Discovery of Small-Molecule Human Immunodeficiency Virus Type 1 Entry Inhibitors That Target the gp120-Binding Domain of CD4†

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The interaction between human immunodeficiency virus type 1 (HIV-1) gp120 and the CD4 receptor is highly specific and involves relatively small contact surfaces on both proteins according to crystal structure analysis. This molecularly conserved interaction presents an excellent opportunity for antiviral targeting. Here we report a group of pentavalent antimony-containing small molecule compounds, NSC 13778 (molecular weight, 319) and its analogs, which exert a potent anti-HIV activity. These compounds block the entry of X4-, R5-, and X4/R5-tropic HIV-1 strains into CD4+ cells but show little or no activity in CD4-negative cells or against vesicular stomatitis virus-G pseudotyped virions. The compounds compete with gp120 for binding to CD4: either immobilized on a solid phase (soluble CD4) or on the T-cell surface (native CD4 receptor) as determined by a competitive gp120 capture enzyme-linked immunosorbent assay or flow cytometry. NSC 13778 binds to an N-terminal two-domain CD4 protein, D1/D2 CD4, immobilized on a surface plasmon resonance sensor chip, and dose dependently reduces the emission intensity of intrinsic tryptophan fluorescence of D1/D2 CD4, which contains two of the three tryptophan residues in the gp120-binding domain. Furthermore, T cells incubated with the compounds alone show decreased reactivity to anti-CD4 monoclonal antibodies known to recognize the gp120-binding site. In contrast to gp120-binders that inhibit gp120-CD4 interaction by binding to gp120, these compounds appear to disrupt gp120-CD4 contact by targeting the specific gp120-binding domain of CD4. NSC 13778 may represent a prototype of a new class of HIV-1 entry inhibitors that can break into the gp120-CD4 interface and mask the gp120-binding site on the CD4 molecules, effectively repelling incoming virions.

Human immunodeficiency virus type 1 (HIV-1) infection of target cells begins with the attachment of virions to its primary receptor, the cell surface CD4 (16, 38). This first step of viral entry into the host is mediated by a highly specific and structurally regulated interaction between the viral envelope glycoprotein gp120 and CD4 molecules. The HIV virion surface is coated with viral envelope spikes, which are composed of trimeric heterodimers of the exterior gp120 and transmembrane gp41 glycoprotein (41). The binding of gp120 to CD4 triggers a cascade of conformational changes in the viral envelope protein: first, the exposure of gp120 coreceptor (CXCR4 or CCR5)-binding site and the subsequent engagement of the coreceptors (41), followed by the formation of gp41 prehairpin intermediates and "fusion-active" trimer-of-hairpins required for the final step of virion entry (10, 45). Thus, of the three distinct sequential events of HIV entry process (i.e., virion attachment to CD4, coreceptor binding, and virion-cell membrane fusion), the binding of gp120 and CD4 molecules clearly dictates the subsequent key steps of viral invasion into the host cells.

The gp120 glycoprotein binds to the most N-terminal domain 1 (D1) of CD4, centering on the second complementarity-determining region (CDR2)-like loop (2, 3, 5). Mounting evidence from the recent X-ray crystal structure analysis and molecular modeling studies indicates that the gp120-CD4 interaction involves relatively small contact surface areas on both proteins. In the crystal structure of HIV-1 gp120 core complexed with an N-terminal two-domain CD4 (D1/D2 CD4) and a Fab fragment of a neutralizing anti-gp120 antibody, direct interatomic contacts were observed between 22 amino acid residues of CD4 and 26 residues of gp120 (41). These critical CD4 residues in contact with gp120 were clustered between positions 25 to 64, whereas the corresponding gp120 residues were spread over six segments (41). More recent structural analysis on primary isolate YU2 gp120 has revealed that the characteristics of the gp120 core structure, as well as gp120-CD4 interaction appear to be highly conserved among different HIV-1 isolates (40). Reaching from the target cell membrane, CD4 obliquely binds into a recess formed at the interface of the outer domain, the inner domain, and the bridging sheet of the gp120 core. This "plug and socket" mode of CD4-gp120 binding, however transient, is highly specific and molecularly conserved (40), rendering this step a compelling antiviral target.

Here we report a group of antimony-containing small molecule compounds, NSC 13778 (molecular weight, 319) and its analogs, which exhibit a potent anti-HIV-1 activity by blocking virus entry into cells. Further mechanistic characterization has revealed that viral entry inhibition appears to be mediated by the disruption of gp120 and CD4 interaction. The compounds not only block binding of gp120 to CD4 but also displace gp120

already bound to CD4. In contrast to gp120-binders that block gp120-CD4 interaction by binding to gp120 and preventing it from interacting with CD4, our data suggest that these compounds compete with gp120 for its specific binding site on the CD4. This group of compounds may represent a new class of HIV-1 entry inhibitors that can break down the gp120-CD4 interface and target the specific gp120 contact site on the CD4 molecules.

MATERIALS AND METHODS

Reagents. NSC 13778 and its analogs, as well as nevirapine, were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI (http://dtp.nci.nih.gov/). Recombinant HIV-1 gp120$_{IIIB}$, soluble CD4 (sCD4), and a truncated N-terminal two-domain CD4 protein (D1/D2 CD4), all produced in a baculovirus expression system, and fluorescein isothiocyanate (FITC)-conjugated murine anti-gp120$_{IIIB}$ monoclonal antibody (MAb) were purchased from ImmunoDiagnostics, Inc. (Woburn, MA). The following agents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120$_{BaL}$, bicyclam JM-2987 (hydrobromide salt of AMD-3100) (18, 32), and HIV-1 C34 Peptide (gp41) (9, 10). Peptides derived from CD4 residues, i.e., CD4 peptide#1 (QKKSIQFHW KNSNQIKILGNQGSFLTKGPSKLNDRADSRRS) and CD4 peptide #2 (KIEDSDTYICEVEDQKEEVQLLVFGLTANSDTHL), 20-mer lysine repeat (K20), and 20-mer aspartate repeat (D20) were synthesized by Global Peptide Services (Fort Collins, CO). Phycoerythrin (PE)-conjugated anti-CD4 MAb clone SK3, FITC-conjugated anti-CD4 MAb, MT310, purified anti-CD4 OKT4 MAb, and PE-conjugated goat anti-mouse immunoglobulin G (IgG) F(ab')$_2$ fragment (heavy and light chain) were from BD Biosciences (San Jose, CA), DakoCytomation (Carpinteria, CA), Ortho Diagnostics (Raritan, NJ), and Jackson Immunoresearch Laboratories, Inc. (West Grove, PA), respectively.

Evaluation of anti-HIV activity. Primary screening for anti-HIV activity was performed in a human T-cell line CEM-SS infected with HIV-1$_{RF}$ in the absence or presence of increasing concentrations of the test compound as described previously (64, 74). The toxicity of the compounds was determined simultaneously on the same plate in uninfected CEM-SS cells.

The multinuclear activation of a galactosidase indicator (MAGI) assay was conducted to investigate anti-HIV activity of test compounds at early steps of viral replication using the HeLa–CD4–long terminal repeat (LTR)–β-Gal indicator cells (37) (contributed by Michael Emerman [AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH]), with modifications (60). MAGI cells were infected with HIV-1$_{NL4-3}$ for no more than 31 h to limit HIV-1 replication to one round. The assay utilizes the ability of the viral Tat protein to transactivate an integrated galactosidase indicator gene driven by the HIV-1 LTR (37). Thus, one-round HIV-1 infection of MAGI cells with or without test compounds permits the elucidation of anti-HIV activity at early stages of viral replication up to viral integration. Similarly, MAGI-CCR5 cells (8) (contributed by Julie Overbaugh [AIDS Research and Reference Reagent Program]) were infected with various HIV-1 strains isolated from infected individuals: 92US077 (X4/R5-tropic), 92US727 (R5-tropic), 93IN101 (R5-tropic), and 93BR019 (R5-tropic) (AIDS Research and Reference Reagent Program). HIV-1-infected target cells visualized as blue foci (37) were counted under a light microscope and are expressed as blue focus units (BFU) per well. All assays were performed in triplicate.

