SUPPLEMENTARY METHODS

Reagents

AMD3100, a small-molecule inhibitor of HIV-1 entry via the CXCR4 coreceptor was purchased from Sigma-Aldrich. Peridinin chlorophyll protein-Cy5.5 mouse anti-human CD3 (Catalog #560835, clone UCHT1, 1:40 dilution), PE-Cy7 mouse anti-human CD4 (Catalog #557852, clone SK3, 1:40 dilution), and allophycocyanin-H7 mouse anti-human CD8 antibodies (Catalog #560179, clone SK1, 1:40 dilution), used for flow cytometry experiments, were purchased from BD Biosciences and stored at 4°C in the dark. The Aqua-Amcyan stain was a component of the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific, Catalog #L34957, 1:1,000 dilution) and stored frozen at -20ºC in the dark. The plasmid pLAI-RenLuc was made by inserting a synthetic a human codon-optimized Renilla luciferase gene into a pLAI.2-based vector (obtained from the National Institute for Biological Standards and Control) immediately downstream of the env coding sequence while also deleting the nef sequence via BamHI-XhoI ligation.

HIV-1, HIV-2 and SIV

HIV-1_{BaL} was obtained from Advanced Biotechnologies. Clinical isolates from the Southern Research virus collection included HIV-2 (CBL-20) and SIV (mac239, mac251).

Reporter HIV-1

Replication-competent reporter HIV-1 encoding Renilla luciferase was made by transfecting HEK293T cells with pLAI-RenLuc plasmid. Cell-free viral supernatant was isolated 3 days post-

transfection, clarified using a 0.45 µm syringe filter and stored at -80°C. The amount of HIV in the virus preparation was quantified by p24 antigen ELISA (Perkin Elmer)

Antiviral assays

Antiviral assessments in primary human CD4+ T-lymphocytes and macrophage cultures infected with HIV-1_{BaL} were conducted using a p24 endpoint assay as previously described[1]. A 7-day RT endpoint antiviral assay using fresh human PBMCs independently infected with HIV-2 (CBL-20) and SIV (mac239, mac251) strains was performed by Southern Research as a contracted research study.

For time-of-addition antiviral assays, MT-2 cells were infected with single-cycle reporter HIV-1 for 1 hour at 37ºC, washed, and 75 µL aliquots seeded into 96-well plates (1.5 x 10^4 cells/well). A 25 µL aliquot of complete RPMI media was added to 8 uninfected and 8 infected wells as respective minimum (0%) and maximal (100%) infection controls. Twenty-five µL aliquots of 4x drug-media were added to 8 replicate wells at indicated times post-infection. Assay plates were kept in a humidified 37ºC incubator before and after drug-media additions. Plates were developed 48 hpi by ONE-Glo addition and the resulting chemiluminescence measured using an Envision plate reader. Data analysis was performed using GraphPad Prism 7.0 to calculate EC_{50} values. GS-CA1 and control ARVs were tested at concentrations equivalent to 10x and 100x their EC_{50}, producing similar results.

For the antiviral assays performed in the presence of different serums, MT-4 cells were first infected with LAI-RenLuc reporter HIV-1 for 3 hours at 37ºC. Infected cells were then washed,

suspended in RPMI containing 40% FBS and 20 µL aliquots (5 x 10^4 cells) added to 96-well plates. Twenty µL aliquots of 4-fold serially-diluted test compound were added in triplicate to the assay plates, followed by addition of 40 µL complete RPMI, 100% human serum (Sigma Aldrich), or 100% mouse serum (Bio-Rad). Assay plates were kept in a humidified 37ºC incubator and developed 3 days later by addition of Dual-Glo Luciferase Assay reagent (Promega).

