ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2004, p. 4349–4359 Vol. 48, No. 11
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.11.4349–4359.2004

N-Substituted Pyrrole Derivatives as Novel Human Immunodeficiency Virus Type 1 Entry Inhibitors That Interfere with the gp41 Six-Helix Bundle Formation and Block Virus Fusion

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Received 14 April 2004/Returned for modification 28 May 2004/Accepted 13 July 2004

A recently approved peptidic human immunodeficiency virus type 1 (HIV-1) fusion inhibitor, T-20 (Fuzeon; Trimeris Inc.), has shown significant promise in clinical application for treating HIV-1-infected individuals who have failed to respond to the currently available antiretroviral drugs. However, T-20 must be injected twice daily and is too expensive. Therefore, it is essential to develop orally available small molecule HIV-1 fusion inhibitors. By screening a chemical library consisting of "drug-like" compounds, we identified two N-substituted pyrroles, designated NB-2 and NB-64, that inhibited HIV-1 replication at a low micromolar range. The absence of the COOH group in NB-2 and NB-64 resulted in a loss of anti-HIV-1 activity, suggesting that this acid group plays an important role in mediating the antiviral activity. NB-2 and NB-64 inhibited HIV-1 fusion and entry by interfering with the gp41 six-helix bundle formation and disrupting the α-helical conformation. They blocked a D-peptide binding to the hydrophobic pocket on surface of the gp41 internal trimeric coiled-coil domain. Computer-aided molecular docking analysis has shown that they fit inside the hydrophobic pocket and that their COOH group interacts with a positively charged residue (K574) around the pocket to form a salt bridge. These results suggest that NB-2 and NB-64 may bind to the gp41 hydrophobic pocket through hydrophobic and ionic interactions and block the formation of the fusion-active gp41 core, thereby inhibiting HIV-1-mediated membrane fusion and virus entry. Therefore, NB-2 and NB-64 can be used as lead compounds toward designing and developing more potent small molecule HIV-1 fusion inhibitors targeting gp41.

Human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) transmembrane subunit gp41 plays a crucial role in the early steps of viral entry into target cells (39) and may serve as an important target for the development of HIV-1 entry inhibitors (4, 26). The gp41 molecule consists of three domains, i.e., the cytoplasmic domain, the transmembrane domain, and the extracellular domain (ectodomain). The ectodomain contains three major functional regions: the fusion peptide (FP), the N-terminal heptad repeat (NHR or HR1), and the C-terminal heptad repeat (CHR or HR2). Peptides derived from the NHR and CHR regions of gp41, designated N and C peptides, have potent antiviral activity against HIV-1 infection (23, 34, 52, 53). One of the C peptides, T-20 (Fuzeon; Trimeris Inc.), has shown potent in vivo anti-HIV-1 activity in clinical trials for treatment of patients with HIV-1 infection and AIDS (27, 28, 30, 53) and was recently licensed by the U.S. Food and Drug Administration as the first member of a new class of anti-HIV drugs, known as HIV fusion inhibitors. Discovery of this drug is a great breakthrough in the development of anti-HIV drugs since it can be used for treatment of HIV-infected individuals who fail to respond to the currently available antiretroviral drugs, such as HIV reverse transcriptase and protease inhibitors (27, 30). However, the future application of T-20 may be constrained due to its lack of oral availability, the high cost of production, and the rapid emergence of resistant HIV-1 strains in patients receiving T-20 (50). Therefore, it is essential to develop small-molecule anti-HIV-1 compounds with a mechanism of action similar to that of C peptides but without the disadvantages of the peptidic drugs.

In studies of the mechanism by which C peptides inhibit HIV-1 fusion, it has been demonstrated that the gp41 N and C peptides mixed at equimolar concentrations form a stable α-helical trimer of antiparallel heterodimers, representing the fusion-active gp41 core (34, 36). Crystallographic analysis has revealed that this is 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 this coiled coil (3, 47, 51). The C helix interacts with the N-helix mainly through the hydrophobic residues in the grooves on the surface of the central coiled-coil trimer. Each of the grooves on the surface of the N-helix trimer has a deep hydrophobic pocket that accommodates three conserved hydrophobic residues in the gp41 CHR region (3), suggesting that this pocket is an attractive target for designing new class of anti-HIV-1 drugs, which may prevent the early fusion events (2, 3).

Using the gp41 pocket as the target structure, we previously identified two small-molecule compounds, ADS-J1 (9, 20) and XTT formazan (54), by using a computer-aided molecular docking techniques and a sandwich enzyme linked immunosorbent assay (ELISA) (24) with a monoclonal antibody (MAb), NC-1, that specifically recognizes the fusion-active gp41 core structure (21). These compounds inhibit HIV-1 fusion possibly by docking into the gp41 pocket and interfering with the formation of the gp41 six-helix bundle formation. However, they

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may not be good lead compounds for the development of anti-HIV-1 drugs since both are dyes and contain several reactive groups. Nevertheless, the identification of these compounds is useful as proof of the concept that a small-molecule organic compound might block the fusion-active gp41 six-helix bundle formation and inhibit HIV-1 entry. Here we report the identification of two pyrrole derivatives, NB-2 and NB-64, as novel HIV-1 fusion inhibitors, which may interact with gp41 at the fusion-intermediate conformation, possibly binding to the gp41 hydrophobic pocket and surrounding area and block the gp41 six-helix bundle formation, thereby inhibiting the fusion between the viral and target cell membranes. NB-2 and NB-64 are "drug-like" compounds and may be used as leads for designing more potent HIV-1 fusion inhibitors, which are expected to be developed as a new class of anti-HIV-1 drugs.

MATERIALS AND METHODS

Reagents. MT-2 cells, HIV-1_{IIIB}-infected H9 cells (H9/HIV-1_{IIIB}), U87-T4-CXCR4 cells (expressing both CD4 and CXCR4), U87-T4-CCR5 cells (expressing both CD4 and CCR5), and Cf2Th/synCCR5 cells (expressing CCR5, but no CD4), laboratory-adapted and primary HIV-1 strains, anti-p24 MAb (183-12H-5C), HIV immunoglobulin (HIVIG), and AMD3100 were obtained from the NIH AIDS Research and Reference Reagent Program. Lymphoid cell line CEMx174 5.25M7, kindly provided by C. Cheng-Mayer, is stably transduced with an HIV-1 long terminal repeat (LTR)-green fluorescent protein (GFP) reporter and luciferase reporter construct. These cells, expressing CD4 and both coreceptors, CXCR4 and CCR5 (18), were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1 μg of puromycin/ml, and 200 μg of G418/ml. Recombinant soluble CD4 (sCD4) and gp120 were obtained from Immunodiagnostics, Inc. (Woburn, Mass.). Peptides N36, C34 (3, 36), and IQN17 (11) were synthesized by a standard solid-phase Fmoc (9-fluorenylmethoxy carbonyl) method in the MicroChemistry Laboratory of the New York Blood Center. A biotinylated D peptide, D10-p5-2K (11), was also synthesized in-house with D-amino acids and was oxidized as previously described (11). The peptides were purified to homogeneity by high-performance liquid chromatography. The identity of the purified peptides was confirmed by laser desorption mass spectrometry (PerSeptive Biosystems). Rabbit antisera directed against the mixture N36-C34 and against IQN17 were prepared as previously described (21). Mouse MAb NC-1 specific for the gp41 six-helix bundle was prepared and characterized as previously described (21). Rabbit and mouse immunoglobulin G (IgG) were purified by using protein A/G beads (Pierce, Rockford, Ill.). Mouse MAb 12G5 specific for CXCR4 was purchased from R&D Systems (Minneapolis, Minn.). Compounds used for screening were purchased from ChemBridge Corp. (San Diego, Calif.). NB-177 and NB-178 were purchased from Maybridge plc (Trevillett, England). Chloropeptin was a generous gift from Satoshi Omura and Haruo Tanaka of The Kitasato Institute, Tokyo, Japan.

