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A Salt Bridge between an N-terminal Coiled Coil of gp41 and an Antiviral Agent Targeted to the gp41 Core Is Important for Anti-HIV-1 Activity

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HIV-1 envelope glycoprotein transmembrane subunit gp41 play a critical role in the fusion of viral and target cell membranes. The gp41 C-terminal heptad repeat region interacts with the N-terminal coiled-coil region to form a six-stranded core structure. Peptides derived from gp41 C-terminal heptad repeat region (C-peptides) are potent HIV-1 entry inhibitors by binding to gp41 N-terminal coiled-coil region. Most recently, we have identified two small organic compounds that inhibit HIV-1-mediated membrane fusion by blocking the formation of gp41 core. These two active compounds contain both hydrophobic and acidic groups while the inactive compounds only have hydrophobic groups. Analysis by computer modeling indicate that the acidic groups in the active compounds can form salt bridge with Lys 574 in the N-terminal coiled-coil region of gp41. Asp 632 in a C-peptide can also form a salt bridge with Lys 574. Replacement of Asp 632 with positively charged residues or hydrophobic residues resulted in significant decrease of HIV-1 inhibitory activity. These results suggest that a salt bridge between an N-terminal coiled coil of the gp41 and an antiviral agent targeted to the gp41 core is important for anti-HIV-1 activity.

Key Words: **HIV-1; gp41; salt bridge; coiled coil; antiviral agent; anti-HIV-1 activity.**

Infection by the human immunodeficiency virus type 1 (HIV-1) involves fusion of the viral membrane with the target cell membrane, followed by the subsequent transfer of viral genetic material into the cell. HIV-1 envelope glycoproteins gp120 and gp41 play critical roles in these initial events of viral infection, *i.e.* gp120 recognizes the target cell by binding to both CD4 and a co-receptor (CCR5 or CXCR4) and gp41 then promotes the fusion of viral and cellular membranes (1). The gp41 ectodomain contains three major functional regions, the fusion peptide region located at the N-terminus of gp41, followed by two 4–3 heptad repeat (HR) regions predicted to form coiled coils (2). Peptides derived from the N- and C-terminal HR regions, designated N- and C-peptides, can interact with each other to form a six-stranded α-helical bundle, 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 (3–6). The six-stranded α-helical bundle was proposed to be the fusion-active gp41 core (3, 4). This was confirmed by using a monoclonal antibody (mAb), NC-1, which specifically recognizes conformational epitopes on the complexes formed between N- and C-peptides and binds to gp41-expressing cells only in the presence of CD4 molecules (7).

C-peptides are potent HIV-1 inhibitors (8, 9). They inhibit the membrane fusion step of HIV-1 infection, in a dominant-negative manner, by binding to the N-terminal HR region of gp41 and blocking the formation of the gp41 core (3, 4, 10). Clinical trials demonstrated that C-peptides can be potentially used for chemotherapy of HIV-1 infection (11). However, their future clinical application may be constrained because of proteolytic degradation and lack of oral bioavailability. Therefore, it is essential to develop low molecular weight HIV-1 inhibitors having a similar mechanism of action as C-peptides.

X-ray crystallographic analysis of the gp41 core structure demonstrated that there is a hydrophobic deep pocket in each groove on the surface of the inner core of gp41 and it was suggested to be an important target for development of novel HIV-1 inhibitors (12). Eckert *et al.* (13) recently constructed several short cyclic peptides with D-amino acid residues. These pep-

Abbreviations used: BCECF, 2′, 7′-bis-(2-carboxyethyl)-5-and-6-carboxyfluorescein acetoxyethyl ester; CD, circular dichroism; CHR, C-terminal heptad repeat; CPE, cytopathic effect; ELISA, sandwich enzyme-linked immunosorbent assay; HIV-1, the human immunodeficiency virus type 1; HPLC, high-performance liquid chromatography; HR, heptad repeat; NHR, N-terminal heptad repeat; IC_{50}, 50% effective concentration; MAb, monoclonal antibody.