For antiviral assays evaluating the effect of MOI, CEM-NK^R CCR5+Luc+ cells were infected at a density of 3 x 10^6 cells per mL with 0.08 µL, 0.43 µL, 2.14 µL, and 10.68 µL of the replication competent HIV-1 NL4-3-JRFL-secNLuc reporter virus per 1 x 10^6 cells, corresponding to MOIs of 0.01, 0.05, 0.25, and 1.25, respectively. A mock infection in which no virus was added to the cells was also set up and incubated similarly as an uninfected control. Cells were infected in complete RPMI medium supplemented with 10 µg/mL DEAE-dextran by mixing the suspension for 4 hours at 37°C on a rocking platform. Infected cells were washed twice with complete RPMI medium and then resuspended at 1 x 10^6 cells per mL in complete RPMI medium containing 20 µg/mL DEAE-dextran. Fifty microliter aliquots of the virus-infected cell suspensions (5 × 10^4 cells) were then added to 54 of the inner wells of a 96-well plate containing 50 µL of 4-fold serially-diluted test compounds assayed in triplicate, as well as 6 wells of vehicle-containing media as the uninfected, no drug controls. The final DMSO concentration in the assay was 0.5%. After 5 days in a humidified 37ºC incubator with 5% CO_2, assay plates were centrifuged for 5 minutes at 1,200 rpm and 50 µL of cell-free supernatant was discarded. Fifty microliters of Nano-Glo Luciferase Assay Reagent was then added to each well of the assay plate, the well contents were mixed by pipetting, and the resulting chemiluminescence was measured using an

Envision plate reader. Data analysis was performed using GraphPad Prism 7.0 to calculate EC_{50} values.

HIV-1 capsid population sequences

Unique full-length capsid population sequences (n = 10,512) from 8 major HIV-1 subtypes (A1, B, C, D, F1, G, CRF01_AE, and CRF02_AG) were downloaded from the HIV Los Alamos database (www.hiv.lanl.gov, 8/3/2016 build). Downloaded sequences were aligned using Clustal Omega (EMBL-EBI) and consensus sequences generated for each subtype using the Simple Consensus Maker within the HIV Los Alamos database. Sequence identity calculations at the residue level across genotypes were done in Unipro UGENE. The BioEdit Sequence Alignment Editor was used to generate and analyze HIV-1/HIV-2/SIV capsid sequence alignments.

Preparation of recombinant HIV-1 protease and Gag proteins

For the preparation of recombinant HIV-1 protease (PR) protein, a synthetic, codon-optimized DNA fragment encoding HIV-1 PR was cloned into BamHI-digested pET3b bacterial expression vector to form pET3b-PR. This plasmid was transformed into BL21AI cells. The expression strain was grown in 2x YT media until log phase of growth and protein expression was induced by the addition of 14 mM arabinose for 3 hours. To purify this protein, bacterial cell pellets were resuspended in 20 mM Tris (pH 7.5), 1 mM EDTA, 5 mM DTT and lysed by four repeated passages through a microfluidizer at 18,000 psi. Inclusion bodies containing HIV protease were pelleted and washed with 25 mM Tris (pH 7.5), 2.5 mM EDTA, 0.5 M NaCl, 10 mM DTT, 1 M urea wash buffer. The washed inclusion bodies were harvested by centrifugation, solubilized in 8 M urea, 25 mM Tris (pH 7.5), 1 mM EDTA, 10 mM DTT and clarified by centrifugation. The

denatured protease was loaded onto an equilibrated Q-Sepharose anion exchange column and recovered in the flow-through fraction. The sample was further clarified by centrifugation at 100,000 x g for 1 hour. HIV protease was refolded by rapid 15-fold dilution into 10 mM sodium acetate (pH 4.5), 3 mM DTT at 25ºC. The pH was raised to 6.0 with addition of 1 M NaOH and the sample was rapidly cooled to 4ºC. The sample was filtered through a 0.45 um filter and loaded onto a cation exchange column equilibrated with 10 mM sodium acetate (pH 6.0), 10% (v/v) glycerol, 1 mM DTT (SP Buffer A). Bound protein was eluted with a linear gradient of 20 column volumes to 100% SP Buffer B (SP BufferA + 0.5 M NaCl). Fractions containing protease were snap-frozen and stored in liquid nitrogen.

