Online Supplement

Supplemental Materials and Methods

Cell culture: TZM-bl cells (NIH AIDS Reagent Program, catalogue number: 8129) were maintained with DMEM medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS), HEPES 25mM and 0.5% Gentamycin. HEK293T cells (ATCC, CRL-11268) were maintained in DMEM supplemented with 10% heat-inactivated FCS and 1% penicillin-streptomycin (P/S). Lymphoid MT-2 cells, Jurkat cells and PM1 cells, as well as HIV-1 latently infected ACH2 cells (NIH AIDS Reagent Program, catalogue numbers: 237, 177, 3038 and 349, respectively) and isolated human peripheral blood mononuclear cells (PBMC) from anonymous healthy blood donors were cultured with RPMI medium (Gibco) supplemented with 10% heat-inactivated FCS and 1% of P/S. PBMC were activated with 5 μg/ml of PHA during 3 days prior to infection. All cell types were incubated at 37ºC and 5% CO_2.

Plasmids: pGag-EGFP (NIH AIDS Reagent Program, catalogue number: 11468) expresses HIV-1 Gag fused to EGFP. pNLE-ΔEnv (a kind gift from Yasuko Tsunetsugu-Yokota, Tokyo University of Technology, National Institute of Infectious Diseases, Tokyo, Japan) expresses HIV-1 with a mutation in Env. It was used to pseudo-type HIV-1 with several envelope proteins. pHXB2-env (provided by Prof. Dr. Christian Jassoy, Institute for Virology, University of Leipzig, Lepizig, Germany) expresses HIV-1 Lai envelope. Plasmids pW61D_TCLA.71, pWITO4160, pSS1196.1, pBal.26 and pMN.3 (a kind gift from Kelli Green, Duke University Medical Center, Durham, USA) express different HIV-1 envelope proteins. pMM310 (a kind gift from Yasuko Tsunetsugu-Yokota) expresses E. coli β-lactamase fused to the amino terminus of HIV-Vpr. Transfections in HEK 293T and TZM-bl cells were performed with Lipofectamine 2000 (Invitrogen, Paisley, UK) or JetPEI, respectively, according to the manufacturer's manual.

Drugs and lipids: Soraphen A (SorA) is part of the library of myxobacterial secondary metabolites belonging to the Helmholtz Centre for Infection Research (HZI) in Braunschweig, Germany. The ACC inhibitor 5-(Tetradecyloxy)-2-furoic acid (TOFA) (Cayman Chemical company, Michigan, USA) was used as a control drug. The HIV protease inhibitor Lopinavir (LPN, Sigma, Missouri, USA), the non-nucleoside reverse transcriptase inhibitor Nevirapine (Sigma) and the entry inhibitor enfuvirtide (Fuzeon, Roche, Basel, Switzerland) were used as controls in several assays. The histone deacetylase inhibitor Vorinostat and the fatty acid palmitic acid were obtained from Sigma.

Virus stocks: HIV-1 viral stocks were produced by transfecting HEK293T cells with the HIV molecular clone pNL4.3 (NIH AIDS Reagent Program, catalogue number: 114) or via propagation of HIV_{LAI} in PM1 cells or PBMC. The viruses were titrated in TZM-bl cells and stored at -80°C. HIV pseudoparticles carrying different envelope proteins (see above) were produced by transfection of HEK293T cells.

Dose-response assays: TZM-bl cells were seeded (10^4 cells/well) in 96-well flat-bottom plates and PBMC were seeded (50000 cells/well) in 96-well V-bottom plates. Unless state otherwise, cells were incubated for 1h with the drug or the vehicle control (DMSO) prior to infection. Five 10-fold dilutions of the drug were tested in triplicate. After incubation, the plates were washed with medium and infected with HIV_{LAI} at MOI=0.5. Fresh drug was added to maintain the drug concentration. After incubation for 48h (TZM-bl) or 72h (PBMC) at 37°C 5% CO_2, supernatant from infected cells was used to re-infect fresh TZM-bl cells. 48h after TZM-bl cell re-infection, the luciferase activity was measured using Britelite Plus™ (PerkinElmer, Waltham, USA). In parallel, the cytotoxicity of the compounds were assessed using the commercial ATP-based system CellTiter-Glo® Lu-

minescent Cell Viability Assay (Promega, Madison, USA). Mean luciferase values were normalized to untreated controls and Effective Concentration 50 (EC_{50}) and Cytotoxic Concentration 50 (CC_{50}) were calculated in GraphPad Prism (GraphPad Software, San Diego, CA, USA) by analyzing the log of the drug concentration vs. the normalized response.