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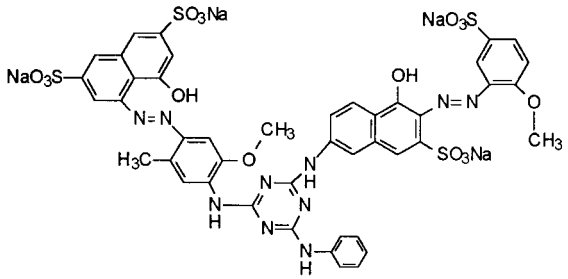
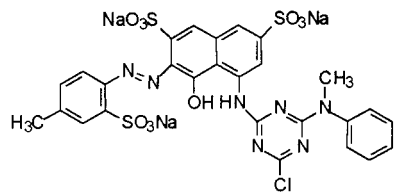


ADS-J1



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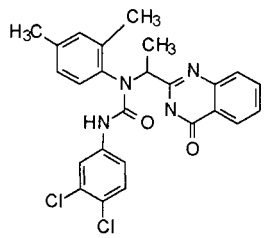

ADS-J13

FIG. 1. Chemical structures of the compounds.

tides, like the C-peptides, also inhibit HIV-1-induced membrane fusion by binding to the gp41 pocket. A small molecular synthetic moiety attached to the N-terminus of a C-peptide (p38, aa 628–665 of gp41) was synthesized and found to be an HIV-1 entry inhibitor (14). We used a different approach to search for the small molecular HIV-1 entry inhibitors targeted to the gp41 core structure (15). We first developed an immunological assay to screen for antiviral compounds that block the interaction between the N- and C-peptides to form the fusion-active gp41 core, thus inhibiting gp41-mediated membrane fusion (16). In this screening assay, the complex of the gp41 core formed between N- and C-peptides is detectable by the conformation-specific mAb NC-1 (16). Using this screening assay, we have identified two small antiviral compounds targeted to the HIV-1 gp41 core structure (17). Here we compare the structures of the active and inactive compounds tested and those of peptide C34 and its analogs in order to determine the role of the salt bridges formed between the gp41 N-terminal coiled coil and the antiviral agents targeted to the gp41 core.

MATERIALS AND METHODS

Peptides. Peptides N36 (gp41 residues 546–581), C34 (gp41 residues 628–661) (4) and the C34 analogues were synthesized using an Advanced Chemtech 396 synthesizer with Fmoc-amino acids and HBTU as the coupling agent. The N-termini of the peptides were acetylated and their C-termini were amidated. All peptides were purified to homogeneity by reverse-phase high-performance liquid chromatography (HPLC). The identity of the purified peptides was confirmed by mass spectrometry (PerSeptive Biosystems).

Detection of complex formation between the N- and C-peptides by a sandwich enzyme-linked immunosorbent assay (ELISA). The sandwich ELISA assay was carried out as previously described (16). Briefly, IgG purified from rabbit antisera directed against the N36/C34 complex, dissolved in 0.1 M Tris (pH 8.8), was coated onto wells of a 96-well polystyrene plate (Immulon II, Dynatech Laboratories, Inc., Chantilly, VA), followed by addition of the peptide complexes formed by mixing N36 and the C-peptides in PBS at equimolar concentrations. Then, the mAb NC-1, biotin-labeled goat-anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN), streptavidin-labeled horseradish peroxidase (Zymed, South San Francisco, CA), and the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO) were added sequentially. Optical density (OD) at 450 nm was read in an ELISA reader (Dynatech Laboratories, Inc., Chantilly, VA). Each sample was tested in triplicate. The 50% effective concentration (EC_{50}) of a C-peptide for forming a complex with N36 was calculated using the Calcusyn computer program (18).