Syncytium-formation assay for screening HIV-1 fusion inhibitors. HIV-1_{IIIB}-infected H9 cells (H9/HIV-1_{IIIB}) at 2×10^5/ml were cocultured with MT-2 cells (2×10^6/ml) in the presence of compounds to be screened (final concentration of compound, 25 μg/ml) in a 96-well plate at 37°C for 2 days. HIV-1-induced syncytium formation was observed under an inverted microscope and scored as "−" (no syncytium was observed), "±" (ca. 50% syncytia were inhibited), and "+" (no syncytium formation was inhibited). The compounds scored with "−" and "±" were selected for further screening by ELISA for inhibitors against the gp41 six-helix bundle formation.

ELISA for screening for compounds that inhibit the gp41 six-helix bundle formation. A sandwich ELISA as previously described (24) was used to screen for compounds that inhibit the gp41 six-helix bundle formation. Briefly, 2 μM peptide N36 was preincubated with a test compound at the indicated concentrations at 37°C for 30 min, followed by addition of 2 μM C34. After incubation at 37°C for 30 min, the mixture was added to wells of a 96-well polystyrene plate (Costar; Corning, Inc., Corning, N.Y.), which were precoated with IgG (2 μg/ml) purified from rabbit antisera directed against the N36-C34 mixture. Then, MAb NC-1, biotin-labeled goat anti-mouse IgG (Sigma Chemical Co., St. Louis, Mo.), streptavidin-labeled horseradish peroxidase (SA-HRP; Zymed, South San Francisco, Calif.), and the substrate 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) were added sequentially. The A_{450} was measured by using an ELISA reader (Ultra 384; Tecan, Research Triangle Park, N.C.). The percent inhibition by the compounds was calculated as previously described (22) and the effective concentration for 50% inhibition (EC_{50}) was calculated by using the software Calcusyn (6), kindly provided by T. C. Chou (Sloan-Kettering Cancer Center, New York, N.Y.).

Assessment of anti-HIV-1 infectivity. The inhibitory activity of compounds on infection by laboratory-adapted HIV-1 strains was determined as previously described (22). In brief, 10^4 MT-2 cells were infected with HIV-1 at 100 50% tissue culture infective doses in 200 μl of RPMI 1640 medium containing 10% FBS in the presence or absence of compounds at graded concentrations overnight. The culture supernatants were replaced with fresh medium without the addition of testing compounds. For the time-of-addition assay, MT-2 cells were incubated with HIV-1_{IIIB} at 37°C for 0, 1, 2, 3, 4, 6, and 8 h, respectively, before the addition of a test compound (44 μM). Zidovudine (AZT) at 10 μM was used as a control. After culture for another 2 h, the cells were washed to remove the free virus and compounds. Fresh medium without testing compounds was added. On the fourth day postinfection, 100 μl of culture supernatants was collected from each well, mixed with equal volumes of 5% Triton X-100, and assayed for p24 antigen, which was quantitated by ELISA (54). Briefly, the wells of polystyrene plates (Immulon 1B; Dynex Technology, Chantilly, Va.) were coated with HIVIG in 0.85 M carbonate-bicarbonate buffer (pH 9.6) at 4°C overnight, followed by washes with PBS-T buffer (0.01 M phosphate-buffered saline [PBS] containing 0.05% Tween 20) and blocking with PBS containing 1% dry fat-free milk (Bio-Rad, Inc., Hercules, Calif.). Virus lysates were added to the wells and incubated at 37°C for 1 h. After extensive washes, anti-p24 MAb (183-12H-5C), biotin-labeled anti-mouse IgG1 (Santa Cruz Biotech., Santa Cruz, Calif.), SA-HRP, and TMB were added sequentially. Reactions were terminated by the addition of 1 N H_2SO_4. The A_{450} was recorded in an ELISA reader (Ultra 384). Recombinant protein p24 (U.S. Biologicals, Swampscott, Mass.) was included for establishing a standard dose-response curve.

The inhibitory activity of compounds on infection by primary HIV-1 isolates was determined as previously described (40). Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors at the New York Blood Center by standard density gradient centrifugation by using Histopaque-1077 (Sigma). The cells were plated in 75-cm^2 plastic flasks and incubated at 37°C for 2 h. The nonadherent cells were collected and resuspended at 5×10^6 in 10 ml of RPMI 1640 medium containing 10% FBS, 5 μg of phytohemagglutinin (PHA)/ml, and 100 U of interleukin-2/ml, followed by incubation at 37°C for 3 days. The PHA-stimulated cells were infected with the corresponding primary HIV-1 isolates at a multiplicity of infection (MOI) of 0.01 in the absence or presence of a compound at graded concentrations. Culture media were changed every 3 days. The supernatants were collected 7 days postinfection and tested for p24 antigen by ELISA as described above. The percent inhibition of p24 production and EC_{50} values were calculated as described above.

Cell-cell fusion. A dye transfer assay was used for detection of HIV-1 mediated cell-cell fusion as previously described (23, 25, 35). H9/HIV-1_{IIIB} cells were labeled with a fluorescent reagent, Calcein-AM (Molecular Probes, Inc., Eugene, Oreg.) and then incubated with MT-2 cells (ratio = 1:5) in 96-well plates at 37°C for 2 h in the presence or absence of the compounds tested. The fused and unfused calcein-labeled HIV-1-infected cells were counted under an inverted fluorescence microscope (Zeiss, Oberkochen, Germany) with an eyepiece micrometer disk. The percent inhibition of cell-cell fusion and the EC_{50} values were calculated as previously described (23).

Inhibition of fusion between PBMC infected by primary HIV-1 strains (X4 and R5 viruses) with CEMx174 5.25M7 cells, which express CD4 and both coreceptors, CXCR4 and CCR5, was determined by a luciferase assay. Briefly, PHA-stimulated PBMC were infected with corresponding primary HIV-1 strains, respectively, for 7 days as described above. The infected PBMC were washed and resuspended in culture medium to 10^5/ml. Then, 50 μl of a compound at graded concentration in triplicate was incubated with equal volume of HIV-1-infected PBMC at 37°C for 30 min. Next, 100 μl of CEMx174 5.25M7 cells (2×10^5) was added, followed by incubation at 37°C for 3 days. The cells were collected, washed, and lysed with the lysing reagent included in the luciferase kit (Promega, Corp., Madison, Wis.). Aliquots of cell lysates were transferred to 96-well flat-bottom luminometer plates (Costar), followed by the addition of luciferase substrate (Promega). The luciferase activity was measured in an Ultra 384 luminometer.

Virus-cell fusion. Inhibition of fusion of HIV-1_{NL4-3}-*luc* pseudotyped viruses expressing Env of the HIV-1_{HXB2} (X4) or HIV-1_{SF162} (R5) strains with U87-T4-CXCR4 and U87-T4-CCR5 cells, respectively, was measured as previously described (17). Briefly, 100 μl of U87-T4-CXCR4 and U87-T4-CCR5 cells, respectively, at 10^5 cells/ml was added to the wells of a 96-well tissue culture plate, followed by culture at 37°C overnight. Then, 50 μl of a test compound at graded concentrations was mixed with 50 μl of the HIV-1_{NL4-3}-*luc* viruses pseudotyped with the HIV-1_{HXB2} or HIV-1_{SF162} Env, prepared as described

previously (17), at a final p24 concentration of 0.5 ng/ml. After incubation at 37°C for 30 min, the mixtures were added to the cells and incubated at 37°C overnight. The supernatants were removed, and fresh culture medium without testing compounds was added. After incubation at 37°C for 3 days, the cells were harvested and lysed for measuring luciferase activity as described above.

Detection of in vitro cytotoxicity. The in vitro cytotoxicities of compounds for U87-T4-CXCR4, MT-2, CEMx174 5.25M7, and U373-MAGI-CXCR4$_{CEM}$ cells and PBMC were measured by a colorimetric method with XTT tetrazolium dye as previously described (9). Briefly, 100 μl of a compound at a graded concentration was added to equal volumes of cells (5×10^5/ml) in each well of 96-well plates. After incubation at 37°C for 4 days, XTT (1 mg/ml; 50 μl/well; Polysciences, Inc., Warrington, Pa.) was added. Four hours later, the soluble intracellular formazan was quantitated colorimetrically at 450 nm with a reference at 650 nm. The percent cytotoxicity (40) and the 50% cytotoxicity concentrations (CC_{50}) were calculated by using the software Calcusyn (7).

