Expedited Articles

Structure-Based Identification of Small Molecule Antiviral Compounds Targeted to the gp41 Core Structure of the Human Immunodeficiency Virus Type 1

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Recent X-ray crystallographic determination of the HIV-1 envelope glycoprotein gp41 core structure opened up a new avenue to discover antiviral agents for chemotherapy of HIV-1 infection and AIDS. We have undertaken a systematic study to search for anti-HIV-1 lead compounds targeted to gp41. Using molecular docking techniques to screen a database of 20 000 organic molecules, we found 16 compounds with the best fit for docking into the hydrophobic cavity within the gp41 core and with maximum possible interactions with the target site. Further testing of these compounds by an enzyme-linked immunosorbent assay and virus inhibition assays discerned two compounds (ADS-J1 and ADS-J2) having inhibitory activity at micromolar concentrations on the formation of the gp41 core structure and on HIV-1 infection. These two compounds will be used as leads to design more effective HIV-1 inhibitors targeted to the HIV-1 gp41 core structure.

Introduction

Several antiviral drugs targeted to HIV-1 reverse transcriptase (RT) and protease have been approved by the U.S. Food and Drug Administration (FDA) in recent years for the treatment of HIV-1 infection and AIDS.[1] Combination therapy using these two types of inhibitors has been remarkably successful in reducing viral load and has led to a decline in morbidity and mortality.[2−6] However, these drugs have a number of shortcomings: (1) emergence of HIV-1 mutant strains having single or multiple resistance to the drugs used;[7−9] (2) adverse side effects;[1] and (3) high cost.[10] In addition, these drugs are targeted to later stages of infection. Therefore, it is essential to develop compounds with higher effectiveness and lower side effects which can prevent early steps of HIV-1 infection.

The gp41 transmembrane glycoprotein is involved in virus−host cell fusion and virus entry into the host cell.[11,12] gp120 binding to CD4 and co-receptors (i.e., CXCR4, CCR5, etc.) on T lymphocytes and macrophages triggers a conformational change in the gp41 glycoprotein and exposes the fusion peptide at the gp41 amino termini that mediates fusion between the viral envelope protein and cell membranes.[11,13,14] In addition, gp41 plays an important role in the noncovalent oligomerization of the envelope glycoproteins.[15,16] The complex role of gp41 in viral entry and the oligomerization process makes it an attractive target for developing antagonists against early steps of the HIV infection process.

In addition to the fusion peptide, the gp41 ectodomain also contains two 4−3 heptad repeats (HR) adjacent to the N- and C-terminal portions, designated NHR and CHR, respectively. The hydrophobic amino acid sequences in both NHR and CHR regions have the propensity to form α-helices, denoted N and C helices.[17] Peptides derived from the NHR and CHR regions are named N-peptides, such as DP-107[18] and N-36,[19] and C-peptides, including SJ-2176,[20] DP-178,[21] and C-34.[19] N- and C-peptides mixed at equimolar concentrations form stable α-helical trimers of antiparallel heterodimers, representing the fusion-active (fusogenic) core domain of gp41.[19,22]

Recent X-ray crystallographic studies[23,24] have demonstrated that the structure of the fusion-active conformation of the gp41 core is a six-stranded helical bundle. Three N helices associate to form the internal coiled-coil trimmer, while three C helices pack against this trimer in an antiparallel fashion into the hydrophobic grooves formed on the surface of the trimer. Each of the grooves has a deep cavity that accommodates three highly conserved hydrophobic residues (Trp628, Trp631, and Ile635) in C helices. Structure−function analysis demonstrated convincingly that mutation of these residues drastically reduced association of C-peptides with N-peptides to form the gp41 core and the antiviral activity of C-peptide, suggesting that these hydrophobic cavities are critical for gp41-mediated membrane fusion and are attractive targets for designing new anti-HIV drugs preventing the early fusion events.[25]