Cell fusion assay. A fluorescent dye transfer assay was used for detection of HIV-1-mediated cell fusion (8). HIV-1_{IIIB}-infected H9 cells were labeled with a fluorescent reagent, 2′,7′-bis-(2-carboxyethyl)-5-and-6-carboxyfluorescein acetoxyethyl ester (BCECF-AM, Molecular Probes, Inc., Eugene, OR) and incubated with MT-2 cells (ratio = 1:10) in a 96-well plate at 37°C for 2 h in the presence or absence of peptides. The fused and unfused BCECF-labeled HIV-1 infected cells were counted under an inverted fluorescence microscope (Zeiss, Germany) with an eyepiece micrometer disc. The percentage of inhibition of cell fusion and the 50% inhibitory concentration (IC_{50}) were calculated as previously described (8).

Hardware and software. All molecular modeling studies were performed in a Silicon Graphics Indigo^2 Extreme computer. Automated docking simulations were run using the DOCK3.5 Suit of programs (19, 20). Sybyl6.5 from Tripos Associates, Inc. (21) was used for all other modeling purposes including molecular visualizations. CrystalEyes2 (22) stereographic eyeglasses were used along with Sybyl6.5 software for stereo visualization. Three-dimensional structures of the compounds used for molecular docking studies were generated by the CONCORD

