Palmitic Acid Is a Novel CD4 Fusion Inhibitor That Blocks HIV Entry and Infection

David Y.-W. Lee,¹ Xudong Lin,² Elena E. Paskaleva,² Yanze Liu,¹ Shadakshara S. Puttamadappa,³ Carol Thornber,⁴ James R. Drake,² Maja Habulin,⁵ Alexander Shekhtman,³ and Mario Canki²

Abstract

The high rate of HIV-1 mutation and the frequent sexual transmission highlight the need for novel therapeutic modalities with broad activity against both CXCR4 (X4) and CCR5 (R5)-tropic viruses. We investigated a large number of natural products, and from *Sargassum fusiforme* we isolated and identified palmitic acid (PA) as a natural small bioactive molecule with activity against HIV-1 infection. Treatment with 100 μ M PA inhibited both X4 and R5 independent infection in the T cell line up to 70%. Treatment with 22 μ M PA inhibited X4 infection in primary peripheral blood lymphocytes (PBL) up to 95% and 100 μ M PA inhibited R5 infection in primary macrophages by over 90%. Inhibition of infection was concentration dependent, and cell viability for all treatments tested remained above 80%, similar to treatment with 10⁻⁶ M nucleoside analogue 2', 3'-dideoxycytidine (ddC). Micromolar PA concentrations also inhibited cell-to-cell fusion and specific virus-to-cell fusion up to 62%. PA treatment did not result in internalization of the cell surface CD4 receptor or lipid raft disruption, and it did not inhibit intracellular virus replication. PA directly inhibited gp120-CD4 complex formation in a dose-dependent manner. We used fluorescence spectroscopy to determine that PA binds to the CD4 receptor with $K_d \sim 1.5 \pm 0.2 \mu$ M, and we used one-dimensional saturation transfer difference NMR (STD-NMR) to determined that the PA binding epitope for CD4 consists of the hydrophobic methyl and methylene groups located away from the PA carboxyl terminal, which blocks efficient gp120-CD4 attachment. These findings introduce a novel class of antiviral compound that binds directly to the CD4 receptor, blocking HIV-1 entry and infection. Understanding the structure-affinity relationship (SAR) between PA and CD4 should lead to the development of PA analogs with greater potency against HIV-1 entry.

Introduction

IN MOST PATIENTS, highly active antiretroviral therapy (HAART) substantially improves clinical outcomes in treated populations.^{1,2} However, the high rate of HIV-1 mutation increases the likelihood and frequency of generating drug-resistant HIV-1 strains.³ Consequently, some reports show that as many as 20% of all new HIV-1 infections are with viruses resistant to the currently available drugs,^{4,5} which highlight the need for continued discoveries of novel inhibitors of HIV-1 infection.⁶ New classes of drugs, including fusion inhibitors, provide new therapeutic options, especially in the antiretroviral (ARV) treatment-experienced population. Inhibitors of virus entry are desirable therapeutic modalities,

since in addition to limiting viral spread in the already infected host, they also block the virus from entering its target cell and thereby prevent *de novo* infection.^{6,7} Worldwide, heterosexual intercourse remains the principal route of new HIV infections, with women bearing a disproportionate burden of new infections,⁷ and viral entry inhibitors can be used in microbicide formulations aimed at the prevention of sexual transmission.⁸

Products derived from natural sources represent a potential source of novel and therapeutic agents that have been shown to inhibit HIV-1 infection at different stages of the virus life cycle.^{9,10} We investigated a large number of different natural products (NP), including plants and marine products, and identified an aqueous extract from *Sargassum*

¹Mailman Research Center, McLean Hospital, Harvard Medical School, Belmont, Massachusetts 02478.

²Center for Immunology and Microbial Disease, Albany Medical College, Albany, New York 12208.

³Department of Chemistry, State University of New York at Albany, Albany, New York 12208.

⁴Department of Biological Sciences, University of Rhode Island, Kingston, Rhode Island 02881.

⁵Department of Chemistry and Chemical Engineering, University of Maribor, Maribor, Slovenia.

fusiforme (*S. fusiforme*) as a potent inhibitor of HIV-1 infection.¹¹ We undertook a large-scale, bioactivity-guided fractionation on a complex *S. fusiforme* mixture that generated 600 fractions, with one bioactive fraction (SP4-2) exhibiting activity against global HIV-1 infection by ~87%.¹² The SP4-2 fraction blocked viral entry by 53%, and it also inhibited postentry replication up to 71% that was specific against reverse transcriptase (RT). Inhibition of entry was reversed by the addition of the sCD4 receptor, suggesting an interaction of the SP4-2 bioactive molecule with the CD4 receptor.¹² From the SP4-2 fraction we isolated and identified two unsaturated fatty acids, oleic and linoleic acid, and two saturated fatty acids, myristic and palmitic acid (PA).¹³ Our initial results showed that the two unsaturated fatty acids, oleic and linoleic acid, inhibit HIV-1 reverse transcriptase.¹³ However, to date, only myristic acid has been reported to have activity against HIV-1 infection, which inhibits virus budding.^{14,15}

In the present study we undertook a detailed investigation of HIV-1 inhibition of infection by PA, a saturated 16-carbon (16:0) fatty acid. Here we report that treatment of cells with PA results in specific PA-to-CD4 receptor binding and subsequent inhibition of HIV-1 entry.