By immunization of mouse with a subdomain of the gp41 core, consisting of N-36 and C-34 connected by a six-residue hydrophilic linker, designated N36(L6)C34,[19] a monoclonal antibody (mAb), designated NC-1, was recently generated.[26] This mAb specifically recognizes

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discontinuous epitopes present on the six-helix subdomain formed by the association of N- and C-peptides and binds to oligomeric forms of gp41 expressed on the HIV-1-infected cells in the presence of soluble CD4.[26]

C-Peptides block in vitro HIV-1 infection and cell fusion at nanomolar concentrations.[20,21] In a recent phase I/II clinical trial in humans, T-20, one of the C-peptides, showed potent in vivo inhibition of HIV-1 replication, resulting in viral load reduction comparable to that obtained by the 3−4 combination therapies with RT and protease inhibitors.[27] Despite this early success, the application of this peptide therapy may be limited due to the high production cost of the peptide and lack of its oral availability. Thus, identification of small molecule inhibitors reacting with the same or overlapping target sites on gp41 recognizing the antiviral peptides is needed. To achieve this goal, we screened a commercial small molecule database by automated molecular docking techniques utilizing the X-ray crystal structure of the gp41 core. This method has been shown to be effective in identifying lead compounds against many other targets.[28−32]

In this report, we present results of a systematic study starting with theoretical docking of compounds to the deep cavity by a computer-aided molecular docking technique that led to the identification of two small molecule anti-HIV-1 inhibitors. These inhibitors also block the formation of the N/C helix complex suggesting that they inhibit HIV-1 infection by targeting to the gp41 core structure.

Materials and Methods

Cells, Viruses, and Antibodies. MT-2 cells, HIV-1_{IIIB}-infected H9 cells (H9/HIV-1_{IIIB}), and the HIV-1_{IIIB} isolate were obtained from the NIH AIDS Research and Reference Reagent Program. Rabbit polyclonal antibody (pAb) and mouse monoclonal antibody (mAb) NC-1 directed against the recombinant N36(L6)C34 polypeptide were generated, purified, and characterized as previously described.[26] Rabbit and mouse IgG were purified using protein-A kits (Pierce, Rockford, IL).

Peptides and Compounds. Peptides were synthesized by a standard solid-phase FMOC method in The New York Blood Center in-house facility. The N-termini of the peptides were acetylated, and their C-termini were amidated. The peptides were purified to homogeneity by high-performance liquid chromatography (HPLC). The identity of the purified peptides was confirmed by laser desorption mass spectrometry (PerSeptive Biosystems). The small organic compounds (ADS-J1 through ADS-J16) were purchased from ComGenex, Inc. (Budapest, Hungary). Chemical structures of these compounds are shown in Chart 1.

Hardware and Software. A Silicon Graphics Indigo^2 Extreme computer was used for all molecular modeling studies. The DOCK3.5 suit of programs[33,34] was used for automated docking simulations. Sybyl6.5 from Tripos Associates, Inc.[35] was used for all other modeling purposes including molecular visualizations. CrystalEyes2[36] stereographic eye glasses were used along with Sybyl6.5 software for stereo visualization. CONCORD 4.0[37] from Tripos Associates, Inc. was used to generate 3D structures of the compounds used for molecular docking studies.

Automated Docking of Small Molecules to the Hydrophobic Cavity of the gp41 Core Structure. The DOCK suit of programs has been successfully used to identify lead compounds against several targets, and the methods were described in great detail.[28−32] The important steps for docking using these programs are as follows:

1. Identification of the target site in a well-defined receptor structure (preferably, X-ray crystal structures but NMR and homology modeled structures are also used).
2. Creation of the molecular surface of the target site.
3. Identification of the important residues for possible interaction with the ligand molecule.
4. Generation of spheres to fill the active site that serve as the guide to locate ligands whose interatomic distance matches the intersphere−center distance.
5. Generation of a grid box encompassing the spheres to save the steric and electrostatic information at each grid point so that the ligand orientation can be scored during docking.
6. Searching of thousands of orientations of ligands to match the center of the spheres.
7. Evaluation of the ligand orientation by shape or force-field scoring function. The shape scoring function resembles van der Waals attractive energy, whereas the force-field scoring function approximates at best an interaction enthalpy and is approximately the sum of van der Waals attractive, dispersive, and Coulombic electrostatic energies.
8. Location of local minima by simplex minimization.