Inhibition of gp120 binding to CD4. Wells of polystyrene plates were coated with 100 μl of sheep anti-gp120 antibody D7324 (Cliniqa, Fallbrook, Calif.) at 2 μg/ml in 0.85 M carbonate-bicarbonate buffer (pH 9.6) at 4°C overnight and blocked with 1% dry fat-free milk in PBS at 37°C for 1 h. Then, 100 μl of recombinant gp120 molecule (Immunodiagnostics) at 0.5 μg/ml was added, followed by incubation at 37°C for 1 h, followed by three washes with PBS-T. sCD4 at 0.25 μg/ml was added in the presence of a compound, followed by incubation at 37°C for 1 h. After three washes, rabbit anti-sCD4 IgG (0.25 μg/ml, 100 μl/well) was added, followed by incubation at 37°C for 1 h. Binding of rabbit anti-sCD4 IgG was determined by sequential addition of biotinylated goat-anti-rabbit IgG, SA-HRP, and TMB. After the reactions were terminated, the A_{450} was recorded in a Tecan ELISA reader.

Binding of anti-CXCR4 antibody to CXCR4-expressing cells. The inhibition of the binding of anti-CXCR4 antibody to CXCR4-expressing cells was determined by using a cell-based ELISA as described previously (56). Briefly, U373-MAGI-CXCR4$_{CEM}$ cells (10^5/well) that express CXCR4 molecules were cultured in a 96-well plate at 37°C overnight. The cells were fixed with 5% formaldehyde at room temperature for 15 min and washed with PBS-T. The cells were incubated with anti-CXCR4 MAb 12G5 at 37°C for 1 h in the presence or absence of the compounds tested. Isotype IgG2a was used as a control. After the addition of biotin-labeled goat anti-mouse IgG, SA-HRP, and the substrate TMB sequentially, the A_{450} was measured by using an ELISA reader (Tecan).

Binding of FLSC of gp120-CD4 recombinant protein to CCR5-expressing cells. A plasmid expressing the full-length single chain protein (FLSC), consisting of full-length HIV-1 BaL gp120 joined at their C termini to the D1D2 domains of CD4 by a 20-amino-acid linker (14), was kindly provided by A. Pinter at the Public Health Research Institute with permission from A. L. DeVico at the University of Maryland. Stable cell lines expressing recombinant FLSC were established by transfecting plasmid into 293T cells by using FuGene 6 (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's protocol. Soluble FLSC was purified from cell culture medium by lectin chromatography with *Galanthus nivalis* snowdrop agglutinin (Sigma) as described previously (16). Inhibitory activity of compounds on the binding of FLSC to Cf2Th/synCCR5 cells, which express CCR5, but no CD4, was determined by a cell-based ELISA established in our laboratories (55). Each sample was tested in triplicate.

CD spectroscopy. A test compound at 100 μg/ml was incubated with 10 μM N36 in phosphate buffer (pH 7.2) at 37°C for 30 min before addition of C34 in the same buffer at an equimolar concentration. After incubation at 37°C for 30 min, the mixture was cooled down, and circular dichroism (CD) spectra of the mixture were measured on the J-715 CD spectrometer equipped with a thermoelectric temperature controller (Jasco, Tokyo, Japan). The instrument was calibrated by using a two-point calibration method with (+)-10-camphorsulfonic acid. The wavelength dependence of molar ellipticity (θ) was monitored at 4°C by 5 scans in 1-nm increments with a sampling time of 10 s as previously described (12, 54).

FN-PAGE. Fluorescence native polyacrylamide gel electrophoresis (FN-PAGE) was performed as previously described (33). Briefly, 18% precast Tris-glycine gels and a Novex X-Cell II Mini Cell (Invitrogen, Carlsbad, Calif.) were used for native PAGE. Peptide N36 (40 μM in PBS) was incubated with a compound at 200 μg/ml at 37°C for 30 min, and the peptide C34-fluorescein isothiocyanate (C34-FITC; 40 μM in PBS) was added. In the control, N36 was incubated with C34-FITC at 37°C for 30 min before addition of the compounds. After incubation at 37°C for another 30 min, the mixture was diluted in Tris-glycine native sample buffer and then loaded onto 10-by-1.0-mm wells. Gel electrophoresis was carried out with 125-V constant voltages at room temperature for ca. 90 min in Tris-glycine native running buffer. Immediately after electrophoresis, an image of the fluorescence bands in the gel was obtained by using the FluorChem 8800 Imaging System (Alpha Innotech Corp., San Leandro, Calif.) with a transillumination UV light source using an excitation wavelength at 302 nm and a fluorescence filter with emission wavelength at 520 nm.

Inhibition of a biotinylated D-peptide binding to IQN17. To measure the binding of a D peptide, D10-p5-2k, to IQN17, an ELISA was established. In brief, wells of a 96-well polystyrene plate were coated with IgG (10 μg/ml) purified from rabbit antisera directed against IQN17. The peptide IQN17 (10 μM) was incubated with a compound at graded concentrations at 37°C for 30 min before addition of the peptide D10-p5-2K-biotin (5 μM). After incubation at 37°C for 30 min, the mixture was added to the plate, followed by incubation at 37°C for 30 min. After extensive washes, D10-p5-2K-biotin bound to the IQN17 was quantitated by the addition of SA-HRP and TMB sequentially. The absorbance at 450 nm was read in a Tecan ELISA reader.

Automated docking of compounds to the hydrophobic pocket on the surface of the internal trimeric coiled coil of gp41. Glide 2.5 software from Schrödinger (Portland, Oreg.) was used for docking in flexible (ligand) docking mode. This docking method is a fast and accurate docking program consisting of two steps: (i) generation of receptor grids and (ii) ligand docking and scoring. The initial three-dimensional coordinates of the compounds were generated by using Concord software (Tripos Associates). The compounds were minimized by using the "premin option" in Glide with an MMFF94s force field. The ligands were prepared by following a series of steps available within the Glide software, which includes addition of hydrogens to the ligands, followed by assigning appropriate ionization states of each ligand by using the "ionizer" option. Since NB-2 and NB-64 both have carboxylic acid moiety, they are expected to be ionized in physiological pH. These structures were then minimized by using the "premin" option with the MMFF94s force field. The X-ray crystal structure of the gp41 core was retrieved from the protein data bank (pdb code = 1aik) (3). The hydrophobic pocket in the gp41 core (3) was used as the target site for docking simulations. The protein was prepared through a series of steps as described in the Glide operating manual. In brief, one of the C helices and the structural waters were removed from the six-helix bundle to expose the hydrophobic pocket. Initially, all of the hydrogens were removed from the core structure and the modified protein structure (five helices) was run through a procedure called "pprep," which neutralizes residues that do not participate in forming salt bridges and those which are more than a certain distance away from the ligand atom. The modified core structure was then optimized by restrained minimization in Macromodel by using MMFF94s force field. Hydrogens were added during the optimization steps. The minimized structures of ligands and target protein were subsequently used in docking simulation. The conformational flexibility of ligands were considered by exhaustive conformational search within Glide augmented by a heuristic screen that removes conformations which are not suitable for receptor binding or has long-range hydrogen bonds, whereas the protein conformations remained fixed. Glide performs an exhaustive systematic search of the conformational space and uses a series of hierarchical filters to locate the possible position of the ligand in the receptor-binding site during docking simulations. The shape and properties are represented on a grid by different fields, which provide more accurate position and orientation of ligand (termed "pose") in the receptor. A flexible Monte-Carlo sampling and minimization were used to identify the best ligand poses for scoring. Multiple poses for each ligand were saved, and the poses with the lowest G-score, E-Model, Energy, H bond, and VDW characteristics were used for in-depth interaction analysis. Schrödinger's proprietary scoring function, GlideScore, was used in the docking experiment.

