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Supplementary Information

α-Aminoisobutyric Acid Incorporation Induces Cell Permeability and Antiviral Activity of HIV-1 Major Homology Region Fragment

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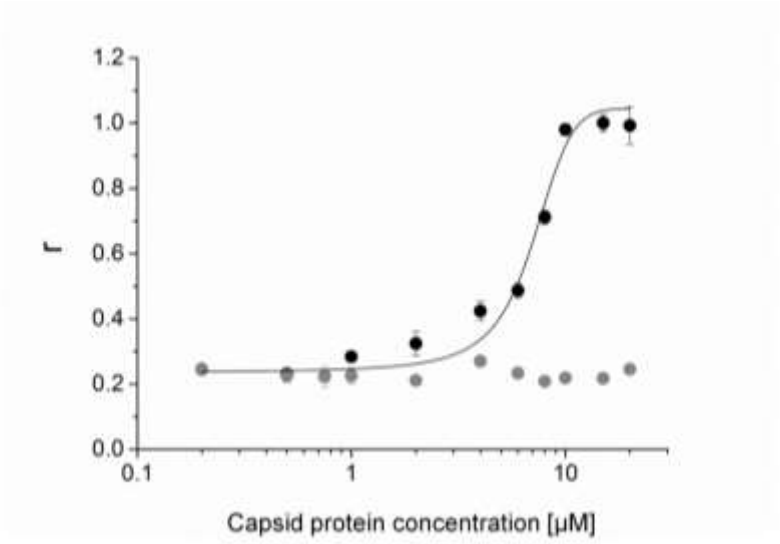

Fig. S1 Fluorescence anisotropy (r) binding profile of dansyl-labeled Pep-V (grey) or Pep-Aib (black) upon titration of HIV-1 capsid protein.

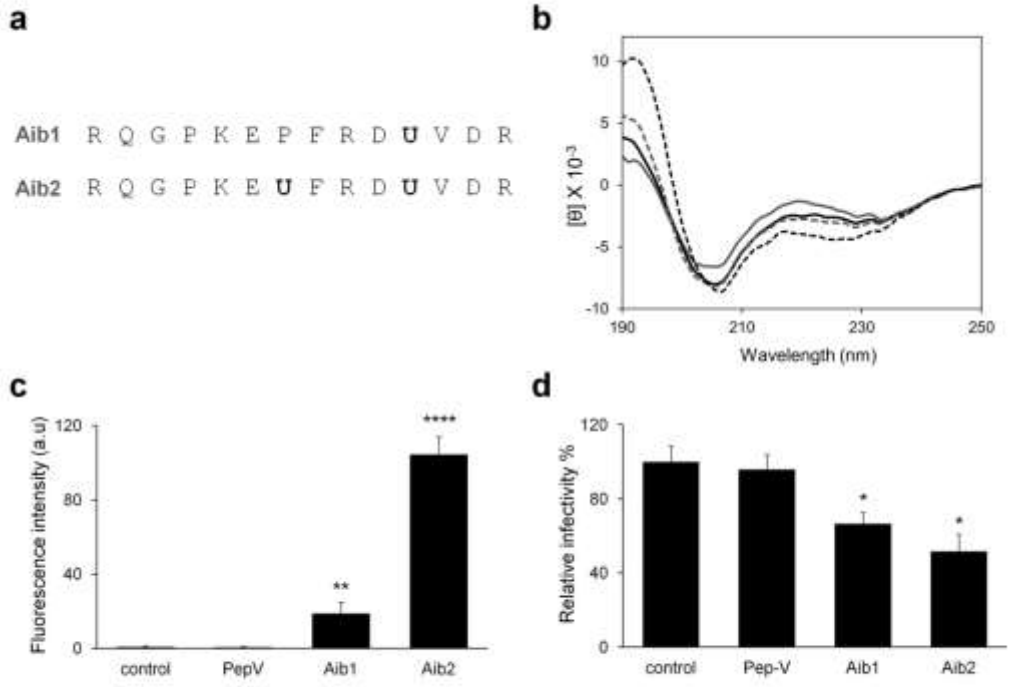

Fig. S2 (a) Sequences of Aib1 and Aib2. (b) CD spectra of Pep-V (solid grey), Pep-Aib (solid black), Aib1 (dashed grey) and Aib2 (dashed black) (50 μM) incubated

with 70% TFE. (c) Quantitative analysis of live-cell imaging confocal fluorescence microscopy of HEK 293T cells incubated either in the absence (control) or presence of the indicated rhodamine B-labeled peptides (5 μM) for 1 h. (d) Antiviral activity of the indicated peptides (5 μM) analyzed by FACS. The relative infectivity is the percentage of infectivity for peptide-treated samples, compared to untreated samples. For (c) and (d), values are means ± SD, Student's t-test, *$p<0.05$, **$p<0.01$, ****$p<0.0001$.