Materials and Methods

Palmitic acid

Purification of *S. fusiforme* and the SP4-2 fraction to its individual components and identification of a straight-chain saturated PA (hexadecanoic acid, CH₃(CH₂)₁₄COOH, MW = 256.42) by nuclear magnetic resonance (NMR) was previously described.^{12,13} PA (Sigma) was solubilized at 100 mM in ethanol (EtOH)¹⁶ and stored at -20°C. Working aliquots were kept at 4°C for up to 4 weeks.

Cells and treatments

SupT1, GHOST (3) X4/R5, CEM, and 1G5 cells were obtained from the HIV AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, and were cultured and maintained as specified by the reagent protocol. Monocyte-derived human macrophages (Mφs) and peripheral blood leukocytes (PBL) were recovered from peripheral blood mononuclear cells (PBMCs) by countercurrent centrifugal elutriation as previously described.¹⁷ Monocytes were cultured as adherent monolayers (1 × 10⁶ cells/well in 24-well plates), differentiated for 7 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% human serum, and recombinant human macrophage colony-stimulating factor (rhM-CSF, Wyeth, Cambridge, MA). PBL were cultured in RPMI and 10% fetal bovine serum (FBS), stimulated for 24 h with 4 μg/ml phytohemagglutinin (PHA), and cultured in the presence of 10 U interleukin (IL)-2.

Cell cultures were treated overnight (18 h) with different PA concentrations, or with different treatment as indicated in the figure legends for each experiment, washed three times, and HIV-1 infected in the absence of PA, except for macrophage cultures that were infected in the presence of PA. Cells were infected with HIV-1 at the indicated multiplicity of infection (moi), washed three times, and returned to culture with same concentration of each treatment for the duration of the experiment, and then analyzed as indicated.

gp120-CD4 ELISA and lipid rafts

Inhibition of gp120-CD4 complex formation was investigated by gp120 capture ELISA, in accordance with the manufacturer's instructions (ImmunoDiagnostics, Inc., MA). Envelope gp120 (IIIB) protein was captured on 96-well plates, washed, and incubated in the presence of CD4-biotin alone or in the presence of increasing concentrations of PA, as indicated. Streptavidin-HRP was added, and then developed by addition of a chemiluminescent substrate, *o*-phenylenediamine dihydrochloride (OPD). The reaction was stopped by adding 1 N HCl and read at 490 nm. Percent of gp120-CD4 binding was calculated from gp120-CD4 complex formation in the absence of any inhibitor.

Lipid rafts of 1G5 T cells were labeled with 1 μg/ml cholera toxin-B subunit (CTB)-HRP and isolated as previously described.¹⁸ Briefly, labeled cells were then incubated for 1 h in media containing 20 μM PA, 20 μM myristic acid (MA), or no fatty acid (No Treatment). Cells were then lysed in cold 0.1% Triton X-100 and lipid rafts isolated by sucrose density gradient centrifugation. Five gradient fractions were collected (top to bottom) and assayed for HRP activity. The (CTB-HRP) distribution was reported as a percent of total activity.

HIV-1 molecular clones and BlaM-Vpr chimera

HIV-1 X4-tropic molecular clone NL4-3, which expresses all known HIV-1 proteins,¹⁹ and the R5-tropic molecular clone 81A,²⁰ which has Ba-L Env sequences on the backbone of NL4-3, were obtained from the HIV AIDS Research and Reference Reagent Program. Macrophage HIV-1 R5-tropic primary isolate ADA was prepared as previously described.^{11,17} Envelope expression-deficient and luciferase-positive pNL4-3.HSA.R⁺E⁻ (HIV-Env⁻Luc⁺) was obtained from Dr. Nathaniel Landau^{21,22} and was pseudotyped with the VSV-G envelope. The pL-VSV-G vector was obtained from Dr. M. Emerman; it contains a VSV-G insert in the pcDNA expression vector modified by replacing the cytomegalovirus promoter with the HIV-1 long terminal repeat.²³ We generated native and pseudotyped virus by calcium phosphate transfection of 293T cells, as previously described.²⁴ Cell-free viral stock was quantified for HIV-1 p24 core antigen content by enzyme-linked immunosorbent assay (ELISA, AIDS Vaccine Program, NCI-Frederick), and was also quantified for titers of infectious virus by multinuclear activation of a β-galactosidase indicator (MAGI) assay.²⁵