The DOCK suit of programs has been used to screen one commercially available database from ComGenex, Inc., Budapest, Hungary, consisting of 20 000 small molecule compounds. The 3D coordinates of the small molecules were generated by the CONCORD program.

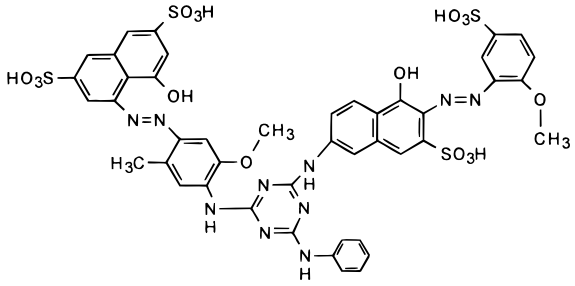
One of the C helices from the coiled-coil trimer of heterodimers was removed to generate the target site on the N-peptide for docking of small molecule compounds. According to information from X-ray crystallography, two indole rings from the C-peptide (Trp628 and Trp631) dock into a deep hydrophobic cavity. The negative image of this cavity (target site) was created by selecting all residues (8.0 Å radius) surrounding Trp628. The molecules were then docked into the cavity, and the quality of the ligand binding was evaluated by a force-field scoring function. Two hundred top-scoring compounds were selected for further analysis by visual inspection using the Sybyl program and stereoscopic eye wears (CrystalEyes). Irrespective of the score, 20 compounds with the best fit and maximum possible interactions (hydrophobic, electrostatic, H-bond, etc.) with the target site were selected for biological assays (16 compounds were available from the supplier and their chemical structures are shown in Chart 1).

ELISA. A sandwich ELISA was established to screen for compounds that interfere with the formation of the N-36/C-34 complex as described previously.[38] Briefly, N-36 (2 μM) was preincubated with compounds at graded concentrations at 37 °C for 30 min, followed by addition of C-34 (2 μM). After incubation at 37 °C for 30 min, the mixture was added to wells of a 96-well polystyrene plate (Immulon I, Dynatech Laboratories, Inc., Chantilly, VA) which were precoated with IgG (10 μg/mL) purified from rabbit antisera directed against N36(L6)-C34. Then, the mAb NC-1, biotin-labeled goat-anti-mouse IgG (Boehringer Mannheim), streptavidin-labeled horseradish peroxidase (Zymed, S. San Francisco, CA), and the substrate 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO) were added sequentially. Absorbance at 450 nm (A_{450}) was read using an ELISA reader (Dynatech Laboratories, Inc., Chantilly, VA). The percentage of inhibition by the compounds of the binding of NC-1 to the peptide complexes was calculated as previously described,[39] and the concentration for 50% inhibition (IC_{50}) was calculated using a computer program, designated Calcusyn.[40]

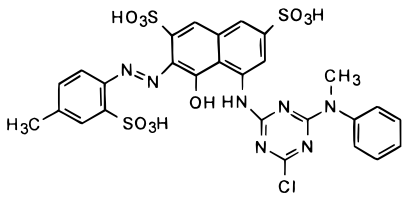
HIV-1-Mediated Cell Fusion. A dye transfer assay was used for detection of HIV-1-mediated cell fusion as previously described.[41] H9/HIV-1_{IIIB} cells were labeled with a fluorescent reagent, 2′,7′-bis(2-carboxyethyl)-5(and 6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM, Molecular Probes, Inc., Eugene, OR), and then incubated with MT-2 cells (ratio = 1:10) in 96-well plates at 37 °C for 2 h in the presence or absence of compounds tested. The fused and unfused BCECF-labeled HIV-1-infected cells were counted under an inverted fluorescence microscope (Zeiss, Germany) with an eyepiece micrometer disk. The percentage of inhibition of cell fusion and the IC_{50} values were calculated as previously described.[41]