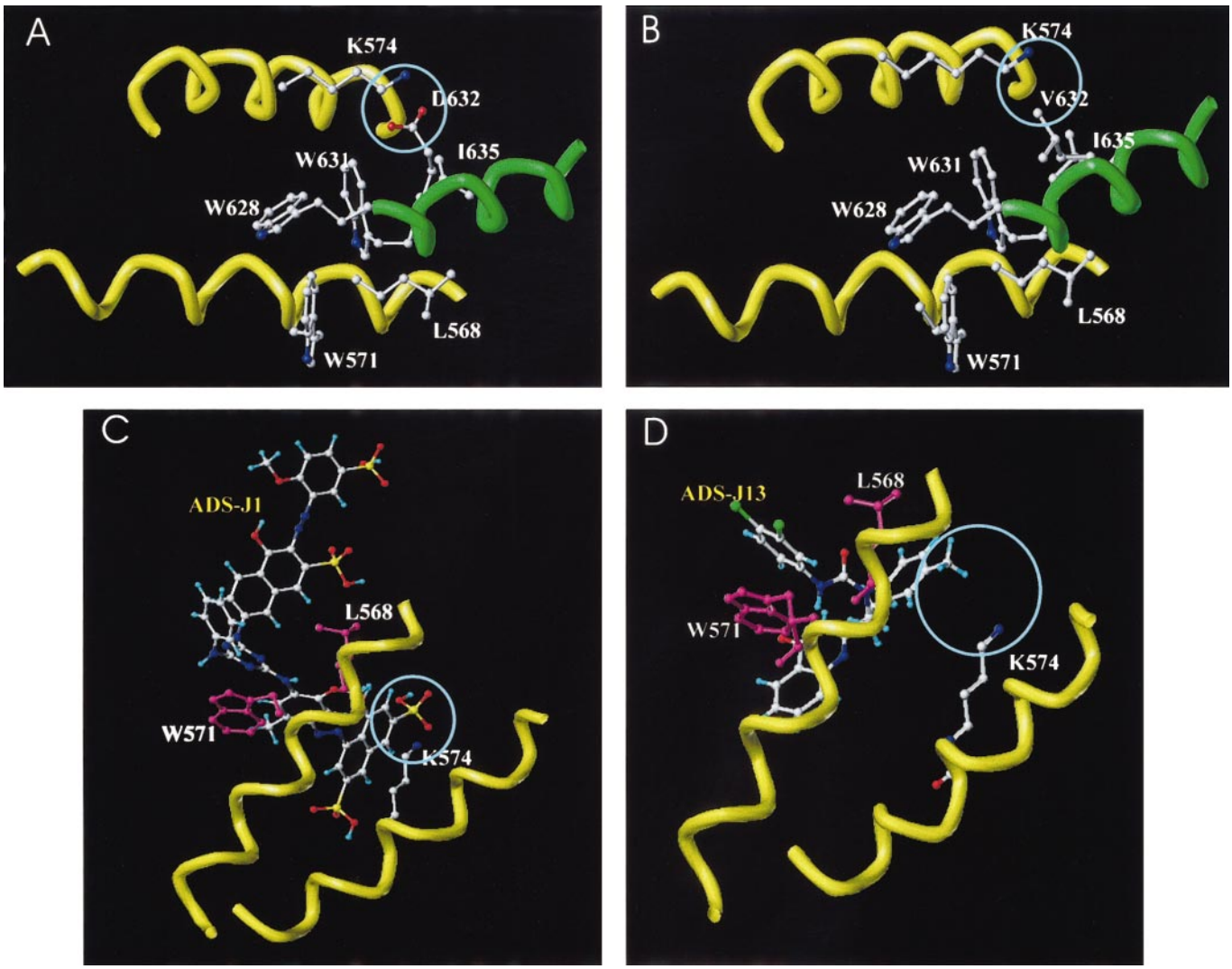

FIG. 2. The hydrophobic and ionic interactions of N-peptides with C-peptides or small compounds. (A) Interaction between C34 and N36 (only the pocket region is shown for clarity). Hydrophobic interaction between residues Trp 628, Trp 631, and Ile 635 in C34 (green) and residues Leu 568, and Trp 571 in N36 (yellow) and the ionic interaction between Asp 632 in C34 and Lys 574 in N36 to form a salt bridge (circled) are indicated. (B) Interaction between N36 (yellow) and an analog of C34 with a non-conserved mutation (D632V) (green). Although D632V has hydrophobic residues to interact with the hydrophobic residues in N36, it does not have a negatively charged residue at position 632 to form a salt bridge with Lys 574 in N36 (circled). (C) Interaction between N36 and ADS-J1. The hydrophobic groups (phenyl and naphthalene) in ADS-J1 interact with the hydrophobic residues in N36. ADS-J1 also has a negatively charged group (sulfonic acid) which is in close proximity to Lys 574 in N36. A salt bridge may be formed through this ionic interaction (circled). (D) Interaction between N36 and ADS-J13. The inactive compound ADS-J13 has hydrophobic groups to interact with the hydrophobic residues in N-peptides, but lacks the important ionic interaction site to form a salt bridge with Lys 574 (circled).

4.0 (23) program from Tripos Associates, Inc. The details of the docking simulations have been published (17).

RESULTS AND DISCUSSION

Although the sandwich ELISA can be used for large-scale screening, it is not cost-effective to randomly screen compounds. Therefore, we used a computer-aided molecular docking method by the DOCK3.5 suit of programs (19, 20) at the first step to screen three-dimensional databases of chemical libraries for compounds that potentially dock into the hydrophobic pocket on the surface of the inner N-helix core of gp41. By screening a database of 20,000 small organic molecules, we selected 200 top scoring compounds for in-depth inspection of the interactions at the hydrophobic pocket and neighboring regions by molecular visualization techniques (24). Sixteen of the 200 best scoring

compounds (named ADS-J1 to ADS-J16) (17) were selected for further screening by the immunological assay and tested for their inhibitory activity on HIV-1 infection, including HIV-1-mediated cell fusion and cytopathic effect (CPE) as previously described (8). We found that ADS-J1 (7-[6-phenylamino-4-[4-[(3,5-disulfo-8-hydroxynaphthyl)azo]-2-methoxy-5-methylphenylamino]-1,3,5-triazin-2-yl]-4-hydroxy-3-[(2-methoxy-5-sulfophenyl)-azo]-2-naphthalene sulfonic acid) (Fig. 1) had the best inhibitory activity against formation of the complex between N36 and C34 and against HIV-1 infection (16, 17). Its specificity to block the interaction between N- and C-peptides was confirmed by using circular dichroism (CD) spectroscopy (data not shown). ADS-J2 (5-[(4-chloro-6-phenylamino-1,3,5-triazine-2-yl)-amino]-4-hydroxy-3-[(4-methyl-6-sulfophenyl)azo]-2,7-naphthalene disulfonic acid) (Fig. 1) is the second active compound. The remaining compounds (see their structures in reference 17) had much lower or no anti-HIV-1 activity. The inhibitory activity of these compounds on the formation of the gp41 core is correlated with their antiviral activity against HIV-1 infection ($r = 0.993$), indicating that the immunological screening assay is a valid method for identification of HIV-1 inhibitors targeted to the gp41 core.