In order to examine possible steps of viral replication inhibited by the compounds, the addition of test compounds was delayed for 0, 1, 2, or 4 h after MAGI cells were inoculated with HIV-1$_{NL4-3}$ (MAGI time-of-addition assay). In this time-of-addition assay format, various viral entry blockers (sCD4, 10 μg/ml; bicyclam, 100 nM; C34 peptide, 10 nM; dextran sulfate, 10 μg/ml) and HIV-1 reverse transcriptase inhibitor nevirapine (1 μM) were included as reference compounds. Although viral entry blockers substantially lost antiviral activity if the compound addition was delayed for more than 2 h, nevirapine remained significantly active even when the addition was delayed for 4 h.

Antiviral activity of selected compounds was also examined in chronically HIV-1-infected H9$_{IIIB}$ and tumor necrosis factor alpha-induced U1 cells as described previously (60). All cell-based anti-HIV activity studies were carried out in the presence of 5% (CEM-SS assay) or 10% (MAGI, MAGI-CCR5, and chronically HIV-1-infected cell-based assays) fetal bovine serum (FBS) in the culture media.

Evaluation of virucidal activity. HIV-1$_{NL4-3}$ virions produced by in vitro transfection of COS7 cells were pelleted by a microcentrifugation at 32,800 × g for 2 h (60) and resuspended in Dulbecco's modified Eagle medium-based complete

media with 5% FBS with or without various concentrations of NSC 13778 and its selected analogs. After a 1-h incubation at 37°C, the virions were repelleted to remove the compounds and resuspended in Dulbecco's modified Eagle medium-based complete medium with 5% FBS. Virus inocula thus prepared were used to infect MAGI cells as described above with a final FBS concentration of 10%.

Cell-based fusion assay. HeLa-derived HL2/3 cells (contributed by Barbara Felber and George Pavlakis; AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), which express HIV-1$_{HXB2}$ Env on the cell surface in addition to Tat, Gag, Rev, and Nef proteins in the cytoplasm (12), were cocultured with CD4 and CXCR4-expressing MAGI cells (above) at a 1:1 cell density ratio (5 × 10^4 cell/well each) for 8 h in the absence or presence of test compounds in order to examine whether the compounds interfered with the binding process of HIV-1 Env and cell surface receptor or coreceptor. Upon fusion of MAGI and HL2/3 cells, Tat protein from HL2/3 cells activates β-galactosidase indicator gene expression in MAGI cells. The fused cells, seen as blue foci, were counted under a light microscope.

Evaluation of viral entry inhibition by NSC 13778 against HIV-1 enveloped versus VSV-G pseudotyped virions. Infectious virus stocks were generated by transfection of 293T cells with methods and plasmids described in detail by Buckman, et al. (6). Briefly, stocks of HIV-1 with the native glycoprotein were produced by transfection of the wild-type proviral plasmid pNL$_{4-3}$ or an integrase negative mutant pIN-D116N (6, 24) by the CaPO$_4$ method. Stocks of HIV-1 pseudotyped with the vesicular stomatitis virus G protein (VSV-G) were produced by cotransfection of the envelope-negative mutants pNL$_4$·3Env(−) or pIN-D116N/Env(−) (6) in combination with pHCMV-G (7). CD4-positive HeLa cell-derivative, HCLZ cells (28) (kindly provided by D. Waters, NCI-Frederick) and human osteosarcoma cells (HOS) were used as target cells for HIV-1 enveloped and VSV-G pseudotyped virions, respectively. Infections were carried out in the absence or presence of 100 μM NSC 13778 for 4 h, after which the cells were extensively washed to remove extracellular virions, transfected plasmid DNA, and the compound, followed by further incubation for 24 h. In order to evaluate possible differences in cell entry between HIV-1 enveloped and VSV-G pseudotyped virions in the presence of NSC 13778, quantitative real-time PCR was used to determine the 2-LTR circle copy numbers by using total cellular DNA extracted from infected cells as described previously (6).

HIV-1 infection of CD4-negative B cells. Various B-cell lines have been reported to be susceptible to CD4-independent HIV-1 entry via chemokine receptor CXCR4 in vitro (20). Of various B-cell lines screened, CD4-negative Akata cells (EBV+), a Burkitt's lymphoma cell line (61, 68) (kindly provided by K. Takada), were selected to evaluate inhibitory activity of NSC 13778 against wild-type HIV-1$_{RF}$. Two million Akata cells were incubated with 0.5 ml of HIV-1$_{RF}$ (∼20,000 BFU as determined by MAGI assay [see above]) at 37°C in 5% CO$_2$-containing humidified air overnight. Similarly, CD4$^+$ CEM-SS cells were infected with the identical HIV-1 inoculum in parallel. On the following day, cells were washed with RPMI 1640 medium three times and resuspended in RPMI 1640 complete medium with 10% FBS. Culture supernatants collected immediately after vigorous virus washout at time zero (baseline) and 48 to 72 h after infection were subjected to p24 antigen level determination by enzyme-linked immunosorbent assay (ELISA; AIDS Vaccine Program, NCI-Frederick, Frederick, MD). Cells harvested at 48 to 72 h postinfection were subjected to the low-molecular-weight (LMW) DNA extraction (33). The presence of HIV-1 early RT transcript, minus-strand cDNA, in the LMW DNA was examined by PCR with the primer pair 5′R-3′U5 as described previously (69). In addition, a mitochondrial DNA primer pair, MTC/F (5′-TGGAGCCGGAGCACCCTATG TC-3′) and MTC/R (5′-ATGGGCGGGGGTTGTATTGATG-3′), was used as an internal control for each PCR sample.

HIV-1 gp120 capture ELISA. Recombinant HIV-1$_{IIIB}$ gp120 protein (referred to as rgp120 hereafter unless otherwise noted) capture ELISA was performed according to the manufacturer's instructions (ImmunoDiagnostics, Inc., Woburn, MA). Briefly, various concentrations of free ligand rgp120 (up to 5 μg/ml) were added to a CD4-coated plate in the absence or presence of test compounds diluted in the kit component C diluent buffer. After a 60-min incubation at room temperature, the amounts of captured rgp120 were detected by peroxidase-conjugated murine anti-gp120 MAb. Prior to testing the rgp120-capture inhibitory activity of the test compounds, we determined that rgp120 binding reached saturation in 30 min with the ligand concentration of 100 to 200 ng/ml. All assays were performed in duplicate and were independently repeated at least three times. In separate experiments, the test compounds were added after a 60-min preincubation of rgp120 in CD4-coated plates. After rgp120-preadsorbed plates were washed three times to remove unbound rgp120, the compounds diluted in diluent buffer (or diluent buffer only) were incubated in the plates for another 15 min, followed by detection of remaining bound rgp120 by the anti-gp120 antibody. In mechanistic characterization of rgp120-displacing activity of NSC 13778,
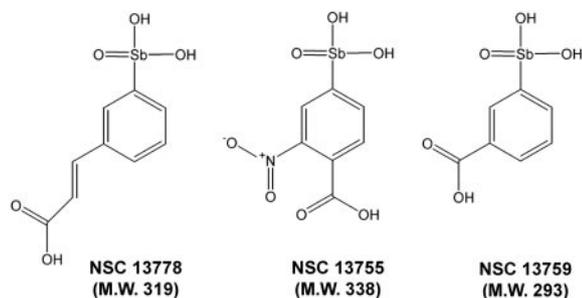
FIG. 1. Structures of NSC 13778 and its analogs NSC 13755 and NSC 13759.

the four different peptides listed above were dissolved in the kit buffer and added with NSC 13778 to gp120 capture ELISA plates.