Detection of HIV-1-Mediated Cytopathic Effect (CPE) and in Vitro Cytotoxicity. The inhibitory activity of the

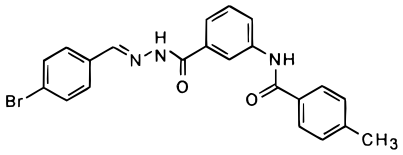
Chart 1. Chemical Structures of the Compounds



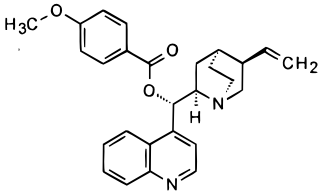
ADS-J1



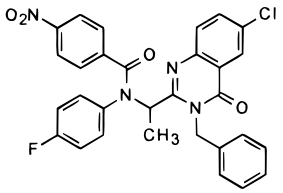
ADS-J2



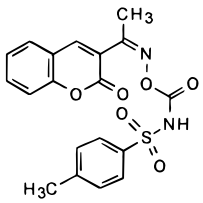
ADS-J3



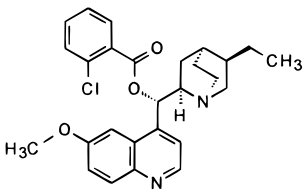
ADS-J4



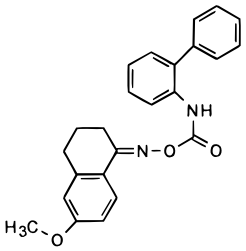
ADS-J5



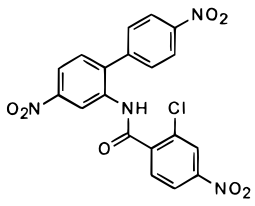
ADS-J6



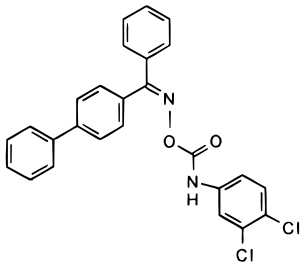
ADS-J7



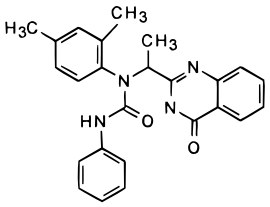
ADS-J8



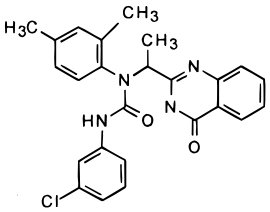
ADS-J9



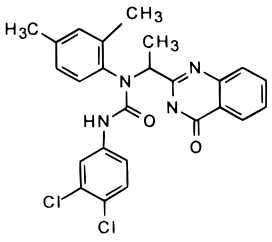
ADS-J10



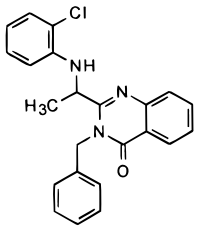
ADS-J11



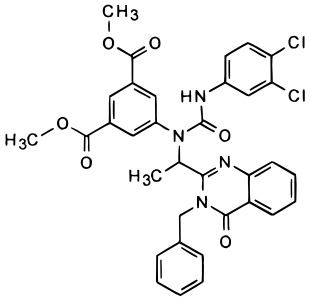
ADS-J12



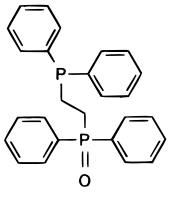
ADS-J13



ADS-J14



ADS-J15



ADS-J16

compounds was determined by a colorimetric method based on protection of cells against HIV-1-mediated CPE, as previously described.[39] Briefly, 1×10^4 MT-2 cells in 96-well plates were infected with diluted HIV-1_{IIIB} in 200 mL of RPMI 1640 medium containing 10% FBS in the presence of compounds at graded concentrations. After 1 h, 24 h, and 4 days, half of

