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Amino acid derivatives of the (−) enantiomer of gossypol are effective fusion inhibitors of human immunodeficiency virus type 1

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ABSTRACT

T20 and maraviroc are the only two currently available entry inhibitors that have shown efficacy in treating HIV-1-infected individuals who have failed to respond to first-line antiretroviral drugs. Gossypol is a polyphenolic aldehyde extracted from cotton plants. By modifying the (−) enantiomer of gossypol with a series of small molecules, we have found that neutral amino acids with aliphatic group derivatives of (−) gossypol show the strongest inhibitory activity and the lowest cytotoxicity *in vitro* among all the derivatives tested. Additionally, the selectivity index of the (−) gossypol-neutral amino acid conjugates is increased 100-fold when compared with (−) gossypol alone. It is widely accepted that gossypol and gossypol derivatives inhibit HIV-1 replication by targeting reverse transcriptase. However, from the results of our time-of-addition assay, HIV-1-mediated cell fusion assay and VSV-G pseudotyped virus assay, we demonstrate that the alanine-(−) gossypol derivative ((−)G-Ala) is an effective HIV-1 entry inhibitor. Further mechanistic analysis revealed that (−)G-Ala neither blocks gp120-CD4 binding nor interacts with the HIV-1 co-receptor CXCR4. Results from sandwich ELISA, native-PAGE and circular dichroism (CD) show that (−)G-Ala inhibits the cell fusion-activated gp41 core domain. Moreover, (−)G-Ala binds to the HIV-5-Helix protein and blocking D-peptide (PIE7) binding to the hydrophobic pocket on the surface of the gp41 internal trimeric coiled-coil domain. The contraceptive properties of (−) gossypol and amino acid derivatives of (−) gossypol are also discussed. Collectively, our results indicate that (−)G-Ala may bind to the gp41 hydrophobic pocket and block the formation of the cell fusion-activated gp41 core to inhibit HIV-1-mediated membrane fusion and subsequent viral entry.

1. Introduction

Since the human immunodeficiency virus type 1 (HIV-1) was isolated from a patient with acquired immune deficiency syndrome (AIDS) in 1983, the most important milestones achieved in HIV/AIDS research have been the development of anti-HIV drugs (Fauci, 2003). Of the 32 anti-retroviral drugs currently used in the treatment of HIV infection, most are reverse transcriptase and protease inhibitors. Although the success of these drugs in improving and extending the lives of patients infected with HIV is remarkable, the toxicity and the emergence of drug-resistant strains of HIV greatly restrict the liberal usage of these drugs. Currently, there are two FDA-approved HIV-1 entry inhibitors, T20 (brand name Fuzeon), which targets the viral envelope glycoprotein gp41 (Chen et al., 1995; Huang et al., 2003; Lalezari et al., 2003; Wild et al., 1994), and maraviroc (brand name Selzentry), which blocks the binding of HIV-1 gp120 to CCR5 to prevent the events leading to membrane fusion (Dorr et al., 2005). Limitations to T20 use include low oral bioavailability as well as a high cost of production, while limitations of maraviroc include a lack of activity against patients infected with the T-cell tropic X4 virus.

The mechanism of HIV-1 entry into host cells is a complex process that involves the triggering of a series of nonspecific interactions between the positively charged domains on the gp120 viral surface protein and the negatively charged proteoglycans present on the target cellular membrane (Mondor et al., 1998; Moulard et al., 2000). An alternative mechanism of HIV-1 entry involves a cascade of specific interactions between the HIV-1 and host cell surface lectin binding proteins, such as DC-SIGN (Tilton and Doms, 2010), followed by gp120 subunit binding with cellular CD4 receptors. Subsequent conformational changes in gp120 facilitate the binding of gp120 to chemokine co-receptor CCR5 or CXCR4. There is an additional structural conformational change that enables the insertion of the fusion peptide into the host cell membrane, which facilitates the final fusion step of the HIV-1 and host cell membranes by an unknown mechanism (Chan and Kim, 1998; Lin

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et al., 2003; Wyatt and Sodroski, 1998). The ectodomain of gp41 contains three major functional domains: the fusion peptide (FP), the N-terminal heptad repeat (NHR or HR1), and the C-terminal heptad repeat (CHR or HR2) (Jiang et al., 2004). Previous studies have shown that the gp41 NHR and CHR peptides form a stable α-helical trimer that then forms an antiparallel heterodimer and represents the cell fusion-activated gp41 core (Lu and Kim, 1997). Crystallographic analysis confirms the formation of a six-stranded α-helical bundle (6-HB) in which three N-helices associate to form the central trimeric coiled-coil and three C-helices pack obliquely in an antiparallel manner into the highly conserved hydrophobic grooves on the surface of the coiled-coil trimer (Chan et al., 1997; Tan et al., 1997; Weissenhorn et al., 1997). Each of the grooves on the surface of the N-helix trimer has a deep pocket that harbors three conserved hydrophobic residues in the gp41 CHR region (Chan et al., 1997). Thus, the conserved pocket is an attractive target for drugs that could potently bind to and block 6-HB formation, resulting in the interruption of the fusion between the viral and cellular membranes.

Gossypol is a polyphenolic aldehyde extracted from cottonseed that has been used as a male contraceptive (Anon., 1978). Gossypol has been shown to inactivate HIV in an *in vitro* system (Polsky et al., 1989), albeit with poor efficiency. Previous studies have suggested that the (−) enantiomer of gossypol has good antiviral activity against HIV-1 in peripheral blood mononuclear cells against human immunodeficiency virus type 1 at a concentration 20-fold lower than the concentration at which cytotoxicity is observed; however, there is also evidence that in the cutaneous T cell H9 cell line, the (−) enantiomer is not only more potent against HIV-1 than in PBMCs, but also more cytotoxic (Lin et al., 1989). The present study focuses on amino acid-(−) gossypol derivatives in an attempt to develop potential drugs that can better inhibit HIV-1 activity, decrease cellular cytotoxicity, and avoid the male antifertility activity of gossypol. Furthermore, we investigate the mechanism of inhibition of HIV-1-induced membrane fusion by amino acid-modified (−) gossypol as well as the effect of amino acid modification on the bioavailability of (−) gossypol with the ultimate goal of designing a more potent HIV-1 fusion inhibitor.

2. Materials and methods

2.1. Cell culture and reagents

MT-2 cells and HIV-1 IIIB chronically infected H9 (H9/HIV-1 IIIB) cells were grown in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin–streptomycin (Gibco, USA). U373-MAGI-CXCR4CEM cells were grown in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, 0.1% hygromycin, 0.01% puromycin and 0.2% G418. Monoclonal antibodies (mAb) specific to the gp41–6-HB complex (isotype NC-1), CXCR4 (isotype 12G5) and primary HIV-1 93TH051 (Env subtype E, X4R5) were obtained from the NIH AIDS Research and Reference Reagent Program. Peptides N36 (SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL), C34 (WMEWD REINNYTSLIHSLIEESQNQQEKNEQELL) IQN17 (RMKQIEDKIEEIESKQ KKIENEIARIKKLLQLTVWGIKQLQARIL) and biotinylated-PIE7 (KGA CDYPEWQWLCAA) with D-amino acids were obtained from GL Biochem Ltd. (Shanghai) (Chan et al., 1997; Eckert et al., 1999; Lu and Kim, 1997; Welch et al., 2007). Rabbit antisera against the mixture of N36/C34 and against IQN17 were obtained as previously described (Jiang et al., 1998). AMD-3100 was purchased from Sigma–Aldrich (USA). Recombinant soluble CD4 (sCD4), gp120 and gp120 antibody were obtained from eEnzyme (USA). Gossypol (7-(8-formyl-1,6,7-trihydroxy-3-methyl-5-propan-2-ylnaphthalen-2-yl)-2,3,8-trihydroxy-6-methyl-4-propan-2-ylnaphthalene-1-carbaldehyde, $C_{30}H_{30}O_8$) was purchased from China Cotton-Unis (China).

2.2. Chemical synthesis of amino acid-(−) gossypol derivatives

The (−) enantiomer of gossypol was prepared by hydrolysis of the Schiff base of the intermediate (−) gossypol bis-(L-tryptophan methyl ester) with an 88.1% yield with an enantiomer excess (ee) of 97%. A series of (−) gossypol bis-Schiff bases were synthesized by treating (−) gossypol with the corresponding amino acid in a suitable solvent (Fig. 1A). The desired products were obtained in good yield and relatively high purity (greater than 95%). The analytical and spectral data of all synthesized compounds agreed with the desired chemical structures.