Surface plasmon resonance biosensor analysis. Surface plasmon resonance analyses were performed by using a Biacore 2000 optical biosensor (Biacore International AB, Uppsala, Sweden). Soluble CD4, rgp120, and D1/D2 CD4 were immobilized onto CM5 sensor chips by using standard amine-coupling chemistry as previously described (26). The surfaces densities for sCD4, rgp120, and D1/D2 CD4 were ca. 1,000, 1,000, and 2,800 response units, respectively. Noncovalently attached protein was removed by injection of 50 mM sodium hydroxide. NSC 13778 and its analogs were diluted in phosphate-buffered saline (PBS [pH 7.5]) to concentrations starting from 0.11 up to 14 μM and injected over each flow cell at a flow rate of 50 μl/min. The compounds were allowed to associate and dissociate for 30 s. After the injection, the surface was washed by 50% dimethyl sulfoxide for 30 s at a flow rate of 50 μl/min, followed by additional 30-s wash with 3% dimethyl sulfoxide in PBS to regenerate the surface.

Trp fluorescence quenching study. Binding of NSC 13778 to D1/D2 CD4 was examined by monitoring changes in the emission intensity of intrinsic tryptophan (Trp) fluorescence of the protein as described previously (64, 73). Briefly, increasing amounts of NSC 13778 were added to D1/D2 CD4 protein diluted to 200 nM in PBS in a quartz microcuvette (5 by 5 mm; Thermo Spectronic, Rochester, NY) containing a total reaction volume of 180 μl at ambient temperature. Measurements were made by using an Aminco Bowman Series 2 Fluorimeter (Rochester, NY) with excitation at 280 nm (5-nm bandwidth) and emission at 350 nm (5-nm bandwidth). Corrections were made for intrinsic fluorescence of the buffer and for the loss of fluorescence intensity due to protein adsorption and nonspecific quenching effects. The percent fluorescence reduction in the presence of test compound is defined as $100 \times [1 -$ intensity (drug)/intensity (buffer only)].

Flow cytometric analysis. The effects of NSC 13778 on binding of rgp120 or anti-CD4 MAbs to cell surface CD4 were evaluated by flow cytometry. Briefly, CEM-SS cells (10^6 cells/ml) were incubated with or without 10 μg of rgp120/ml in Opti-MEM medium (Invitrogen) in the absence or presence of increasing concentrations of NSC 13778 at 37°C in 5% CO_2-containing humidified air for 30 min. The centrifuged cells were incubated with FITC-anti-gp120 MAb, PE-SK3 MAb, or FITC-MT310 MAb on ice for 15 min; washed first in Dulbecco's PBS without calcium and magnesium (D-PBS; Invitrogen) supplemented with 2% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and 0.5% sodium azide (Sigma) (BSA-buffer); and then washed in D-PBS and resuspended in 2% BSA buffer for analysis. For unconjugated anti-CD4 OKT4 MAb, the antibody binding on the cell surface CD4 was detected by PE-conjugated goat anti-mouse IgG F(ab')$_2$ fragment (heavy and light chain). Flow cytometric analysis was performed on a Coulter XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Events were collected by gating on forward and 90° light scatter. Positive fluorescence was determined by using a matched isotype control IgG conjugated with an appropriate fluorophore.

Lymphoproliferation assays. In order to examine whether NSC 13778 and its analogs interfere with CD4 functions, lymphoproliferative responses to a recall antigen, 2.5 μg of tetanus toxoid (EMD Biosciences, Inc., San Diego, CA)/ml, and phytohemagglutinin (1:100; Invitrogen, Carlsbad, CA) were determined as described previously (52) in the absence or presence of increasing concentrations of the test compounds by using peripheral blood mononuclear cells obtained from healthy blood-bank donors. Cell growth was determined by XTT assay (74). Proliferative responses were expressed as stimulation indices, which were calculated as follows: (optical density [OD] of cells cultured in the presence of mitogens – OD media background)/(OD of cells cultured in the absence of mitogens – OD media background).

RESULTS

Anti-HIV-1 activity of NSC 13778 and its analogs. NSC 13778 (molecular weight, 319; Fig. 1) is an aromatic pentavalent antimony compound originally submitted to the Developmental Therapeutics Program of the National Cancer Institute more than 30 years ago. The antiviral activity of NSC 13778 and five other analogs was recently discovered through a cell-based anti-HIV screen. Further characterization on mode of anti-HIV-1 action was carried out by using the active compounds with observed purity of >95% ($n = 3$, Fig. 1) as determined by mass spectrometry (62). All three active compounds (NSC 13778, NSC 13755, and NSC 13759) studied shared a similar pentavalent stibono-benzoic acid structure (Fig. 1). In CEM-SS cells, EC50 values ranged from 0.94 ± 0.14 μM (mean ± the standard deviation [SD]) to 8.68 ± 2.49 μM without any discernible cytotoxicity at the concentrations tested up to 400 μM (Table 1).

In order to determine possible step(s) of viral replication inhibited by the compounds, we evaluated the activity of NSC 13778 and two analogs in one-round of HIV-1 infection assay by using a MAGI assay. All three compounds exerted potent antiviral activity in MAGI system with 50% inhibitory concentration (IC_{50}) ranging from 0.64 ± 0.40 to 2.47 ± 1.65 μM (Table 1). In contrast, no antiviral activity was demonstrated in chronically HIV-1-infected cells (data not shown). These data indicated that the compounds inhibited HIV-1 replication most likely prior to viral integration.

NSC 13778 inhibits HIV-1 infection by blocking gp120-CD4 interaction. We then examined the impact of delayed drug addition in MAGI-based time of addition assay, wherein MAGI cells were treated with the compounds upon HIV-1 inoculation (time zero) or at various time points postinoculation. Although NSC 13778 and two analogs effectively blocked HIV-1 infection at 3 to 10 μM if added at time zero, a substantial decline in antiviral activity was observed when the compound addition was delayed for more than 2 h after HIV inoculation (Fig. 2). These data suggested that the compounds exerted anti-HIV activity at an early stage of viral replication, most likely as a virucidal agent or viral entry inhibitor. However, we found that HIV-1 virions pretreated with the compounds at 10 μM still remained highly infectious (data not shown), indicating a virucidal effect did not play a fundamental role in the anti-HIV activity of NSC 13778 and its analogs.

To further elucidate how NSC 13778 and the active analogs interfered with the HIV entry process, we used various assays

TABLE 1. Summary of antiviral activity of NSC 13778 and its analogs[a]

NSC no.	CEM-SS-based HIV-CPE protection assay			MAGI assay	
	Mean EC_{50} (μM) ± SD	CC_{50} (μM)	TI	Mean IC_{50} (μM) ± SD	Mean IC_{90} (μM) ± SD
13778	0.94 ± 0.14	>400	>426	0.64 ± 0.40	2.70 ± 0.95
13755	8.68 ± 2.49	>400	>46	2.47 ± 1.65	9.07 ± 3.13
13759	1.88 ± 0.15	>400	>213	1.66 ± 0.71	5.98 ± 1.93