Fusion-sensitive BlaM-Vpr chimera DNA plasmid was a kind gift from Dr. W. Greene,²⁶ and HIV-1 virions containing the BlaM-Vpr chimera were produced by cotransfection of 293T cells with pNL4-3 proviral DNA (60 μg), pCMV-BlaM-Vpr (20 μg), and pAdVantage vectors (10 μg) (Invitrogen), as previously described.²⁶ After 48 h at 37°C, the virus-containing supernatant was centrifuged at low speed to remove cellular debris and then concentrated at 72,000 × g for 90 min at 4°C, and viral stocks were normalized by p24 content measured by ELISA, and stored at -80°C before use.

HIV-1 fusion assay and flow cytometry

Standard HIV-1 fusion assay was done as previously described.²⁶ Briefly, Sup T1 cells were first infected for 2 h with BlaM-Vpr-X4 (NL4-3) chimera at 0.5 moi, washed in CO₂-independent media, loaded for 1 h at room temperature (rt)

with the CCF2/AM dye as specified by the manufacturer (Gibco), and washed in developing buffer; the reaction was allowed to develop overnight. After development, cells were washed in phosphate-buffered saline (PBS) and fixed in 1.2% paraformaldehyde solution. The BlaM reaction was detected by the change in the emission fluorescence of CCF2 after cleavage by the BlaM-Vpr chimera, which was monitored by FACS with a three-laser Vantage SE (Becton Dickinson, San Jose, CA). A coherent krypton laser operating at 200 mW and generating light at 406.7 nm was used to excite the CCF2 dye. Blue emission was detected with an HQ455/50 filter and green emission was detected with an HQ545/90 BP filter; for light splitting, a 505 SP filter was used. Data were collected with CellQuest and analyzed with FlowJo software (Treestar, San Carlos, CA).

GHOST X4/R5-expressing cells were plated in 24-well plates at a concentration of 5×10^4 cells/well in DMEM, 10% FBS, 500 mg/ml G418, 100 mg/ml hygromycin, 1 mg/ml puromycin, and 1% penicillin/streptomycin. The next day, cells were treated with dilutions of PA for 1.5 h, and cells were infected at 0.3 moi with either X4-tropic (NL4-3) or with R5-tropic (81A) HIV-1 clones; cells were then washed and returned to media containing each respective treatment. Cells were collected 40–48 h after infection, washed in PBS, and incubated in 200 μ l 1.2% paraformaldehyde in PBS for 2–3 h at 4°C prior to FACS analysis. Cell counting was performed on a BD FACSCanto FACS system and analyzed with BD FACS-Diva software.

SupT1 cells were pretreated overnight with different concentrations of PA or treated for 5 h with 10 ng/ml phorbol 12-myristate 13-acetate (PMA), which served as a positive control for CD4 internalization.²⁷ All systems were surface labeled with phycoerythrin (PE)-conjugated anti-CD4 antibody (Santa Cruz, CA) and analyzed on a BD LSR II flow cytometer. Internalization of CD4 was measured by the shift in the mean fluorescent intensity of labeled cells.

NMR experiments

To perform the STD-NMR experiment, we used commercially available sCD4 (200 μ g, 4.4 nmol, Progenics Pharmaceuticals, Inc.), which was exchanged into the NMR buffer (160 μ l of D₂O and 40 μ l *d*₆-DMSO, containing 10 mM KPO₄ buffer, pH 7.0). A 1 mM solution of PA was dissolved in the NMR buffer and used for the titration of the NMR sample containing 18 μ M sCD4; 20% of *d*₆-DMSO in the NMR buffer was used to prevent formation of PA micelles. All NMR experiments were performed at room temperature. The data were acquired on a Bruker Avance 700-MHz spectrometer equipped with a z-gradient cryoprobe. The on-resonance irradiation of the protein during the 1D STD NMR experiment was performed at a chemical shift of 0 ppm. Off-resonance irradiation was applied at 30 ppm. A sequence of Gaussian-shaped pulses with a strength of 86 Hz and a length of 50 ms separated by a 1 ms delay was applied for 2.04 s during selective presaturation of the sCD4. The total number of scans was 1024. The NMR data were processed and analyzed using Topspin 2.0 (Bruker, Inc.).