2.3. Anti-HIV-1 activity assays

The inhibitory activity of the amino acid-(−) gossypol derivatives against the laboratory-adapted HIV-1_{IIIB} strain were examined using the HIV-1_{IIIB}/TZM-bl indicator cell culture system as previously described (Pusch et al., 2006). Briefly, 1.5×10^3 TZM-bl cells were seeded in a volume of 300 μl into each well of a 48-well plate and incubated overnight at 37 °C. The cells were then infected with HIV-1_{IIIB} at a multiplicity of infection (M.O.I.) of 0.1 and incubated at 37 °C for 2 h in the presence or absence of the amino acid-(−) gossypol derivatives. After the incubation period, the culture medium was replaced with fresh medium lacking virus and amino acid-(−) gossypol derivatives. The cells were incubated for two days at 37 °C, after which they were lysed in lysis buffer. Luciferase substrate was added to the cell lysates as recommended by the manufacturer's protocol (Promega). The luciferase activities were quantified using a luciferase reporter assay system (Promega, Madison, WI). The percentage of the compound inhibitory activity was calculated using the following formula: [1-(E-N)/(P-N)]×100%, where N represents the negative control, P represents the positive control and E corresponds to the experimental group. The EC_{50} values (effective concentration for 50% inhibition) were calculated using the computer program Calcusyn (Martinez-Irujo et al., 1998).

The inhibitory activity of the experimental compounds on primary HIV-1 replication was determined as previously described (Neurath et al., 1996). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by standard density gradient centrifugation using Histopaque-1077 (Sigma). PBMCs were cultured in RPMI 1640 medium containing 15% FBS, 1% penicillin–streptomycin and IL-2 (20 units/ml). Three-day PHA-stimulated (5 μg/ml) and polybrene-treated (2 μg/ml) cells were infected with the primary HIV-1 strain 93TH051 (Env subtype E, X4R5) at a M.O.I. of 0.01 in the presence or absence of the tested compounds. The supernatants were collected 4 days post-infection and tested for p24 antigen expression by ELISA using the RETRO-TEK HIV-1 p24 Antigen ELISA Kit (USA). The absorbance of the samples at 450 nm (A450) was read using a Tecan GENios microplate reader (Tecan, Switzerland), and the EC_{50} values were calculated as described above.

2.4. Cytotoxicity assays

The *in vitro* cytotoxicity of the amino acid-(−) gossypol derivatives on TZM-bl cells, GHOST cells, MT-2 cells and PBM cells was determined by MTT colorimetric assay as previously described (Pang et al., 2008). Briefly, 100 μl of several concentrations of each tested compound was added to an equal volume of cells (5×10^5 cells/ml) in each well of a 96-well plate. After incubation at 37 °C for 72 h, 100 μl of the supernatant of each sample was discarded, and 10 μl of MTT solution suspended in phosphate-buffered saline (5 mg/ml), was added to each well, followed by incubation at 37 °C

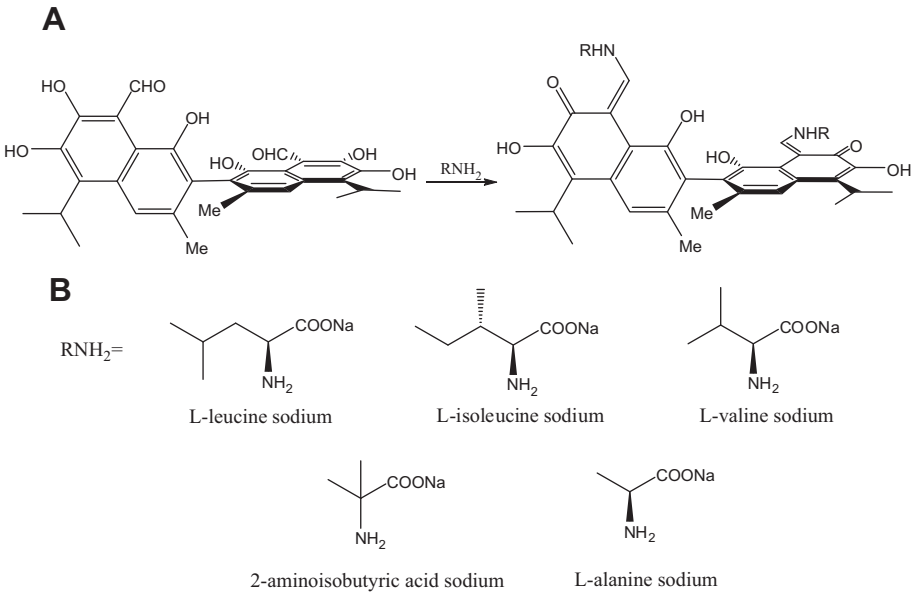

Fig. 1. The scheme of synthesizing amino acid derivatives of (−) gossypol and its chemical structures. A schematic diagram (A) for the synthesis of amino acid (represented by RNH2) derivatives of (−) gossypol as described in Section 2, and showing the amino acids (B) which produced the most effective derivatives of (−) gossypol.

for 4 h. Subsequently, 100 μl of a 50% DMF, 20% SDS solution was added to each well. After the formazan was dissolved completely, the absorbance at 595/630 nm was recorded using a Tecan GENios microplate reader (Tecan, Switzerland). The percent cytotoxicity and the 50% cytotoxicity concentrations (CC_{50}) were calculated using the software Calcusyn.

2.5. In vitro spermicidal assays

In vitro spermicidal assays were conducted as previously described (Manmade et al., 1983) to investigate the spermicidal activity of the amino acid derivatives of the (−) enantiomer relative to (−) gossypol. Briefly, the test compounds (100 μM) were incubated with mouse sperm in Hank's Balanced Salt Solution (HBSS), and then, the percent motility of the sperm was measured using a hemocytometer at 5, 10, 20, 40 and 60 min after co-culture.

2.6. Time-of-addition assays

TZM-bl cells were seeded in 48-well plates and then incubated with HIV-1_{IIIB} at 37 °C for 0, 1, 2, 3, 4, 6, and 8 h (Jiang et al., 2004) before adding each compound at a concentration of 10 μM. Then, the cells were co-cultured with virus at 37 °C for 2 h. After co-culture, the culture medium was exchanged with medium without compound and incubated at 37 °C for 2 days. The cells were then harvested, lysed and assayed for luciferase activity as described above. AZT and T20 were used as negative and positive control treatments, respectively.

2.7. Cell–cell fusion assay

The inhibition of HIV-1-mediated cell–cell fusion was estimated as previously described (Liu et al., 2005b). Briefly, 6×10^4 MT-2 cells were suspended in a volume of 50 μl and co-cultured with 2×10^4 H9/HIV-1 IIIB cells suspended in 50 μl in 96-well plates and incubated at 37 °C for 2 h in the presence or absence of the derivatives. After 6 h incubation, the number of syncytia was counted. The percentage inhibition of cell–cell fusion and the EC_{50} values were calculated as described above.

2.8. Infectivity assay

Inhibition of fusion between two virus-infected cell lines by the amino acid-(−) gossypol derivatives was analyzed as previously described (Pang et al., 2008). The first cell line used was GHOST-CXCR4 cells infected with HIV-1 NL4-3-*luc* pseudotyped virus expressing the env gene of the HIV-1 HXB2 (X4) strain. The second cell line used was human 293T cells infected with a VSV-G viral strain, which is a chimeric virus composed of the HIV-1 NL4-3-*luc* core and the envelope glycoprotein of vesicular stomatitis virus. GHOST-CXCR4 cells or human 293T cells (300 μl at 10^5/ml) were seeded into the 48-well plates and then incubated overnight at 37 °C. A series of mixtures of each of the viruses (50 μl) and a test amino acid derivative (50 μl at increasing concentrations) were prepared and incubated at 37 °C for 30 min. They were then added to the cells and incubated at 37 °C for 2 h. The supernatants of the cells were replaced with fresh culture medium without the derivatives and the cells then incubated at 37 °C for 2 days, harvested and lysed with the lysis buffer. The luciferase activity due to virus infection was measured and EC50 values were calculated as described above.

2.9. Inhibition of gp120 binding to CD4

An enzyme-linked immunosorbent assay (ELISA) experiment to assess the ability of (−)G-Ala to inhibit binding between gp120 and CD4 was performed according to a previously published protocol with minor modifications (Zhao et al., 2005b). Briefly, U373-MAGI-CXCR4CEM cells were cultured in 96-well plates overnight at 37 °C. The culture medium was removed, and cells were fixed with 5% formaldehyde diluted with 0.01 M PBS at room temperature for 15 min. The cells were incubated in a blocking solution of 5% non-fat milk overnight at 4 °C and then washed once with PBS with 0.05% Tween-20 (PBS-T). Then, 100 μl of a mixture of the appropriate concentrations of (−)G-Ala and recombinant gp120 (at a final concentration of 1 μg/ml) was diluted in 1% non-fat milk and added to the cells. After incubating the plate at 37 °C for 1 h, the plate was washed three times with PBS-T. Mouse anti-gp120 IgG (0.25 μg/ml) was added to each well, and the samples were incubated at 37 °C for 1 h and subsequently washed

three times with PBS-T. Then, biotinylated goat anti-mouse IgG (0.25 μg/ml) and streptavidin-labeled horseradish peroxidase (SA-HRP) (0.25 μg/ml) were added to each sample and incubated at 37 °C for 1 h. The samples were developed by adding the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) to each well, followed by incubation at room temperature for 20 min. Color development was stopped with 2 M H_2SO_4, and the absorbance of each sample was read at 450 nm (A450) as described above.