[a] CPE, cytopathic effect; EC_{50}, effective concentration to achieve 50% protection against HIV-CPE; CC_{50}, cytotoxic concentration resulting in 50% cell death; TI, therapeutic index.
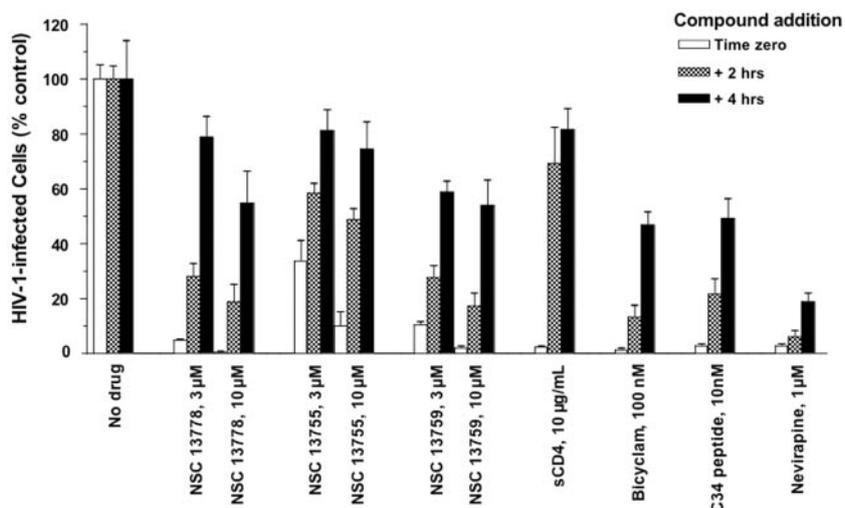
FIG. 2. MAGI-based time of addition assay. The effect of delayed compound addition on antiviral activity was investigated in one round of HIV-1 infection of MAGI cells to determine possible steps of viral replication affected by the test compounds. NSC 13778 and its analogs, NSC 13759 and NSC 13759, were added at 3 or 10 μM to HIV-1$_{NL4-3}$-infected MAGI cells upon virus inoculation (time zero), 2 h (+ 2 h), or 4 h (+ 4 h) postinoculation. A substantial loss in antiviral activity, depicted as HIV-1-infected cells (percentage of no drug control, mean ± SD), was demonstrated for all three test compounds when the drug addition was delayed for more than 2 h, in contrast to their potent activity if added at time zero. Also included in the experiment as reference compounds were virus entry blockers (10 μg of sCD4/ml, 100 nM bicyclam, 10 nM C34 peptide) and reverse transcriptase inhibitor (1 μM nevirapine). Note that whereas viral entry blockers substantially lose antiviral activity if the compound addition is delayed for more than 2 h, nevirapine remains significantly active even when the addition is delayed for 4 h. The results shown here are representative of three separate experiments.

focusing on specific aspects of virus envelope and cell receptor/coreceptor interactions. We cocultured HL2/3 cells, which express HIV-1 Env on the cell surface and Tat protein in the cytoplasm (12), and MAGI indicator cells in the absence or presence of various concentrations of the test compounds. NSC 13778 and the active analogs efficiently blocked fusion between HL2/3 and MAGI cells mediated by gp120 on the HL2/3 cell surface and CD4/CXCR4 on the MAGI cell surface (Fig. 3A). NSC 13778 also exhibited comparable antiviral activity against X4-, R5-, or X4/R5-tropic HIV-1 strains in MAGI cells expressing both CXCR4 and CCR5 viral coreceptors (MAGI-CCR5 cells) (8) (Fig. 3B). In contrast, the compound showed no inhibitory activity against VSV-G pseudotyped virions (Fig. 3C). These data demonstrated that the activity of NSC 13778 and its active analogs was specific to the HIV-1 Env-cell surface receptor/coreceptor-mediated entry process and that the compounds most likely targeted highly conserved HIV-1 Env domains, which are required for viral attachment or fusion process, or the CD4 receptor, rather than serving as a coreceptor antagonist.

We next examined the effect of NSC 13778 on the HIV-1 infection of CD4-negative cells. In order to ascertain whether NSC 13778 blocked HIV-1 cell entry by binding to Env domain(s) critical for viral attachment or fusion process or by masking CD4, we opted to compare its antiviral activity in CD4$^+$ versus CD4$^-$ cells in parallel, with both infected with the same unmodified wild-type HIV-1. Some CD4-negative human B cells have previously been shown to be susceptible to HIV-1 entry via CXCR4 (20). Of various B-cell lines screened for in vitro susceptibility, we selected a Burkitt's lymphoma cell line, Akata (61, 68), which lacked detectable cell surface CD4 expression, as determined by flow cytometry (data not shown). Akata cells were exposed to wild-type HIV-1$_{RF}$ in the absence

or presence of NSC 13778. Supernatant p24 antigen levels from a 3-day-old culture indicated Akata cells were productively infected with HIV-1 despite the treatment with NSC 13778, which completely blocked HIV-1 infection in CEM-SS cells exposed to the identical virus inoculum in the same manner (Fig. 4A). The presence of reverse-transcribed product of HIV-1 genome was also confirmed inside Akata cells (Fig. 4B). The lack of anti-HIV activity of NSC 13778 in CD4-negative B cells thus suggested that the compound did not significantly affect HIV-1 Env interactions with chemokine receptors or subsequent fusion process but most likely interfered with HIV-1 gp120-CD4 interaction.

NSC 13778 competes with gp120 for CD4 binding. The inhibitory activity of NSC 13778 against gp120-CD4 interaction was investigated in a competitive gp120-capture ELISA, in which increasing amounts of rgp120 were added to an sCD4-coated microplate in the absence or presence of various doses of NSC 13778, and the amounts of rgp120 bound to the plate were determined. As shown in Fig. 5A, significant decreases in OD values were observed in the presence of NSC 13778 within the rgp120 concentrations tested (up to 5,000 ng/ml [≅41.67 nM]). We confirmed that NSC 13778 neither interfered with the binding affinity of anti-gp120 antibody used in the assay nor stripped off coated sCD4 from the ELISA plate after the incubation (data not shown). Thus, the observed decreases in absorbance indicated that NSC 13778 reduced the amounts of rgp120 captured on a solid-phase CD4. Saturation binding curves were compared between no drug control and NSC 13778-dosed groups. Apparent dissociation equilibrium constant (K_d) values for rgp120 increased with the NSC 13778 concentration, whereas the maximal binding capacity (B_{max}) remained relatively constant (Fig. 5A, bottom panel). Similar patterns of gp120-binding inhibition by NSC 13778 were also
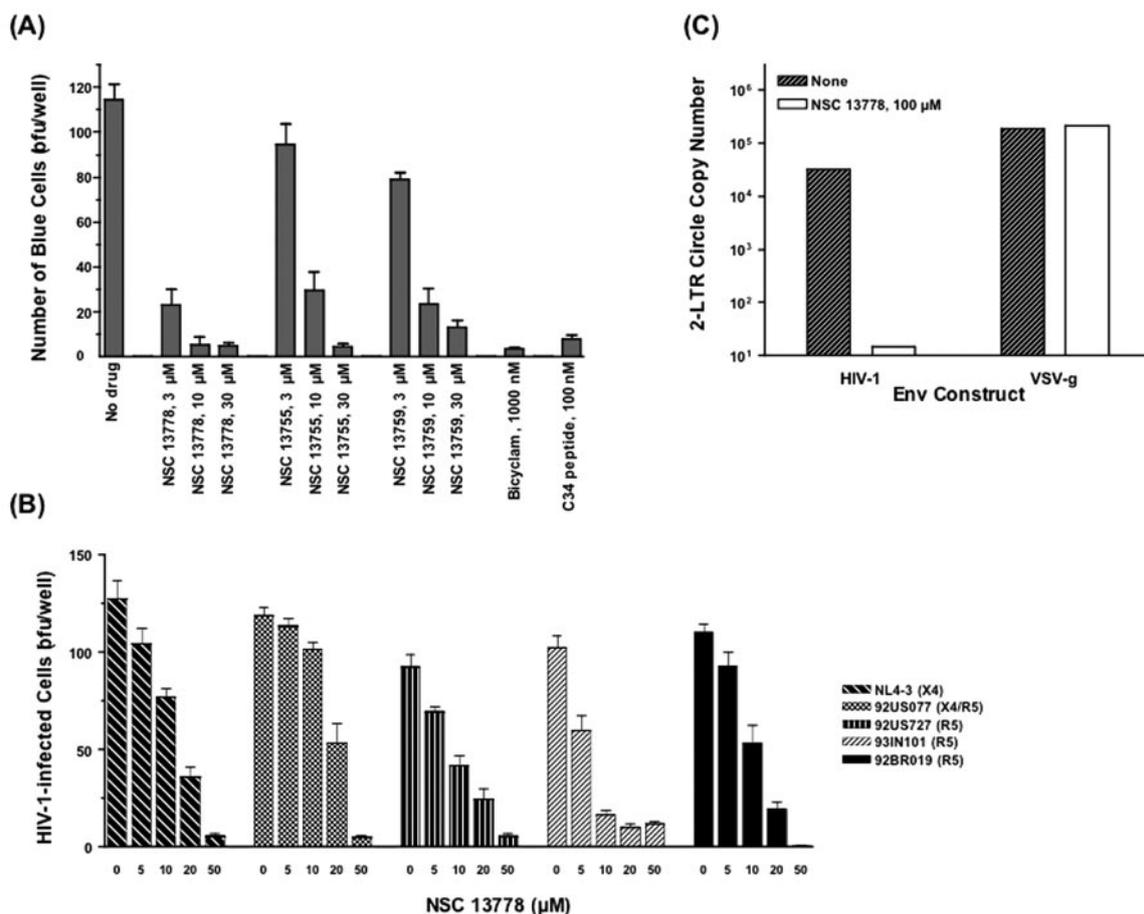
FIG. 3. Effects of NSC 13778 and its analogs on viral envelope and cell surface receptor-coreceptor interaction. (A) Env (HXB2)- and Tat-expressing HL2/3 donor cells (12) were cocultured with MAGI indicator cells (see the text) in the absence or presence of NSC 13778, NSC 13755 or NSC 13759. The number of fused cells, seen as blue foci under a microscope, is expressed as BFU/well (mean ± SD). NSC 13778 and its analogs effectively inhibited fusion of HL2/3 and MAGI cells at ca. 3 to 30 μM. Also included as reference compounds are 1,000 nM bicyclam and 100 nM C34 peptide. The experiment shown is representative of three separate experiments. (B) Antiviral activity of NSC 13778 was compared against X4 (NL4-3)-, R5 (92US727, 93IN101, and 92BR019)-, and X4/R5-tropic (92US077) HIV-1 strains by using MAGI-CCR5 cells that expressed both CXCR4 and CCR5 coreceptors (8). Shown are the numbers of HIV-1-infected MAGI-CCR5 cells (BFU/well, mean ± SD). NSC 13778 exhibited comparable antiviral activity against five different HIV-1 strains requiring CXCR4, CCR5 or both coreceptors to enter target cells, suggesting that the compound did not appear to act as a coreceptor antagonist. The experiment shown is representative of four separate experiments. (C) Comparison of inhibitory activity of NSC 13778 against HIV-1-enveloped versus VSV-G pseudotyped virus entry. HCLZ or HOS cells were exposed to HIV-1$_{NL4-3}$ enveloped or VSV-G pseudotyped virions in the absence or presence of NSC 13778 at 100 μM. The efficiency of cell entry was evaluated by the 2-LTR circle copy numbers determined by quantitative real-time PCR (6). The experiment shown is from infections with integrase deficient virions with respective envelope proteins (see methods) and is representative of four separate experiments.

