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Structure-Based Identification and Neutralization Mechanism of Tyrosine-Sulfate Mimetics that Inhibit HIV-1 Entry

Priyamvada Acharya[†], **Cajetan Dogo-Isonagie**[#,‖], **Judith M. Lalonde**[§,‖], **Son N. Lam**[#,‖], **George J. Leslie**[¶,‖], **Mark K. Louder**[†,‖], **Leah L. Frye**[‡], **Asim K. Debnath**[&], **Jeremy R. Greenwood**[‡], **Timothy S. Luongo**[†], **Loïc Martin**[⊥], **K. Shawn Watts**[‡], **James A. Hoxie**[¶], **John R. Mascola**[†], **Carole A. Bewley**[#,*], and **Peter D. Kwong**[†,*]

[†]Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

[#]Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

[§]Department of Chemistry, Bryn Mawr College, Bryn Mawr, PA 19010, USA

[¶]Department of Medicine, Hematology-Oncology Division, University of Pennsylvania, Philadelphia, PA 19104, USA

[‡]Shrödinger, LLC, Portland, OR 97204, USA

[&]Laboratory of Molecular Modeling and Drug Design, Lindsley F. Kimball Research Institute, New York Blood Center, 310 E 67th Street, New York, NY 10021, USA

[⊥]CEA, iBiTecS, Service d'Ingénierie Moléculaire des Protéines, Gif sur Yvette F-91191, France

Abstract

Tyrosine sulfate-mediated interactions play an important role in HIV-1 entry. After engaging the CD4 receptor at the cell surface, the HIV-1 gp120 glycoprotein binds to the CCR5 co-receptor via an interaction that requires two tyrosine-sulfates, at positions 10 and 14 in the CCR5-N terminus. Building on previous structure determinations of this interaction, here we report the targeting of these tyrosine sulfate-binding sites for drug design through *in silico* screening of small molecule libraries, identification of lead compounds, and characterization of biological activity. A class of tyrosine sulfate-mimicking small molecules containing a “phenyl sulfonate-linker-aromatic” motif was identified that specifically inhibited binding of gp120 to the CCR5-N terminus as well as to antibodies that recognize the co-receptor-binding region on gp120. The most potent of these compounds bound gp120 with low μM affinity and its CD4-induced conformation with K_Ds as tight as ~50 nM. Neutralization experiments suggested the targeted site to be conformationally inaccessible prior to CD4 engagement. Primary HIV-1 isolates were weakly neutralized, pre-incubation with soluble CD4 enhanced neutralization, and engineered isolates with increased dependence on the N terminus of CCR5 or with reduced conformational barriers were neutralized with IC_{50} value as low as ~ 1 μM. These results reveal the potential of targeting the tyrosine-sulfate interactions of HIV-1 and provide insight into how mechanistic barriers, evolved by HIV-1 to evade antibody recognition, also restrict small molecule-mediated neutralization.

[*]Correspondence should be addressed to -CAB, Laboratory of Bioinorganic Chemistry, NIDDK/NIH, 8 Center Drive; Building 8A, Room 1A02, Bethesda, MD 20892; Tel: 301-594-5187, Fax: 301-402-4182, caroleb@mail.nih.gov; PDK, Vaccine Research Center, NIAID/NIH, 40 Convent Drive; Building 40, Room 4508, Bethesda, MD 20892; Phone: (301) 594-8685; Fax: (301) 480-2658, pdkwong@nih.gov.
[‖]These authors contributed equally

