HXB2 RT protein (8 μ L) at 0.79mg/mL and 2 μ L of the various drug concentrations were prebound for 10 minutes at 37°C. Reactions were initiated by adding prebound RT and drug to substrate mix and incubated at 37°C for 5 minutes. Each assay reaction (40 μ L) contained 0.2 pmol of T/P (5' ³²P-labeled 17-mer DNA primer, CGCGCCGAATTCCCGCT annealed to 40-mer RNA template, GCGCGGCUUAAGGGCGAUCGUUAUAAGACGUCGGUUCGAA), 4 μ L dNTPs, 25 mM Tris-HCl, pH 8.0, 100 mM KCl, 2 mM dithiothreitol, 5 mM MgCl₂, 5 μ M (dT)₂₀, and 0.1 mg/ml bovine serum albumin. DMSO made up 5% of the reaction. The T/P extended by RT was dependent on the inhibition ability of Nevirapine and our 3G11. Reactions were terminated with 100 μ L of 40 mM EDTA, 99% formamide. Reaction products were immediately denatured by incubating at 95°C for 5 minutes. Of each 140 μ L reaction, 4 μ L of final reaction products were separated on a 20% polyacrylamide gel and imaged on a Bio-Rad Molecular Imager FX.

Computational compound docking

To model interaction of compound 3G11 with HIV-1 capsid we used crystal structure of the CA-NTD in the complex with BD 3 solved at 1.7 Å resolution (PDB ID: 4E91)¹⁵. Structure of the CA-NTD/BD 3 complex (PDB ID: 4E91) was selected as the starting template because of the similarities between the 3G11 structure and the benzodiazepine core of BD 3. All modeling studies were performed using Schrodinger 2015-3 software suite and its graphical interface, Maestro. The protein pdb file was subjected to Protein Preparation Wizard and side chains of missing atoms being restored. The cyclophilin binding loop remained unmodified since it is not involved into BD3 binding. All water molecules were removed and the binding site was set as a box around BD3 molecule with a minimal distance of 10 Å from any ligand atom to the box boundary. Two stereo isomers of 3G11 were generated and optimized with Ligprep procedure. Docking was performed with Glide using first standard precision protocol. Straight docking into the crystal structure of both isomers did not return plausible poses due to steric clashes. Therefore an alternative approach was taken. Each of the isomers was placed manually followed by induced fit minimization of side chains of residues involved in the direct contact with the ligand. Only R-isomer produced a pose with minimal perturbations whereas S isomer failed to fit. The resulted complex was assessed by redocking using extra precision level of Glide accuracy returning Glide score of -8.663. For docking of 3G11 into HIV-2 CA, the homology model of HIV-2 capsid was generated using structure of HIV-1 CA complex with 3G11 as a template. The structure of the 3G11/HIV-2 CA complex was then optimized by energy minimization. The minimized structure was evaluated using the same docking procedure as the structure of the 3G11/HIV-1 CA complex.

For docking of 3G11 to HIV-1 RT the structure of the complex of HIV-1 RT with efavirenz²⁶ (PDB ID: 1FK9) was selected because of the structural similarities between efavirenz and the S-isomer of 3G11. 3G11-S was docked.

NMR Analysis

¹⁵N-labeled HIV-1 CA-NTD was prepared and purified as previously described²⁷, ¹⁵N HSQC spectra were collected on a Bruker Avance 700 MHz spectrometer. The NMR buffer was: 50 mM Na phosphate pH 8, 50 mM NaCl, 2 mM DTT, and 4.5 % DMSO. CA-NTD concentration was 120 μM and titration spectra were collected with a [CA-NTD]:[3G11] ratio of 1:1, 1:2, 1:3 and 1:5. Only the highest concentration spectrum is shown in Figure 4A.

RESULTS

The small molecule 6-(tert-butyl)-4-phenyl-4-(trifluoromethyl)-1H,3H-1,3,5-triazin-2-one (3G11) blocks an early step of HIV-1 infection

The chemical compound 6-(tert-butyl)-4-phenyl-4-(trifluoromethyl)-1H,3H-1,3,5-triazin-2-one (3G11) was discovered as a small molecule that potentially targets capsid and inhibits infection of replication competent HIV-1 on a cell-based screen using the T cell line MT-2 (Japanese Patent #4CO86 and a recently submitted manuscript: Urano E, Miyauchi K, Kojima Y, Hamatake M, Ablan SD, Fudo S, Freed EO, Hoshino T, Komano J. A triazinone derivative inhibits HIV-1 replication by interfering with reverse transcriptase activity) (Figure 1). To test whether the ability of 3G11 to inhibit HIV-1 infection is early during infection, we challenged Cf2Th cells with single round replication HIV-1-GFP viruses. As shown in Figure 2, 3G11 blocked HIV-1 infection as potent as the small molecule inhibitor PF74, and is much stronger when compared to the small molecule BI-2^{9,10,23}. The small molecule 3G11 used at 10 μM completely inhibited infection of single-round replication HIV-1 viruses; these results suggested that 3G11 blocks HIV-1 infection at an early step such as reverse transcription, nuclear import, or integration.