observed when increasing amounts of the compound were added after the gp120-CD4 binding reached equilibrium in 60 min (Fig. 5B), indicating that NSC 13778 displaced rgp120 preadsorbed to the sCD4-coated plate. These findings suggested that NSC 13778 competitively inhibited gp120-binding to CD4. Of note, sCD4, a natural ligand for gp120, was also tested for the ability to block gp120-binding in the same gp120 capture ELISA with or without delayed addition. Although sCD4 blocked the binding of rgp120 to CD4 when added at time zero as expected, addition of sCD4 after 60-min preincubation of rgp120 in the sCD4-plate had no effect on saturation binding of rgp120 to CD4 (data not shown).

We next compared the levels of inhibitory activity of NSC 13778 and its analogs in a gp120-capture ELISA. The test compounds were incubated at various concentrations with

rgp120 (100 ng/ml) in sCD4-coated plates. A dose-dependent inhibition of gp120 capture was clearly demonstrated with NSC 13778 and two active analogs (Fig. 5C), whereas three inactive analogs with observed purities of at least 84% (62) showed no or little inhibition (data not shown). In addition, three other active and three inactive analogs, although with lesser purity, were tested in the same gp120 capture ELISA, which demonstrated virtually an identical correlation between gp120-CD4 disrupting activity and anti-HIV activity in cells (data not shown). IC$_{50}$s in the gp120 capture ELISA ranged from 1.29 ± 0.62 μM (mean ± SD) to 2.66 ± 0.95 μM for three active compounds (Fig. 5C, bottom panel). NSC 13778 exerted equally potent inhibitory activity against recombinant HIV-1$_{BaL}$ gp120 (rgp120$_{BaL}$) binding to CD4 in a rgp120$_{BaL}$-capture ELISA (data not shown).
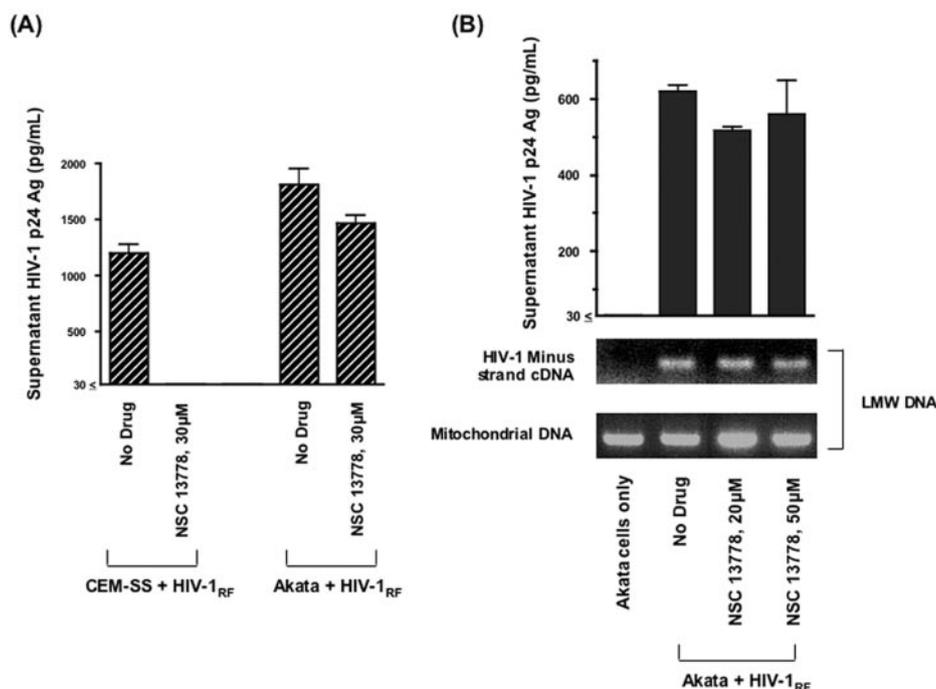
FIG. 4. Antiviral activity of NSC 13778 in CD4-positive versus CD4⁻ target cells. Anti-HIV activity of NSC 13778 was compared between CD4⁺ CEM-SS versus CD4⁻ Akata, a Burkitt's lymphoma cell line (61, 68), infected with wild-type HIV-1$_{RF}$. (A) Levels of p24 antigen (mean ± SD) in the culture supernatants obtained from CEM-SS or Akata cells 3 days after HIV-1 infection in the absence or presence of NSC 13778 at 30 µM. The experiment shown is representative of three separate experiments. (B) In order to examine the presence of reverse-transcribed product of HIV-1 RNA genome, minus-strand cDNA, inside Akata cells, LMW DNA extracted from HIV-1-infected Akata cells in the absence (no drug) or presence of 20 or 50 µM NSC 13778 was subjected to PCR by using a primer pair, 5′R-3′U5, as described previously (69). As an internal control, the mitochondrial DNA fragment was amplified by using a primer pair, MTC/F and MTC/R (see the text), for each sample. Also shown are the levels of p24 antigen in the culture supernatants from the corresponding samples.