2.10. Cell-based ELISA for determination of anti-CXCR4 antibody binding to CXCR4-expressing cells

A cell-based ELISA experiment was performed as previously described (Zhao et al., 2002). Briefly, U373-MAGI-CXCR4CEM cells were seeded in a 96-well plate, fixed with 5% formaldehyde and blocked with 5% non-fat milk overnight at 4 °C. A mixture of appropriate concentrations of (−)G-Ala and mouse anti-CXCR4 antibody (isotype 12G5, 0.25 μg/ml) was diluted with 1% non-fat milk and added to each sample, and then the samples were incubated at 37 °C for 1 h. Samples were washed three times with PBS-T, then biotin-labeled rabbit anti-mouse IgG (0.25 μg/ml) was added and incubated at 37 °C for 1 h. SA-HRP (0.25 μg/ml) and TMB were subsequently added to each sample as described above. The A450 value for each sample was read using a Tecan GENios microplate reader (Tecan, Switzerland). AMD3100 was used as a positive control.

2.11. Sandwich ELISA for detecting gp41 6-HB formation

To identify whether (−)G-Ala specifically targeted gp41 6-HB formation, a sandwich ELISA was performed as previously described (Jiang et al., 1998, 1999). Briefly, equimolar amounts of the N36 and/or C34 peptides were incubated with or without (−)G-Ala (at the appropriate concentrations) at 37 °C for 30 min. The peptide-(−)G-Ala mixtures were added to a 96-well plate that was previously coated with rabbit anti-6-HB serum diluted in a carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. The samples were then blocked with 5% non-fat milk at 37 °C for 2 h. After incubating the samples at 37 °C for 1 h, the samples were thoroughly washed with PBS-T. Then, mAb NC-1 (1 μg/ml) diluted with 1% non-fat milk was added to each well, and the samples were incubated at 37 °C for 1 h followed by PBS-T washes. Biotinylated goat anti-mouse IgG (0.25 μg/ml), SA-HRP (0.25 μg/ml) and TMB were added to each sample as described above. The A450 was read by a Tecan GENios microplate reader (Tecan, Switzerland). The percentage of inhibition of gp41–6-HB formation by (−)G-Ala was calculated as described above (Martinez-Irujo et al., 1998). T20 was included as a positive control.

2.12. Native polyacrylamide gel electrophoresis (native-PAGE)

Native-PAGE was performed as previously described (Liu et al., 2003) to determine the effect of (−)G-Ala on 6-HB formation by the N- and C-peptides. To this end, 50 μM N36 and C34 were incubated with or without (−)G-Ala at 37 °C for 30 min. To each sample, 2× loading buffer was added, and then the mixture was loaded onto 15 × 1.0 mm precast 18% Tris–glycine polyacrylamide gels. Samples were separated electrophoretically using a constant voltage setting of 80 V for 30 min at 4 °C. Then the voltage was increased to 120 V for an additional 120 min at 4 °C. After electrophoresis, the gel was stained with Coomassie blue and imaged by an Epson scanning system.

2.13. Western blotting

Western blotting was used to identify gp41–6-HB complex formation. For Western blot experiments, samples were separated as described in the native-PAGE method section, followed by transfer to nitrocellulose filter membranes. The membranes were blocked in TBS with 5% non-fat milk for 30 min at room temperature, then the membranes were incubated for 1 h at room temperature in the presence of an antibody specific for the gp41–6-HB complex (isotype NC-1). After washing three times for ten minutes each with TBS, HRP-conjugated goat anti-mouse antibody was added to the membranes for 30 min incubation at room temperature. The membranes were washed three times in TBS for 5 min each. A chemiluminescent substrate was then added to the membrane for 3 min at room temperature, and the membranes were exposed to autoradiographic film. The developed films were scanned using an Epson scanning system.

2.14. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed to determine the conformation of the NHR and CHR peptide complexes using a previously described method (Liu et al., 2003). First, the N36 and the C34 peptides were suspended in PBS to obtain the mother liquid from which the concentrations were calculated based on the following calculation: mg of peptide per ml = (A280 × DF × MW)/e. A_{280} represents the absorbance of the solution at 280 nm in a 1-cm cell, DF represents the dilution factor, MW represents the molecular weight of the peptide and e represents the molar extinction coefficient of each chromophore at 280 nm. Then, equimolar amounts of the two peptides were mixed in PBS with 10 μM (−)G-Ala. Separate reactions were performed in which (−)G-Ala was pre-incubated with N36 peptide before the addition of C34. After incubating the mixtures at 37 °C for 30 min, the samples were cooled on ice and analyzed using CD spectroscopy. Sample CD spectra were obtained using a Jasco J-810 CD spectropolarimeter (Jasco Inc., Japan) from 240 to 200 nm using a quartz cuvette with a 0.1 mm optical path. Other parameters include a 0.2 nm step size, 5 nm bandwidths, and 5 s signal averaging time. Scans were performed in triplicate, and spectra were corrected based on the spectra obtained from the buffer.

2.15. Nuclear magnetic resonance

All nuclear magnetic resonance (NMR) experiments were performed on a Bruker Avance III 500-MHz spectrometer equipped with a 5 mm BBI-Z H-X-D probe head at 300 K. The water proton signal was suppressed using the W5 water suppression by gradient tailored excitation. The 5-Helix protein was over-expressed in an *Escherichia coli* strain BL21 (DE3) that was transformed with the recombinant plasmid pGEX-6p-1-5-Helix and purified by glutathione–sepharose 4B affinity column purification. Samples were prepared for NMR by suspending the 1 mM 5-Helix protein and 10 μM (−)G-Ala in 500 μl of PBS containing 10% D_2O. Then, 10 μM (−)G-Ala and 1 mM 5-Helix protein were incubated in 500 μl PBS containing 10% D_2O at 37 °C for 30 min. ^1H NMR spectra were acquired after 256 scans with a 2 s relaxation delay over a spectral width of 8000 Hz. A sequence of Gaussian-shaped pulses with a frequency of 86 Hz and a length of 50 ms separated by 1 ms delays was carried out for 2.04 s. NMR data acquisition was performed using the software Topspin 2.0 (Bruker, Inc.) and analyzed using the software MestReC 4.9.9.9.

2.16. Inhibition of biotinylated PIE7 binding to IQN17

To measure binding between the D peptide, a biotinylated version of PIE7, and IQN17, an ELISA assay was performed as previously described (Jiang et al., 2004). Briefly, a 96-well polystyrene plate was pre-coated with 2 μg/ml rabbit anti-IQN17 IgG serum overnight at 4 °C. Then, 10 μM IQN17 peptide was incubated with

various concentrations of (−)G-Ala at 37 °C for 30 min, followed by the addition of 5 μM biotinylated PIE7 and incubated again at 37 °C for 30 min. The mixture was subsequently added to the ELISA plate and incubated at 37 °C for 30 min. After three times washes with PBS, biotinylated-PIE7 that remained bound to IQN17 was quantitated by the addition of SA-HRP (0.25 μg/ml) and TMB sequentially. The A450 was read using a Tecan GENios microplate reader (Tecan, Switzerland), and the EC_{50} values were calculated as described above. AMD3100 was used as a control.

3. Results

3.1. Selection of the most effective amino acid derivatives of (−) gossypol for the inhibition of HIV-1 entry

A series of small molecules including essential amino acids, non-essential amino acids, small peptides and other chemical groups was covalently attached to gossypol, and their inhibitory activities were tested using the TZM-bl indicator cell culture system (data not shown). We identified five derivatives that had stronger inhibitory activity than any of the other derivatives and chose these for subsequent study (Fig. 1B). The (−) enantiomer of gossypol but not the (+) enantiomer had good antiviral activity in peripheral blood mononuclear cells against human immunodeficiency virus type 1 at a concentration more than 20-fold lower than that required for cytotoxicity (Lin et al., 1989). To further enhance its activity, we modified (−) gossypol using the five amino acids listed in Table 1. The results show that the activity of the amino acid-modified (−) gossypol had been improved by a factor of approximately ten when compared to (−) gossypol alone.