Immunofluorescence: MT-2 cells were treated with SorA 10 μM, TOFA 10 μM or vehicle control (DMSO). After 1h incubation, cells were spinoculated 75 min 1200g 4°C with HIV_{LAI} at MOI=0.5. 48h after spinoculation, cells were incubated 1h on poly-L-lysine-coated microscopy glass slides (Thermoscientific) and the supernatant was added to fresh MT-2 cells and incubated 48h further. Samples were fixed (30 minutes in PBS containing 4% paraformaldehyde (PFA)), permeabilized (20 minutes in 0.2% Triton X-100; Sigma) and blocked (30 minutes with FCS 10%). Samples were stained for 1h with the anti-HIV p24 antibody ARP3243.3 clone 05-009 provided by the Centre for AIDS Reagents, NIBSC (UK), and a secondary anti-mouse IgG antibody labelled with Alexa Fluor 647 (Invitrogen) for 45 minutes in the dark, followed by a 15 minutes nuclear staining with 4,6-diamidino-2-phenylindole (DAPI). Glasses were placed in microscopy slides with Mowiol (Sigma) and images were acquired on the Leica TCS SP5 at 63x.

TZM-bl infectivity assay and Immunoassay: Infectivity of supernatants from PBMC, ACH2 cells, and transfected 293T was determined with the TZM-bl assay. Clarified cell culture supernatants were added to fresh TZM-bl cells. 48h after supernatant addition luciferase activity was measured. HIV p24 and gp120 proteins were detected using the ELISA kits INNOTEST® HIV Antigen mAb to detect p24 (Fujirebio, Gent, Belgium) and the HIV-1 gp120 antigen capture assay (ABL, Rockville, USA). To obtain cell lysates, treated (±10 μM SorA and ±50 μM palmitic acid) and transfected 293T cells pro-

ducing HIVpp were washed with PBS and lysed with passive lysis buffer 10min at 4°C. Lysates were centrifuged (14000g 5´ 4°C) to remove cell debris.

Transmission electron microscopy: ACH2 cells were treated with 10 μM SorA, 10 μM Lopinavir or DMSO (vehicle). After 1h incubation, HIV production was activated with 10 μM Vorinostat. 48h after activation, cells were fix with 2.5% glutaraldehyde in 0.1 M phosphate buffer incubating 2x 30 minutes at RT. After washing, samples were incubated with 1.1% osmium tetroxide + 0.8% potassium ferricyanide in phosphate buffer 1-2h at 4°C. Samples were dehydrated and embedded in the resine Eponate 12 as previously described (1). Pictures were taken with a transmission electron microscope (TEM) JEM 1010 100 kV (JEOL, Tokyo, Japan) with CCD Megaview 1kx1k at 80 kV and analysed with Imagej software (National Institutes of Health, NIH).

CD4 binding and fusion assay: TZM-bl cells were spinoculated (2095xg 4°C 30 min) with 10 ng p24-containing supernatant from SorA- or vehicle (DMSO)- treated ACH2 cells that were previously activated with Vorinostat. Cells were washed with PBS and lysed with M-PER for 10 min at 4°C. p24 was detected with the ELISA kit INNOTEST® HIV Antigen mAb. Cells were maintained at 4°C throughout the assay to prevent virus-cell fusion. The fusion assay was performed as in (2). Briefly, HIVpp containing β-lactamase activity were produced by transfection in the presence of SorA, Lopinavir or DMSO. Jurkat cells ($2.5x10^5$ cells/condition) were spinoculated (1200xg, 25°C, 2h) with 50 ng or 200 ng p24 of HIVpp. Cells were incubated 2h at 37°C 5% CO_2. After washing, cells were loaded with the substrate for β-lactamase (CCF2-AM) at 1 μM. After 1h at room temperature in the dark, cells were washed, re-suspended in CO_2 independent medium (Gibco) containing 5% heat-inactivated FCS and incubated overnight at room temperature in the dark. Cells were washed, stained with propidium iodide (0.5 μg/ml) and