Electrostatic potential has been shown at the gp120-CD4 contact surfaces between negatively charged gp120 residues and positively charged CD4 residues (41). We sought to determine whether the disruption of gp120-CD4 by NSC 13778 was caused by electrostatic interference or a competition for the specific gp120-binding site. Two different peptides derived from CD4 residues, one including the gp120-binding domain (CD4 peptide #1 [QKKSIQFHWKNSNQIKILGNQGSFLTK GPSKLNDRADSRRS], mostly positively charged; see Fig. S1A in the supplemental material) and the other, an adjacent segment not involved in the gp120-binding (CD4 peptide #2 [KIEDSDTYICEVEDQKEEVQLLVFGLTANSDTHL], mostly negatively charged; see Fig. S1B in the supplemental material), as well as a 20-mer lysine repeat (K20) and a 20-mer aspartate repeat (D20), were evaluated for the potential to interfere with NSC 13778 in a gp120 capture ELISA. NSC 13778, and various concentrations of each peptide were simultaneously incubated with rgp120 in a sCD4-coated plate. The gp120-CD4 disrupting activity of NSC 13778 was dramatically reduced in the presence of positively charged CD4 peptide #1 or K20 (Fig. S1A), whereas negatively charged CD4 peptide #2 or D20 showed no interference with the NSC 13778 activity (Fig. S1B), suggesting the involvement of electrostatic elements in the gp120-CD4 disruption rather than competition for the specific binding motif. In the absence of NSC 13778, these peptides showed no interference in the gp120 capture itself, except for CD4 peptide #2, which reduced the amounts

of bound rgp120 in a dose-dependent manner (data not shown). The CD4 peptide #2-mediated inhibition of gp120 capture in the current ELISA may have resulted from nonspecific binding of the peptide to positively charged gp120-binding domain of CD4, for it did not block the anti-gp120 antibody binding to rgp120 (data not shown).

NSC 13778 binds to D1/D2 CD4 near tryptophan residues within the gp120-binding domain. To further explore the binding target of NSC 13778, we examined the binding affinities of NSC 13778 to sCD4 and rgp120 by using SPR biosensor technology. Our preliminary data indicated that the compound consistently bound to sCD4 and rgp120 immobilized on a sensor chip (Fig. S2 in the supplemental material). However, in these preliminary experiments, an inactive analog NSC 13771 used as a reference repeatedly showed some degrees of binding to both proteins (Fig. S2 in the supplemental material), suggesting that our preliminary SPR data represented, at least in part, nonspecific bindings of the analyte to the surface, as has previously been recognized in various SPR applications (17, 50). We then investigated the binding affinities of NSC 13778 and NSC 13771 to a truncated N-terminal two-domain CD4 protein, D1/D2 CD4, by SPR. The sensorgram overlays for the drug binding to immobilized D1/D2 CD4 clearly demonstrated that NSC 13778 bound to D1/D2 CD4, whereas NSC 13771 showed little affinity (Fig. 6A), despite that the binding capacity of the D1/D2 CD4 surface was higher than the sCD4 or rgp120 surface (see above).
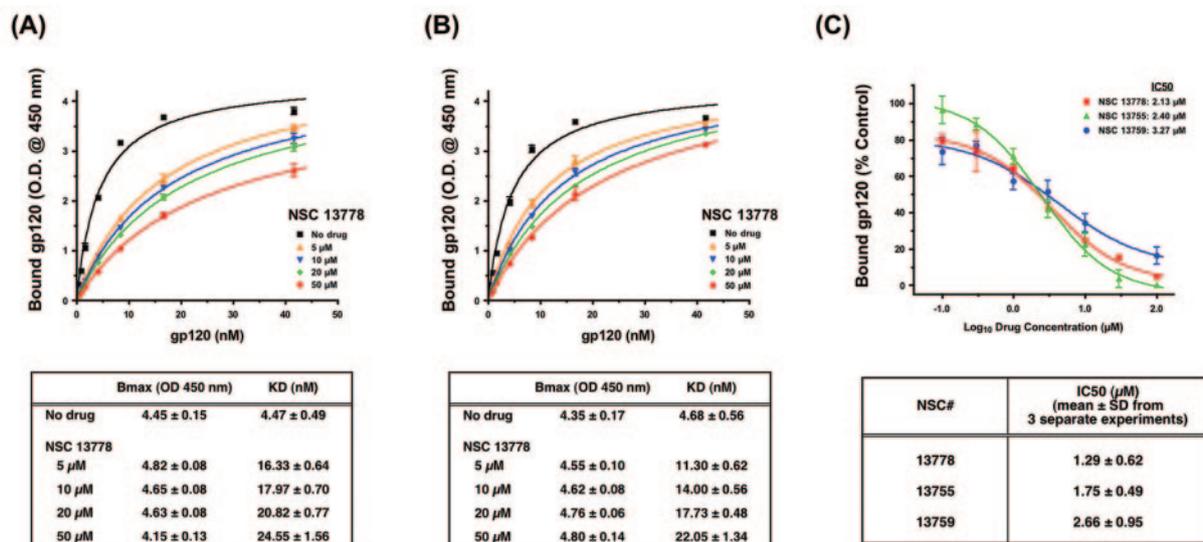
FIG. 5. Inhibition of gp120 capture by NSC 13778 and its active analogs. (A) Saturation binding curves of rgp120 captured onto a solid-phase sCD4 in the absence (no drug) or presence of 5, 10, 20, or 50 μM NSC 13778 in a competitive gp120-capture ELISA. The amounts of captured rgp120 are indicated by the OD values at 450 nm (O.D. @ 450 nm). The data were analyzed by using a GraphPad Prism (GraphPad Software, Inc., San Diego, CA) one-site binding model. Note that the apparent K_d values for rgp120 (nM; mean ± the standard error of the mean [SEM]) increased with the NSC 13778 concentration, whereas the B_{max} values (OD 450 nm [mean ± the SEM]) remained relatively constant (lower panel). (B) Comparison of saturation binding curves of rgp120 in the absence or presence of increasing concentrations of NSC 13778 (5, 10, 20, and 50 μM) in a competitive gp120-capture ELISA similar to that used for panel A but with the addition of the compound after rgp120-CD4 binding reached equilibrium in 60 min. NSC 13778 dose dependently displaced rgp120 prebound to CD4. Again, the data were analyzed by using a GraphPad Prism one-site binding model. Similar to the results for panel A, apparent K_d values for rgp120 (nM [mean ± the SEM]) increased with the NSC 13778 concentration, whereas the B_{max} values (OD 450 nm [mean ± the SEM]) remained relatively constant (inset). (C) Fifty percent gp120-binding inhibition concentrations (IC_{50}) of NSC 13778, NSC 13759, and NSC 13759 were determined by coincubation of rgp120 (100 ng/ml) and increasing concentrations of the test compounds in a gp120-capture ELISA. IC_{50} values ranged from 1.29 ± 0.62 μM to 2.66 ± 0.95 μM (mean ± SD from three independent experiments) (lower panel).

There are two Trp residues within the gp120-binding domain of CD4 (Trp28 and Trp62). D1/D2 CD4 contains a total of three Trp residues, the first two in the gp120-binding domain of D1 and the third at position 157 in the D2 domain. We hypothesized that NSC 13778 would affect the tryptophan fluorescence emission of the protein, if the compound bound to the gp120-binding domain of CD4. As shown in Fig. 6B, a dose-dependent reduction in the intrinsic tryptophan fluorescence emission was observed when D1/D2 CD4 was incubated with NSC 13778 but not with the inactive analog NSC 13771, suggesting NSC 13778 specifically bound to D1/D2 CD4 near Trp residues. Similar levels of Trp fluorescence quenching
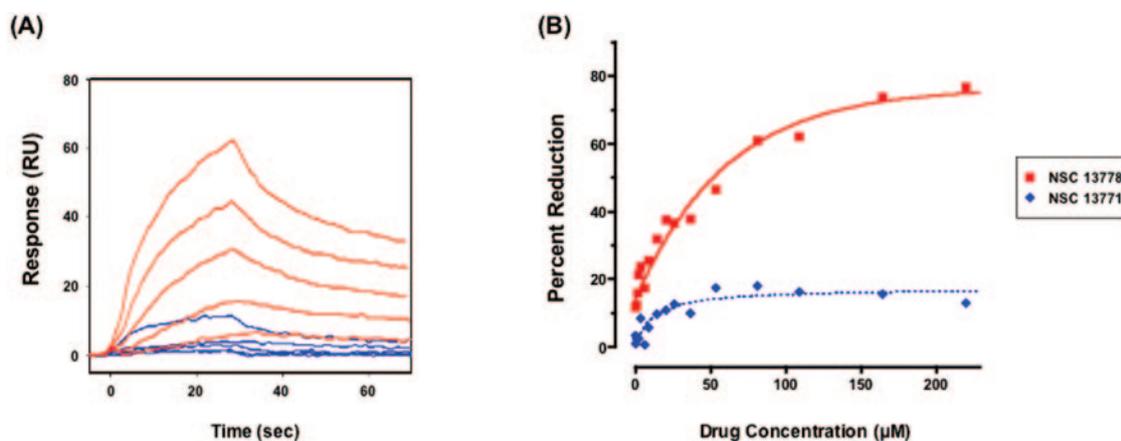