Table 1. Inhibitory Activities of Compounds Selected from the ComGenex Database by Docking to the Cavity within the gp41 Core Domain

compd	MW	CC_{50}[a] ± SD (μg/mL)	IC_{50}[b] ± SD (μg/mL)			SI[c]
			NC-1 binding	cell fusion	CPE	
ADS-J1	1177	292.16 ± 2.27	0.73 ± 0.08	4.95 ± 1.31	8.29 ± 1.13	35.24
ADS-J2	802	289.94 ± 12.39	3.18 ± 0.36	21.85 ± 1.38	30.76 ± 6.74	9.43
ADS-J3	436	24.91 ± 2.62	>100	>100	>100	≤1.00
ADS-J4	429	74.12 ± 0.76	>100	>100	>100	≤1.00
ADS-J5	557	30.17 ± 4.84	>100	>100	>100	≤1.00
ADS-J6	400	285.98 ± 19.81	>100	>100	>100	≤1.00
ADS-J7	465	41.33 ± 2.38	>100	59.78 ± 8.69	>100	≤1.00
ADS-J8	386	46.83 ± 4.93	>100	>100	>100	≤1.00
ADS-J9	443	47.22 ± 5.48	>100	>100	>100	≤1.00
ADS-J10	461	74.67 ± 6.04	>100	>100	>100	≤1.00
ADS-J11	412	40.77 ± 1.67	>100	>100	>100	≤1.00
ADS-J12	447	54.02 ± 8.14	>100	>100	>100	≤1.00
ADS-J13	481	39.22 ± 7.60	>100	>100	>100	≤1.00
ADS-J14	390	86.82 ± 5.79	>100	>100	>100	≤1.00
ADS-J15	660	20.95 ± 0.67	>100	>100	>100	≤1.00
ADS-J16	414	28.88 ± 5.97	>100	52.27 ± 2.85	>100	≤1.00

[a] CC_{50}, 50% cytotoxic concentration. [b] IC_{50}, 50% inhibitory concentration. [c] Selectivity index (SI) = CC_{50}/IC_{50} for CPE.

the culture media were changed. On the sixth day postinfection, an indicator XTT tetrazolium dye (1 mg/mL, 50 mL/well; PolySciences, Inc., Warrington, PA) was added to the cells. After 4 h, the soluble intracellular formazan was determined colorimetrically at 450 nm. The percent of inhibition of CPE and the IC_{50} values were calculated as described above.

The in vitro cytotoxicity for MT-2 cells of the compounds was determined in 96-well plates using the XTT dye to measure cell viability in the absence of virus. 5% Triton X-100 (10 μL) was added to the wells corresponding to positive controls (P), and 10 μL of medium was added to wells corresponding to negative controls (N). The percent cytotoxicity was calculated using the following formula: % cytotoxicity = $[(E - N)/(P - N)] \times 100\%$, where E represents experimental data in the presence of compounds. The concentration corresponding to 50% cytotoxicity (CC_{50}) for MT-2 cells was calculated using the Calcusyn computer program. The selectivity index (SI = CC_{50}/IC_{50}) for each compound was calculated.