analyzed with a LSR Fortessa cytometer (BD bioscience). Data were analyzed with Flow Jo software (Tree Star).

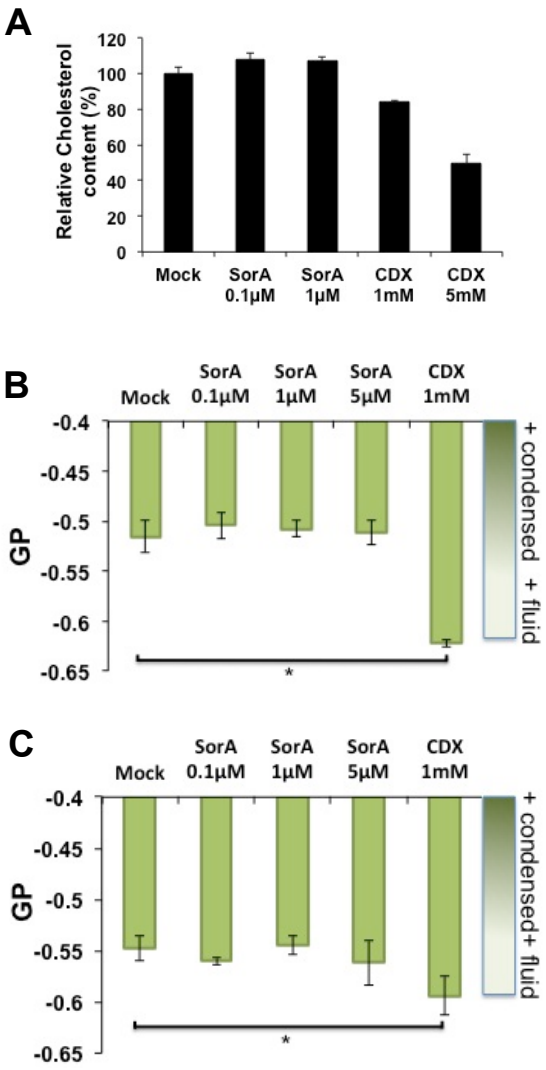
qPCR. 10 μM Soraphen A was added to SAHA-activated ACH2 cells, 293T cells co-transfected with the plasmids HIV pNLE-Env and pHXB2-Env, and to HIV_{LAI}-infected PBMC. 48 h after treatment, cell culture supernatants were clarified by centrifugation and filtered and cells were washed three times in PBS. RNA from virus-containing supernatants and from cells was extracted with QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and the Rneasy Micro Kit (Qiagen), respectively. HIV cDNA was generated with the SuperScript First-Strand Synthesis System (Invitrogen) using an HIV-specific primer spanning the U3 region of the HIV LTR and tagged with an unique nucleotide sequence: 5′-CTGATCTAGAGGTACCGGATCCAAAGCTCGATGTCAGCAGTCTT-3′. qPCR was performed with SYBR-Green Select Master Mix (Applied Biosystems, Foster City, California, USA) using the primers: Fw 5'-GCCGCCTAGCATTTCATCAC-3' and Tag: 5'-CTGATCTAGAGGTACCGGATCC-3' in the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Standard curves were generated with serial dilutions of amplicons using the same primers. qPCR was analysed with the ExpressionSuite Software (Applied Biosystems).