3G11 is a specific inhibitor of HIV-1 infection

This section explores the range of viruses that 3G11 is affecting. To test the ability of 3G11 to block the replication of other lenti- and retro-viruses, we challenged Cf2Th cells using the indicated viruses in the presence of 3G11. As shown in Figure 3, 3G11 only blocks HIV-1 infection. The specific ability of 3G11 to block HIV-1 infection is shown by its inability to block HIV-2, simian immunodeficiency virus (SIV_{mac}), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), equine infectious anemia virus, N-tropic murine leukemia virus (N-MLV), B-tropic leukemia virus (B-MLV), Moloney murine leukemia virus (Mo-MLV). As a control, we performed similar infections in the presence of the small inhibitor PF-74. These experiments suggested that 3G11 is a specific inhibitor of HIV-1 infection.

3G11 binds to CA-NTD at the site facing the interior of the viral core

We used solution state NMR spectroscopy to identify the interaction site of 3G11 with the CA protein (Figure 4A). ¹⁵N HSQC spectra of CA-NTD revealed that a subset of CA-NTD residues was broadened upon addition of 3G11 (Figure 4A and B). Most affected residues are located in helices 1, 2 and 3 of CA-NTD and form part of the CA-NTD structure facing the interior of the assembled core particle. Affected residues are located in the vicinity of a CA-

NTD pocket that was previously identified as the binding site of small-molecule CA ligands identified in drug discovery efforts carried out by Boehringer Ingelheim such as the compound BD3¹⁵ (Figure 4B and C). Furthermore, 3G11 displays certain structural similarities to compounds shown to bind at this particular site. We used the previously published structure of a benzodiazepine compound BD 3 bound to CA-NTD (PDB ID: 4E91) to dock 3G11 into the same binding pocket and to generate a model of the CA-NTD/3G11 complex (Figure 4B and C) (see Materials and Methods). The docked model yields a favorable Glide score (-8.663) and is in good agreement with experimentally observed NMR perturbations (Figure 4A and B). Similarly to the CA-NTD/BD 3 complex, bound 3G11 is largely buried in the deep interior pocket of CA-NTD surrounded by residues located in the vicinity of the helix 1 to helix 2 linker, helix 3 to helix 4 linker and the C-terminal end of helix 7 (Figure 4C). 3G11 is smaller in size than BD 3 or other benzodiazepine and benzimidazole CA ligands developed by Boehringer Ingelheim, and in our model it is almost completely submerged in the CA-NTD interior and the impact of its binding on the overall CA-NTD structure is minimal, which is in agreement with our results suggesting that 3G11 does not change the stability of the HIV-1 core or in vitro assembled HIV-1 CA-NC complexes (see below). As shown in Figure 4B, 3G11 binds to a pocket composed of capsid residues V27, A31, F32, S33, M39, L56, V59, and G60. Our model reveals that 3G11 is not expected to make any direct contacts with other CA-NTD or CA-CTD domains located in the vicinity of the binding site in the assembled capsid structure (Figure 4D). These observations suggest that 3G11 might be interfering with interactions that occur between the inside surface of the HIV-1 core with components of the HIV-1 core, such as Viral RNA, reverse transcriptase, integrase and others.

We also used computational modeling and docking to assess whether 3G11 can bind to the HIV-2 CA (Figure 4C). We find that whereas the differences in the amino acids composition of HIV-2 CA in the vicinity of the 3G11 binding site result in a slightly different orientation of the compound in the binding pocket, the docking score for the 3G11/HIV-2 CA complex (-8.739) is similar to that of the 3G11/HIV-1 CA complex.

The role of Capsid in the sensitivity of HIV-1 to 3G11

The small molecule 3G11 binds to capsid and potently blocks HIV-1 infection suggesting that 3G11 might be affecting the uncoating process of HIV-1. To measure whether 3G11 affects the uncoating process of HIV-1, we performed the fate of the capsid assay in the presence of 3G11. The fate of the capsid assay distinguishes assembled from disassembled capsid during infection²⁵. For this purpose, we challenged HeLa cells with HIV-1 in the presence of 3G11 (Figure 5A). Eight hours post-infection infected cells were harvested, and assembled cores (PELLET) were separated from disassembled capsid (SOLUBLE). As shown in Figure 5A, 3G11 did not change the stability of the HIV-1 core during infection. As controls, we used the small molecule HIV-1 inhibitors PF74 and BI-2, which are known to destabilize the core during infection^{8,16}. As shown in Figure 5A, 3G11 did not change the stability of the HIV-1 core during infection when compared to the use of PF74 or BI-2. These experiments allowed us to conclude that 3G11 does not affect the stability of the HIV-1 core during infection, which is in agreement with our results showing that 3G11 does not perturb capsid-capsid interaction in the hexamer.