For fluorescence titration experiments, 2 μ M sCD4 (Progenics Pharmaceuticals, Inc.) dissolved in the NMR buffer was used, and a 1 mM solution of PA dissolved in the NMR buffer was added in 1 μ M steps. Titrations in the absence of

sCD4 were performed as a reference. Tryptophan fluorescence was measured using an excitation wavelength of 290 nm. Measurements were performed on a Fluorolog-3 fluorescence spectrophotometer (HoribaJobin Yvon) at 25°C in a 1-ml stirred cuvette. The fluorescence emission signal was subtracted from the signal of the reference titrations, and the differences adjusted by the dilution factor were plotted against the final concentration of added PA. Curve fitting (OriginLab) was performed to find the best values for K_d using a single-site binding isotherm approximation.²⁸

Results

Palmitic acid inhibits X4 and R5-tropic HIV-1 infection

Previous studies with whole *S. fusiforme* extract and with the bioactive SP4-2 fraction demonstrated inhibition of HIV-1 infection in several primary and transformed cell lines.^{11,12} To test the ability of PA, which was isolated from the SP4-2 bioactive fraction,¹³ to specifically block productive X4 and R5-tropic HIV-1 infection, we first tested for virus inhibition in GHOST X4/R5-expressing human osteosarcoma cells by flow cytometry (Fig. 1A). Cells were treated with increasing micromolar PA concentrations and infected with HIV-1, NL4-3 (X4 infection, upper panels), or 81A (R5 infection, lower panels). In the X4 virus-infected cells, PA treatment gradually reduced the total number of infected cells from 20% to 5.9% (X4 infection, upper panels). This reduction in the total number of infected cells translated to 13, 24, 40, 52, and 70% inhibition of X4 infection due to the corresponding PA treatment of 10, 20, 40, 60, and 100 μ M, respectively. Similarly, infection with R5 virus gradually reduced the total number of infected cells from 52.5% to 14.45%, which translated to 15, 31, 43, 56, and 73% inhibition of infection due to the corresponding PA treatment of 10, 20, 40, 60, and 100 μ M, respectively (R5 infection, lower panels). This result demonstrated that treatment with free PA blocks both X4 and R5 tropic HIV-1 infection to similar levels, which is in agreement with our previous results for the SP4-2 fraction inhibition of both X4 and R5 infection in the same cell line.¹² To ascertain the specificity of PA for inhibition of HIV-1, we also tested MA, which did not inhibit HIV-1 infection as compared to PA (data not shown).

Human peripheral blood mononuclear cells (PBL) and macrophages (M ϕ s) are the primary target for HIV-1 infection *in vivo*; we examined PA inhibition of HIV-1 infection in these primary cells (Fig. 1B and C). In PBL, measurements of HIV-1 p24 antigen levels in cell culture supernatants revealed a peak of p24 production at 8897 pg of p24/ml for NL4-3-infected and untreated cultures (0 μ g PA), which was followed by a gradual decline to approximately 435 pg p24/ml (Fig. 1B, left panel). Treatment with 1.5, 3, 6, 12, and 22 μ M PA resulted in 70, 75, 77, 84, and 95% inhibition of NL4-3 infection (Fig. 1B, middle panel). Treatment with 10⁻⁶ M ddC inhibited infection by 84%, somewhat lower than 95% maximal PA inhibition. In parallel, we measured cell viability, which remained above 96% for all PA treatments tested and measured slightly above 89% viability for 10⁻⁶ M ddC treatment (Fig. 1B, right panel).

In human M ϕ s, peak p24 production was quantified at 30,717 pg p24/ml for HIV-1 primary R5 isolate ADA-infected and untreated (0 μ g PA) cells, which was approximately 3.4-fold higher as compared to NL4-3 infection in PBL. This p24 production was followed by a gradual decline to 373 pg

p24/ml (Fig. 1C, left panel). Treatment with 25, 50, 100, 150, and 200 μ M PA resulted in 35, 67, 91, 95, and 99% inhibition of ADA infection, and treatment with 10^{-6} M ddC resulted in 99% inhibition (Fig. 1C, middle panel). Cell viability increased with the increase in inhibition of infection and was similar to the viability of ddC-treated cells (Fig. 1C, right panel).

In both cell types, a dose-dependent inhibitory effect of PA was observed throughout productive HIV-1 infection, most notably at the peak of virus replication. However, it is not clear why PA inhibitory concentrations differed between these two primary cell types. Possible differences in the level of infection between laboratory-adapted NL4-3 and primary

isolate ADA, and expression of receptor or coreceptor between these primary cells, may account for these differences.