Surprisingly, ADS-J13 [N′-(3,4-dichlorophenyl)-N-[1-(4-oxo-3,4-dihydroquinazolin-2-yl)ethyl]-N-2,4-dimethyl phenylurea] (Fig. 1) had higher docking score than ADS-J1 and ADS-J2, but it had no HIV-1 inhibitory activity. It was of interest to know why ADS-J13, unlike ADS-J1, does not inhibit HIV-1-mediated cell fusion. We initiated computer-aided modeling analyses on the interaction pattern of these compounds at the cavity and surrounding area to gain deeper insight on the structural characteristics that could justify the differences in activity of these compounds. The analyses indicated that all the compounds studied here had the potential to dock into the deep hydrophobic pocket on the surface of the central N-helix core. Close visual inspection of the possible interaction pattern of these compounds by 3D stereoscopic eye glasses revealed that ADS-J1 was positioned in such a way that its hydrophobic groups (phenyl and naphthalene) were able to interact with the hydrophobic residues (Leu 568, Val 570, Trp 571) in the pocket. In addition, one of its negatively charged groups (sulfonic acid groups) is in close proximity to a positively charged group of Lys 574 in the N-helix located outside the pocket (Fig. 2), suggesting that these two oppositely charged groups may interact with each other to form a salt bridge. ADS-J2 also has a similar positively charged group to interact with Lys 574 in the N-helix. ADS-J3 to ADS-J16 have hydrophobic groups, which may interact with the hydrophobic residues in the pocket. But they do not have any negatively charged group(s) to interact with Lys 574 (only ADS-J13 is shown in Fig. 2 as an illustrative example). Though this ionic interaction is not expected to fully explain the inactivity of these compounds, it suggests that along with hydrophobic groups, negatively charged group(s) may be important to interact with positively charged residue, especially Lys 574, in the surrounding areas to form the salt bridge. This interaction may play a key role to hold the compounds in appropriate positions so that they can effectively block the interaction between the N- and C-terminal HR regions to form the gp41 core.

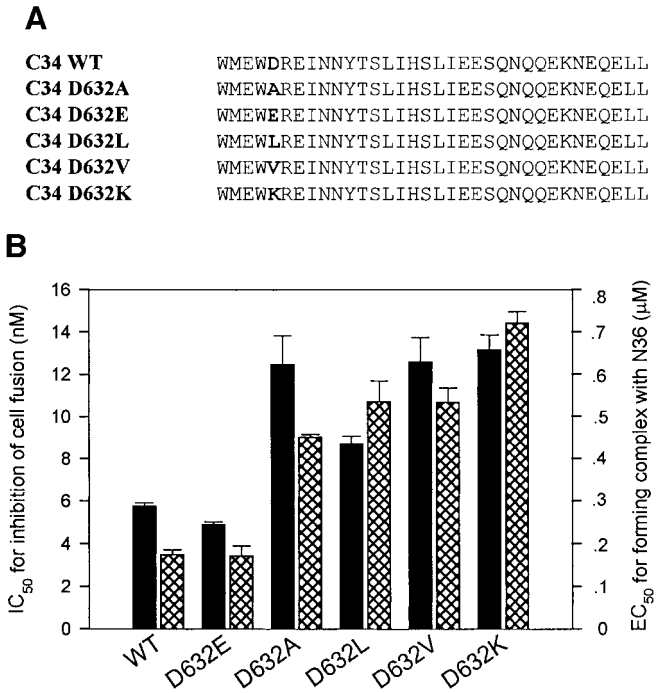

FIG. 3. The sequence of C34 and its analogues (A) and their activity to inhibit HIV-1-mediated cell fusion (filled bars) and to form NC-1 detectable complexes with N36 (crossed bars) (B). A fluorescent dye transfer assay was used for detection of HIV-1-mediated cell fusion (8). A sandwich ELISA was applied for determination of the activity of C-peptides to form complexes with N36 as previously described (16).