Next we studied the ability of 3G11 to modulate the stability of in vitro assembled HIV-1 capsid-nucleocapsid (CA-NC) complexes, which recapitulate the surface of the HIV-1 core, as previously described⁹. As shown in Figure 5B, 3G11 did not increase the stability of in vitro assembled HIV-1 capsid-nucleocapsid complexes at any of the tested concentrations. In addition, 3G11 was not able to destabilize in vitro assembled HIV-1 CA-NC complexes at any of the indicated concentrations (Figure 5C). These results indicated that 3G11 does not modulate the stability of in vitro assembled HIV-1 CA-NC complexes. The inability of 3G11 to affect core stability as measured by fate of the capsid and CA-NC stability is in agreement with our structural findings suggesting that 3G11 binds in a pocket that does not affect capsid-capsid interactions in the hexamer.

To assess the functional role of capsid as the determinant for the sensitivity to 3G11, we utilized capsid HIV-1/SIV_{mac} recombinant chimeras²⁸. For this purpose, we tested whether HIV-1 containing the capsid protein of SIV_{mac} [HIV(SCA)] is sensitive to inhibition by 3G11. Although 3G11 did not inhibit SIV_{mac} infection, it potently inhibited HIV(SCA) (Figure 5D). 3G11 did not block an SIV_{mac} virus containing the capsid of HIV-1[SIV_{mac}(HCA-p2)]. To control for the bona fide origin of the HIV-1/SIV_{mac} recombinant chimeras, we showed that SIV_{mac} and HIV(SCA) viruses were not restricted by TRIM5 α_{rh} when compared to HIV-1 or SIV(HCA-p2) (Figure 5D)²⁸. In addition, we also utilized PF74, which blocks HIV-1 and SIV_{mac} infection. Although 3G11 interacted with capsid and was isolated in a screen to target capsid, these experiments suggested that capsid is not the functional determinant for the sensitivity of HIV-1 infection to 3G11.

3G11 blocks the occurrence of HIV-1 reverse transcription during infection

To identify the stage at which the small molecule inhibitor 3G11 blocks HIV-1 infection, we tested the formation of late reverse transcripts and 2-LTR circles in the presence of 3G11 using real-time PCR, as described in Materials and Methods. For this purpose, we challenged HeLa cells using HIV-1-GFP at a MOI of 0.2 in the presence of 3G11. As a control, we also included the reverse transcription inhibitor nevirapine. Cells were collected 7 and 24 hours post infection, and the levels of late reverse transcripts and 2-LTR circles were determined using real-time PCR, respectively. In parallel similar infections were performed, and cells were collected 48 hours post infection to determine the amount of infected cells by measuring the percentage of GFP-positive cells. As shown in Figure 6A, 3G11 inhibits HIV-1 infection by preventing the occurrence of reverse transcription as potently as the reverse transcription inhibitor nevirapine (Nev). In agreement with a block on reverse transcription, 3G11 blocked the formation of 2-LTR circles (Figure 6A). Overall, these results indicated that 3G11 blocks the occurrence of HIV-1 reverse transcription.

To strengthen the finding that 3G11 blocks HIV-1 reverse transcription, we measured the formation of HIV-1 late reverse transcripts and infection using increasing concentrations of 3G11. As shown in Figure 6B, HIV-1 reverse transcription and infection was completely blocked by using 3G11 at 3, 5, or 10 μ M. Similarly, these results showed that 3G11 blocks HIV-1 before the occurrence of reverse transcription.

Next we investigated whether 3G11 prevents the formation of the minus-strand DNA, which is the earliest measurable step on the HIV-1 reverse transcription process. To measure

formation of the minus-strand DNA, we challenged HeLa cells using HIV-1-GFP. Cells were collected 7 and 48 hours post infection, and the levels of minus-strand DNA and infection were determined using real-time PCR and flow cytometry, respectively. As shown in Figure 6C, the use of different concentrations of 3G11 prevents the formation of minus-strand DNA. As a control we performed similar infections in the presence of nevirapine. Overall, these results demonstrated that 3G11 blocks reverse transcription prior to the synthesis of the minus-strand DNA.

3G11 blocks HIV-1 reverse transcriptase activity in vitro measured by a primer extension assay

3G11 blocks HIV-1 reverse transcription during infection suggesting that is blocking the reverse transcriptase activity. To test the effect of 3G11 on HIV-1 reverse transcriptase activity, we utilized an in vitro assay that measures HIV-1 reverse transcriptase activity in a reaction that extends a radiolabeled primer, as described in materials and methods. As shown in Figure 7, 3G11 inhibits the primer extension reaction similar to the known reverse transcriptase inhibitor nevirapine. These results suggest that 3G11 has the ability to inhibit HIV-1 reverse transcriptase activity.