Palmitic acid inhibits cell-to-cell and virus-to-cell fusion

HIV-1 infection is spread either by free viral particles or 100 times more efficiently by direct cell-to-cell fusion,²⁹ and we previously demonstrated that whole *S. fusiforme* extract was capable of inhibiting cell-to-cell fusion in GHOST cells.¹¹ We next determined the ability of PA to block cell-to-cell fusion by treating uninfected GHOST cells with 20 or 40 μ M PA and cocultivating these cells together with NL4-3-infected CEM cells for 24 h (Fig. 2A). Tat-driven GFP expression measured in uninfected GHOST cell cultures can arise only from fusion of these cells with HIV-1-infected CEM cells, and we monitored this mixed cell culture system quantitatively for GFP expression by flow cytometry. Untreated (0 μ M PA) GHOST mixed cell cultures resulted in 6% GFP fusion-positive cells. Treatment with 20 and 40 μ M PA resulted in GFP reduction to 2.94% and 2.19%, which translated to 56% and 70% inhibition of cell-to-cell fusion (indicated at the top of each panel).

The SP4-2 fraction was also shown to block HIV-1 binding and fusion, which was reversed by the addition of sCD4, suggesting interaction between the SP4-2 bioactive molecule and CD4 receptor.¹² To begin to assess the mechanism by which PA inhibits HIV-1 replication we investigated virus-to-cell fusion in a standard and specific HIV-1 fusion assay^{30,31} as the first step in the infection process (Fig. 2B). In contrast to untreated (0 μ M PA) T cells that allowed HIV-1 particles to fuse and enter into 37.6% of the cells, treatment with 2, 4, and 8 μ M PA restricted virus fusion to 24.6, 19.7, and 14.4% cells, which translated to 3.5, 48.2, and 62.5% inhibition of HIV-1 fusion, respectively (indicated inside each panel). Inhibition of fusion in this system was confirmed by the addition of X4 coreceptor inhibitor AMD3100 that limited virus fusion to 2.8% of cells, which translated to 93% inhibition.

Palmitic acid does not internalize cell surface CD4 receptor and does not disrupt lipid rafts

Because CD4 is the main receptor responsible for HIV-1 attachment, fusion, and entry into the cell, and PA-mediated inhibition of HIV-1 fusion could result from internalization of the cell surface CD4 receptor, we checked this possibility by treating SupT1 cells overnight with increasing PA concentrations, or treating cells for 5 h with positive control for receptor internalization, 10 ng/ml PMA²⁷ (Fig. 3A). Cells were surface labeled with PE-conjugated anti-CD4 antibody (Santa

Cruz, CA) and analyzed on a BD LSR II flow cytometer. Percent internalization (Int) or percent surface (Sur) CD4 was measured by the shift in the mean fluorescent intensity of labeled cells, and is indicated inside each box. Treatment with 0, 25, 50, and 100 μ M PA internalized the CD4 receptor by 1.3, 1.4, 4.7, and 7.4%, respectively. Cell surface CD4 receptor expression changed from 98, 98, 94, and 91%, which corresponded to the same micromolar PA treatment. In contrast, PMA treatment internalized the cell surface CD4 receptor by 94%, and cell surface receptor expression was 1.8%. Based on these results we conclude that treatment with up to 100 μ M PA does not appear to significantly alter CD4 receptor cell surface expression.

To evaluate whether the effect of PA on HIV infectivity might be due to the impact on lipid rafts, which are necessary for HIV infection, we investigated whether PA treatment has the ability to isolate cholera toxin-B (CTB)-labeled rafts from 1G5 T cells (Fig. 3B). As shown by the results presented in Fig. 3B, treatment of T cells with 20 μ M PA, which inhibited HIV infection by 65% (Fig. 4B) or treatment with 20 μ M MA, which failed to inhibit HIV infection (not shown), failed to inhibit the ability to isolate CTB-HRP-labeled lipid rafts by sucrose density gradient centrifugation.¹⁸ Treatment with 100 μ M PA also failed to inhibit isolation of lipid rafts (not shown). These results are consistent with the hypothesis that PA inhibits HIV infection by binding to the CD4 receptor and blocks HIV uptake, and also suggest that the inhibitor effect of PA is not due to disruption of the lipid raft structure or perturbation of the cellular membrane.

Palmitic acid inhibits gp120-CD4 complex formation and CD4-dependent infection

We next investigated the ability of PA to specifically inhibit envelope gp120-CD4 receptor complex formation by gp120 capture ELISA (Fig. 4A). Then 96-well plates were coated with gp120 (IIIB) and incubated with biotin-sCD4 in absence or presence of increasing PA concentrations. Percent inhibition was calculated in the absence of PA, which was taken as 100% gp120-CD4 complex formation. Treatment with 25, 50, and 100 μ M PA inhibited gp120-CD4 binding by 23, 45, and 71%, respectively. This result clearly indicated that PA inhibits HIV-1 infection by blocking gp120-CD4 interaction. In reverse experiments, we coated plates with sCD4, incubated gp120 with increasing concentrations of PA and tested for inhibition of gp120-CD4 complex formation, which was not inhibited, indicating that PA does not bind to soluble gp120 (not shown).