Western Blots. Viral supernatants were clarified, pelleted and aliquoted at -80°C. Standard curves for quantification were produced with serial dilutions of the recombinant HIV proteins p24 (Abcam, ab127888) and gp120 (Abcam, ab73769). Samples were mixed with Laemmli buffer, heated at 97°C for 5 minutes and transferred to nitrocellulose membrane by western blotting. Mouse monoclonal antibody for p24 was purchased from Centre for AIDS Reagents (Ref.: ARP3243.3) and diluted 1:5000 in 5% BSA with TBS-T buffer. For gp120, a rabbit polyclonal antibody (Abcam, ab106578) diluted 1:1000 in

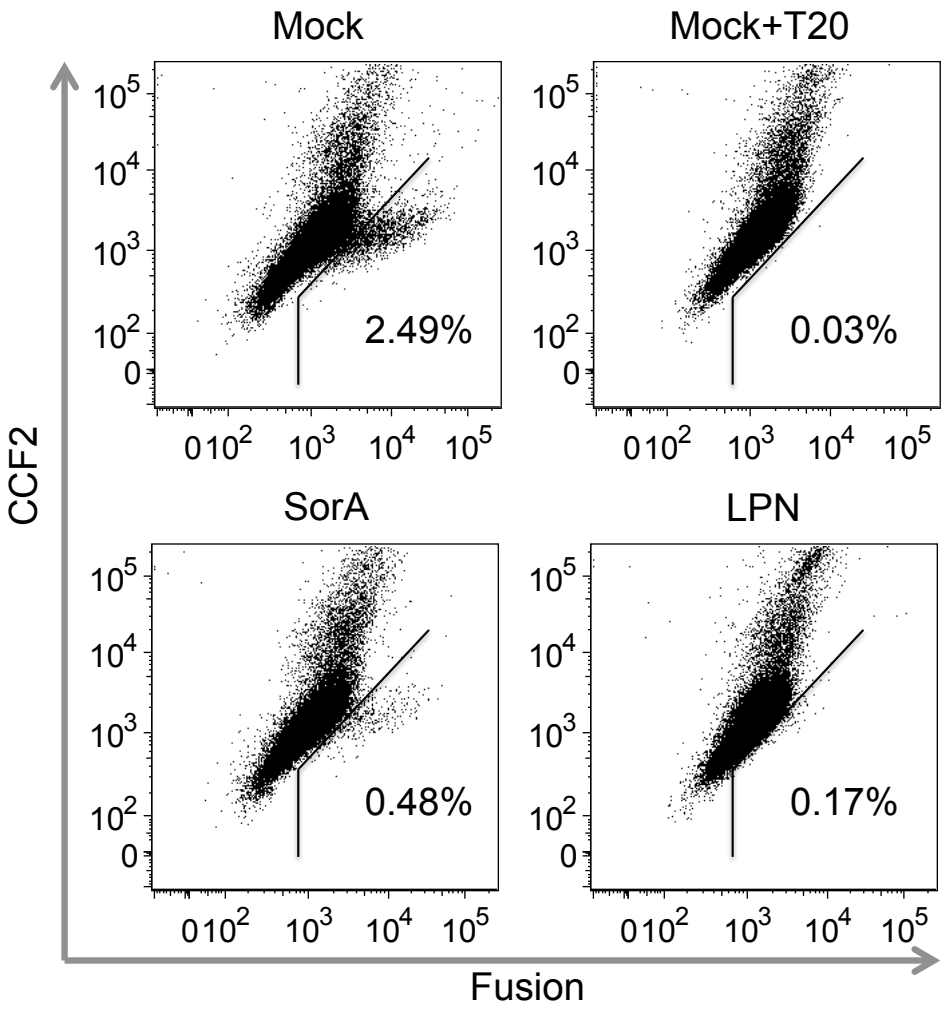
the same solution was used. For detection we used horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham ECL Anti-Rabbit IgG, Horseradish Peroxidase-Linked Species-Specific Whole Antibody, Ref.: NA934). Protein bands were developed using West Femto Maximum or ECL Plus Western Blotting Substrate (Thermo Scientific). Protein quantification and analysis were performed using J-Image software (BioRad).