3.2. The amino acid-(−) gossypol derivatives have strong inhibitory activities on infection by laboratory-adapted HIV-1 strains and primary HIV-1 isolates, but low in vitro cytotoxicity

The TZM-bl indicator cell culture system and the p24 antigen ELISA were used to confirm the inhibitory activity of (−) gossypol and its amino acid derivatives against the laboratory-adapted HIV-1 strain $HIV\text{-}1_{IIIB}$ as well as primary HIV-1 isolate. The EC_{50} values of HIV-1 infection are displayed in Table 1. All of the amino acid derivatives were able to effectively inhibit the laboratory-adapted strain $HIV\text{-}1_{IIIB}$ and primary HIV-1 isolates with EC_{50} values ranging from 0.4 to 1 μM and from 0.3 to 0.8 μM, respectively. These results suggest that the amino acid moiety of the (−) gossypol derivatives have significant inhibitory activities on HIV-1 entry, albeit with different potency. Concentrations of drug that did not cause detectable changes in cell morphology or loss of monolayer adherence to the culture plate were considered to be below the threshold of cytotoxicity (Royer et al., 1995). All experiments conducted in our study used drug concentrations that were below the cytotoxicity threshold. The (−) enantiomer of gossypol caused nearly 20% of monolayer destruction at a concentration of 5 μM. At a concentration of 2.5 μM, (−) gossypol showed only approximately 40% inhibition of HIV-1 fusion. Collectively, these results imply that although (−) gossypol shows some anti-HIV-1 activity, it is overall less active and more cytotoxic than its amino acid derivatives.

Compared with (−) gossypol, the amino acid derivatives had low *in vitro* cytotoxicity as determined by MTT colorimetric assays. The amino acid derivatives lowered *in vitro* cytotoxicity in TZM-bl, GHOST-CXCR4, MT-2 and PBMCs with a CC_{50} approximately 5-fold lower than that of (−) gossypol (Table 2).

3.3. The amino acid-(−) gossypol derivatives have low spermicidal activity in vitro

To compare the spermicidal activity of the amino acid-(−) gossypol derivatives and unmodified (−) gossypol, an *in vitro* spermicidal assay was conducted. Our results show that 250 μM is the minimum concentration of (−) gossypol required for completely inhibition of mouse sperm motility within 10 min (data not shown). Next, we compared the effect of the amino acid derivatives with (−) gossypol on sperm motility and found that exposure of sperm to either the amino acid derivatives or (−) gossypol at a concentration of 100 μM for up to 1 h resulted in less spermicidal activity when the amino acid derivatives were used than when (−) gossypol was used (Fig. 2).

3.4. The alanine-(−) gossypol derivative inhibits HIV-1 replication by interfering with membrane fusion

By comparing the activity and cytotoxicity profiles of the various amino acid-(−) gossypol derivatives, we found that the alanine-(−) gossypol conjugate ((−)G-Ala) had the highest selectivity index (Table 1). We suspect that the increased activity against HIV-1 is attributable less to the amino acid moiety and more to the (−) gossypol moiety (see discussion for further explanation). We also suspect that the mechanism of the various amino acid derivatives must be the same. Thus, we chose to use (−)G-Ala in the following experiments.

We used a time-of-addition assay to identify which phase of HIV-1 life cycle is interfered by (−)G-Ala. Our results show that (−)G-Ala inhibits HIV-1 in a manner very similar to T20. (−)G-Ala was only effective against HIV-1 infection when added to the cells simultaneously with virus, but we noticed no inhibitory activity if (−)G-Ala was added more than one hour after the cells were exposed to the virus. As a control, reverse transcriptase inhibitor AZT was shown to still be effective in inhibiting HIV-1 replication even if it was added 8 h post-infection (Fig. 3A). These results suggest that (−)G-Ala is an effective inhibitor of HIV-1 infection that targets an early phase of the HIV-1 life cycle.

Table 1
Inhibition of HIV-1 infection by (−) gossypol and its amino acid derivatives.

Compounds	Molecular weight	EC_{50}[a] (μM ± SD)[b]		
		Inhibition of $HIV\text{-}1_{IIIB}$ activity	Inhibition of HIV-1 NL4-3 *luc* activity	Inhibition of P24 production
(−)Gossypol	518.55	–[c]	0.64 ± 0.24	–[c]
(−)G-Ile	788.83	0.52 ± 0.06	0.10 ± 0.05	0.51 ± 0.03
(−)G-Aib	732.73	1.08 ± 0.25	0.34 ± 0.01	0.85 ± 0.01
(−)G-Val	760.78	0.56 ± 0.05	0.17 ± 0.02	0.68 ± 0.05
(−)G-Ala	704.67	0.41 ± 0.04	0.16 ± 0.01	0.36 ± 0.02
(−)G-Leu	788.83	0.76 ± 0.06	0.22 ± 0.03	0.71 ± 0.02

[a] Effective concentration for 50% inhibition by (−) gossypol and its amino acid derivatives to different HIV-1 strains.
[b] The data presented were means ± standard deviations from three independent experiments where each treatment was tested in triplicate.
[c] "–"Means no inhibition.

Table 2
In vitro cytotoxicity and selectivity index by (−) gossypol and its amino acid derivatives.

Compounds	CC_{50}[a] (μM ± SD)[b]				IS[c]	
	TZM-bl	GHOST-CXCR4	MT-2	PBMCs	TZM-bl	PBMCs
(−)-Gossypol	9.74 ± 0.29	6.85 ± 0.61	16.27 ± 3.02	12.86 ± 0.87	–[d]	–[d]
(−)-G-Ile	62.34 ± 12.09	26.48 ± 0.95	65.62 ± 13.45	73.36 ± 4.68	119.88	143.84
(−)-G-Aib	65.10 ± 7.06	53.27 ± 8.21	77.35 ± 4.45	81.43 ± 2.8	60.28	95.80
(−)-G-Val	49.29 ± 6.03	44.30 ± 6.19	62.41 ± 3.46	70.67 ± 2.02	88.02	103.93
(−)-G-Ala	64.10 ± 7.71	63.46 ± 10.65	84.64 ± 12.09	95.08 ± 6.32	156.34	264.11
(−)-G-Leu	53.99 ± 1.44	43.66 ± 3.83	59.17 ± 9.68	68.12 ± 2.24	71.04	95.94

[a] Concentration required to induce cell death by 50% as measured by the MTT method in TZM-bl, GHOST-CXCR4, MT-2 and PBM cells.
[b] The data presented were means ± standard deviations from three independent experiments where each treatment was tested in triplicate.
[c] The selectivity index (CC_{50}/EC_{50}).
[d] "–" Means no calculation.

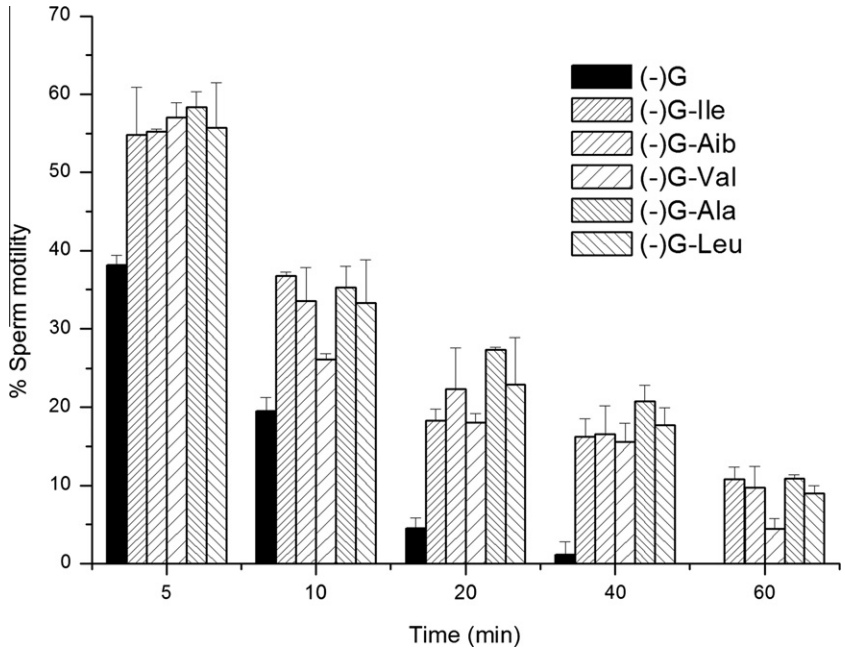

Fig. 2. *In vitro* spermicidal activities of the top five amino acid-(−) gossypol derivatives compared to the unmodified (−) gossypol. The percentage motility of sperms observed after incubation in the presence of the derivatives at a concentration of 100 μM is plotted against the time period of exposure to the derivatives.

Fusion between virus and host cell membranes or between HIV-infected cells and uninfected cells is a critical step for HIV entry into new target cells (Jiang et al., 2004). Therefore, we examined whether (−)G-Ala inhibited virus infectivity and cell–cell fusion. The fusion between H9/HIV-1_{IIIB} cells and uninfected MT-2 cells was detected with a cell–cell fusion assay. As shown in Fig. 3B, (−)G-Ala efficiently inhibited HIV-1-mediated cell–cell fusion with an EC_{50} value of 3.73 ± 0.08 μM. Then, we tested the inhibition of infectivity between the HIV-1 NL4-3-*luc* pseudotyped virus expressing the env gene of the HIV-1 HXB2 (X4) strain and GHOST-CXCR4 cells. Our results show that (−)G-Ala strongly inhibits virus infectivity with an EC_{50} value of 0.16 ± 0.01 μM (Table 1). In summary, these results indicate that (−)G-Ala effectively inhibits HIV-1 entry into host cells.