RESULTS

Identification of two anti-HIV-1 compounds, NB-2 and NB-64, from a chemical library by screening. Using a syncytium formation assay and the sandwich ELISA with the MAb NC-1, we screened a chemical library from ChemBridge Corp. (San Diego, Calif.) consisting of 33,040 compounds at a single dose (25 μg/ml). These compounds are "drug-like" molecules which were rationally preselected to form a "universal" library that covers the maximum pharmacophore diversity with the minimum number of compounds. We found that at this concentration two compounds, NB-2 and NB-64, significantly inhibited HIV-1 mediated syncytium formation and the six-helix bundle formation between the gp41 N peptide N36 and the C peptide C34. Both NB-2 and NB-64 are N-substituted pyrrole deriva-

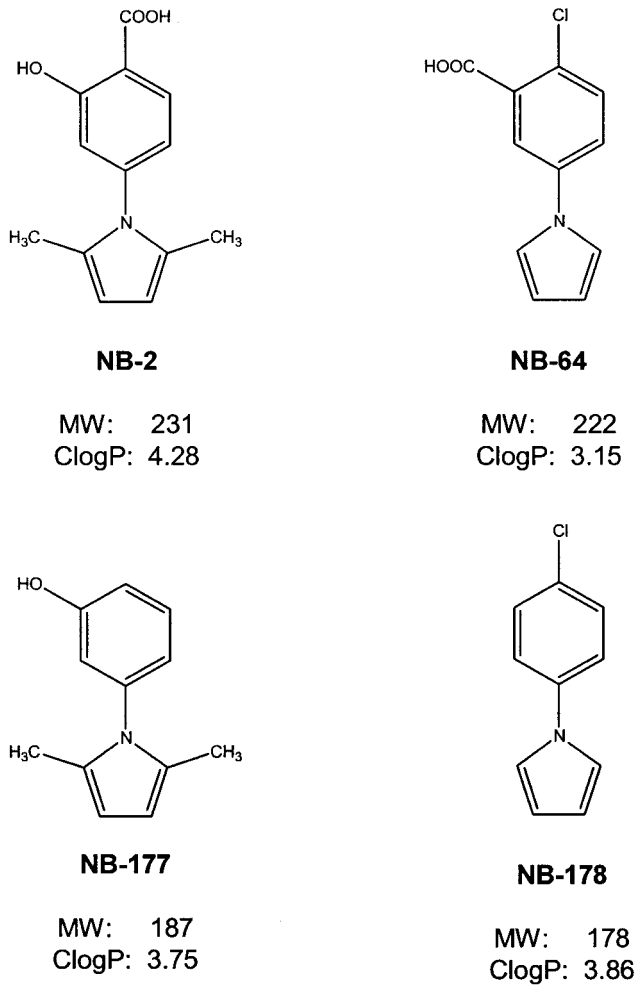

FIG. 1. Chemical structures of NB-2, NB-64, NB-177, and NB-178. Log *P* values were calculated by using the ClogP software (Biobyte Corp., Claremont, Calif.).

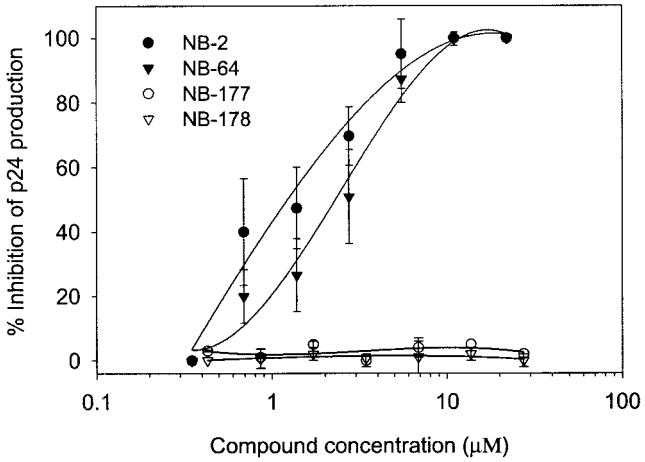

FIG. 2. NB-2 and NB-64 inhibited HIV-1 replication in MT-2 cells as indicated by suppression of p24 production. HIV-1_{IIIB} was used in this assay. Each sample was tested in triplicate. Error bars indicate standard deviations.

tives with molecular masses of 231 and 222 Da, respectively, and ClogP (a measure of partition of a drug in water and octanol phase) of 4.28 and 3.15, respectively (Fig. 1). We obtained from Maybridge another two N-substituted pyrrole derivatives, NB-177 and NB-178, as controls. These two compounds have the same parent structure as NB-2 and NB-64, respectively, except that they do not have the COOH group (Fig. 1). However, NB-177 and NB-178 had no inhibitory activity in the above two screening assays.

NB-2 and NB-64 had low in vitro cytotoxicity. The in vitro cytotoxicity of NB-2 and NB-64 to cells that were used in the present study for evaluation of anti-HIV-1 activity was determined by using XTT colorimetric assay. Both NB-2 and NB-64 had low in vitro cytotoxicity to U87-T4-CXCR4, MT-2, CEMx174 5.25M7, and U373-MAGI-$CXCR4_{CEM}$ cells and PBMC (Table 1).

NB-2 and NB-64 have potent inhibitory activities on infection by laboratory-adapted and primary HIV-1 strains. The inhibitory activities of NB-2 and NB-64 on infection of MT-2 cells by laboratory-adapted HIV-1 strains and of PBMC by primary HIV-1 strains were determined as previously described (22, 40, 54). Both NB-2 and NB-64 significantly inhibited HIV-1_{IIIB} replication with EC_{50} at ca. 1 to 2 μM (Fig. 2), whereas NB-177 and NB-178 have no inhibitory activity at concentrations up to 400 μM. NB-2 and NB-64 also inhibited, in addition to HIV-1_{IIIB}, other laboratory-adapted HIV-1 strains, including one strain resistant to AZT (AZT-R). Both compounds had potent inhibitory activity on infection by primary HIV-1 strains with distinct genotypes and biotypes, although some primary HIV-1 strains (e.g., RU570) were less sensitive than other strains (Table 2). These results suggest that NB-2 and NB-64 have potent antiviral activity against a broad spectrum of HIV-1 strains.

NB-2 and NB-64 inhibit HIV-1 entry by blocking membrane fusion. A time-of-addition assay was carried out to determine whether NB-2 and NB-64 are HIV-1 entry inhibitors. NB-2 and NB-64 inhibited HIV-1 replication when they were added to the cells with virus together but showed no inhibitory activity if they were added 1 h or longer after virus was added to the cells. However, AZT was still effective in inhibiting HIV-1 replication even when it was added 8 h postinfection (Fig. 3).

Fusion between virus and target cell membranes or between HIV-infected cells and uninfected cells is the critical step of HIV entry into a new target cell. Therefore, it is essential to determine whether NB-2 or NB-64 inhibits virus-cell and cell-cell fusion. As shown in Fig. 4A, both NB-2 and NB-64 inhibited fusion of HIV-1_{NL4-3}-*luc* pseudotyped viruses expressing HIV-1_{SF162} (R5) Env with U87-T4-CCR5 (EC_{50} = 14.79 ± 1.41 and 12.32 ± 2.52 μM, respectively), whereas NB-177 and NB-178 had no inhibitory activity. A similar result was obtained with pseudotyped viruses expressing HIV-1_{HXB2} (X4) Env and U87-T4-CXCR4 cells (data not shown). NB-2 and NB-64 also significantly inhibited fusion of HIV-1_{IIIB}-infected H9 cells with uninfected MT-2 cells, with EC_{50} values of 6.74 ± 0.16 and 29.92 ± 2.61 μM, respectively. However, NB-177 and NB178 had no inhibitory activity on cell-cell fusion (Fig. 4B). Furthermore, NB-2 and NB-64 inhibited fusion between CEMx174 5.25M7 cells and PBMC infected by primary HIV-1 strains with distinct genotypes and phenotypes (Table 3).

TABLE 1. In vitro cytotoxicity of NB-2 and NB-64

Cell type	Mean cytotoxicity ± SD[a]			
	NB-2		NB-64	
	CC_{50} (μM)	CC_{90} (μM)	CC_{50} (μM)	CC_{90} (μM)
U87-T4-CXCR4	>4,000	>4,000	>4,000	>4,000
MT2	2,755 ± 283	>4,000	>4,000	>4,000
CEMx174 5.25M7	2,491 ± 196	3,714 ± 501	>4,000	>4,000
U373-MAGI-$CXCR4_{CEM}$	1,855 ± 569	3,258 ± 780	>4,000	>4,000
PBMC	834 ± 144	1,557 ± 13	1,521 ± 194	3,378 ± 640

[a] The assay was done in triplicate and the data are presented as means ± the standard deviations where applicable.