For the preparation of recombinant Gag protein, a DNA fragment encoding the HIV-$1_{NL4.3}$ Gag gene was first PCR amplified from the pKS13 plasmid using primers that added an 8xHis-tag, Avitag sequence (GLNDIFEAQKIEWHE) and stop codon to its C-terminus. This fragment was then cloned into NdeI/XhoI digested pET30a vector and the resulting plasmid was transformed into BL21-DE3 cells containing a Bir-A plasmid. The expression strain was grown in 2x YT media until log phase of growth and protein expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside and 50 µM D-Biotin. To purify this protein, a 20 gram cell pellet was lysed in 200 mL of Lysis Buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM TCEP). The lysate was then centrifuged at 30,000 x g for 1 hour at 4°C and the clarified supernatant analyzed by SDS-PAGE analysis using an anti-Streptavidin-HRP antibody.

Virus entry assay

The virus-host cell membrane fusion assay and preparation of HIV-1-BlaM virus were each performed as previously described[1], with minor modifications. Briefly, human PBMCs from each of three independent donors were seeded in 96-well plates (1×10^6 per well) and treated with GS-CA1, DTG, AMD3100, or DMSO (vehicle control) for 48 hours. Cell cultures were then infected by spinoculation at 1,200 × g for 2 hours with BlaM-Vpr/HIV-1 (400 ng p24 equivalent virus input per 10^6 cells) containing copackaged Vpr protein covalently linked to the β-lactamase enzyme, followed by a 1 hour incubation at 37°C. Cells were washed in CO_2-independent medium (Gibco) and then loaded with CCF2-AM dye for 1 hour at room temperature per the manufacturer's protocol (Life Technologies). Cells were washed twice with CO_2-independent medium and resuspended in 200 μL of CO_2-independent medium containing 10% FBS and 2.5 mM probenecid (Sigma-Aldrich) before the β-lactamase reaction was allowed to develop for 16 hour at room temperature in the dark. Cells were washed once with PBS before immunostaining for 20 minutes at room temperature in the dark. Cells were washed once with PBS, fixed in a 4% solution of paraformaldehyde for 20 minutes at room temperature in the dark, and washed again. The change in the emission fluorescence of the CCF2-AM dye after cleavage by cytoplasmic BlaM-Vpr protein was measured in live CD3+ CD4+ CD8− cells using an LSRFortessa flow cytometer (Becton Dickinson). Green (520 nm, uncleaved CCF2) and blue (447 nm, cleaved CCF2) emissions were detected using HQ545/90 and HQ455/50 filters (BD Biosciences), respectively. Data were analyzed using FlowJo 10.0.7 software (TreeStar).

Western blotting

HEK293T cells were co-transfected with pKS13ΔEnv and pHCMV-G plasmids in the presence of fixed concentrations of GS-CA1 (0-100 nM). Cells were harvested 2 days later, pelleted, and

sonicated on ice in RIPA buffer supplemented with protease inhibitors. Protein samples (10 µg) were subjected to SDS-PAGE under reducing conditions, transferred to PVDF membranes, and probed with mouse monoclonal anti-p24 (Catalog #MA1-71516. 1:1,000 dilution) and rabbit monoclonal anti-tubulin primary antibodies (Catalog #2125, clone 11H10, 1:2,000 dilution) and species-appropriate HRP-conjugated goat-anti-mouse (Catalog #31430) and goat anti-rabbit (Catalog #31460) IgG secondary antibodies (1:5,000 dilution). Blots were developed using Pierce ECL substrate, scanned with the ImageQuant LAS4000 (GE Healthcare Life Sciences) and quantified using NIH ImageJ version 1.46 software. Full uncropped blots are available as Source data.