FIG. 6. Binding of NSC 13778 to D1/D2 CD4 protein. (A) Sensorgram overlays for the drug binding to D1/D2 CD4 immobilized on a Biacore sensor chip. Shown are the binding responses of NSC 13778 (red) and NSC 13771 (blue) injected at 0.22, 0.44, 0.88, 1.76, and 3.52 μM over the surface. (B) Changes in the emission intensity of intrinsic tryptophan fluorescence of D1/D2 CD4 protein (200 nM) were monitored in the presence of increasing amounts of NSC 13778 (red) or NSC 13771 (blue) and depicted as the percent inhibition compared to untreated control after correction for the buffer and nonspecific quenching effects. The experiment shown is representative of three separate experiments.
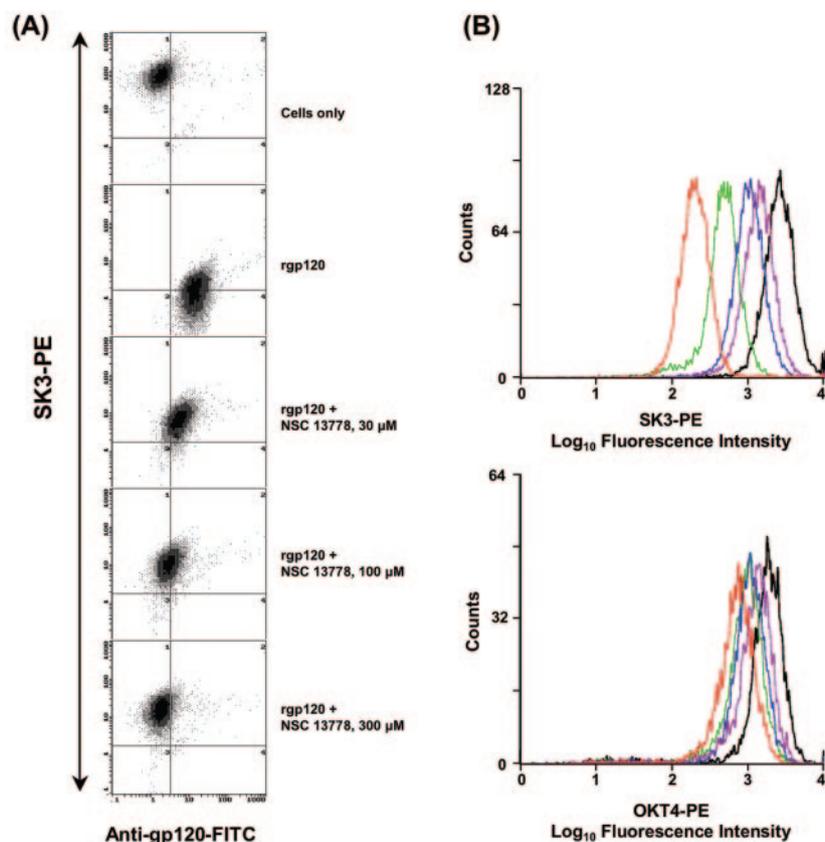
FIG. 7. Effects of NSC 13778 on binding of rgp120 or anti-CD4 antibodies to cell surface CD4. (A) CEM-SS cells were incubated with 10 μg of rgp120/ml in the absence of NSC 13778 or in the presence of 30, 100, or 300 μM NSC 13778, followed by staining with FITC-conjugated anti-gp120 MAb and PE-conjugated anti-CD4 antibody clone SK3. Scattergrams obtained by flow cytometric analysis are shown. (B) CEM-SS cells were incubated with rgp120 or NSC 13778 alone at 30, 100, 300 μM and stained with anti-CD4 MAb SK3 (top) or OKT4 (bottom). Histograms of fluorescence intensity shown include cells only (control) (black), rgp120 (red), 30 μM NSC 13778 (purple), 100 μM NSC 13778 (blue), and 300 μM NSC 13778 (green). Note that the the binding of SK3 to cell surface CD4 (top) was dose dependently reduced in the presence of NSC 13778, whereas the OKT4-binding was not as significantly reduced (bottom).

were observed with an active analog NSC 13755 tested at various concentrations, whereas two other inactive analogs showed no or lesser interference (data not shown).

Effects of NSC 13778 on the cell surface CD4 receptor. We then investigated whether NSC 13778 could block gp120 binding on cell surface CD4, using flow cytometric analysis. CEM-SS cells were incubated with 10 μg of rgp120/ml in the absence or presence of increasing concentrations of NSC 13778. Addition of rgp120 to CEM-SS cells resulted in anti-gp120-FITC reactivity on the cell surface, whereas it substantially blocked the binding of anti-CD4 antibody, Leu-3a clone SK3, known to recognize the gp120-binding domain of CD4 (54) (Fig. 7A, second from top). In the presence of NSC 13778, there was a dose-dependent decrease in the amounts of rgp120 bound on the cell surface, as indicated by the decreasing reactivity of anti-gp120 MAb and a concurrent shifting of SK3 MAb signal toward baseline (cells only control) (Fig. 7A). These findings suggested that NSC 13778 blocked rgp120-binding on the cell surface and, as a result, the gp120-binding domain of CD4 became readily accessible to SK3 anti-CD4 antibody recognition. However, despite a complete abrogation of rgp120 binding at the highest concentration of NSC 13778 tested, SK3 reactivity did not return to the baseline level (Fig. 7A). When

CEM-SS cells were incubated with NSC 13778 alone, the compound appeared to reduce the binding of SK3 itself (Fig. 7B). Similar results were obtained with another anti-CD4 MAb, MT310, also known to recognize the gp120-binding site of CD4 (54) (data not shown). Of note, slightly reduced fluorescence intensities of OKT4 were observed in CEM-SS cells incubated with rgp120 or NSC 13778 alone (Fig. 7B), presumably reflecting the early phase of CD4 downmodulation induced by the ligand-binding to CD4 (27). However, the reduced reactivity of SK3 or MT310 in the presence of NSC 13778 was probably not due to compound-induced downmodulation of CD4 but was more likely due to the binding of NSC 13778 to the SK3 (or MT310) recognition site, the gp120-binding domain of CD4, since the shift in OKT4 staining was significantly smaller than the reduction in the SK3 binding (Fig. 7B).

Because NSC 13778 appeared to bind to the cell surface CD4 receptor, we examined whether NSC 13778 would hinder CD4-mediated normal T-cell functions. Lymphoproliferative responses to a recall antigen, tetanus toxoid, and phytohemagglutinin were examined in peripheral blood mononuclear cells obtained from three different normal donors in the absence or presence of increasing concentrations of NSC 13778 and its analogs. None of the compounds reduced lymphoproliferative

responses to either tetanus toxoid or phytohemagglutinin at concentrations tested up to 100 μM (Fig. S3 in the supplemental material).