These results suggest that NB-2 and NB-64 inhibit HIV-1 entry by blocking HIV-1-mediated membrane fusion.

NB-2 and NB-64 do not block gp120-CD4 binding, nor do they interact with the coreceptors. The process of HIV-1 entry into a $CD4^+$ target cell can be divided into three steps: (i) the virus Env surface subunit gp120 binds to the CD4 molecule; (ii) the gp120-CD4 complex interacts with a coreceptor (CXCR4 or CCR5) on target cells; and (iii) the transmembrane subunit gp41 changes conformation to form the fusion-active six-helix bundle, resulting in the fusion of viral envelope with the target cell membranes (43). In the following experiments, we investigated which step of the HIV-1 entry is blocked by NB-2 and NB-64.

We first determined whether NB-2 and NB-64 block sCD4 binding to gp120 interaction. HIV-1 envelope glycoprotein gp120 was captured by the anti-gp120 antibody coated onto wells of polystyrene plates. Chloropeptin, a gp120-CD4 binding inhibitor having a potent anti-HIV-1 activity (37), was used as a control. The results indicated that chloropeptin at 10 μg/ml markedly inhibited gp120-CD4 binding, whereas NB-2 and NB-64 at the same concentration did not significantly inhibit the interaction between CD4 and gp120, suggesting that NB-2 and NB-64 are not targeted to the gp120-CD4 binding step.

Then we determined whether NB-2 and NB-64 bind to the HIV-1 coreceptor CXCR4 by a cell-based ELISA as previously described (56) by using MAb 12G5, which specifically recognizes CXCR4 and blocks HIV-1 infection of $CXCR4^+$ cells (38). AMD3100, a potent CXCR4 antagonist (44, 45), was used as a control. Indeed, AMD3100 at 10 μM significantly inhibited 12G5 binding to U373-MAGI-$CXCR4_{CEM}$ cells, a CXCR4-expressing cell line. However, at the same concentration, NB-2 and NB-64 had no inhibitory activity in this assay. In another experiment, we tested whether NB-2 and NB-64 inhibited FLSC binding to cells expressing CCR5, Cf2Th/synCCR5 cells, by using TAK-779, a CCR5 antagonist (1), as a control. TAK779 at 10 μM completely blocked binding of FLSC to Cf2Th/synCCR5 cells, whereas, at the same concentration, NB-2 and NB-64 had no significant inhibitory activity. These results suggest that NB-2 and NB-64 may not interact with the HIV-1 coreceptors CXCR4 and CCR5.

NB-2 and NB-64 interfere with the gp41 six-helix bundle formation. Subsequently, we determined the effect of NB-2 and NB-64 on the gp41 six-helix bundle formation, a critical conformational change during HIV-1 fusion with the target cells. A model system of the gp41 six-helix bundle was established by mixing the N and C peptides at equal molar concentrations (34). This model gp41 core structure can be detected by sandwich ELISA with a conformation-specific MAb, NC-1 (21, 24). Using this system, we tested the inhibitory activities of NB-2, NB-64, NB-177, and NB-178 on the gp41 six-helix bundle formation. NB-2 and NB-64 inhibited the six-helix bundle

TABLE 2. Inhibitory activity of NB-2 and NB-64 on infection of MT-2 cells and PBMC by laboratory-adapted and primary HIV-1 strains, respectively

HIV-1 strain	Mean inhibitory activity ± SD[a] of:			
	NB-2		NB-64	
	EC_{50} (μM)	EC_{90} (μM)	EC_{50} (μM)	EC_{90} (μM)
Laboratory adapted				
IIIB (clade B, X4)	1.04 ± 0.21	2.81 ± 0.17	2.21 ± 0.40	5.85 ± 0.63
RF (clade B, X4)	1.69 ± 0.46	2.65 ± 0.47	3.58 ± 1.04	8.73 ± 2.01
SF2 (clade B, X4R5)	1.86 ± 0.37	5.92 ± 0.59	4.75 ± 0.85	9.13 ± 0.93
AZT-R (clade B, X4)	11.57 ± 0.49	15.07 ± 0.64	51.37 ± 2.29	94.28 ± 5.55
Primary				
94UG103 (clade A, X4R5)	25.09 ± 0.25	31.24 ± 0.38	35.77 ± 4.32	78.15 ± 5.85
92US657 (clade B, R5)	44.13 ± 7.97	126.7 ± 18.40	29.73 ± 8.10	133.06 ± 17.3
93IN101 (clade C, R5)	4.45 ± 0.97	10.94 ± 1.48	19.73 ± 0.86	39.91 ± 3.65
93TH051 (clade E, X4R5)	4.87 ± 0.59	17.30 ± 1.02	23.60 ± 8.10	91.04 ± 20.41
93BR020 (clade F, X4R5)	10.60 ± 0.04	15.22 ± 0.04	4.72 ± 0.81	8.55 ± 0.45
RU570 (clade G, R5)	98.00 ± 5.21	209.15 ± 6.74	39.68 ± 1.08	105.34 ± 5.44
BCF02 (group O, R5)	0.89 ± 0.50	8.94 ± 1.69	5.90 ± 4.05	54.50 ± 7.07

[a] The assay was done in triplicate.

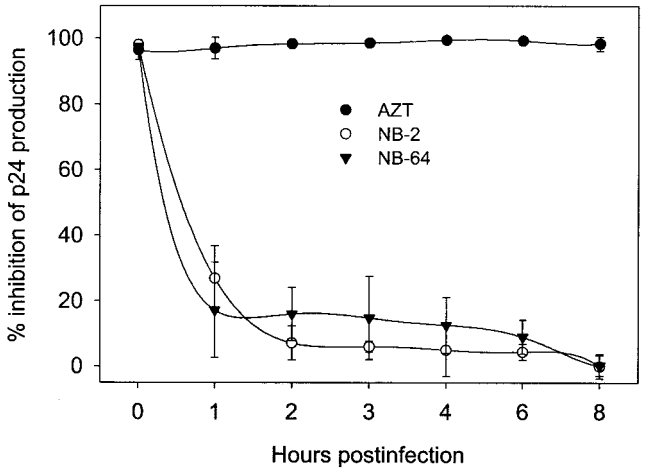

FIG. 3. NB-2 and NB-64 inhibited HIV-1 entry. Inhibition of HIV-1 entry was determined by a time-of-addition assay. The assay was done in triplicate, and the data are presented as means ± the standard deviations.

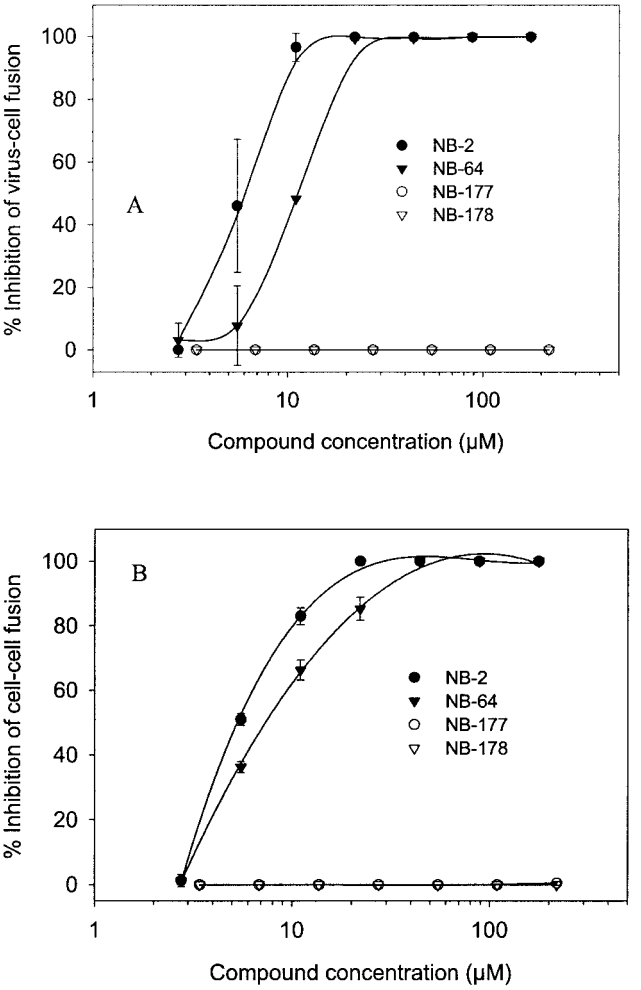

FIG. 4. NB-2 and NB-64 inhibited HIV-1-mediated membrane fusion. (A) virus-cell fusion. Inhibition of fusion of HIV-1_{NL4-3}-*luc* pseudotyped viruses expressing Env of the HIV-1_{SF162} (R5) strain with U87-T4-CCR5 cells was determined by a luciferase assay. Each sample was tested in triplicate, and the data are presented as means ± the standard deviations. (B) Cell-cell fusion assay. Inhibition of fusion between H9/HIV-1_{IIIB} and MT-2 cells was assessed by a dye transfer assay as described in Materials and Methods. Each sample was tested in quadruplicate, and the data are presented as means ± the standard deviations.