If this assumption is correct, the C-terminal HR region of gp41 may also need a negatively charged residue at the right position to interact with Lys 574 in the N-helix to form a salt bridge. Analysis of the crystal structure of the gp41 core reveals that the C-terminal HR region indeed contains an Asp at position 632 which is in close proximity to Lys 574 in the N-helix (Fig. 2) (4). Analyzing the currently available HIV-1 gp41 sequences, we found that both the Lys 574 and Asp 632 in gp41 are highly conserved. Out of 214 sequences analyzed, 100% of the sequences contain positively charged residues (Lys or Arg) at positions corresponding to Lys 574 in the HIV-1_{HXB2} and 98% of the sequences have negatively charged residues (Asp or Glu) at positions corresponding to Asp 632 in the HIV-1_{HXB2} (25). These data suggest that these highly conserved, oppositely charged residues located in the N- and C-terminal HR regions of gp41 may play an important role in HIV-1-mediated membrane fusion

and in C-peptide-mediated inhibition of HIV-1 infection (8, 9, 11).

In order to further verify this assumption, we synthesized a series of C34 peptide analogues by replacement of Asp 632 with Glu (D632E), Lys (D632K), Leu (D632L), Val (D632V), and Ala (D632A), respectively. Their activity to inhibit HIV-1-mediated cell fusion and to form NC-1 detectable complexes with N36 was compared. As shown in Fig. 3, a conserved mutation of Asp 632 with a negatively charged residue Glu (D632E) did not change the inhibitory activity on cell fusion and the ability to form a complex with N36. However, replacement of Asp 632 with a positively charged residue Lys (D632K) and with hydrophobic residues Leu and Val (D632L and D632V) as well as with Ala (D632A) resulted in significant decrease of the activity of the corresponding peptides to inhibit HIV-1 infection and to form complexes with N36. These results confirm that the salt bridge formed between the negatively charged residue at position 632 in a C-peptide and the positively charged residue at the position 574 in the N-terminal HR region is important for the C-peptide-mediated inhibitory activity against HIV-1-mediated membrane fusion.

As discussed before, Ferrer *et al.* (14) used a structure-based combinatorial approach to identify a hybrid molecule, in which the pocket-binding fragment (aa 628–635 of gp41) located at the N-terminus of a C-peptide (p38, aa 628–665 of gp41) was replaced by three non-peptide elements (C7-Mn34-Mn42) selected from a biased library, can bind to the gp41 pocket and inhibit HIV-1 mediated membrane fusion (14). The three-position non-peptide elements may mimic the three hydrophobic residues (Trp 628, Trp 631, and Ile 635) in a C-peptide to bind to the hydrophobic residues in the pocket formed by the inner N-helices. However, the interaction of the non-natural portion with the inner N-helix core is much weaker than the native amino acid sequence of the pocket-binding fragment in a C-peptide (14). Removal of the peptide may result in loss of anti-HIV-1 activity. Based on above observation on the importance of the interaction between the positively charged residue Lys 574 in the NHR region of gp41 and the negatively charged groups in the anti-HIV-1 compound ADS-J1, we predict that in addition to the hydrophobic interaction in the pocket, the ionic interaction along the pocket may also be required for the binding of a small molecule to the inner coiled coil. If a fourth non-peptide element is added to C7-Mn34-Mn42 at a right position to interact with Lys 574 in the N-terminal HR region of gp41, the small molecular synthetic moiety may have better pocket-binding activity and HIV-1 inhibitory activity.

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