Results and Discussion

Recent determination of the X-ray crystal structures of the gp41 core and identification of a deep hydrophobic pocket within the core opened up a new avenue to target this site for structure-based drug design. As gp41 plays an important role in fusion of the HIV-1 envelope with the target cell membrane, inhibition of this early event may lead to inhibition of infection. Drugs targeted to this site may be useful against mutant viruses resistant to RT and/or protease inhibitors. Though high-throughput screening (HTS) and combinatorial libraries have paved the way for rapidly screening millions of compounds in a short period of time,[42−44] it requires substantial resources and is not cost-effective if libraries are not designed rationally. Structure-based drug design by docking has shown promise when using a large library for screening.[45−48] This theoretical screening method, if judiciously used, may screen out compounds that interact effectively with the target sites. Using computer-aided molecular docking by the DOCK suit of programs, we have screened a database of 20 000 small organic molecules for compounds which dock into the deep hydrophobic cavity on the trimer created by three N helices. The force-field scoring method was used to rank best possible compounds for docking potentials into the cavity instead of just a shape-based scoring method because other charged groups surrounding this cavity may also play an important role in interacting with ionic groups present in the inhibitor molecules. We selected 200 top-scoring compounds from a dock run for in-depth inspection of the interactions at the hydrophobic cavity and neighboring regions by molecular visualization techniques. The dock scores cannot be a quantitative predictor of activity because many approximations are involved in its search technique and scoring methods. Therefore, close visual inspection with stereoglasses of the top-scoring molecules individually for appropriate interactions is necessary.[29,49]

Sixteen of the 200 best-scoring compounds were tested by ELISA for inhibitory activity on the formation of the N-36/C-34 complex using mAb NC-1 and on HIV-1 infection, including HIV-1-mediated cell fusion and CPE, and for in vitro cytotoxicity (Table 1). Two of the compounds, 7-[6-phenylamino-4-[4-[(3,5-disulfo-8-hydroxynaphthyl)azo]-2-methoxy-5-methylphenylamino]-1,3,5-triazin-2-yl]-4-hydroxy-3-[(2-methoxy-5-sulfophenyl)-azo]-2-naphthalenesulfonic acid (ADS-J1) and 5-[(4-chloro-6-phenylamino-1,3,5-triazin-2-yl)amino]-4-hydroxy-3-[(4-methyl-6-sulfophenyl)azo]-2,7-naphthalene-disulfonic acid (ADS-J2), have promising inhibitory activity against the formation of mAb NC-1 detectable N-36/C-34 complex and against HIV-1-mediated cell fusion and CPE. To find out the energetic feasibility of the CONCORD program-generated conformations used for the dock runs, conformational analysis of both ADS-J1 and ADS-J2 was performed by a systematic conformational search in the Sybyl program. The best 1000 conformers for each compound were selected for docking runs. In the case of ADS-J1, the energy of the best-docked conformation is 13 kcal/mol higher than the CONCORD-generated docked conformation, confirming the validity of the conformer used for the study. In the case of ADS-J2, the energy of the best-docked conformer from the systematic search is only 1.6 kcal/mol better than that corresponding to the docked conformer used in this study. Therefore, the docked conformations of ADS-J1 and ADS-J2 are energetically feasible conformers.

The specificity of the ELISA for detecting the complex formed by the N- and C-peptides using mAb NC-1 has been confirmed by showing that (1) NC-1 only binds to the complex N-36/C-34 but does not react with the