Recombinant HIV-1 protease activity assay

Recombinant HIV-1 protease enzyme (20 nM) was added to a 96-well plate preloaded with 3-fold serially diluted test compounds (11 concentrations in duplicate) and incubated for 15 minutes at room temperature. Protease was added to the plates in 1x assay buffer (pH 5.3), consisting of 100 mM ammonium acetate, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.25 mg/mL bovine serum albumin. An equal volume of 1x assay buffer (pH 5.3) containing 80 µM (2x K_m) of a fluorogenic HIV-1 protease substrate (Catalog # H-2992, Bachem Americas, Inc.) was then added to each well of the assay plate. The final DMSO, enzyme and substrate concentration in each well was 1%, 10 nM, and 40 µM, respectively. Substrate cleavage was quantified for 10 minutes by reading the assay plates in kinetic mode on a TECAN Infinite M1000 plate reader with excitation and emission wavelengths set to 320 nm and 420 nm, respectively. Cleavage rates were expressed as a percentage of untreated wells and the IC_{50}

calculated using a four parameter curve fitting algorithm. Five independent experiments were performed and the data reported as mean ± s.d.

HIV-1 for resistance selections

The emergence of resistant HIV-1 in the presence of fixed concentrations of GS-CA1 was assessed in MT-4 cells acutely infected with HIV-1_{IIIB} and in human PBMCs independently infected with each of five clinical HIV-1 isolates (BaL, 92US657, 92HT593.1, 7467, and 7576). HIV-1 isolates 92US657 and 92HT593.1 were obtained through the NIH AIDS Reagent Program. Primary HIV-1 isolates 7467 and 7576 were propagated at Gilead Sciences from archived pre-ART plasma samples obtained from Gilead-sponsored clinical trials study participants (studies GS-US-216-0114, GS-US-236-0128 and GS-US-236-0103). Plasma was obtained from adult HIV-infected subjects with informed consent and the approval of the institutional review board and stored frozen at −80°C. Working virus stocks of all primary HIV-1 isolates were prepared at ImQuest Biosciences by brief expansion (5-9 days) in PHA-activated human PBMCs followed by concentration by tangential flow filtration (TFF). Virus aliquots were frozen at -80°C and assessed for RT enzyme activity, p24 concentration and $TCID_{50}$.

Fixed-dose GS-CA1 selections in MT-2 cells infected with HIV-1_{IIIB}

MT-2 cells were infected with HIV-1_{IIIB} at an MOI of ~ 0.05 and at a density of 1×10^6 cells per mL by gently mixing the virus/cell suspension at 37ºC on a rocking platform. After 2 hours of virus adsorption, the cells were pelleted, re-suspended in complete RPMI and seeded into 24-

well plates at a cell density of 2×10^5 cells per well. Approximately 16 hpi, each of four different fixed concentrations of GS-CA1 was independently added to 24 replicate wells to achieve a final volume of 1 mL per well. GS-CA1 was tested at fixed GS-CA1 concentrations equal to 2.5-fold, 5-fold, 10-fold, and 20-fold its EC_{95} value of 0.63 nM, corresponding to 1.58 nM, 3.15 nM, 6.3 nM, and 12.6 nM GS-CA1, respectively. Viral breakthrough was assessed over a period of 35 days, during which cell cultures were maintained by routine passaging (1:5 dilution) every 3 to 4 days by discarding 800 µL of the culture and replenishing with 800 µL fresh drug-containing RPMI. Prior to passaging of the culture, wells were visually inspected on a light microscope for the development of virus-induced cytopathic effect (CPE). Wells in which >80% of the cell culture showed evidence of CPE, the viral supernatant was harvested and stored frozen at -80ºC.

Fixed-dose GS-CA1 selections in PBMCs infected with HIV-1 clinical isolates

PHA-activated human PBMCs from 4 healthy donors were mixed at 1:1:1:1 ratios and independently infected with each of five different HIV-1 isolates (BaL, 92US657, 92HT593.1, 7467 and 7576). Cells were infected at a virus concentration of 2 ng p24 equivalent per million PBMCs. Infections were performed by gently mixing the virus/cell suspension at 37ºC on a rocking platform and were done at a density of 2×10^6 cells per mL in complete RPMI supplemented with 10 µg/mL DEAE-dextran (Sigma-Aldrich) and 5 ng/mL IL-2. After 4 hours of virus adsorption, each virus/cell suspension was transferred to a T-75 flask and maintained in a humidified 37ºC incubator for 2 days. After two days of virus/reservoir amplification in the culture, infected cells were harvested, washed extensively with complete RPMI before resuspension at 2×10^6 cells per mL in complete RPMI supplemented with 20 µg/mL DEAE-dextran and 10 ng/mL IL-2. One mL aliquots of each infected cell suspension were seeded into