FIG. 1. Inhibition of X4 and R5-tropic HIV-1 infection and cell viability. (A) Flow cytometry analyses of GHOST X4/R5 GFP-expressing cells that were treated overnight with increasing micromolar concentrations of PA indicated on top of each box, washed, and infected for 1.5 h at 0.3 MOI in replicates ($n = 3$), with NL4-3 (X4 infection) or with 81A (R5 infection). The percentage of infected cells is indicated inside each box, and % inhibition is indicated below each box. Uninfected and untreated control is superimposed over each histogram by a dotted line. (B) Human PBL were treated overnight with increasing micromolar concentrations of PA or 10^{-6} M ddC, washed, and infected with NL4-3 at 0.1 MOI for 2 h in the absence of each treatment, washed three times, and returned to culture with each respective treatment for the duration of the experiment; (left panel) p24 pg/ml antigen production in cell-free supernatants at the peak of infection on day 6; (middle panel) calculated % inhibition; and (right panel) % viability. (C) Human macrophages (M ϕ) were treated overnight with increasing micromolar concentrations of PA or with 10^{-6} M ddC, infected with HIV-1 R5 primary isolate ADA at 0.2 MOI for 18 h in the presence of each treatment, washed three times, and returned to culture with each respective treatment for the duration of the experiment; (left panel) p24 pg/ml antigen production in cell-free supernatants at the peak of infection on day 10; (middle panel) calculated % inhibition; and (right panel) % viability. Bars indicate $\pm$ SD, representative of three experiments.

FIG. 2. Inhibition of cell-to-cell and virus-to-cell fusion. (A) GHOST GFP-expressing cells were pretreated overnight with 0, 20, or 40 μ M PA and cocultivated at a 1:1 ratio with NL4-3-infected CEM cells for 24 h. Mixed cell cultures were examined by flow cytometry and percent of GFP-expressing fused cells is indicated inside each panel. Mock cells were cocultivated with uninfected CEM cells and analyzed for background GFP expression. Percent PA-mediated inhibition is indicated on top of each panel. (B) SupT1 cells were treated overnight as indicated on the top of each box, washed, loaded with CCF2/AM dye, and then infected with fusion competent BlaM-Vpr NL4-3 in the absence of treatment. Virus-cell fusion was measured by cleavage of CCF2 dye. Cells were analyzed by multiparameter flow cytometry using a violet laser for excitation of CCF, gated from 10,000 cells. Percent virus-to-cell-fused cells (FUS) and % inhibition (INH) are indicated inside each box. Results are representative of three independent experiments.

FIG. 3. Analysis of cell surface CD4 receptor and lipid rafts distribution. **(A)** Sup-T1 cells were treated overnight with increasing concentrations of PA, or for 5 h with 10 ng/ml PMA that is a positive control for CD4 internalization. All systems were washed and surface labeled with PE-conjugated anti-CD4 antibody, results of which were superimposed over negative control (gray) unstained cells. Cells were analyzed on a BD LSR II flow cytometer. Internalization of cell surface CD4 was measured by the shift in the mean fluorescent intensity, and percent internalized (Int) and percent surface (Sur) CD4 receptor are marked inside each box. Representative of two independent experiments. **(B)** Lipid rafts of 1G5 T cells that were labeled with 1 μg/ml CTB-HRP. Labeled cells were then incubated for 1 h in media containing 20 μM palmitic acid (PA), 20 μM myristic acid (MA), or no fatty acid (No Treatment). Cells were then lysed in cold 0.1% Triton X-100 and lipid rafts were isolated by sucrose density gradient centrifugation. Five gradient fractions were collected and assayed for HRP activity. The CTB-HRP distribution is reported as a percent of total activity. Lipid rafts are expected to be present in fractions 1 and 2, which contain the 5%/35% sucrose interface. Shown is the average CTB-HRP distribution for three independent experiments. Bars indicate $\pm$ 1 SD.

To confirm that PA specifically inhibits CD4-dependent entry, we utilized infectious envelope-deficient and luciferase-positive HIV-1 (HIV-Env⁻Luc⁺), which we pseudotyped with a CD4-independent vesicular stomatitis virus (VSV-G) protein envelope,²⁴ or with a CD4-dependent HXB2 envelope (Fig. 4B). To examine single-cycle viral replication, CEM cells were treated with 10 or 20 μM PA and infected with each pseudotyped virus; luciferase expression was quantified 72 h after infection. Inhibition of infection with CD4-dependent HXB2-enveloped virus was quantified at 32% and 65%. However, inhibition of productive infection with CD4-independent VSV-G-enveloped virus was limited to 2% and 12%, also suggesting that PA treatment posed no intracellular

restriction to CD4-independent virus replication once the virus was allowed to bypass CD4 receptor restriction and enter the host cell. Collectively, these results indicate that PA blocks HIV-1 infection by associating with the CD4 receptor and thus limiting virus entry into cells.