Docking of 3G11 on HIV-1 reverse transcriptase revealed an HIV-1 mutant virus resistant to 3G11

Inhibition of RT activity by 3G11 suggests that the compound binds directly to the reverse transcriptase enzyme. Indeed, the chemical structure of the S-isomer of 3G11(3G11-S) bears similarities to the chemical structure of efavirenz (Figure 1), a potent non-nucleoside reverse transcriptase inhibitor. We performed docking of 3G11-S into the efavirenz binding pocket using the crystal structure of the efavirenz/HIV-1 RT complex²⁶ (PDB ID: 1FK9). We found that 3G11 can be very well accommodated in the efavirenz binding site with a docking score of -12.802, considerably higher than the score obtained for the 3G11/HIV-1 CA complex. Many compound/protein interactions observed in the efavirenz/HIV-1 RT complex are recapitulated in the complex of 3G11 with the enzyme (Figure 8A). Side chains of residues L100, Y181 and Y188 line the efavirenz binding site and their mutations are frequently associated with resistance to non-nucleoside reverse transcription inhibitors (NNRTIs)²⁹. Furthermore, the HIV-2 reverse transcriptase contains I and L in positions corresponding to Y181 and Y188, respectively, which is thought to be the reason why HIV-2 is resistant to NNRTI compounds. We were not able to dock 3G11 into the HIV-2 reverse transcriptase binding pocket containing residues L181 and L188, which is in agreement with our observations that 3G11 does not inhibit HIV-2 replication. These observations suggested that an HIV-1 virus containing a change in residue L100 on the reverse transcriptase enzyme will overcome the inhibition imposed by 3G11. For this purpose, we selected the mutation L100I (HIV-1-L100I), which has been described to render HIV-1 resistant to NNRTIs²⁹. To functionally test our computational findings, we tested the ability of HIV-1-L100I to infect mammalian cells in the presence of increasing concentrations of 3G11 in dog (Cf2Th) and human cells(CHME5). As shown in Figure 8B and C, the mutant virus HIV-1-L100I is completely resistant to the inhibitory effects of 3G11. These results suggested that reverse transcriptase is the genetic determinant for the sensitivity of HIV-1 to the small molecule 3G11.

Discussion

This work describes the ability of a novel small molecule inhibitor to block HIV-1 infection. Because 3G11 inhibits infection of single round HIV-1 viruses, we thought to compare its ability to inhibit HIV-1 against known inhibitors such as PF74 and BI-2^{8–10}. 3G11 showed to be as potent as PF74 when comparing inhibition of HIV-1 infection. Interestingly, 3G11 seems to be very specific for HIV-1 infection since it did not inhibit any of the other tested viruses, including HIV-2, SIV_{mac}, BIV, FIV, EIAV, N-MLV, B-MLV, Mo-MLV.

Because the capsid protein is involved in the early steps of HIV-1 replication^{5,6,30–35}, and 3G11 was found on a screen designed to isolate capsid inhibitors (Japanese Patent #4CO86 and a recently submitted manuscript: Urano E, Miyauchi K, Kojima Y, Hamatake M, Ablan SD, Fudo S, Freed EO, Hoshino T, Komano J. A triazinone derivative inhibits HIV-1 replication by interfering with reverse transcriptase activity), we tested whether 3G11 interacts with the HIV-1 capsid protein by NMR. Interestingly, 3G11 binds to a pocket in the protein that faces the inside compartment of the HIV-1 core. This particular pocket has been previously described for other drugs¹⁵. Although 3G11 binds to capsid, we were unable to detect an effect of the drug on capsid stability by using the fate of the capsid and capsid stability assays^{23,25}. Furthermore, by using HIV-1/SIV_{mac} capsid chimeras, we attempted to functionally test the role of capsid in the sensitivity of HIV-1 to 3G11; these experiments suggested that capsid is not the determinant for the sensitivity of HIV-1 to 3G11. In separate experiments, we tested the ability of 3G11 to affect HIV-1 particle assembly, release and production to find that none of these steps were affected (data not shown). These results were in agreement with our findings demonstrating that 3G11 docks to the HIV-2 capsid but does not restrict HIV-2 infection.

Next using real-time PCR, we analyzed the step on the viral life cycle at which HIV-1 is blocked during infection. These experiments demonstrated that 3G11 blocks the formation of HIV-1 late reverse transcripts during infection of human cells suggesting that 3G11 is a reverse transcription inhibitor.

Furthermore, we measured whether 3G11 affects the formation of the HIV-1 minus-strand DNA, which is the first step of reverse transcription. Our investigations revealed that 3G11 blocks formation of the HIV-1 minus-strand DNA suggesting that 3G11 blocks reverse transcription at a very early stage.

To further characterize the ability of 3G11 to block reverse transcription, we tested the ability of 3G11 to block the HIV-1 reverse transcriptase enzyme by using a primer extension assay *in vitro*. In agreement with a block to reverse transcription during infection, 3G11 inhibits the enzymatic activity of the HIV-1 reverse transcriptase in an *in vitro* assay.