Transgenic HIV-1 virions expressing VSV-G envelope proteins are thought to enter cells through an endocytic pathway because this is the typical route of VSV infection (Matlin et al., 1982). We used the HIV-1 NL4-3-*luc* pseudotyped virus expressing the envelope proteins of the VSV-G strain to establish whether (−)G-Ala inhibits HIV-1 activity by selectively targeting gp41. As shown in Fig. 3C, (−)G-Ala does not block the replication of VSV-G envelope protein-expressing HIV-1 strains in human 293T cells, even when the (−)G-Ala concentrations used were far in excess of the CC_{50} value. When T20, a known inhibitor of gp41, was used as a positive control, we found an identical inhibition curve with (−)G-Ala, supporting the idea that (−)G-Ala targets gp41. When AZT was used as a negative control, a dramatically different inhibition curve was observed (Fig. S1). These results suggest that (−)G-Ala selectively targets on HIV-1 entry, but not other process.

3.5. (−)G-Ala neither blocks gp120-CD4 binding nor interacts with the HIV-1 co-receptor CXCR4

Three major steps are involved in HIV-1 entry: the interaction between HIV-1 gp120 and the host cell surface receptor CD4 forming the gp120-CD4 complex, the binding between HIV-1 and a co-receptor, such as CXCR4, and membrane fusion mediated by the HIV-1 transmembrane glycoprotein gp41 (Berger et al., 1999; Hu et al., 2005, 2000). We performed a cell-based ELISA to investigate whether (−)G-Ala blocked the interaction between CD4 and gp120, which is an early step during the HIV-1 entry process. Fig. 4A shows that (−)G-Ala does not block the interaction between CD4 and gp120 at 50 μM, similar results were observed when (−)G-Ala was omitted. However, we observed a large difference between (−)G-Ala and the blank control, suggesting that (−)G-Ala does not target gp120-CD4 binding.

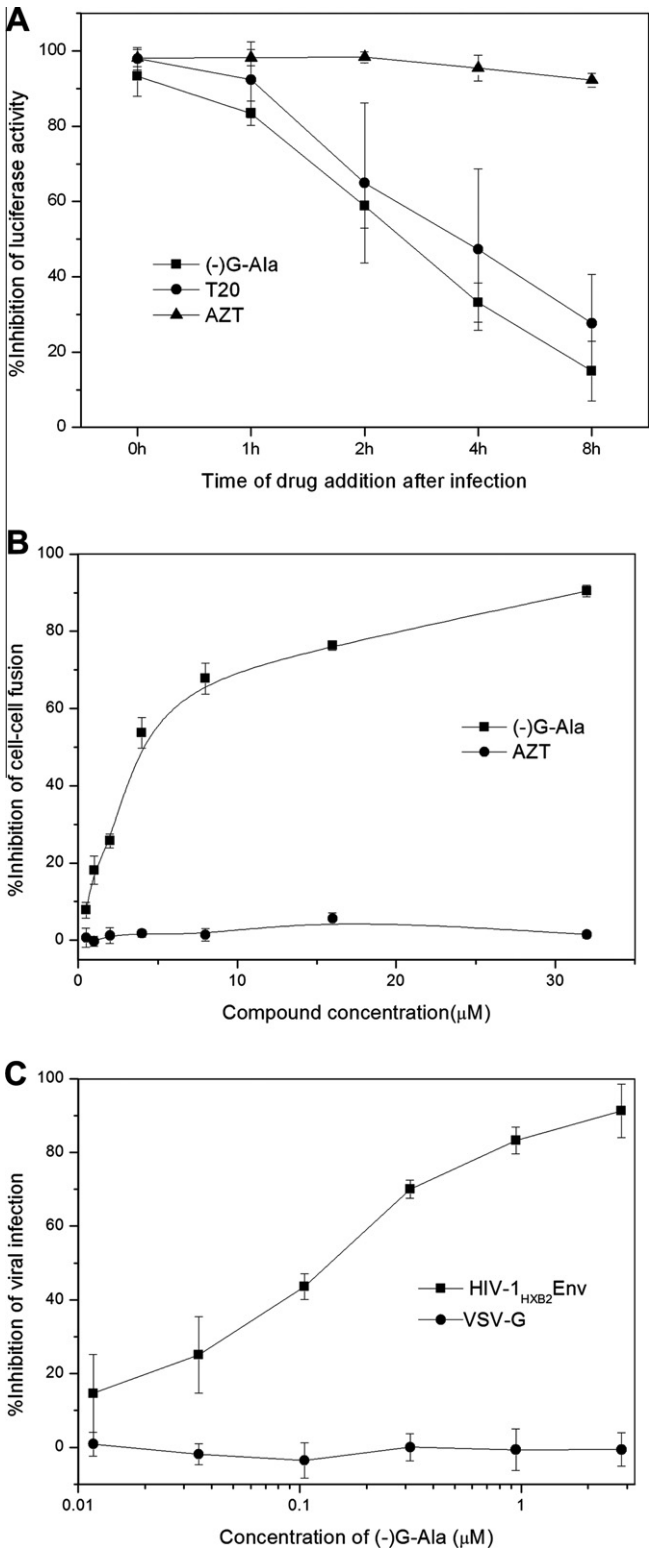

Fig. 3. Results of experiments indicating that (−)G-Ala inhibited HIV-1 entry. Each data point, presented as a mean ± standard deviation bar, was derived from two independent experiments done in triplicate. (A) The time-of-addition experiments showing the possible target stage in the HIV-1 life cycle of (−)G-Ala compared to the drugs AZT and T20. The % inhibition of luciferase activities observed at various times of drug treatments is plotted. (B) The cell–cell fusion assay experiments showing inhibitory activities of (−)G-Ala, compared to that of AZT, on HIV-1 mediated cell–cell fusion between H9/HIV-1_{IIIB} and MT-2 cells under increasing concentrations of the drugs. (C) Results of the experiments comparing the antiviral activities against HIV-1NL4-3-*luc* and VSV-G of increasing concentrations of (−)G-Ala.

A separate cell-based ELISA was used (Zhao et al., 2004) to determine whether (−)G-Ala binds to the HIV-1 co-receptor CXCR4. In this assay, we used an anti-CXCR4 mAb (isotype 12G5) (Wang et al., 2009b) that specifically recognizes CXCR4 and blocks infection of cells expressing CXCR4+ due to HIV-1 infection (McKnight et al., 1997) and the CXCR4-expressing cells, U373-MAGI-CXCR4CEM (Zhao et al., 2002). The small molecule AMD3100, a bicyclam derivative that acts as a potent CXCR4 antagonist and strongly inhibits the binding between the 12G5 anti-CXCR4 antibody and CXCR4 (Donzella et al., 1998; Zhao et al., 2005a), was used as a positive control. C34 peptide, a highly effective inhibitor of HIV-1 entry and cell–cell fusion (Chan et al., 1998) by targeting 6-HB, was used as a negative control. Fig. 4B shows that while AMD3100 potently blocks the binding of the 12G5 anti-CXCR4 antibody to CXCR4 at 10 μM, (−)G-Ala and C34 showed no effect on this interaction even at a concentration of 50 μM, with inhibitory activities of 13.8% at 10 μM and 11.5% at 50 μM. These results support the idea that (−)G-Ala does not interact with the HIV-1 co-receptor CXCR4.

3.6. (−)G-Ala inhibits the interactions between the NHR and CHR peptides to inhibit the formation of the cell fusion-activated gp41 core

HIV-1 env-mediated membrane fusion is thought to occur through a mechanism whereby HIV-1 binding to CD4 and/or a co-receptor triggers a conformational change that forms a coiled-coil in HIV-1 protein gp41 (Bonhaus et al., 1998; Chan et al., 1997). This binding is thought to expose the gp41 fusion peptide, leading to the formation of the six-helix bundle culminating in membrane fusion (Tien, 2005). The formation of the six-helix bundle represents an attractive candidate for the (−)G-Ala mechanism of action. We performed a sandwich ELISA to determine whether (−)G-Ala blocks gp41 cell fusion-activated core formation. This model was tested by mixing equimolar concentrations of the NHR and CHR peptides N36 and C34, respectively, and using the gp41 conformation-specific mAb NC-1 to detect a conformational change during the fusion of HIV-1 with the host cell (Jiang et al., 1998, 1999). The results are shown in Fig. 5A. When (−)G-Ala was first added to a solution of N36 and pre-incubated for 30 min before the addition of C34 and a subsequent 30 min incubation, (−)G-Ala was able to inhibit 6-HB formation in a dose-dependent manner with an EC_{50} of approximately 2 μM. We noticed no inhibitory activity if we incubated (−)G-Ala with pre-assembled 6-HB complexes. Our results indicate that (−)G-Ala can block the activation of the gp41 core and does not disrupt the preformed gp41 core.