DISCUSSION

Inhibitors of HIV-1 entry had actively been pursued for clinical applications long before the interaction of gp120-CD4 was ultrastructurally elucidated. One of the first entry inhibitor agents to be administered to HIV-1-infected patients was sCD4, devised as a decoy for HIV-1 gp120 protein. Although sCD4 exhibited a potent antiviral activity against laboratory-adapted HIV-1 strains in vitro (19, 25, 34, 63, 70), no significant antiviral efficacy was observed in patients (58); this was presumably due to the relative resistance of primary HIV-1 isolates to sCD4-mediated neutralization (15, 48, 51). A number of various CD4-derived products have since been proposed to date, including CD4-mimicking proteins and CD4-immunoglobulin hybrid molecules, in an attempt to optimize the pharmacokinetics and gp120-binding in vivo (1, 35, 46, 71, 72, 76). Other HIV entry inhibitors pursued for development include chemokine receptor antagonists (4, 21, 56, 65), various chemotypes of gp120-binders (31, 44), and fusion inhibitors (22, 75). Despite such persistent and intense efforts for drug development, T20, a fusion inhibitor (36, 42, 43), is the only HIV entry inhibitor approved by the U.S. Food and Drug Administration for the treatment of HIV/AIDS at the present time.

HIV-1 gp120 protein undergoes extensive structural rearrangements upon binding to CD4. It has been suggested that the specific CD4-binding pocket within the gp120 core does not exist in the absence of CD4 (40). However, HIV can efficiently initiate the attachment to the cell surface CD4 because of the ability of CD4 to bind multimeric gp120 spikes simultaneously (39). Likewise, the chemokine receptor-binding domain is deeply buried away from the protein surface until gp120-CD4 binding takes place (39). Such conformational masking of critical and potentially vulnerable receptor-binding sites of gp120 has also been demonstrated for the neutralizing antibody-recognition domains (39). This remarkable degree of highly coordinated gp120 domain-masking and sequential refolding during viral entry illustrates the superb resilience of HIV to evade host immune surveillance and presents a formidable obstacle for the development of effective entry blockers. Conceivably, sCD4 was found to be virtually ineffective in previous clinical trials, because sCD4-binding triggered gp120 rearrangements in situ, resulting in the prompt exposure of the chemokine receptor-binding domain and permitting accelerated viral entry process through chemokine receptor, especially via CCR5 (55, 59, 66, 67). HIV-1 gp120 binders targeting a portion of gp120 residues that line the CD4-binding pocket may deter gp120 rearrangements and thus viral entry. However, such agents will quickly be overcome by resistant HIV-1 strains (44).

Rather than aiming for a "moving target," gp120, it may be more efficient to specifically mask the gp120-binding domain of CD4, so as not to allow incoming HIV-1 virion gp120 to undergo conformational changes required to begin the specific receptor-mediated viral entry process. Although such a CD4-masking strategy will almost certainly foster resistant HIV-1 strains that can enter the host cells independently of CD4,

selection pressure to attain CD4 independence may also force HIV to expose neutralizing antibody recognition domains, rendering the virus more susceptible to immune clearance in vivo (23). To date, various forms of polyanionic compounds have been extensively studied in vitro as potential inhibitors of HIV-1 Env-CD4 interaction (13, 14, 29, 47, 53, 57). However, the majority of such agents nonspecifically suppress adsorption of different viruses to the cell surface by binding to positively charged segments of virion envelope proteins, such as the V3 loop in the case of HIV-1 (49). The agents that presumably bind to CD4 have demonstrated little potency or are highly toxic to the host cells (29, 53). The prospects of specifically targeting CD4 by polyanionic macromolecular compounds have not improved, especially in light of the small gp120 contact surface area of CD4 revealed by ultrastructural analysis (41).

In the current study, we identified a group of small-molecule stibonic acid-containing compounds that blocked de novo HIV-1 infection by disrupting the gp120 and CD4 interaction without discernible in vitro cytotoxicity at the concentrations tested up to 400 μM. In contrast to gp120-binding compounds (30), these compounds competed with gp120 for binding to CD4, either immobilized on a solid phase (sCD4) or on the T-cell surface (native CD4 receptor), and displaced gp120 preadsorbed to CD4. The specificity of the targeted step, gp120-CD4 interaction, was suggested by several lines of evidence: viral entry inhibition against X4-, R5-, and X4/R5-tropic HIV-1; no activity against VSV-G pseudotyped virions; and little activity against infection of CD4-negative B cells by wild-type HIV-1. Furthermore, NSC 13778 bound to D1/D2 CD4 protein immobilized on a Biacore sensor chip and dose dependently induced significant levels of Trp fluorescence quenching of D1/D2 CD4, which contains two of a total of three Trp residues in the gp120-binding domain. These data suggested that the compound most likely bound to or near the gp120-binding domain of N-terminal CD4.

Based on all of the findings described, we concluded that the antiviral activity associated with NSC 13778 and its active analogs was predominantly mediated by specific masking of the gp120-binding domain of CD4, which prevented HIV-1 entry into the target cells. However, the exact mechanism of CD4-targeting has yet to be fully elucidated at this time. The gp120-CD4 disrupting activity of NSC 13778 in a competitive gp120 capture ELISA was greatly reduced in the presence of peptides with positively charged amino acid residues but not by negatively charged peptides, demonstrating the importance of electrostatic component of the interfering activity. It has been suggested that NSC 13778 and some analogs exhibit a preferential binding to positively charged amino acid residues (A. Stephen, unpublished observations). A surface plasmon resonance study preliminarily conducted to examine the binding affinity of NSC 13778 showed that the compound bound not only to sCD4 but also to rgp120 protein immobilized on the sensor chip surface (Fig. S2). It is possible that NSC 13778 binds to positively charged domains of gp120, such as the V3 loop, a critical determinant of chemokine receptor specificity, and thus inhibits the interaction of gp120 and coreceptor. However, the inhibition of V3 loop-chemokine receptor interaction does not seem to be a primary mode of viral entry inhibition by NSC 13778, which did not block entry of wild-type HIV into CD4⁻ cells (20).

An inevitable question regarding NSC 13778 and its analogs is whether electrostatically interfering compounds can exert the intended biological activity in vivo, where various proteins present in body fluids may nonspecifically diminish the activity of the compounds. Of note, NSC 13778, whose binding affinities to other proteins were demonstrated in cell-free biochemical assays (A. Stephen, unpublished observations), did not seem to exert any significant cytotoxicity in living cells over a 6-day culture period even at higher concentrations (up to 400 μM). Thus, electrostatic interference of NSC 13778 with various macromolecules observed in biochemical assays may not always translate to what will happen to cells exposed to the compound in physiological environment. It is conceivable that the specific gp120-CD4 disrupting activity of NSC 13778 is not merely mediated by electrostatic interference but also involves exploitation of structural vulnerability in the gp120-CD4 association. For example, the gp120-CD4 interface has been found to be cavity-laden because of an unusual mismatch in surface topography (41). The presence of these cavities may present a window of opportunity for certain small molecule inhibitors to invade the gp120-CD4 interface. It would be of great interest to investigate the activity of NSC 13778 and its analogs against HIV infection of cells expressing mutant CD4 known to permit HIV entry (11), since the structural relationship between gp120 and mutant CD4 may be dramatically altered. Future ultrastructural analysis will be critical to shed light on the molecular positioning of the compound NSC 13778 within the context of gp120-CD4 binding. Such information will help us understand how NSC 13778 can disrupt gp120-CD4 interaction and how best to optimize the lead compound for further development. Comprehensive structure-activity relationship studies are also needed to determine the required pharmacophore, whether antimony is its key component and, if so, whether it is the best element to be used. Another important consideration in developing CD4 CDR2-targeting small molecule HIV entry inhibitors is its potential interference with host T-cell immunity. Although none of the compounds examined here reduced lymphoproliferative responses to tetanus toxoid or phytohemagglutinin, one must always be cognizant of the possible risk associated with its prolonged use and carefully monitor potential adverse effects if it is to be developed further for potential HIV therapeutics. In summary, NSC 13778 appears to represent a prototype of a new class of HIV-1 entry inhibitors that can break down the gp120-CD4 interface by targeting the specific gp120-binding domain of CD4 and effectively repel incoming HIV-1 virions.

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