formation between N36 and C34 in a dose-dependent manner, with EC_{50}s of 13.48 and 15.69 μM, respectively, whereas NB-177 and NB-178 at concentrations up to 140 μM had no inhibitory activity on the six-helix bundle formation. These results suggest that NB-2 and NB-64 may bind to a component in the gp41 coiled-coil domains and interfere with the association between the gp41 NHR and CHR regions.

We previously developed a convenient biophysical method, FN-PAGE, for revealing the visible bands of gp41 core formed by N36 and C34-FITC (33). We used this method to detect the inhibitory activity of NB-2 and NB-64 on gp41 core formation. As shown in Fig. 5, in the absence of N36, C34-FITC showed a clear band at the lower position (lane 1). When N36 and C34-FITC were mixed together, two bands were revealed (lane 2). The major band at the upper position corresponds to the gp41 six-helix bundle formed by N36 and C34-FITC as confirmed by Western blotting with the MAb NC-1 (33). The minor band at the lower position is the isolated C34-FITC. When the compounds NB-2 (lane 3) and NB-64 (lane 5) were preincubated with N36 before the addition of C34-FITC, the intensities of the upper bands were significantly decreased, while those of the lower bands were increased, suggesting that the gp41 six-helix bundle formation between N36 and C34 were inhibited by these two compounds and more isolated C34-FITC was accumulated. However, if N36 was preincubated with C34-FITC before the addition of NB-2 (lane 4) and NB-64 (lane 6), the six-helix bundle formation was not inhibited, indicating that NB-2 and NB-64 may interact with a component in the gp41 NHR region, thus blocking the formation of the fusion-active gp41 core. However, once the six-helix bundle is formed, these two compounds cannot disrupt it.

NB-2 and NB-64 interfere with the conformational change during the interaction between N and C peptides. Previous studies demonstrated that the isolated N peptide tended to aggregate and that the C peptide had a random coil structure in aqueous solution. However, mixture of the N and C peptides shows a typical α-helical conformation, as measured by CD spectroscopy (34), suggesting that the interaction between N and C peptides results in the change of their secondary structure to an α-helical coiled-coil conformation. Using a CD spectrophotometer (model J-715; Jasco, Inc., Japan), we found that NB-2 and NB-64 at 100 μg/ml (ca. 440 μM) significantly disrupted the α-helicity of the N36/C34 mixture, whereas NB-177 and NB-178 had no effects on the α-helicity of the N36/C34 complex (Fig. 6). These results confirm that these two compounds interfere with the conformational change during the interaction between the N and C peptides.

NB-2 and NB-64 blocked binding of a D peptide to the pocket presented on the gp41 trimer modeled by IQN17. During the process of HIV-1 fusion with the target cell membrane, the gp41 NHR and CHR regions associate to form a fusogenic

TABLE 3. Inhibitory activity of NB-2 and NB-64 on HIV-1-mediated cell-cell fusion

Fusion type	Mean inhibitory activity ± SD[a] of:			
	NB-2		NB-64	
	EC_{50} (μM)	EC_{90} (μM)	EC_{50} (μM)	EC_{90} (μM)
MT-2 and H9/HIV-1_{IIIB} (clade B, X4)	6.74 ± 0.16	15.68 ± 0.93	29.92 ± 2.61	39.28 ± 0.45
5.25M7 and PBMC/SF162 (clade B, R5)	20.61 ± 1.44	69.65 ± 10.08	93.06 ± 0.90	150.32 ± 16.24
5.25M7 and PBMC infected by:				
92UG029 (clade A, X4)	10.59 ± 0.08	29.03 ± 0.18	9.94 ± 2.52	31.58 ± 0.18
92US657 (clade B, R5)	28.19 ± 4.79	165.32 ± 22.34	38.11 ± 4.91	99.01 ± 10.14
93IN101 (clade C, R5)	13.86 ± 0.76	37.51 ± 2.28	82.25 ± 0.04	142.03 ± 2.43
93UG065 (clade D, X4)	25.73 ± 0.29	32.59 ± 0.76	93.56 ± 8.73	135.84 ± 0.81
CMU02 (clade EA, X4)	34.50 ± 4.61	85.20 ± 10.38	22.00 ± 4.86	129.14 ± 11.04
93TH051 (clade E, X4R5)	7.46 ± 0.30	17.76 ± 0.72	57.30 ± 1.44	127.88 ± 0.77
93BR020 (clade F, X4R5)	7.04 ± 0.30	29.42 ± 12.30	10.08 ± 1.39	20.32 ± 13.06
BCF02 (group O, R5)	3.94 ± 2.41	10.89 ± 2.25	46.40 ± 13.06	88.74 ± 2.39

[a] The assay was done in triplicate.

core structure. There are three highly conserved symmetrical hydrophobic grooves on the surface of the internal trimeric coiled coil (3, 47, 51), and each of the grooves contains a deep hydrophobic pocket, which is an attractive target for HIV-1 fusion inhibitors (2, 3). However, this hydrophobic pocket in the six-helix bundle core is covered by C peptide and cannot be used to determine the binding affinity of a compound. The N36 cannot form soluble N-trimer since it has a tendency to aggregate in solution. Eckert et al. (11) constructed a hybrid molecule, IQN17, by conjugating GCN4 sequence with a short N-peptide involved in formation of the gp41 hydrophobic pocket. Therefore, IQN17 is soluble and can present the hydrophobic pocket of gp41. Using IQN17, Eckert et al. identified a short circular anti-HIV-1 peptide consisting of D-amino acids, designated D10-p5-2K. This peptide specifically binds to the pocket presented on the IQN17. We used a similar approach to determine whether NB-2 and NB-64 bind to the pocket and block D10-p5-2K binding to the pocket on IQN17 trimer. As shown in Fig. 7, both NB-2 and NB-64 significantly inhibited biotinylated D10-p5-2K binding to IQN17, but NB-177 and NB-178 had no significant inhibitory activity. These results suggest that NB-2 and NB-64 may interact with the hydrophobic pocket in the gp41 central trimer and block the interaction

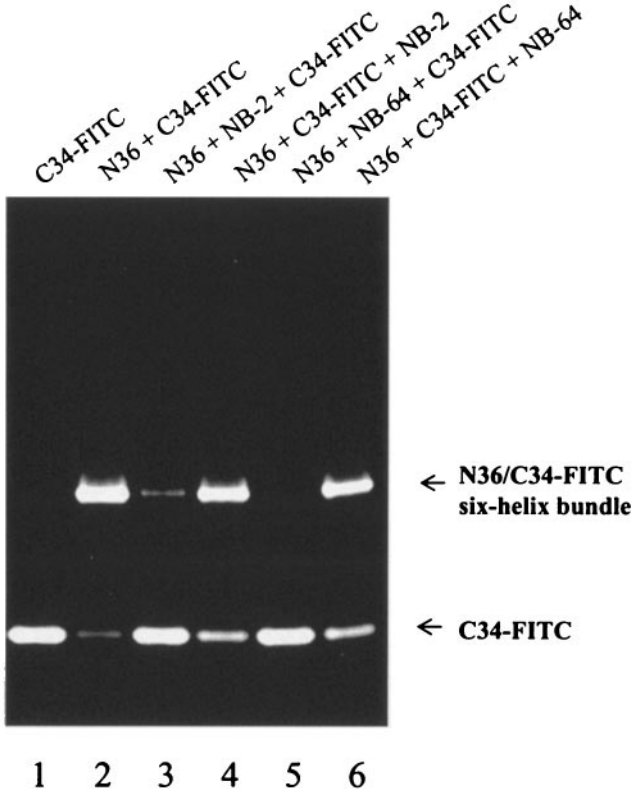

FIG. 5. NB-2 and NB-64 inhibited the gp41 six-helix bundle formation. Inhibition of NB-2 and NB-64 on six-helix bundle formation was determined by FN-PAGE. The peptide N36 was incubated with the NB-2 (lane 3) and NB-64 (lane 5) at 37°C for 30 min before addition of the peptide C34-FITC or with C34-FITC at 37°C for 30 min before addition of the compounds NB-2 (lane 4) and NB-64 (lane 6). After incubation for another 30 min, the mixtures were analyzed by FN-PAGE as described in Materials and Methods.