Experimental procedures

Peptides

Peptides were synthesized by Peptron Inc. The peptides were purified to 96-98% and their identity was confirmed by mass spectrometry.

Circular Dichroism (CD) Spectroscopy

CD spectra were recorded with a Chirascan spectrometer (Applied Photophysics) using a cuvette with an optical path length of 1 mm. Data was acquired from 190 to 260 nm with 0.2 sec integration time, 1 nm step resolution and 1 nm bandwidth. Data from three scans were averaged.

Pep-V and Pep-Aib were dissolved in 50 mM sodium phosphate buffer (pH 8.0). The CD spectra of the peptides were recorded at a final concentration of 50 μM in a solution of 40% TFE and 0.0125 mg/ml of liposomes composed of phosphatidylcholine and cholesterol at a molar ratio of 2.3:1, respectively. The liposomes were a kind gift from Prof. Dan Peer (Tel Aviv University) and were prepared as previously described.[1] To avoid high absorbance due to aggregation of the liposomes, each sample was subjected to ultrasonication for 15 sec.

Fourier Transform Infrared (FTIR) Spectroscopy

Peptide infrared spectra were recorded using a Nexus 470 FTIR spectrometer (Nicolet) with a deuterated triglycine sulfate (DTGS) detector. The peptides were

dissolved in dimethyl sulfoxide (DMSO) to a concentration of 5 mg/ml, deposited on disposable polyethylene IR sample cards (Sigma-Aldrich, Israel) and dried under vacuum. Measurements were made at a 2 cm^{-1} resolution, averaging 64 scans per sample. Spectra were corrected in baseline with DMSO trace as the blank.

Live Cell Imaging Confocal Laser Scanning Microscopy

Human embryonic kidney (HEK) 293T cells were cultured in 35-mm glass-base dishes (Greiner Bio-One) and incubated for 24 h in 300 µl of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, and nystatin (Biological Industries). For imaging, cells were incubated either in the absence or presence of Pep-V or Pep-Aib linked to rhodamine B at the N-terminus. The peptides were dissolved in DMSO and diluted with the growth medium to a final concentration of 5 µM and final DMSO concentration of 2% v/v. The control sample contained cells incubated only with 2% v/v of DMSO. Chromosomes were stained with Hoechst 33342 (bisbenzimide trihydrochloride, Sigma-Aldrich, Israel; 1 µg/ml, 15 min, 37°C). The cells were images using a confocal microscope (Axiovert 200 M, Carl Zeiss MicroImaging), 100× lens (NA 1.45, Zeiss) and Evolve (Photometrics) camera.

Quantification of the intracellular fluorescent signal of the cells was performed using SlideBook software (Intelligent Imaging Innovations). Cells were imaged at the mid-section by confocal microscopy and the picture was deconvolved with the No Neighbors deconvolution algorithm of SlideBook. The specific signal of the rhodamine B-conjugated peptides was identified through intensity based segmentation and the total signal intensity was calculated and divided by the number of cells. The average number of cells that were visualized per time point was 5.

Single-Cycle Infection Assay

293T cells were cultured in 6-well tissue culture dish and incubated for 24 h in 4 ml of DMEM media (as described above). Cells were co-transfected by using the calcium phosphate method with the following plasmids: pCMVΔR8.2[2] (2.5 µg), encodes HIV-

1 proteins except the envelope and the Vpu, VSV-G[2] (0.83 µg), encodes the vesicular stomatitis virus G envelope protein and pHR'CMV-GFP[3] (3.3 µg) encodes an HIV-1-derived retroviral vector carrying the GFP gene. 5 h post-transfection, cell media was replaced with fresh media containing Pep-V or Pep-Aib dissolved in DMSO at final concentrations of 1 µM, 5 µM, 10 µM and 20 µM, or 20 µM of capsid assembly inhibitor CAP-1 (Maybridge) from and final DMSO concentration of 2% v/v. Control cells were treated only with 2% v/v DMSO. 48 h post-transfection, virus-containing media were collected, 50 mM of Hepes buffer was added to the culture supernatants and the mixture was filtered through a 0.45 µm filter. Transfection levels and intracellular protein quantity of the transfected cells were analyzed by fluorescence-activated cell sorting (FACS) and Bradford assay, respectively.