24-well tissue culture plates. A 2X concentration of each test concentration was prepared in complete RPMI and added to 6 replicate wells. An additional 6 wells of each 24-well plate received 1 mL of complete RPMI containing no drug as a control. All 24-well plates were returned to a humidified 37°C incubator for the duration of the selection experiment. GS-CA1 test concentrations were 2.75 nM and 11 nM, corresponding to human plasma-free EC_{95} values of 4.4-fold and 17.5-fold, respectively. At designated weekly intervals (i.e. 7, 14, and 21 days post-infection), a 1.5 mL aliquot of cell-free supernatant was harvested from each well and stored at -80°C, followed by replacement with 1.5 mL of complete RPMI containing 2 $x10^6$ freshly activated PBMCs, 1x concentration of test drug, 5 ng/mL IL-2 and 10 µg/mL DEAE-dextran. After 28 days of selection, an aliquot (150 µL) of cell-free day-28 supernatant from each well was used to infect 2 $x10^6$ freshly activated PBMCs suspended in 1.5 mL of complete RPMI containing 1x concentration of test drug, 5 ng/mL IL-2 and 10 µg/mL DEAE-dextran. This transfer step was done to help dilute the input WT virus from the original reservoir and thus enrich for infectious drug-resistant variants prior to genotypic and phenotypic analyses. After 7 more days in culture (35 total days post-infection), the final cell-free supernatants were collected and analyzed for HIV content by using a p24 antigen capture ELISA and for infectivity on CEM-NK^R CCR5+Luc+ indicator cells. The infectivity of breakthrough (p24+) samples was measured in 96-well plates following the transfer of cell-free supernatants onto CEM-NK^R CCR5+Luc+ indicator cells (1:1 mixture) in complete RPMI supplemented with 10 µg/mL DEAE-dextran. After 3 days in culture, an equal volume of ONE-Glo reagent was added to each well and the amount of chemiluminescence signal measured using an Envision plate reader. Culture supernatants that produced relative light units (RLUs) at least 5-fold over mock-infected controls

were considered infectious in the presence of drug and thus examined for the presence of potential GS-CA1 resistant HIV-1 variants.

Genotyping viral breakthrough variants

Viruses that emerged in the presence of GS-CA1 were genotyped by population sequencing. Total RNA was isolated from mock- and GS-CA1-selected virus-containing supernatant using the QiaAMP Viral RNA Mini Kit (Qiagen). Potential target genes were individually amplified by RT-PCR using the Qiagen OneStep RT-PCR Kit. Primer pairs used for HIV-1 gene amplifications were as follows: (1) MA-forw: 5'-CAGTAGCAACCCTCTATTGTGTGC-3' and NC-rev: 5'-CCTAGGGGCCCTGCAATTT-3' producing a 986-bp fragment encoding HIV-1 capsid and the adjacent p2 spacer peptide, (2) ProF1: 5'- CAACTCCCCCTCAGAAGCAG-3' and ProR3: 5'- CTGCGGGATGTGGTATTCCT-3' producing a 647-bp fragment encoding HIV-1 protease, (3) ProF1, and RtR1: 5'- CCCTGTTAGCTGCCCCATC-3' producing a 1,697-bp fragment encoding HIV-1 reverse transcriptase, and (4) p041: 5'-GCATGGGTACCAGCACACAAAG-3' and p042: 5'-CTAGCTTTCCCTGAAACATACATATGGTG-3 producing a 999-bp fragment encoding HIV-1 integrase. Each RT-PCR product was purified using the QIAquick PCR Purification Kit and sequenced by Elim Biopharmaceuticals, Inc. using the following minimal primer sets for each target coding region: capsid [MA-forw and NC-rev]; protease [ProF1, and ProR3: 5'-CTGCGGGATGTGGTATTCCT-3'], reverse transcriptase [ProF1, ProF4: 5'-TGAGTTTGCCAGGAAGATGGAA-3', RtF9: 5'- AGAACTCAAGACTTCTGGGAAGTTC-3', RtF4: 5'- GTGGGGACTTACCACACCAG-3', p145R: 5'-CTGGCAGCACTATAGGCTGTACT-3' and RtR1]; and integrase [p041, p042, and p039: 5'-