We also used a native PAGE (N-PAGE) assay (Liu et al., 2003) to further verify the inhibition of 6-HB formation by (−)G-Ala. Because the N36 peptide has a net positive charge, we observed no band for N36 under N-PAGE conditions, therefore, this sample was omitted (Wang et al., 2009a). We observed a single band in lane 1 when only C34 peptide was loaded and two bands in lanes 2 and 3 when N36 and C34 were mixed and incubated for different lengths of time (Fig. 5B). The lower band observed in lanes 2 and 3) appeared to resolve at the same position as that of lane 1, suggesting that this band represented C34 in an unbound state. The upper band coincided with the 6-HB complex (lane 8), which was confirmed by Western blotting using 6-HB-specific mAb NC-1. When we pre-incubated the N36 peptide with 50 μM (−)G-Ala and then added the C34 peptide to the mixture at a concentration that was equimolar to N36 followed by another 30 min incubation, the band representing the 6-HB complex disappeared (lane 4). If the C34 peptide was pre-incubated with (−)G-Ala and then N36 was added, then the band corresponding to 6-HB complex reappeared (lane 5). As a negative control, the nucleotide reverse transcriptase inhibitor, AZT, was added to the purified peptides C34 and N46 in a sequential manner similarly to the procedure for (−)G-Ala (lanes

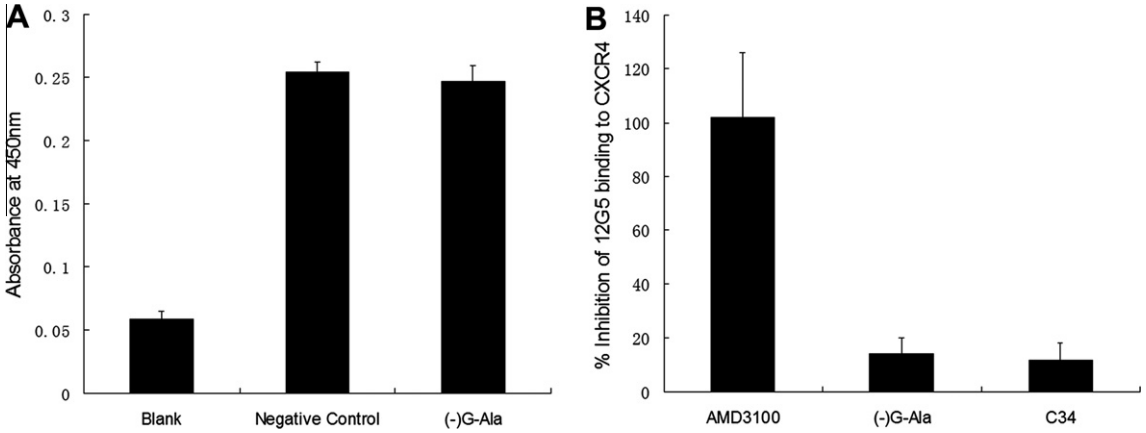

Fig. 4. (−)G-Ala does not block the interaction between gp120 and CD4 or between anti-CXCR4 mAb 12G5 and CXCR4. (A) Results of the CD4-based ELISA experiment investigating whether (−)G-Ala blocked CD4 binding to gp120 at the early phase of HIV-1 entry. Interaction between gp120 and CD4 was detected by using a CD4-based ELISA in the absence or presence of (−)G-Ala, as described in Section 2. The figure shows the absorbance of the samples at 450 nm (A450) as measured by ELISA. (B) Results of the cell-based ELISA experiment investigating whether (−)G-Ala inhibited the binding of anti-CXCR4 antibody 12G5 to U373-MAGI-CXCR4CEM cells. The binding of the anti-CXCR4 mAb, 12G5, to CXCR4-expressing cells, U373-MAGI-CXCR4CEM, was measured in the presence of (−)G-Ala. The figure shows the % inhibition of 12G5 binding to CXCR4 as measured by ELISA. AMD3100, a strong inhibitor of 12G5 binding was used as a positive control, while the peptide C34, which does not inhibit binding of 12G5, was used as the negative control. In both experiments, the data presented were means ± standard deviations from two independent experiments where each treatment was tested in triplicate.

6 and 7). Based on the results above, it appears that (−)G-Ala interrupts the gp41 6-HB formation during its assembly phase but does not disrupt the gp41 6-HB complexes after they have assembled. We also documented a dose-dependence of (−)G-Ala in disrupting 6-HB complex formation by N-PAGE (Fig. 5C). The EC_{50} value observed using N-PAGE was 40 μM, which is much higher than the EC_{50} observed using a sandwich ELISA (3 μM) (Fig. 5A). Wang et al. (2009a) have previously attributed this discrepancy to there being a higher concentration of N36 and C34 peptides used in the N-PAGE assay than in the sandwich ELISA.

3.7. (−)G-Ala greatly suppresses the content of α-helix during the interaction between NHR and CHR peptides

Circular dichroism (CD) experiments were performed to determine whether (−)G-Ala interrupts the α-helical structure of the gp41 6-HB conformation. Lu et al. (1995) demonstrated that the individual NHR peptides have a tendency to aggregate and that CHR peptides have a random coil structure in aqueous solution. We also observed a random coil structure the CHR peptides in aqueous solution (Fig. 6). However, a mixture of the NHR and CHR peptides showed a typical α-helical conformation, measured by CD spectroscopy (Lu and Kim, 1997). This finding suggests that the interaction between the NHR and CHR peptides induces a change in their secondary structure to an α-helical coiled-coil conformation. Based on CD spectra, the α-helical content of 6-HB dramatically decreased by pre-incubating the N36 peptide with (−)G-Ala before adding the C34 peptide (Fig. 6B), indicating that (−)G-Ala interrupts the interaction between N36 and C34 peptides. However, if (−)G-Ala is added to samples containing pre-formed 6-HB complexes, then the impedance of α-helix formation disappears (Fig. 6A). This result suggests that (−)G-Ala blocks the interaction between the gp41 NHR and CHR peptides, thus preventing the formation of 6-HB complexes and inhibiting membrane fusion between HIV-1 and the host cell.

3.8. (−)G-Ala binds to the exposed groove of the HIV-5-Helix protein and also binds to the hydrophobic pocket of the NHR trimer surface when simulated by IQN17

To demonstrate that the (−)G-Ala binds to the gp41 6-HB, NMR experiments were conducted in an HIV-1 strain in which the gp41 6-HB was effectively replaced with the HIV-5-Helix protein, which contains five of the six α-helical coils and only one of the three grooves is exposed because of a missing C-helix (Root et al., 2001). Crucial to our NMR experiments, the N36 and C34 peptide are unable to form this secondary structure separately. Chemical shift mapping is a widely used technique to determine the binding site of a ligand on a protein in which chemical shift changes in the protein (usually of the amide protons and nitrogens) upon addition of the ligand are measured. The largest changes in the NMR spectra are used as an indicator of the likely binding sites (Cioffi et al., 2009). To study drug-protein binding using NMR spectroscopy, methods include measuring the binding-induced chemical shift changes and line width broadening as well as observing any intermolecular nuclear Overhauser effect (NOE) enhancements between the drug and the protein (Roberts, 1993). We observed chemical shift changes and line width changes in the ^1H NMR spectra comparing 5-Helix in the presence of (−)G-Ala with 5-Helix in the absence of (−)G-Ala (Fig. 7A), suggesting an intense binding interaction between (−)G-Ala and the 5-Helix protein.

IQN17 is a peptide chimera that contains the N-terminal portion of GCN4-pIQI (Eckert et al., 1998) and C-terminal fragments of N36. It has a soluble trimeric coiled-coil structure that contains a hydrophobic pocket that mimics the gp41 core. Therefore, IQN17 can be used as a model peptide to screen new fusion inhibitors. Welch et al. (2007) previously demonstrated that PIE7, a short circular D-amino acid peptide, could specifically bind to the pocket presented on IQN17 (Eckert et al., 1999) and inhibit HIV-1 infection (Wang et al., 2009a). We performed an ELISA assays and found that (−)G-Ala could effectively interfere with biotinylated PIE7 binding to the IQN17 peptide with an EC_{50} value of approximately 2.1 μM. The negative control compound, AMD3100, had no effect on the disruption of the interaction between PIE7 and IQN17 (Fig. 7B). This result suggests that (−)G-Ala binds to the hydrophobic pocket on the surface of the gp41 central trimer and blocks the interaction between the viral gp41 NHR and CHR regions, effectively obstructing the formation of the 6-HB and culminating in the inhibition of HIV-1 entry and replication.