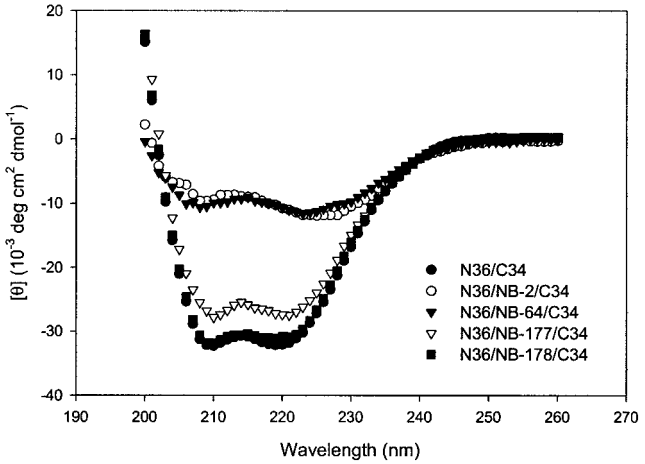

FIG. 6. NB-2 and NB-64 disrupted the α-helical conformation of the complex formed by N and C peptides by CD spectroscopy. NB-177 and NB-178 were used as controls.

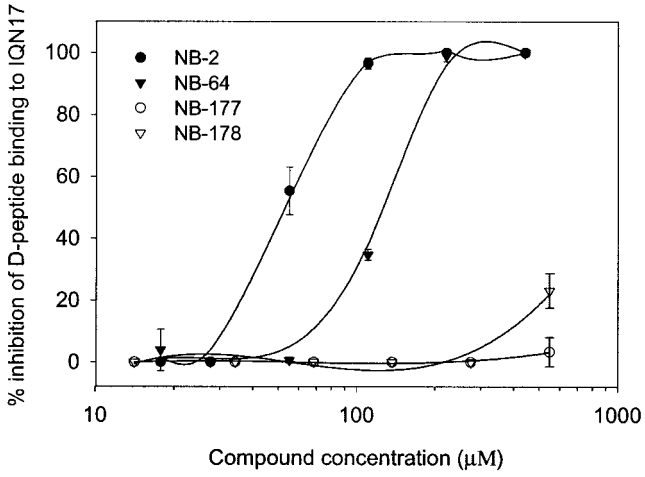

FIG. 7. NB-2 and NB-64 blocked a D-peptide binding to the gp41 pocket. The inhibitory activity of the compounds NB-2, NB-64, NB-177, and NB-178 on binding of the biotinylated D10-p5-2k peptide to IQN17 was determined by ELISA. Each sample was tested in triplicate, and the data are presented as means ± the standard deviations.

between the viral gp41 NHR and CHR regions to form the six-helix bundle, resulting in inhibition of HIV-1 entry and replication.

Interactions of NB-2 and NB-64 with the residues in the hydrophobic pocket on the surface of the internal trimeric coiled coil of gp41 core and surrounding areas. We used a computer-aided molecular docking technique to determine whether NB-2 and NB-64 fit into the hydrophobic pocket and how these molecules interact with the amino acid residues within the gp41 pocket and surrounding area. Our previous docking study with ADS-J1 revealed, in addition to the importance of hydrophobic interactions, a salt bridge formation between the acidic group in ADS-J1, which mimicked the acidic residue aspartic acid 632 (D632) in the CHR region, and the positively charged residue lysine 574 (K574) in the NHR region of gp41 (20). An extensive mutation study confirmed the importance of this salt bridge in eliciting the biological activity of ADS-J1 (20). The docking study of NB-2 and NB-64 revealed that both of these compounds formed salt bridges (3.2 to 3.6 Å) by using their negatively charged carboxylic acid residue with a positively charged residue K574 of the gp41 core structure. However, the hydrophobic interactions of these two compounds were slightly different. The hydrophobic chlorobenzene moiety of the NB-64 was within the hydrophobic cleft formed by Leu565, Leu568, Val570, Trp571, and Ile573. Due to the presence of two methyl groups in NB-2, its orientation was somewhat different than that of NB-64, and the two methyl groups in the pyrrole ring led to hydrophobic interactions with Trp571, Ile573, and Leu576 residues in the gp41 core. The acidic group (COOH) in both NB-2 and NB-64 appears to be important for their anti-HIV activity since NB-177 and NB-178, which have no acidic group, had no inhibitory activity on HIV-1 entry and replication. The docking result with NB-64 is shown in Fig. 8 as an example.

DISCUSSION

During the past 20 years, one of the greatest progresses in HIV/AIDS research is the development of anti-HIV drugs (13). Thus far, 24 anti-HIV drugs (including three combination drugs) have been approved by the U.S. Food and Drug Administration, and more drug candidates are in the pipelines (13, 42). Most of these drugs are targeted to the HIV-1 reverse transcriptase and protease, except T-20 (Fuzeon) which inhibits HIV-1 fusion and entry by targeting the viral envelope glycoprotein gp41 (5, 19, 30, 53). Although T-20 has shown great promise against HIV replication in clinical application (29, 30), it has two major limitations: lack of oral availability (delivered by subcutaneous injection twice daily) and the high cost of production (42). Therefore, the development of small-molecule HIV-1 fusion inhibitors that are targeted to gp41, but orally administered and less expensive, is a pressing need.

We previously reported the identification of a small-molecule HIV-1 fusion inhibitor, ADS-J1, through screening by using computer-aided molecular docking techniques and a sandwich ELISA with MAb NC-1 (9, 20, 24). However, this compound is not a good lead compound since it is a dye with azo bonds and several reactive groups. In addition, it has a molecular size of 1,177 Da, which is larger than most "drug-like" compounds. Therefore, we decided to screen chemical libraries consisting of drug-like compounds by using a syncytium formation assay for the primary screening and an ELISA for the secondary screening. Although the ELISA is convenient for high-throughput screening of HIV-1 fusion inhibitors targeting gp41 (24), it may miss the anti-HIV-1 compounds that are targeted to other steps of HIV-1 entry, such as gp120 binding to CD4 and the coreceptors (CXCR4 or CCR5). Therefore, a syncytium formation assay for primary screening may pick up the HIV-1 fusion inhibitors targeting to any step of HIV-1 entry.

Using this two-step screening assays, we identified two HIV-1 fusion inhibitors, NB-2 and NB-64, from a chemical library consisting of 33,040 drug-like compounds. These two compounds have the same parent structure and are N-substituted pyrrole derivatives. Both are drug-like compounds based on Lipinski's "rule of five" (32), i.e., a molecular mass of <500 Da, a calculated CLogP value of <5, fewer than five H-bond donors, and fewer than ten H-bond acceptors. Therefore, these two compounds may have good permeability and bioavailability.