Docking of 3G11 to the HIV-1 reverse transcriptase enzyme demonstrated that the residue L100 establishes a direct contact with 3G11. These observations immediately suggested that a mutation on L100 might render HIV-1 resistant to the inhibitory effects of 3G11. As predicted, an HIV-1 virus bearing the change L100I in the reverse transcriptase enzyme renders HIV-1 fully resistant to the inhibitory effects of 3G11. This evidence establishes

reverse transcriptase as the HIV-1 genetic determinant for sensitivity to the small molecule 3G11.

Conclusion

These experiments suggested that 3G11 is a non-nucleoside reverse transcription inhibitor. In agreement, an HIV-1 virus bearing the mutation L100I in the reverse transcriptase enzyme is completely resistant to the inhibitory effects of 3G11, suggesting that reverse transcriptase is the genetic determinant for HIV-1 sensitivity to the small molecule 3G11. Overall our results showed that the novel 3G11 inhibitor binds to the HIV-1 capsid and blocks reverse transcription.

Acknowledgments

We are thankful to the NIH/AIDS repository program for providing valuable reagents such as antibodies and drugs. We would like to thank Dr. Jun Komano and Dr. Eric Freed for providing the compound. This work was funded by an NIH R01 AI087390 to F.D.-G., NIH R01 AI104476 and Voelcker Fund Young Investigator Award to D.N.I. The NMR Core Facility at the UT Health Science Center at San Antonio is supported in part by the NIH P30 CA054174 to the Cancer Therapy and Research Center.

References

1. Thenin-Houssier S, de Vera IMS, Pedro-Rosa L, Brady A, Richard A, Konnick B, Opp S, Buffone C, Fuhrmann J, Kota S, Billack B, Pietka-Ottlik M, Tellinghuisen T, Choe H, Spicer T, Scampavia L, Diaz-Griffero F, Kojetin DJ, Valente ST. Ebselen, a Small-Molecule Capsid Inhibitor of HIV-1 Replication. *Antimicrobial Agents and Chemotherapy*. 2016; 60:2195–2208. [PubMed: 26810656]
2. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. The cytoplasmic body component TRIM5 α restricts HIV-1 infection in Old World monkeys. *Nature*. 2004; 427:848–853. [PubMed: 14985764]
3. Stremlau M, Perron M, Lee M, Li Y, Song B, Javanbakht H, Diaz-Griffero F, Anderson DJ, Sundquist WI, Sodroski J. Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5 α restriction factor. *Proc Natl Acad Sci USA*. 2006; 103:5514–5519. [PubMed: 16540544]
4. Sayah DM, Sokolskaja E, Berthoux L, Luban J. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature*. 2004; 430:569–573. [PubMed: 15243629]
5. Yang Y, Fricke T, Diaz-Griffero F. Inhibition of Reverse Transcriptase Activity Increases Stability of the HIV-1 Core. *J Virol*. 2012; 87:683–687. [PubMed: 23077298]
6. Roa A, Hayashi F, Yang Y, Lienlaf M, Zhou J, Shi J, Watanabe S, Kigawa T, Yokoyama S, Aiken C, Diaz-Griffero F. RING Domain Mutations Uncouple TRIM5 Restriction of HIV-1 from Inhibition of Reverse Transcription and Acceleration of Uncoating. *J Virol*. 2012; 86:1717–1727. [PubMed: 22114335]
7. Blair WS, Pickford C, Irving SL, Brown DG, Anderson M, Bazin R, Cao J, Ciaramella G, Isaacson J, Jackson L, Hunt R, Kjerrstrom A, Nieman JA, Patick AK, Perros M, Scott AD, Whitby K, Wu H, Butler SL. HIV capsid is a tractable target for small molecule therapeutic intervention. *PLoS Pathog*. 2010; 6:e1001220. [PubMed: 21170360]
8. Shi J, Zhou J, Shah VB, Aiken C, Whitby K. Small-Molecule Inhibition of Human Immunodeficiency Virus Type 1 Infection by Virus Capsid Destabilization. *J Virol*. 2010; 85:542–549. [PubMed: 20962083]
9. Fricke T, Brandariz-Nunez A, Wang X, Smith AB, Diaz-Griffero F. Human Cytosolic Extracts Stabilize the HIV-1 Core. *J Virol*. 2013; 87:10587–10597. [PubMed: 23885082]
10. Lamorte L, Titolo S, Lemke CT, Goudreau N, Mercier JF, Wardrop E, Shah VB, von Schwedler UK, Langelier C, Banik SSR, Aiken C, Sundquist WI, Mason SW. Discovery of Novel Small-

Molecule HIV-1 Replication Inhibitors That Stabilize Capsid Complexes. *Antimicrobial Agents and Chemotherapy*. 2013; 57:4622–4631. [PubMed: 23817385]