4. Discussion

In this study, we made modifications to the potential small molecule HIV-1 fusion inhibitor (−) gossypol, effectively improving the

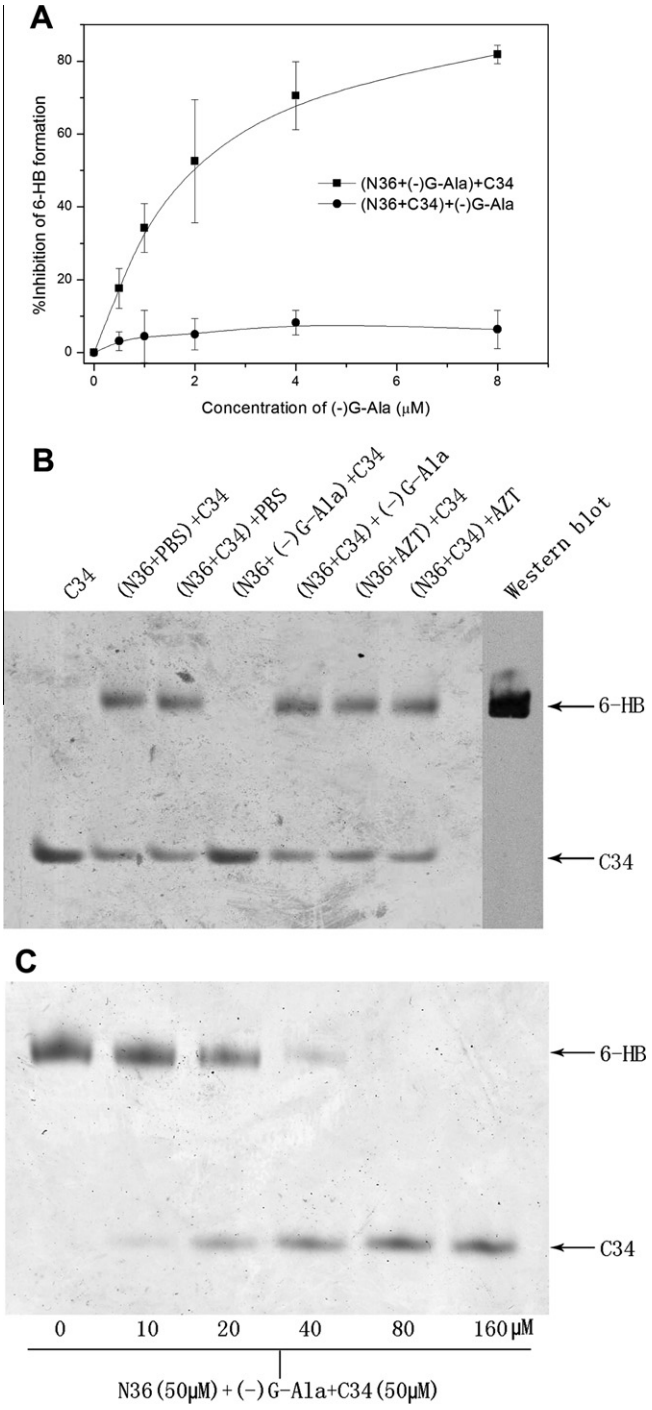

Fig. 5. Experiments showing that (−)G-Ala inhibited 6-HB formation between N36 and C34. (A) Sandwich ELISA experiment showing % inhibition of formation of 6-HB when equimolar amounts of the C34 peptide were incubated with the N36 peptide before the addition of (−)G-Ala or after the N36 peptide was pre-incubated with (−)G-Ala, as described in Section 2. The figure shows the % inhibition of formation of 6-HB under increasing concentrations of (−)G-Ala as measured by ELISA. Each sample was tested in triplicate in two independent experiments, and the data are presented as means ± the standard deviations. (B) N-PAGE experiment showing the inhibition of the formation of the gp41 six-helix bundle by (−)G-Ala. The treatments used are labeled on the top of the lanes. For example, (N36 + (−)G-Ala) + C34 designates the treatment where the peptide N36 was pre-incubated with (−)G-Ala before the addition of the peptide C34 as described in Section 2. After incubation, the mixtures were analyzed by N-PAGE to detect the C34 and 6-HB bands. AZT, which has no effect on gp41 six-helix bundle formation, was used as a negative control. A Western blot of 6-HB is shown in the last lane on the right. C. N-PAGE experiment showing the dose-dependent nature of the inhibition of 6-HB formation by (−)G-Ala. The peptide N36 was incubated with (−)G-Ala at 37 °C for 30 min before the addition of peptide C34. After incubation for another 30 min, the mixtures were analyzed by native PAGE to detect the C34 and 6-HB bands.

anti-HIV-1 potency and increasing the efficacy with a 100-fold improvement in selectivity index. Our results show that mostly neutral, aliphatic amino acid derivatives of (−) gossypol (aminoisobutyric acid, a non-essential amino acid derivative that is not aliphatic, although it demonstrates strong inhibitory activity) demonstrate the strongest inhibitory effect on the laboratory-adapted HIV-1 strain HIV-1_{IIIB} and the primary HIV-1with EC_{50} values ranging from 0.4 to 1.0 μM and from 0.3 to 0.8 μM, respectively (Table 1). The *in vitro* cytotoxicity to TZM-bl, GHOST-CXCR4, MT-2 and PBM cells is much lower in the presence of amino acid derivatives than it is in the presence of (−) gossypol, with the CC_{50} of (−) gossypol being approximately 5-fold higher than that of the derivatives (Table 2). Although we do not present clear evidence that explains this phenomenon, we speculate that this finding may be due to the increased hydrophobicity conferred on the molecule by the aliphatic amino acid moiety. The three N36 helices of the 6-HB complex form an interior, parallel coiled-coil trimer that harbors a hydrophobic groove that is required for C34 helix binding to form the fully active 6-HB (Chan et al., 1997). We suspect that the amino acid moiety of the derivatives may bind to the hydrophobic grooves on the surface of the N36 helices through hydrophobic interaction. We further speculate that the hydrophobic binding functions as a guide for the functional group, (−) gossypol, escorting gossypol to the site of action, which could thus increase the effective concentration of (−) gossypol at the target site. Gossypol is marginally toxic, and this toxicity has been associated with the aldehyde group (Hoffer et al., 1987; Sonenberg et al., 1988). Lin and co-workers have found that the aldehyde groups in compounds similar to gossypol are not necessary for anti-HIV activity but contribute strongly to cytotoxic effects (Lin et al., 1993). With the modifications that we made to (−) gossypol, the aldehyde group is effectively blocked by the amino acid moiety, resulting in the 5-fold decrease in cytotoxicity (Table 2).

The increase in HIV-1 inhibition activity and the decrease in cytotoxicity of the amino acid-(−) gossypol derivatives make possible the studies of the mechanism of gossypol as an antiviral agent. Previous studies have indicated that gossypol targets the enzyme reverse transcriptase (Lin et al., 1989; Polsky et al., 1989). However, it is worth noting that all previous reports of antiviral activity by gossypol and any derivatives thereof involved enveloped viruses (Dorsett et al., 1975; Lin et al., 1989; Radloff et al., 1986; Royer et al., 1991; Vichkanova et al., 1970; Wichmann et al., 1982). Although circumstantial, this fact suggests that gossypol and gossypol derivatives may interfere with interactions between the virus envelope and the plasma membrane of the host cell, thereby inhibiting the invasion process (Royer et al., 1991). In this study, we used (−)G-Ala in time-of-addition assays, cell–cell fusion assays and infectivity assays to clarify the mechanism of the antiviral activity of gossypol. The alanine-(−) gossypol derivative inhibits HIV-1 infection when it is added to the cells at the same time that virus is added, but it shows no inhibitory activity if it is added 1 h or longer after virus is added to the cells (Fig. 3A). (−)G-Ala also significantly inhibits the number of syncytia formed between HIV-1_{IIIB} -infected H9 cells and uninfected MT-2 cells with an EC_{50} of approximately 3.73 μM (Fig. 3B). Fig. 3C shows that (−)G-Ala does not block the replication of VSV-G glycoprotein-expressing HIV-1 cells in human 293T cells but inhibits the replication of HIV-1 NL4-3-*luc* pseudotyped virus in GHOST-CXCR4 cells. Collectively, these data imply that (−)G-Ala inhibits HIV-1 infection during the entry process.