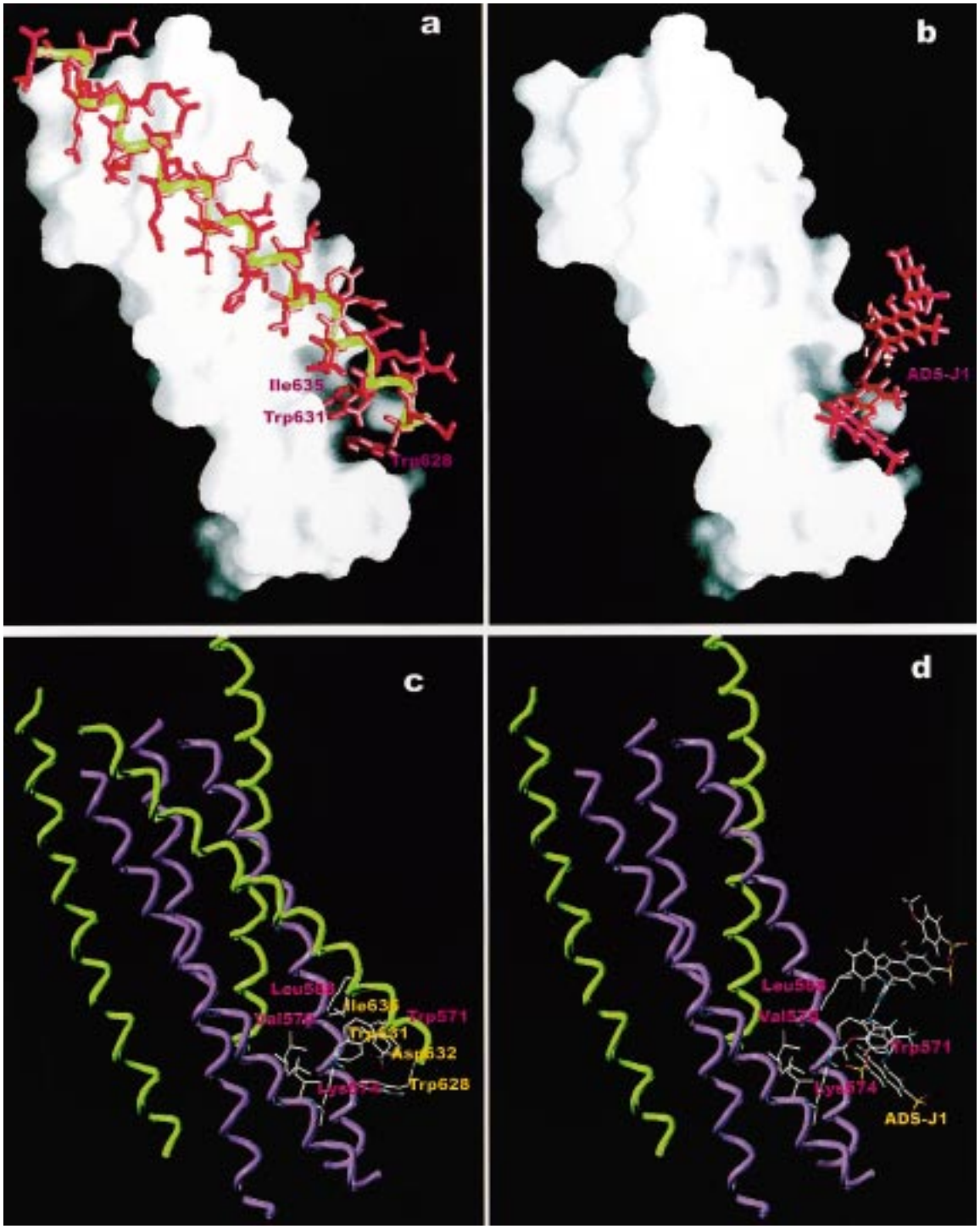


Figure 1. Interaction of C-peptide and ADS-J1 with the hydrophobic cavity within the gp41 core: (a) hydrophobic residues (Trp628, Trp631, and Ile635) of the peptide C-34 penetrating deep into the hydrophobic cavity; (b) ADS-J1 docked into the hydrophobic cavity region; (c) interaction of the residues in and around the cavity formed by the N-peptide coiled-coil (magenta) with C-peptide residues (yellow); (d) interaction of one of the most active compounds, ADS-J1, with the same residues (magenta) in the N-peptide coiled-coil.

individual peptides N-36 and C-34;[26] (2) only peptides derived from the gp41 NHR and CHR regions form complexes detectable by NC-1;[38] and (3) single-point mutations of residues at the interacting sites in the C-peptide abolished its ability to form NC-1 detectable complexes with N-36.[38] The compounds selected by the sandwich ELISA may not block the complex formation by N- and C-peptides but instead block NC-1 binding to the complex. To exclude this possibility, we conducted a control experiment, in which the complex was preformed by mixing N-36 and C-34 without addition of the compounds. Then, the binding of mAb NC-1 to the complex in the presence of compounds was determined. We demonstrated that ADS-J1 and ADS-J2 did not block the NC-1 binding to the preformed N-36/C-34 complex (data not shown). This suggests that inhibition of NC-1 binding by these compounds is due to the blockage of complex formation by N- and C-peptides, rather than to the blockage of antibody binding to the complexes consisting of N- and C-peptides.