11. Kelly BN, Kyere S, Kinde I, Tang C, Howard BR, Robinson H, Sundquist WI, Summers MF, Hill CP. Structure of the antiviral assembly inhibitor CAP-1 complex with the HIV-1 CA protein. *J Mol Biol*. 2007; 373:355–366. [PubMed: 17826792]
12. Tang C, Loeliger E, Kinde I, Kyere S, Mayo K, Barklis E, Sun Y, Huang M, Summers MF. Antiviral inhibition of the HIV-1 capsid protein. *J Mol Biol*. 2003; 327:1013–1020. [PubMed: 12662926]
13. Diaz-Griffero F, Kar A, Lee M, Stremmler M, Poeschla E, Sodroski J. Comparative requirements for the restriction of retrovirus infection by TRIM5 α and TRIMCyp. *Virology*. 2007; 369:400–410. [PubMed: 17920096]
14. Sticht J, Humbert M, Findlow S, Bodem J, Muller B, Dietrich U, Werner J, Krausslich HG. A peptide inhibitor of HIV-1 assembly in vitro. *Nat Struct Mol Biol*. 2005; 12:671–677. [PubMed: 16041387]
15. Lemke CT, Titolo S, von Schwedler U, Goudreau N, Mercier J-F, Wardrop E, Faucher A-M, Coulombe R, Banik SSR, Fader L, Gagnon A, Kawai SH, Rancourt J, Tremblay M, Yoakim C, Simoneau B, Archambault J, Sundquist WI, Mason SW. Distinct effects of two HIV-1 capsid assembly inhibitor families that bind the same site within the N-terminal domain of the viral CA protein. *J Virol*. 2012; 86:6643–6655. [PubMed: 22496222]
16. Fricke T, Buffone C, Opp S, Valle-Casuso J, Diaz-Griffero F. BI-2 destabilizes HIV-1 cores during infection and Prevents Binding of CPSF6 to the HIV-1 Capsid. *Retrovirology*. 2014; 11:120. [PubMed: 25496772]
17. Fricke T, White TE, Schulte B, de Souza Aranha Vieira DA, Dharan A, Campbell EM, Brandariz-Nuñez A, Diaz-Griffero F. MxB binds to the HIV-1 core and prevents the uncoating process of HIV-1. *Retrovirology*. 2014; 11:68. [PubMed: 25123063]
18. Fricke T, Valle-Casuso JC, White TE, Brandariz-Nuñez A, Bosche WJ, Reszka N, Gorelick R, Diaz-Griffero F. The ability of TNPO3-depleted cells to inhibit HIV-1 infection requires CPSF6. *Retrovirology*. 2013; 10:46. [PubMed: 23622145]
19. De Iaco A, Santoni F, Vannier A, Guipponi M, Antonarakis S, Luban J. TNPO3 protects HIV-1 replication from CPSF6-mediated capsid stabilization in the host cell cytoplasm. *Retrovirology*. 2013; 10:20. [PubMed: 23414560]
20. Hilmer JK, Zlotnick A, Bothner B. Conformational equilibria and rates of localized motion within hepatitis B virus capsids. *J Mol Biol*. 2008; 375:581–594. [PubMed: 18022640]
21. Bothner B, Dong XF, Bibbs L, Johnson JE, Siuzdak G. Evidence of viral capsid dynamics using limited proteolysis and mass spectrometry. *J Biol Chem*. 1998; 273:673–676. [PubMed: 9422714]
22. Lienlaf M, Hayashi F, Di Nunzio F, Tochio N, Kigawa T, Yokoyama S, Diaz-Griffero F. Contribution of E3-Ubiquitin Ligase Activity to HIV-1 Restriction by TRIM5 rh: Structure of the RING Domain of TRIM5. *J Virol*. 2011; 85:8725–8737. [PubMed: 21734049]
23. Fricke T, Diaz-Griffero F. HIV-1 Capsid Stabilization Assay. *Methods Mol Biol*. 2016; 1354:39–47. [PubMed: 26714703]
24. Diaz-Griffero F, Perron M, McGee-Estrada K, Hanna R, Maillard PV, Trono D, Sodroski J. A human TRIM5 α B30.2/SPRY domain mutant gains the ability to restrict and prematurely uncoat B-tropic murine leukemia virus. *Virology*. 2008; 378:233–242. [PubMed: 18586294]
25. Yang Y, Luban J, Diaz-Griffero F. The fate of HIV-1 capsid: a biochemical assay for HIV-1 uncoating. *Methods Mol Biol*. 2014; 1087:29–36. [PubMed: 24158811]
26. Ren J, Milton J, Weaver KL, Short SA, Stuart DI, Stammers DK. Structural basis for the resilience of efavirenz (DMP-266) to drug resistance mutations in HIV-1 reverse transcriptase. *Structure*. 2000; 8:1089–1094. [PubMed: 11080630]
27. Biris N, Tomashevski A, Bhattacharya A, Diaz-Griffero F, Ivanov DN. Rhesus Monkey TRIM5 α SPRY Domain Recognizes Multiple Epitopes That Span Several Capsid Monomers on the Surface of the HIV-1 Mature Viral Core. *J Mol Biol*. 2013; 425:5032–5044. [PubMed: 23886867]
28. Owens CM, Yang PC, Gottlinger H, Sodroski J. Human and simian immunodeficiency virus capsid proteins are major viral determinants of early, postentry replication blocks in simian cells. *J Virol*. 2003; 77:726–731. [PubMed: 12477877]