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Human immunodeficiency virus type 1 (HIV-1) continues to be a major global health problem resulting in millions of deaths each year. Although a variety of anti-HIV-1 therapeutics have been developed, the emergence of HIV-1 strains resistant to existing therapies and the side effects of drug regimens make identification of new targets a continuing priority (1). Entry inhibitors are an emerging class of therapeutics that interfere with attachment, fusion, or entry of HIV-1 into cells [reviewed in (2)]. The trimeric HIV-1 envelope protein is composed of two glycoproteins: the attachment-mediating gp120 and the fusion-inducing gp41. The engagement of the cell surface CD4 receptor by gp120 induces structural rearrangements in the viral spike that lead to formation of a highly conserved co-receptor-binding site on gp120 (3-5). Chemokine receptors, CCR5 or CXCR4, function as HIV-1 co-receptors [reviewed in (6)]; upon binding to the CD4-induced conformation of gp120, they trigger additional conformational changes that lead to displacement of the fusogenic N terminus of gp41 into the target cell membrane and subsequent fusion of viral and cell membranes. Although each step in the entry pathway is a potential target for intervention, a number of hurdles, including difficulties with blocking protein-protein interactions, have complicated the development of entry inhibitors. Nonetheless, the promise of entry inhibitors as antiretrovirals is demonstrated by two licensed drugs, fuzeon (7) a fusion inhibitor and maraviroc (8) a CCR5 antagonist.

This study focuses on the interaction of the HIV-1 gp120 and the CCR5 co-receptor. The critical nature of the interaction with CCR5 is demonstrated by the resistance to HIV-1 infection of individuals homozygous for a naturally occurring variant of CCR5, in which 32 residues of the CCR5-N terminus are missing (9). CCR5 is an integral membrane protein with an extracellular N terminus, seven membrane-spanning helices, and three extracellular loops. Interactions between gp120 and CCR5 involve at least the N terminus and the second extracellular loop (ECL-2) (10, 11). We previously determined the structure of the CCR5-N terminus bound to gp120 by a combination of NMR, X-ray crystallography, and docking techniques (12). The CCR5-N terminus adopts an α-helical conformation and binds to a highly conserved region at the base of the third variable loop (V3) on HIV-1 gp120 (Figure 1, panel a; Supplementary Figure 1). This interaction is dominated by two sulfated tyrosines at positions 10 and 14 of CCR5. Disruption of tyrosine sulfation results in loss of binding and viral entry. The importance of this pair of tyrosine-sulfates is further demonstrated by the monoclonal antibody 412d: this antibody also has two tyrosine-sulfates in its heavy chain 3rd complementarity-determining region (CDR H3) (13, 14). The 412d CDR H3 region can substitute for the N terminus of CCR5 to make a functional, entry-competent co-receptor (15). Interestingly, the structure of antibody 412d in complex with HIV-1 gp120 reveals that the CDR H3 of 412d forms an extended conformation (12) (Figure 1, panel b). Despite different modes of binding, the involvement and importance of tyrosine-sulfates is a common feature of both interactions.

Tyrosine-sulfates at positions 10 and 14 in CCR5 and in the CDR H3 loop of antibody 412d occupy similar binding pockets on gp120 (12). Tyrosine sulfation is a post-translational modification that has been implicated in mediating high-affinity protein-protein interactions in a number of systems [reviewed in (16)]; similar to phosphorylation (17-19), these post-translational modifications provide a handle by which molecular mimicry can be used to inhibit biology. Here we present a structure-guided search for small, drug-like molecules that inhibit viral entry by mimicking critical tyrosine sulfate-mediated interactions made by the CCR5-N terminus. Structural information provided the basis for *in silico* screening of compound libraries. Results from the computational analyses were experimentally tested for their ability to bind gp120 as well as to compete with the binding of the CCR5-N terminus and antibodies that recognize a CD4-induced epitope on gp120. Molecules able to inhibit binding of the CCR5-N terminus to gp120 binding were further evaluated in viral entry inhibition assays to determine potency and mechanism of neutralization.