Palmitic acid dose-dependent binding to CD4

To conclusively determine the association of PA and CD4, we used one-dimensional STD-NMR³² to characterize binding of PA to sCD4, *in vitro*. STD-NMR experiments are typically used to probe low-affinity interactions ($K_d \sim 10^{-8}$ to 10^{-3} M) between small molecules (ligand) and proteins,

FIG. 4. Palmitic acid inhibits gp120-CD4 complex formation and infection with CD4-dependent envelope. **(A)** Inhibition of gp120-CD4 complex formation was investigated by gp120 capture ELISA. Envelope gp120 (IIIB) protein was captured on 96-well plates, washed, and incubated in the presence of CD4-biotin alone or in the presence of increasing concentrations of PA, as indicated. Streptavidin-HRP was added, and then developed by the addition of a chemiluminescent substrate, *o*-phenylenediamine dihydrochloride (OPD). Reaction was stopped by adding 1 N HCl and read at 490 nm. Percent inhibition of the complex formation was calculated from untreated (0 μM PA) that was taken as 0% inhibition. **(B)** CEM cells were treated overnight with 10 or 20 μM PA, then washed and infected in the absence of treatment with infectious HIV-Env⁻Luc⁺ virus pseudotyped with either CD4-independent VSV-G or with CD4-dependent HXB2 envelope, to yield single round viral replication. Infection was quantified by luciferase expression 48 h after infection and reported as % inhibition of infection without treatment. Bars indicate ± 1 SD, representative of three independent experiments.

and are routinely used in drug discovery screening tests.³² Saturation transfer from protein to ligand protons identifies ligand binding to a protein, and takes place only when the ligand specifically binds to the protein, with a rate that depends on the protein mobility, ligand-protein complex lifetime, and binding geometry. Protons of the ligand having the strongest contact with the protein show the most intense STD-NMR signals, enabling the mapping of the ligand's binding epitope. During the STD-NMR experiment, increasing concentrations of PA were titrated into the sCD4 solution, and we observed PA binding to sCD4 as shown by an increase in the intensity of the ligand STD-NMR signal (Fig. 5A and B). The amount of STD was quantified by calculating the difference between the intensity of one signal in the off resonance, or reference NMR spectrum (I_0), and the intensity of a signal in the on-resonance NMR spectrum (I_{sat}), for various concentrations of the ligand (Fig. 5C). As the concentration of PA increased, we observed a steady increase of the STD signal from the CH₂ and CH₃ groups of PA (Fig. 5B). Methylene groups located close to the carboxyl end of PA did not exhibit an STD signal during titration. Based on these results, we concluded that PA binds to sCD4 by utilizing a hydrocarbon chain located at a distance from the negatively charged end of the fatty acid. No STD-NMR signal was observed during titration of MA into the sCD4 solution, which was consistent with our results showing no HIV-1 inhibition by MA (data not shown). This negative result indicates that PA binds to sCD4 specifically. We used tryptophan fluorescence of sCD4 to estimate

the binding affinity of PA. Saturating concentrations of PA quenched 30% of the sCD4 tryptophan fluorescence and resulted in a red shift of the emission peak of 2 nm. Based on the fluorescence titration experiments (Fig. 5D and 5D insert), we estimated the dissociation constant (K_d) to be $\sim 1.5 \pm 0.2$ μM.

Discussion

We demonstrated the ability of PA to block X4 and R5-tropic HIV-1 infection and virus replication in T cells, in primary PBL and Mφs (Fig. 1). The reason for the observed differences in the inhibitory concentrations of PA between PBL and Mφs is unclear; it is possibly due to intrinsic differences in receptor and coreceptor frequencies in these primary cells. Differences between laboratory-adapted NL4-3 infection of PBL as opposed to primary isolate ADA infection of Mφs, and the level of infection that was approximately 3.4-fold higher in Mφs as compared to PBL, may also explain differences in inhibition rates between these primary cells. PA also blocked cell-to-cell fusion (Fig. 2A), which has physiological relevance considering that this is one of the mechanisms of T cell depletion *in vivo*.²⁹ Cell-to-cell fusion is normally mediated between infected cells expressing a viral envelope gp120 protein that fuses with the cell surface CD4 receptor of the host cell and thus form syncytia; PA treatment limited this specific gp120-CD4 interaction up to 70%. We further established PA inhibition specificity by demonstrating the ability of PA to explicitly block virus-to-cell fusion (Fig. 2B). We also determined