29. Smith MBK, Ruby S, Horouzhenko S, Buckingham B, Richardson J, Puleri I, Potts E, Jorgensen WL, Arnold E, Zhang W, Hughes SH, Michejda CJ, Smith RH. HIV-1 reverse transcriptase variants: molecular modeling of Y181C, V106A, L100I, and K103N mutations with nonnucleoside inhibitors using Monte Carlo simulations in combination with a linear response method. *Drug Des Discov.* 2003; 18:151–163. [PubMed: 15553926]
30. Di Nunzio F, Fricke T, Miccio A, Valle-Casuso JC, Perez P, Souque P, Rizzi E, Severgnini M, Mavilio F, Charneau P, Diaz-Griffero F. Nup153 and Nup98 bind the HIV-1 core and contribute to the early steps of HIV-1 replication. *Virology.* 2013; 440:8–18. [PubMed: 23523133]
31. Matreyek KA, Yücel SS, Li X, Engelman A. Nucleoporin NUP153 phenylalanine-glycine motifs engage a common binding pocket within the HIV-1 capsid protein to mediate lentiviral infectivity. *PLoS Pathog.* 2013; 9:e1003693. [PubMed: 24130490]
32. Fassati A. Multiple roles of the capsid protein in the early steps of HIV-1 infection. *Virus Research.* 2012; 170:15–24. [PubMed: 23041358]
33. Chen NY, Zhou L, Gane PJ, Opp S, Ball NJ, Nicastro G, Zufferey M, Buffone C, Luban J, Selwood D, Diaz-Griffero F, Taylor I, Fassati A. HIV-1 capsid is involved in post-nuclear entry steps. *Retrovirology.* 2016; 13:28. [PubMed: 27107820]
34. Forshey BM, von Schwedler U, Sundquist WI, Aiken C. Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication. *J Virol.* 2002; 76:5667–5677. [PubMed: 11991995]
35. Lee K, Ambrose Z, Martin TD, Oztop I, Mulky A, Julias JG, Vandegraaff N, Baumann JG, Wang R, Yuen W, Takemura T, Shelton K, Taniuchi I, Li Y, Sodroski J, Littman DR, Coffin JM, Hughes SH, Unutmaz D, Engelman A, KewalRamani VN. Flexible Use of Nuclear Import Pathways by HIV-1. *Cell Host Microbe.* 2010; 7:221–233. [PubMed: 20227665]

Figure 1. 6-(tert-butyl)-4-phenyl-4-(trifluoromethyl)-1H,3H-1,3,5-triazin-2-one(3G11)
Chemical structure of 3G11, BD3 and Efavirenz.

Figure 2. 3G11 blocks HIV-1 infection as potent as PF74

Cf2Th cells were challenged with single round replication HIV-1-GFP viruses in the presence of increasing concentrations of 3G11 (0.1–10), PF74 (0.1–10 μM), and BI-2 (0.1–100 μM). Forty-eight hours post-infection the percentage of GFP-positive cells was measured using a flow cytometer. Experiments were performed in triplicates and a single experiment is shown.

Figure 3. 3G11 is a specific inhibitor of HIV-1 infection

Cf2Th cells were challenged with increasing amounts of the indicated viruses in the presence of 3G11. Forty-eight hours post-infection, the percentage of GFP-positive cells was measured using a flow cytometer. As a control, similar infections were performed in the presence of PF74. Experiments were performed in triplicates and a single experiment is shown.

Figure 4. 3G11 binds to the HIV-1 Capsid

(A) CA-NTD residues perturbed by 3G11 binding identified by NMR spectroscopy. Spectrum of 120 μM CA-NTD alone in red, NMR spectrum of 120 μM CA-NTD + 600 μM 3G11 drug (1:5 ratio) in green. Overlapping peaks appear black. (B) Model of the 3G11/HIV-1 CA-NTD complex. Residues whose NMR signals are most affected by 3G11 binding are highlighted in yellow. (C) Crystal structure of the BD3/HIV-1 CA-NTD complex (PDB ID: 4E91) reveals that the compound binds in a deep interior pocket sandwiched between CA-NTD helices (left panel). 3G 11 can be accommodated in the BD3-binding pocket of HIV-1 CA-NTD (middle panel). Similarly, 3G11 docks into the HIV-2 CA-NTD (right panel). (D) Model of the 3G11-bound CA hexamer viewed from the interior of the core particle. The 3G11- and BD3-binding site is accessible from the core interior and binding of the compound is not expected to generate steric clashes in the assembled core structure.