For infection, 293T cells were cultured in 24-well tissue culture dish and incubated for 24 h in 0.5 ml of DMEM media (as described above). Virions in culture supernatants, normalized by transfection and total protein levels of the transfected cells, were used to infect the naïve 293T cells using 8 µg/ml polybrene (hexadimethrine bromide; Sigma-Aldrich, Israel). GFP fluorescence of the infected cells was analyzed by FACS 48 h post-infection.

Flow Cytometry

Transfected or infected cells with a GFP vector were harvested in 300 µl PBS and the GFP fluorescence was measured using a FACSort apparatus (BDbiosciences). Mock-transfected cells (cells subjected to the transfection procedure without the addition of plasmid DNA) were used to determine the background autofluorescence levels of GFP-negative cells. Total fluorescence of the GFP-positive cell population was calculated by multiplying the percentage of GFP-positive cells with their mean fluorescence intensity. Relative infectivity was calculated by dividing the total fluorescence of the infected cells by the total fluorescence of the transfected cells.

Virus Production, Western Blot and Immunofluorescence

For Western blot analysis of Gag and capsid levels, virions in culture supernatant were purified through 25% sucrose cushion by ultracentrifugation at 25,000 rpm for 2 h (4ºC). Gag and capsid proteins were detected by Western blot, using an anti-HIV-1 capsid antibody (hybridoma clone 183-H12-5C; NIH AIDS Research and Reference Program) and a goat anti-mouse HRP-conjugated secondary antibody (Abcam). Quantification of virus production was performed by calculating the relative amount of Gag and capsid protein levels of virions produced by untreated cells compared to virions produced by treated cells, using the following equation: 100x (extracellular Gag or capsid levels) / (intracellular Gag levels, normalized by actin levels), using the TINA software program.

Transmission Electron Microscopy (TEM)

TEM of purified virions was performed by applying 10 µl samples to 400-mesh copper grids covered by carbon-stabilized Formvar film (Electron Microscopy Science). The samples were allowed to adsorb for 2 min before excess fluid was blotted off. Samples were then negatively stained using 10 µl of 2% uranyl acetate that was deposited on the grid and allowed to adsorb for 2 min before excess fluid was blotted off. TEM micrographs were recorded using JEM-1400 electron microscope (JEOL) operating at 80 kV.

Peptide Toxicity Assay

293T cells were cultured in 96-well micro plates (100 µl/well) and incubated at 37°C. After 24 h incubation, cell media (prepared as described above) was replaced with fresh media without serum, containing Pep-V or Pep-Aib dissolved in DMSO at final concentrations of 1 µM, 5 µM, 10 µM and 20 µM and final DMSO concentration of 2% v/v. Following incubation of 24 h at 37°C, cell viability was evaluated using a cell proliferation kit based on 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Absorbance at 470 nm was measured after 45 min incubation with the reagent, using Synergy HT Microplate Reader (BioTek

Instruments, Inc.). For each well, XTT absorbance was normalized according to cell number which was measured by absorbance at 670 nm. Each measurement was repeated at least three times. The cytotoxic effect of the peptides was calculated as the cell viability in the presence of each peptide relative to the viability of cells that were incubated only with 2% v/v DMSO.

HIV-1 full length CA Protein expression and purification

Pet11a-based expression vector of HIV-1 full length CA protein was kindly provided by W. Sundquist (University of Utah). HIV-1 capsid was expressed and purified in BL21 (DE3) *E. coli* Rosetta cells as previously described[4-5] using two-step purification of ammonium sulfate sedimentation and anion exchange chromatography.

Fluorescence Anisotropy

Fluorescence anisotropy of Dansyl-labeled Pep-V or Pep-Aib (linked via the N-terminus) was measured either in the absence or presence of varying concentrations of HIV-1 capsid protein (purified as described above) in 50 mM Na_2HPO_4 (pH 8.0) buffer. The peptides were dissolved in DMSO and diluted with 50 mM Na_2HPO_4 (pH 8.0) buffer to a final concentration of 100 nM and final DMSO concentration of 10% v/v. The anisotropy was measured by FL3-11 spectrofluorometer (Horiba Jobin Yvon) at 25ºC, using excitation and emission wavelengths of 340 nm and 550 nm, respectively. 5 measurements were collected for each point, and their average values were used for the calculation. Titration curves were fit to a standard four-parameter binding curve using the OriginLab software. The equation used:

$$r = \frac{r_i - r_f}{1 + e^{(x - K_D)/dt}} + r_f \quad (1)$$

Where r is the fluorescence anisotropy at peptide concentration x, r_i is the r at the initial data point, K_D is the peptide concentration at which the r is equal to one-half the r_f.

Statistical Analysis

For all statistical analyses, Student's t-test was performed between a control and a treated group or two individual treatment groups.

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