Results and Discussion

In silico screening and CCR5-N terminus-competition assay

To identify small molecule inhibitors of CCR5-N terminus binding to gp120, we coupled structure-based *in silico* screening to an ELISA-based assay (Figure 2 and Supplementary Figure 2). Two *in silico* screening protocols were used to screen two different small molecule libraries. In the first approach, the ZINC database V7 (20) of drug-like compounds was searched using a consensus method involving the ROCS shape- and electrostatics-matching routine (21) coupled to docking analysis using the Gold algorithm (22) in an effort to identify CCR5-N terminus mimetics. In parallel, the Glide molecular docking algorithm (23) was used to search a Schrödinger database of commercially available screening compounds for small molecules that bind to the co-receptor binding site on gp120.

Structure-guided queries for the shape-based search were generated by centering on the critical tyrosine-sulfate residues of the ligand (Figure 3, panels a and b; and Supplementary Figure 3). Previous biochemical (24) and NMR (25) data showed that the CCR5-N terminus-binding site is selective for tyrosine-sulfate over tyrosine phosphate (Figure 3; panel c). Therefore, compounds that contained phosphates were removed from further analysis. Compounds were filtered for the presence of a sulfur-oxygen bond and using this criterion sulfates, sulfonates and sulfonamides were shortlisted. Search of the ZINC database revealed that it contained very few phenyl sulfates, although there were a large number of phenyl sulfonates (Figure 3, panel c). While missing the oxygen atom linking the SO_3^- and phenyl groups in tyrosine-sulfate, phenyl sulfonate retains the aromatic and anionic properties of the tyrosine-sulfate side-chain.

Compounds were screened for their ability to inhibit the interaction of gp120 with a CCR5-N terminus analog in an ELISA-based inhibition assay (25) (Supplementary Figure 2). Compound **1.1** (Supplementary Figures 3-5), a high scoring hit appearing in both CCR5 Nt:Y14-Y15 and 412D:Y107-A108-P109 queries, showed the best inhibition and was chosen for the next round of ROCS screening. A secondary ROCS search of the Zinc Database was conducted using **1.1** (Supplementary Figure 6) followed by ELISA screening to obtain additional hits (Supplementary Figure 7). These results allowed identification of a chemotype, comprising a phenyl sulfonic acid moiety (Figure 3C) connected to an aryl group by a variable linker, capable of inhibiting gp120-CCR5-N terminus interaction. Chemical structure comparisons on commercial compound libraries were carried out (Supplementary Figure 6) to select compounds based on their similarities with the identified pharmacophore. These were screened in the CCR5-N terminus inhibition ELISA assay to identify additional hits (Supplementary Figure 8).

In an alternative approach, Schrödinger's in-house database of commercially available screening compounds were docked (23) to the CCR5-N terminus binding site on gp120 (Figure 2). Having already identified a binding chemotype, we used this information to filter the hits obtained in the Glide search. A panel of aryl sulfonate compounds were docked to the CCR5-N terminus/412d CDR H3 loop binding site on gp120, the top-scoring compounds were visually inspected and ranked according based on Glide scores. ELISA screening of 60 of these compounds resulted in additional hits (Supplementary Figure 9).

Chemotype Identification

Earlier we showed that a CCR5-N terminus-analog, in which the tyrosine-sulfate (TYS) residues were replaced by tyrosine-sulfonate (TYSN) (Figure 3; panel c), retained significant binding to gp120 (25). In this study, the requirement of a phenyl sulfonate head group in the drug-like compounds was established in the first few cycles of screening. This moiety shares the aromatic and anionic properties of TYS and the criteria used for *in silico*

screening, as well as the direct competition with a CCR5-N terminus analog, ensured that the determinants of this critical interaction were retained in the selected molecules. While the phenyl sulfonate group was critical for binding, the contacts made by this moiety alone were not sufficient to inhibit CCR5-N terminus binding to gp120. A panel of aryl sulfonates that showed less than 20% inhibition in the ELISA assay are shown in Supplementary Figure 10. This is consistent with previous biochemical analyses carried out on CCR5-N terminus binding to gp120 (24) that showed that not only the tyrosine-sulfates but the residues that flanked them were important for binding. The compounds that showed greater than 70% inhibition of CCR5-N terminus peptide binding in the ELISA assay all contained a phenyl sulfonate moiety linked at the para position via a diazo or a hydrazino linker to an aromatic group (Supplementary Figure 7-9). A complete list of compounds screened in the ELISA assay with percent inhibition of gp120-CCR5 N terminus interaction is presented in Supplementary Figure 11.