NB-2 and NB-64 have potent anti-HIV-1 activity with EC_{50} values at low micromolar levels and low cytotoxicity to most cells tested. The compounds have broad specificity against infection by both laboratory-adapted and primary HIV-1 strains with distinct genotypes and phenotypes. The evidence shows that NB-2 and NB-64 interferes with the HIV-1 entry step, more specifically, the process of gp41-mediated fusion between viral and target cell membranes as confirmed by a time-of-addition assay (Fig. 3) and virus-cell and cell-cell fusion assays (Fig. 4). Here we have demonstrated that NB-2 and NB-64 cannot block the interaction between gp120 and CD4 molecules, nor can they interact with CXCR4 or CCR5, but they interfere with the gp41 conformational changes by blocking the formation of the fusion-active gp41 six-helix bundles as demonstrated by several assay systems (Fig. 5 and 6). The

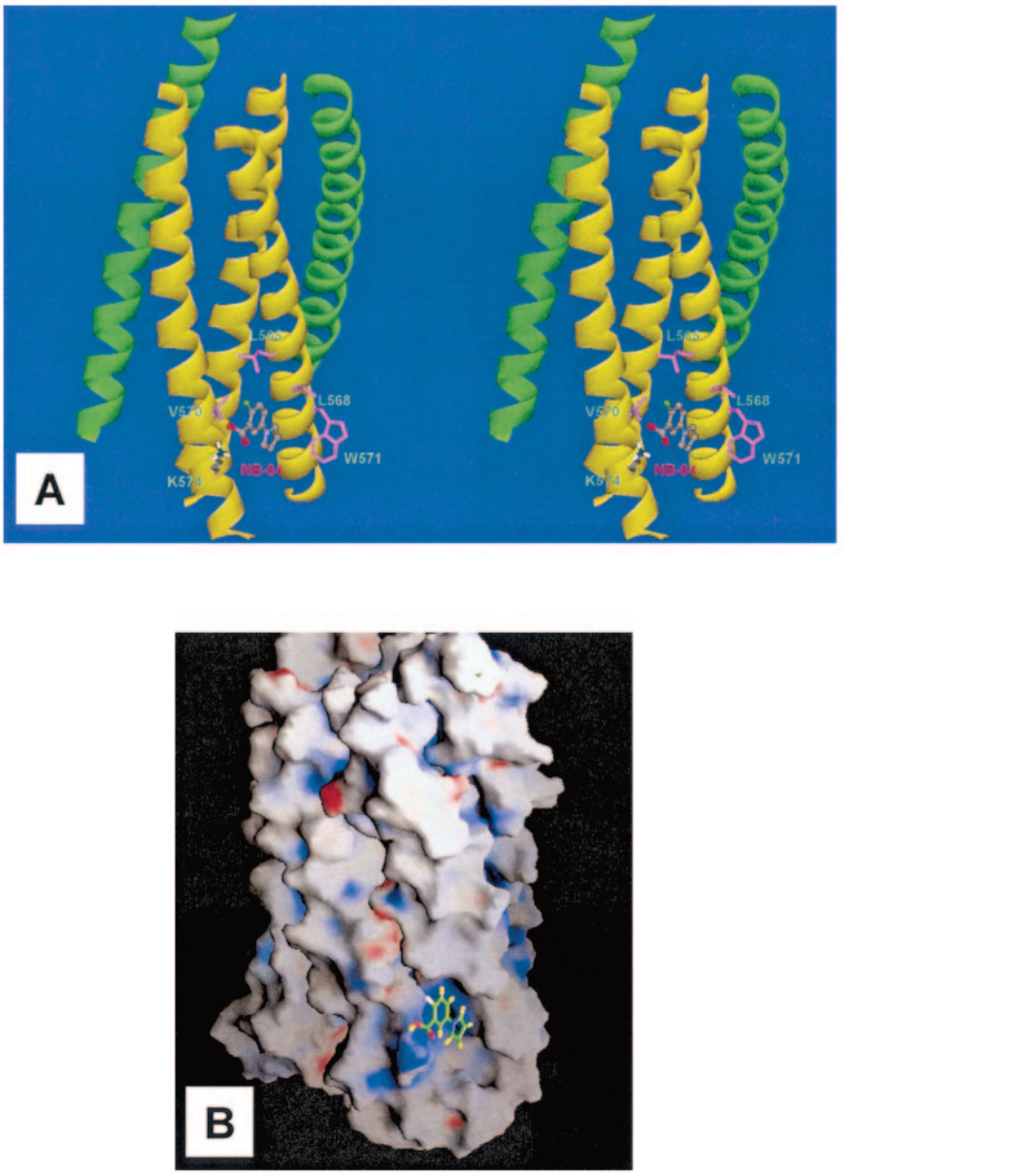

FIG. 8. Docking of NB-64 in the gp41 hydrophobic pocket. (A) The stereo view of NB-64 docked in the hydrophobic pocket showing possible interactions with the neighboring hydrophobic and charged residues. (B) Surface representation of the pocket with the bound ligand NB-64. The compound docked in the deep pocket. The negatively charged carboxylic group of NB-64 is pointing toward the positively charged area (indicated by blue) of the internal gp41 core.

addition of increasing concentrations (15.6 to 1,000 μg/ml) of bovine serum albumin to the culture medium (for virus inhibition assay) or PBS (for ELISA) did not affect the inhibitory activity of NB-2 and NB-64 on HIV-1_{IIIB} infection and on the gp41 six-helix bundle formation (data not shown). This suggests that the activity of NB-2 and NB-64 may not be affected by a serum protein.

Although the design of small-molecule organic compounds to block protein-protein interaction is a challenging approach for drug development (8), the identification of such inhibitors have been reported (15, 41, 48). Recently, Lin et al. identified a highly potent small-molecule HIV-1 entry inhibitor, BMS-378806 (31). This compound, with a molecular mass of 406.5 Da, may block interaction between the viral envelope glycoprotein gp120 and the cellular receptor CD4 by binding to a site within the CD4 pocket of gp120 (49). It may also block CD4-induced exposure of the gp41 NHR region by interacting with a trigger point in gp120 (46). This suggests that a small-molecule compound, if it binds to a "hot spot" in a protein, such as a pocket, may effectively block protein-protein interaction. The deep hydrophobic pocket in the groove on the surface of the gp41 internal trimer formed by the NHR domains has been recognized as a "hot spot" since it plays important roles in the formation and stabilization of the gp41 six-helix bundle (2, 10).

NB-2 and NB-64 may bind to the gp41 pocket since it blocks the binding of D10-p5-2k peptide to the pocket in the grooves formed by IQN17, a hybrid molecule of GCN4 peptide and the pocket-containing N-peptide portion (Fig. 7).

To determine the role of the acidic group in NB-2 and NB-64, we tested two N-substituted pyrrole derivatives, NB-177 and NB-178, that have the same parent structure as NB-2 and NB-64, respectively, except that they do not have the COOH group (Fig. 1). These compounds had no inhibitory activity on HIV-1 replication (Fig. 2), HIV-1-mediated virus-cell and cell-cell fusion (Fig. 4), and gp41 six-helix bundle formation, suggesting that the acid group in the NB-2 and NB-64 is critical for their antiviral activity, a finding consistent with our previous observation on ADS-J1, which has several acid groups and one of them forms a salt bridge with the positively charged residue, lysine 574 (K574) in the gp41 NHR pocket-forming region (20). Computer-aided molecule docking analysis in the present study also suggests that the acid group (COOH) in both NB-2 and NB-64 participate in the formation of a salt bridge with K574 (Fig. 8). These results suggest that NB-2 and NB-64 may interact with a "hot spot" in the gp41 N-helix coiled-coil domain through hydrophobic and ionic interactions and block the formation of the fusion-active gp41 core, resulting in inhibition of HIV-1-mediated membrane fusion and virus entry.

Although the anti-HIV-1 potency of NB-2 and NB-64 are not high enough to be considered as drug candidates, they have broad anti-HIV-1 activity against distinct primary HIV-1 strains and specificity to target gp41. Thus, NB-2 and NB-64 may be used as leads for designing more potent small-molecule HIV-1 fusion inhibitors as a new class of anti-HIV-1 drugs.

ACKNOWLEDGMENTS

We thank Louise Boyer-Chatenet for excellent technical assistance and James Farmer and Jinkui Niu at the MicroChemistry Laboratory for peptide synthesis.

This study was supported by NIH grant RO1 AI46221 (S.J.) and grants from the Glickenhaus Foundation (S.J. and A.K.D.) and the Altria Group, Inc. (A.K.D.).

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