Membrane fluidity assay and cholesterol amount determination: Membrane fluidity was determined as described in (3). Briefly, Jurkat cells or PHA-activated PBMC ($1.5x10^5$ cells/condition) were incubated 48h with SorA at 0.1, 1 and 5 μM or DMSO (mock). Cells were stained with 1 μg/ml of di-4-ANEPPDHQ (30min, 4°C) that changes its fluorescence emission depending on the fluidity of the membrane. Cells were excited at 488nm and emissions at 530/30nm and 670LPnm were simultaneously recorded with a LSR Fortessa. Intensities were converted into a general polarization index (GP) using the equation GP = (I530/30 – I670LP) / (I530/30 + I670LP). GP values range from +1 (more condensed) to -1 (more fluid). To detect changes in cholesterol content, Jurkat cells were similarly incubated with SorA and stained with 0.05 mg/ml of filipin (30min at RT). The intensity of the staining is proportional to the amount of cholesterol in the cell membrane and was determined using a LSR Fortessa. Cyclodextrine (1 and 5 mM, 1h 37°C before staining) was used in both assays as a positive control because it depletes cholesterol resulting in increased membrane fluidity.

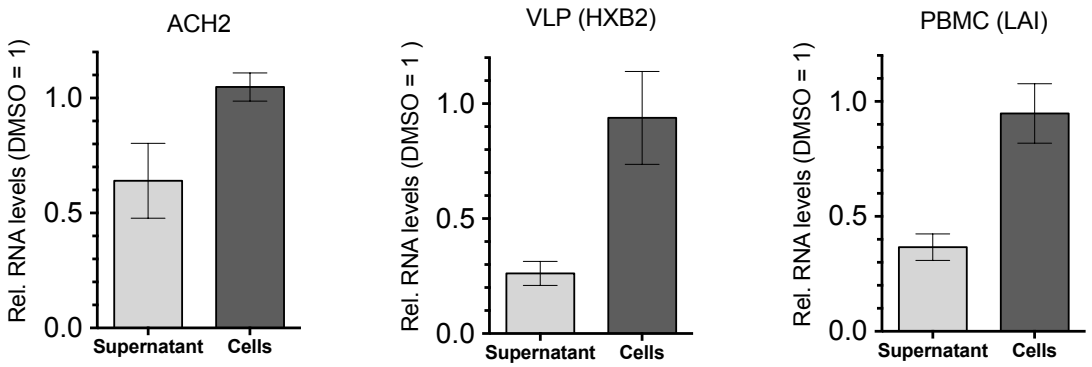
Supplemental figures



Supplemental figure 1. Soraphen A does not change the membrane fluidity or the cholesterol content of Jurkat cells or PBMC. (A) Jurkat cells were incubated with different concentrations of SorA for 48h. Cells were then stained with filipin and analyzed for changes in cholesterol content by flow cytometry. (B) Jurkat cells and (C) PBMC were incubated with different concentrations of SorA for 48h. Cells were then stained with di-4-ANEPPDHQ to detect changes in membrane fluidity by flow cytometry. Cyclodextrin (CDX) was used as a positive control in both assays. Generalized polarization index (GP) values for membrane fluidity and relative cholesterol content are shown. Error bars: standard deviation (SD). (*, $p<0.05$; **, $p<0.01$).



Supplemental figure 2. Reduced target cell fusion activity of HIV particles produced in the presence of Soraphen A. Representative flow cytometry plots of the fusion assay (see figure 3C) are shown. The proportion of cells fused with HIVpp with respect to CCF2-loaded cells in SorA-, LPN-, mock- and mock plus T20-treated samples is given.



Supplemental figure 3. SorA reduces HIV RNA in virus-containing supernatants but not in infected cells. Bars depict HIV RNA levels in SorA-treated samples relative to DMSO control (Set to 1). Left: SAHA-activated ACH2 cells, Middle: 293T cells co-transfected with HIV pNLE-Env and pHXB2-Env, Right: in HIV_{LAI}-infected PBMC. Error bars are ±SEM of the mean of three independent replicates.

Supplemental References

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