Our results show that while (−)G-Ala neither blocks the interactions between gp120 and CD4 nor interrupts HIV-1 binding to co-receptor CXCR4, (−)G-Ala is highly effective in blocking gp41 6-HB core formation. Furthermore, our data suggest that (−)G-Ala most likely binds to the hydrophobic pocket located on the surface of the trimer of N36 helices. The entry of HIV-1 into susceptible target

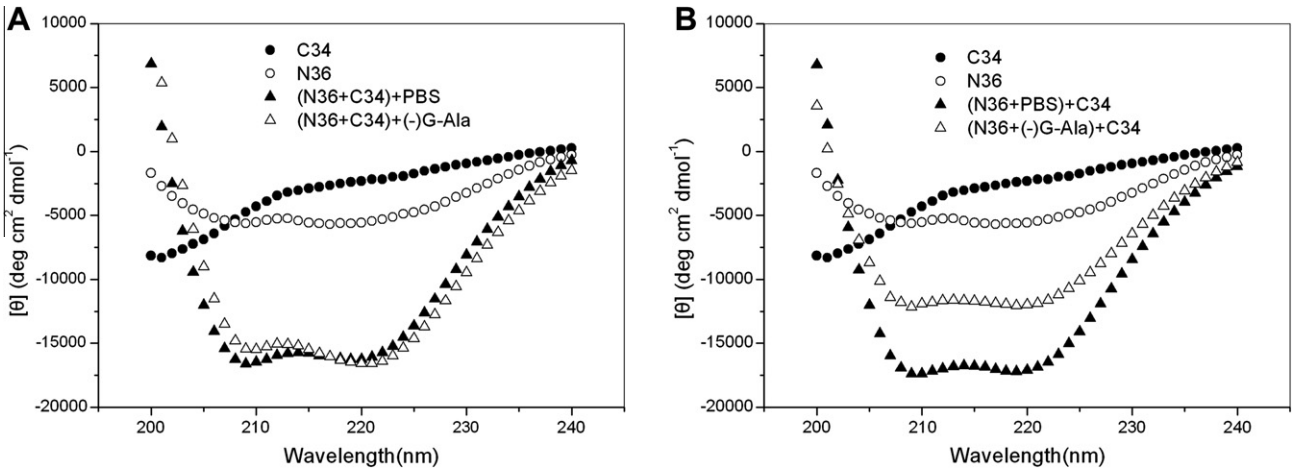

Fig. 6. CD spectroscopy experiments showing that (−)G-Ala suppresses the α-helical content of 6-HB formatted by N36 and C34. (A) CD spectroscopy spectra of samples containing either N36 or C34, or pre-incubated N36 plus C34 peptides with further incubation with or without (−)G-Ala. (B) CD spectroscopy spectra of samples containing either N36 or C34, or N36 pre-incubated in the presence or absence (−)G-Ala followed by incubation with C34 peptide. In both experiments, three scans were acquired for each data set, and buffer-subtraction was used to correct the spectra.

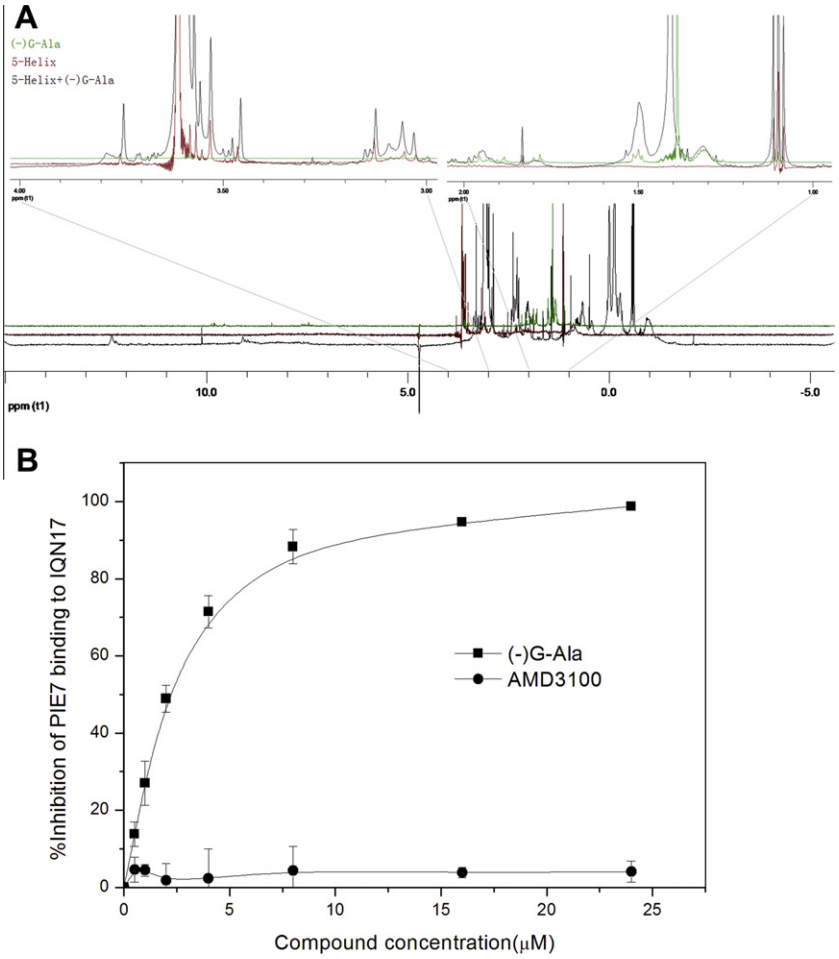

Fig. 7. (−)G-Ala binds to the gp41 hydrophobic pocket. (A) NMR analysis of *in vitro* binding between HIV-5-Helix protein and (−)G-Ala. NMR spectra of free (−)G-Ala (green), free 5-Helix protein (brown) and a mixture of the two (black) obtained as described in Section 2 were compared. The top two graphs shown are the expanded scale of the indicated parts of the bottom graph. These spectra illustrate the chemical shift changes and line width changes when the (−)G-Ala was co-cultured with 5-Helix protein. (B) ELISA analysis of the inhibitory effects of increasing concentrations of (−)G-Ala on the binding of biotinylated PIE17 to IQN17. AMD3100, which has no effects on this interaction, was similarly analyzed as the control. Each data presented were means ± standard deviations from two independent experiments where each treatment was tested in triplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cells is an ordered, multi-step process that ultimately leads to the fusion of viral and cell membranes, each step in the entry process provides a unique opportunity for the development of anti-retroviral drugs (Kuritzkes, 2009). We used ELISA to confirm that (−)G-Ala does not block the interactions between gp120 and CD4 molecules or interact with co-receptor CXCR4 (Fig. 4A and B). The association between the gp41 NHR and CHR domains to form the fusion-active 6-HB core is a critical step during virus-cell fusion (Chan and Kim, 1998). Therefore, a molecule that can block 6-HB formation may inherently have HIV-1 fusion inhibitory activity (Liu and Jiang, 2004; Liu et al., 2007). Our results confirm that (−)G-Ala blocks the formation of 6-HB complexes between the N36 and C34 peptides *in vitro* (Figs. 5 and 6) using a series of experiments, including a sandwich ELISA using the 6-HB-specific mAb NC-1, N-PAGE, and CD spectroscopy. We confirm the interaction between (−)G-Ala and 6-HB using ^{1}H NMR with the 5-Helix protein for comparison. Our results show the chemical shift and line width changes as well as intense binding signals that are indicative of (−)G-Ala binding to gp41. Furthermore, our NMR data support the binding of (−)G-Ala to the hydrophobic grooves on the surface of the N36 helices because the 5-helix protein harbors only one of the three hydrophobic grooves due to the absence of a C-helix (Root et al., 2001). Eckert et al. (1999) have designed a hybrid molecule, IQN17, by conjugating the GCN4 sequence (IQ) with a short NHR peptide (N17) involved in formation of the gp41 hydrophobic pocket. Welch et al. (2007) have identified a short circular anti-HIV-1 peptide consisting of D-amino acids, designated PIE7, which specifically binds to the pocket presented on IQN17. We demonstrated that (−)G-Ala could disrupt PIE7 binding to the hydrophobic pocket present on IQN17 by ELISA (Fig. 7B). Gonzalez-Ortega et al. (Gonzalez-Ortega et al., 2011; Gonzalez et al., 2011) have found that drug-resistance to VIRIP and its analogues is conferred by amino acid changes that are not located within the fusion peptide and may be similar to that of PIE analogues. Thus, we speculate that (−)G-Ala has a similar mode of action as VIRIP and its analogues and inducing a general conformational change that impedes the correct folding of gp41 and prevents fusion.

In summary, in this study, we used an array of biochemical experiments to show that gossypol and its derivatives inhibit HIV-1 replication by targeting the entry process, which contradicts previously proposed mechanisms (Keller et al., 2003). Whether the G-Ala interacts with RT, other it only targets on gp41, or both in a particular situation such as T20, which has a primary binding site in the residues 36–45 (GIVQQQNNLL) region of the gp41 NHR domain and a secondary binding site in the target cell membrane (Liu et al., 2005a), depends, therefore, upon the evidence from the further crystallographic study. We also successfully identified modifications to the fusion inhibitor gossypol that significantly improve its activity. We believe that hydrophobic modifications are the most effective in enhancing the anti-HIV-1 activity of gossypol. Hopefully, our results provide some useful insight for the design and modification of small molecule HIV fusion inhibitors that target gp41.

Acknowledgements

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