CAGGAATTTGGAATTCCCTACAATCC-3']. To identify codon changes, gene sequences for each selected HIV-1 variant were aligned using DNA Sequencher 4.9 Software (Gene Codes Corporation) with that of the input virus and virus passaged in parallel in the absence of GS-CA1.

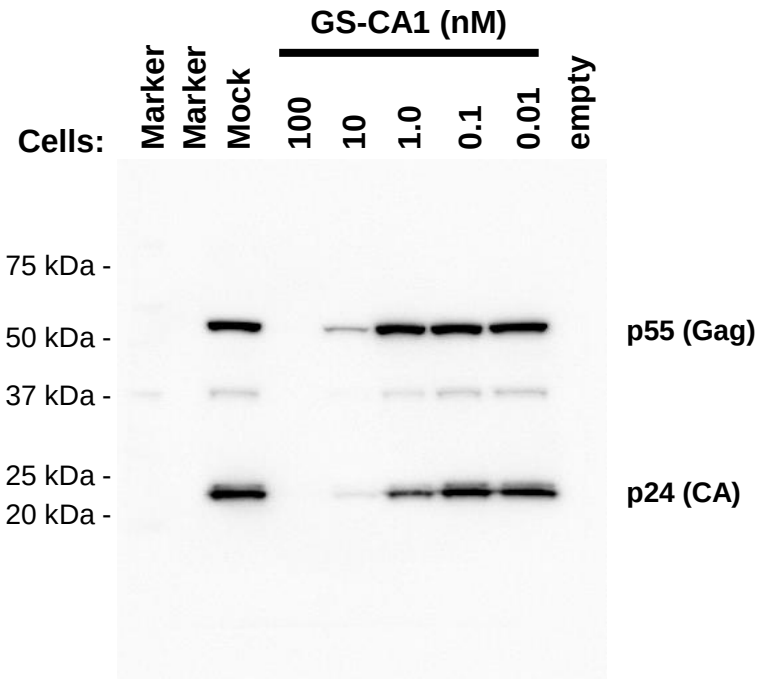
Pharmacokinetics of GS-CA1 and RPV in C57Bl/6 mice