FIG. 5. *In vitro* binding experiments and analysis of sCD4 with PA. (A) Homonuclear NMR spectrum of free 100 μM PA in the NMR buffer (10 mM KPO_4 buffer, pH 7.0, 20% d_6 -DMSO, 80% D_2O). Thirteen PA methylene groups located away from the PA carboxyl terminal resonate at 1.5 ppm. PA methylene groups located close to the PA carboxyl terminal resonate at 2.27 ppm and 1.57 ppm, respectively. (B) STD-NMR signal of PA bound to sCD4. The increase of the methylene STD-NMR signal of PA at 1.5 ppm is observed with the increase of the PA concentration in the sample of 14 μM sCD4 dissolved in the NMR buffer: (1) no PA, (2) molar ratio of sCD4 to PA is 1:0.1, (3) 1:0.6, (4) 1:0.8, (5) 1:1.2, (6) 1:2, (7) 1:3, (8) 1:5, (9) 1:7, and (10) 1:10. The nonzero STD-NMR signal of PA indicates that PA directly binds to sCD4. PA methylene groups $-(\text{CH}_2)_{13}-$ that show a large STD-NMR signal constitute the binding epitope of PA for CD4. Peaks from the PA methylene groups located close to the PA carboxyl terminal that are not part of the binding epitope are suppressed in the STD-NMR spectrum. (C) Fractional STD effect of the $-(\text{CH}_2)_{13}-$ signal at a given PA concentration. The gradual decrease of the STD effect indicates that the PA-sCD4 complex is specific. (D) Fluorescence titration experiment of sCD4 with increasing concentration of PA. This experiment was used to estimate the binding affinity of PA for sCD4. Tryptophan fluorescence was measured using an excitation wavelength of 280 nm. An increase of PA causes a red shift of 2 nm and quenching of the tryptophan fluorescence of sCD4. Inset: Binding isotherm of the normalized sCD4 tryptophan fluorescence with increasing concentration of PA at the emission wavelength of 350 nm. Curve fitting (OriginLab) using a single site binding isotherm approximation resulted in the best value for K_d to be $1.5 \pm 0.2 \mu\text{M}$.

that HIV-1 inhibition by PA was not due to internalization of the cell surface CD4 receptor or to lipid raft disruption (Fig. 3A and B, respectively).

PA treatment inhibited specific gp120-CD4 complex formation in a dose-dependent manner (Fig. 4A), suggesting that

PA blocks HIV-1 infection by interfering with virus gp120 envelope-efficient attachment to the CD4 receptor. Inhibition was mediated against the CD4-dependent HXB2 envelope but not against the CD4-independent VSV-G envelope (Fig. 4B). Productive infection by the CD4-independent HIV-1 envelope

also demonstrated that PA does not pose an intracellular restriction to virus replication, once receptor restriction is bypassed. This is in agreement with data showing partial inhibition of HIV-1 reverse transcriptase by oleic and linoleic acid contained in the SP4-2 fraction but not by PA.^{12,13}

The definitive specificity of the PA and CD4 interaction was demonstrated by STD NMR experiments, which showed that the binding epitope of PA for sCD4 consists of methyl and methylene groups located away from the carboxyl terminal (Fig. 4), suggesting that these groups are responsible for CD4 binding affinity and that, in addition, the PA carboxyl group may block viral attachment to the receptor. The binding affinity between PA and sCD4 was estimated to be $\sim 1.5 \pm 0.2 \mu\text{M}$. MA, which has the same saturated structure and is only two carbon atoms shorter than PA, did not block HIV infection or bind to CD4 (not shown), which demonstrated a high level of specificity of PA to CD4 binding and consequent inhibition of virus entry. Collectively, these results demonstrate that PA binds to sCD4 by utilizing a hydrocarbon chain located away from the negatively charged carboxyl group, which blocks efficient gp120-CD4 interaction.

PA is a natural small molecule that specifically inhibits HIV-1 fusion to the CD4 receptor and probably functions independently of viral coreceptors. However, ARV drugs that interact with host cell receptors could have profound immunomodulatory effects, such as T lymphocyte proliferation, T cell activation, cytokine production, apoptosis, and B cell production of immunoglobulins. In contrast to systemically administered ARV therapeutics, vaginal microbicides that are normally applied topically in a gel formulation may have different pharmacodynamics,³³ as was recently demonstrated with glycerol monolaurate (GML), which prevented mucosal SIV transmission.³⁴ It is of interest to note that GML has physical and chemical properties similar to PA, indicating that PA is a lead molecule for microbicide development. Although PA efficacy of inhibition is limited, understanding the structure-affinity relationship between PA and the CD4 receptor should allow for the rational design and development of PA analogs with enhanced potency against HIV-1 entry; these possibilities with respect to PA applications are currently under investigation.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Mario Canki

Albany Medical College

Center for Immunology and Microbial Disease, MC-151

47 New Scotland Avenue

Albany, New York 12208

E-mail: cankim@mail.amc.edu