A **B** **C**

D

Figure 5. HIV-1 capsid is not the determinant for the sensitivity of HIV-1 to 3G11

(A) The fate of the capsid assay was performed to test whether 3G11 affects the uncoating process of HIV-1. HeLa cells were challenged with HIV-1 in the presence of 3 μM 3G11. Eight hours post-infection cells were lysed and separated into Input, supernatant and pellet fractions, as described in materials and methods. Fractions were analyzed by Western blotting using anti-p24 antibodies. Experiments were performed in triplicates and standard deviations are shown. (B) The stability of HIV-1 CA-NC complexes was measured in destabilization buffer using increasing concentrations of 3G11, as described in Materials and Methods. The goal of this experiment is to measure whether 3G11 has the ability to stabilize HIV-1 CA-NC complexes. Input and Pellet fractions were analyzed by Western blotting using anti-p24 antibodies. As a control, the stability of HIV-1 CA-NC complexes incubated in stabilization buffer was measured. Experiments were repeated three times and a representative experiment is shown. (C) The stability of HIV-1 CA-NC complexes was measured in destabilization buffer supplemented with cellular extracts using increasing concentrations of 3G11. The goal of this experiment is to measure whether 3G11 has the ability to destabilize HIV-1 CA-NC complexes. Input and Pellet fractions were analyzed by Western blotting using anti-p24 antibodies. As a control, the stability of HIV-1 CA-NC complexes incubated in stabilization buffer was measured. Experiments were repeated three times and a representative experiment is shown. (D) Dog Cf2Th were challenged with increasing concentrations of HIV-1, HIV-1(SIV_{ca}), SIV_{mac} and SIV_{mac}(HIV-1_{ca}) in the presence of 3G11. As a control, we performed similar infections in Dog Cf2Th cells expressing rhesus TRIM5 α (TRIM5 α_{rh}) that blocks HIV-1 but not SIV_{mac}. In addition, we performed similar infections in the presence of PF74, which inhibits HIV-1 and SIV_{mac}. Experiments were repeated three times and a representative experiment is shown.

A

B

Figure 6. 3G11 blocks HIV-1 infection before reverse transcription

(A) dog Cf2Th cells were challenged with HIV-1-GFP at an MOI of 0.2 in the presence of 3G11. Cells were harvested at 7, 24 and 48 hours post-infection. Cells harvested at 7 and 24 hours were used for DNA extraction; DNA samples were used to measure formation of late reverse transcripts (upper panel) and 2-LTR circles (middle panel) at 7 and 24 hours post-infection, respectively, by real-time PCR, as described in materials and methods. Infection was determined 48 hours post-infection by measuring the percentage of GFP-positive cells (lower panel). As a control, we performed similar experiments in the presence of nevirapine that inhibits reverse transcription. Experiments were performed three times and standard deviations are shown. (B) Dog Cf2Th cells were challenged with HIV-1-GFP in the presence of increasing concentrations of 3G11. Cells were harvested 7 and 48 hours post-infection and used to determine formation of late reverse transcripts and infection, respectively. Experiments were performed three times and a representative experiment is shown. (C) Similarly, the ability of 3G11 to block formation of the HIV-1 minus-strand was determined. The primers used to measure formation of the minus-strand are depicted in the HIV-1 Long Terminal Repeat (upper panel), as described in materials and methods. 7 hours post-infection by real-time PCR, as described in materials and methods. Dog Cf2Th were challenged with HIV-1-GFP in the presence of increasing concentrations of 3G11. Cells

were harvested 7 and 48 hours post-infection and used to determine formation of the minus-strand (middle panel) and infection (lower panel), respectively. Experiments were performed three times and a representative experiment is shown.

Figure 7. Inhibition of HIV-1 RT by 3G11

5' 32 P-labeled 17-mer primer annealed to 40-mer RNA template was extended by HIV-1 RT (0.79mg/mL) and 50 μ M dNTPs for 5 mins at 37°C with varying concentrations (0, 1, 10 and 100 μ M) of 3G11. Nevirapine was also used for comparison at the same concentrations. Fully extended primer is the fully extended product. No drug control has only DMSO.

Figure 8. HIV-1 viruses bearing the reverse transcriptase change L100I are resistant to the inhibitory effects of 3G11

(A) Crystal structure of the efavirenz/HIV-1 RT complex (PDB ID: 1FK9) (left panel) was used as the template for docking of 3G11 into the same site (right panel). 3G11 displays very high complementarity to the efavirenz binding site with the docking score of -12.8 . The binding pocket is lined by hydrophobic residues L100, Y181 and Y188, mutations of which are associated with resistance to non-nucleoside reverse transcription inhibitors. To functionally test our computational findings that 3G11 interacts with the HIV-1 reverse transcriptase residue L100, we challenged dog Cf2Th

(B) and human CHME5 (C) with HIV-1 viruses bearing the reverse transcriptase mutant L100I (HIV-1-L100I). As a control, we performed similar infections in the presence of the reverse transcription inhibitor AZT. Experiments were performed three times and a representative experiment is shown.