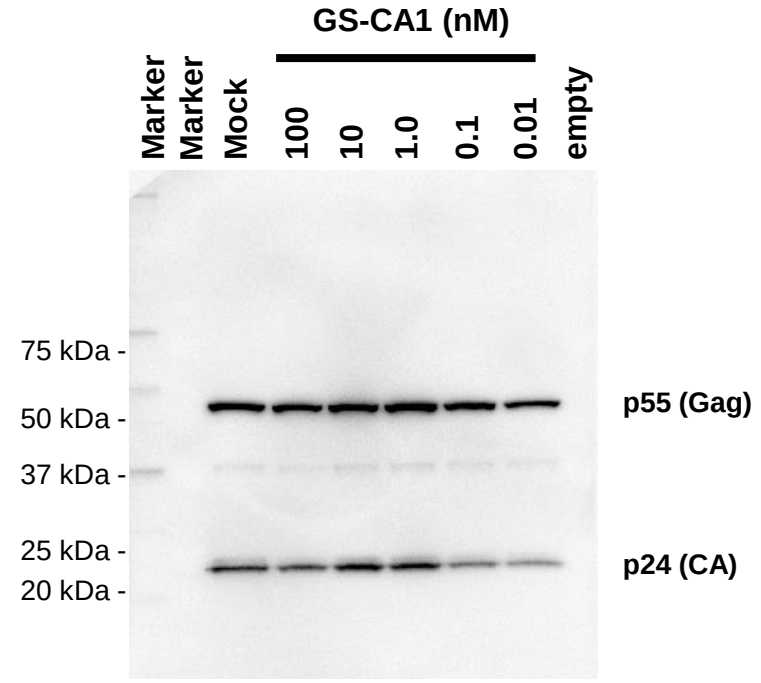
All animal studies conducted in this report were done in strict compliance with all relevant ethical regulations. Nonclinical pharmacokinetic studies were conducted at Covance Labs in a Laboratory Animal Care-accredited facility, and all study protocols were reviewed and approved by their Institutional Animal Care and Use Committee. For drug exposure normalization studies prior to efficacy testing, a 30 mg/mL GS-CA1/100% DMSO solution and a 160 mg/mL RPV suspension formulated in water with 20 mg/mL Poloxamer 338 and micronized using a bead mill was dosed subcutaneously daily for 7 days at 15 mg/kg and 160 mg/kg, respectively, into 15 male 10-week old C57BL/6 mice (Envigo RMS, Inc.). Blood was collected from each animal at designed time points and the plasma drug concentrations determined by LC-MS/MS.

References

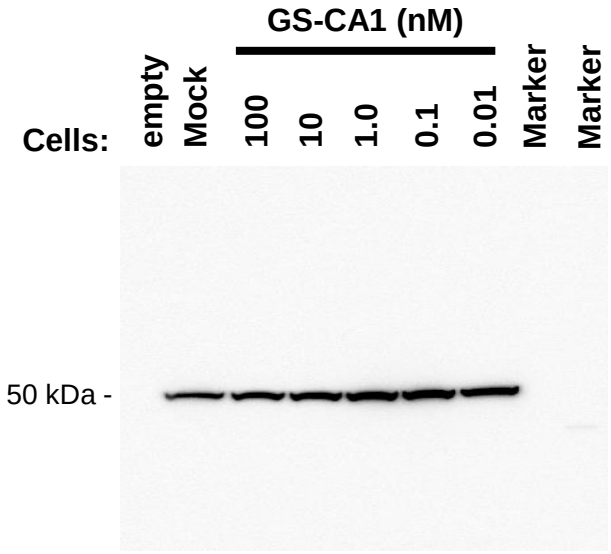
1. Bam, R., *et al.* TLR7 Agonist GS-9620 Is a Potent Inhibitor of Acute HIV-1 Infection in Human Peripheral Blood Mononuclear Cells. *American Society for Microbiology* **61**, 1-14 (2017).

Representative uncropped Anti-p24 blots:

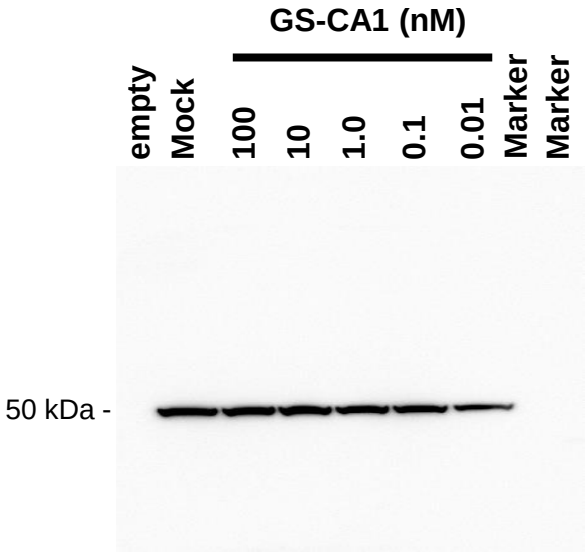




Representative uncropped Anti-α-tubulin blots:



HIV-1, CA_{wt}



HIV-1, CA_{M66I}