Since ADS-J1 and ADS-J2 are polysulfonic acid compounds, it was of interest to determine whether other anti-HIV polysulfonates, such as suramin, Evans blue, and fuchsin acid,[50−52] also block the formation of NC-1 detectable complexes between N- and C-peptides. These compounds, unlike ADS-J1 and ADS-J2, did not inhibit the formation of N-36/C-34 complex (data not shown), suggesting that they are not targeted to the HIV-1 gp41 core. In our previous study,[38] we found that several polyionic compounds, such as aurinetricarboxylic acid (ATA), *meso*-tetra(4-carboxyphenyl)porphin, and 3-hydroxyphthalic anhydride-treated β-lactoglobulin (3HP-α-LG), did not block NC-1 detectable complex formation between N- and C-peptides, although they are potent inhibitors of HIV-1-mediated cell fusion. This confirms that ADS-J1 and ADS-J2, which are different from other polyionic compounds, inhibit HIV-1 infection by binding to the constituents of the HIV-1 gp41 core.

The top-scoring compound ADS-J2 had the second best overall activity in terms of blocking formation of N/C helix complex, cell fusion, and CPE. The best active compound was ADS-J1, and it ranked third in force-field scoring. One of the compounds, ADS-J13, ranked second in force-field scoring but had no activity. The rest of the compounds did not have inhibitory activity. Close visual inspection of the possible interaction pattern of the two active compounds revealed that ADS-J1 is positioned in such a way that hydrophobic groups (phenyl, naphthalene) are interacting with the hydrophobic residues (Leu568, Val570, Trp571) in the hydrophobic cavity. In addition, Lys574 is in close proximity to one of the sulfonic acid groups to make ionic interactions (Figure 1). For ADS-J2 the same interaction with Lys574 exists but there are no detectable hydrophobic interactions possibly explaining its lower activity compared to ADS-J1. The larger size of ADS-J1 may have additional steric effects on the formation of the helical bundle by NHR and CHR fragments in gp41, which could not be predicted from the docking study. It was of interest to know why compound ADS-J13 had a high docking score but did not have inhibitory activity. Close visual inspection by 3D stereoscopic eye glasses revealed that this compound had good hydrophobic interactions with Leu568, Val570, and Trp571 but did not interact with Lys574 (or any other positively or negatively charged residues). This suggests that along with hydrophobic residues in the pocket, other charged groups, especially Lys574, in the surrounding areas may play an important role in interacting with inhibitory compounds. In fact, Asp632 of the CHR peptide is in close proximity to Lys574 for ionic interaction and may also play an important role in the stabilization of the six-stranded bundle formed by the NHR and CHR fragments in gp41. A recent mutation study demonstrated that the charged residues in the gp41 coiled-coil domain may also be important for gp41-mediated membrane fusion.[53]

Conclusion

Recent determination of the X-ray crystallographic structure of the HIV-1 gp41 core and identification of the hydrophobic cavity provided important structural information for developing new class of anti-HIV-1 agents. We have exploited this information and initiated a screening of a large organic molecular library by a combination of theoretical molecular docking, an ELISA-based method, and virus inhibition assays. Two anti-HIV-1 compounds with low micromolar inhibitory activity were identified using these combined techniques and could be used as lead compounds for further development. The critical studies on the interactions of these inhibitors with the target site residues may provide valuable insights into designing new analogues with optimum interactions and improved activity.

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