Assessment of compound purity and structure

During characterization, we observed that some compounds were light-sensitive and/or isomerized. Two subgroups whose structures contained the desired pharmacophore and were not prone to isomerization were identified (Figure 4). Compounds **1-3** and **7** (Figure 4 and Table 1) were selected for further analysis. These were subjected to purity evaluation and structural verification by LC-MS, ^{1}H- and ^{13}C-NMR (Supplementary Figures 12-27). Compounds that were less than 95% pure by LC-MS were purified by RP-HPLC to greater than 95% purity, and their structures confirmed again by NMR and high resolution mass spectroscopy (HR-MS).

Binding affinity of compounds to gp120 by surface plasmon resonance (SPR)

Binding of compounds to immobilized gp120 was measured in the presence and absence of M48-U1(26) (Figure 5; Table 1; Supplementary Figure 28-31). M48-U1 is a member of class of CD4-peptide mimetic that we previously showed induces the CD4-bound conformation of gp120 (27-29). The binding kinetics of all the compounds were too fast for association and dissociation rates to be quantified by SPR. Binding affinities determined by steady-state analyses ranged from 1.5-6 μM (Table 1) and binding was enhanced 15-40 fold in the presence of the CD4-mimetic peptide M48-U1. Compound **1** showed the tightest binding to gp120 in the CD4-bound conformation with a K_d of 49 ± 1 nM.

Inhibition of CD4-induced antibody binding

On the basis of their ability to inhibit CCR5-N terminus binding to gp120, the compounds were expected to occupy one or both tyrosine-sulfate binding pockets on gp120 (Figure 1 and Figure 3, panel a). Specificity of the compounds to this site was established by their ability to compete with an antibody that targets the CD4-induced (CD4i) epitope while not affecting the binding of an antibody that binds to an unrelated epitope. The CD4i antibody, 412d was used for the competition assays since its CDR H3 region recognizes the target site (12) (Figure 1 and Figure 2, panel a). The compounds showed dose-dependent inhibition of 412d binding to gp120 (Figure 5). No inhibition was observed for binding of antibody C11, which binds outside the CD4i epitope.

Inhibition of HIV-1 entry

Compounds **1, 2, 3** and **7** were tested for their ability to inhibit HIV entry, and the CD4i antibody 17b was used as positive control. Compounds were tested against a panel of 27 HIV-1 primary isolates along with HIV-2 strains 7312A and 7312A.V434M, a simian strain SIVmac251 and murine leukemia virus (MuLV) (Figure 6, panel a and Supplementary Table 1). Compound concentrations from 0-250 μM were tested. While compounds **3** and **7** did not

neutralize any of the viruses tested, compounds **1** and **2** inhibited 13 and 25 viruses, respectively, with **1** showing greater breadth and potency than **2**. The activity of the compounds on most strains was weak with IC_{50} values greater than 100 μM; except for HIV-1 Clade B viruses SS1196.01 and 6535.3 which were inhibited by **1** with IC_{50} values of 50.3 μM and 94 μM, respectively. HIV-2 7312A was inhibited by **1**, an effect further enhanced in HIV-2 7312A.V434M, a strain that contains a valine to methionine mutation at position 434 leading to greater sensititvity to HIV-1 CD4i antibodies (30). A similar enhancement of neutralization of HIV-2 7312A.V434M compared to its parental virus was seen with **2** and 17b IgG.

Effect of CD4-induction on neutralization potency

The compounds target a region on gp120 that forms only after CD4 engagement. To test if induction of the CD4-bound conformation of the viral spike would enhance the efficacy of the TYS mimetic compounds to inhibit entry, we tested the activity of the compounds on viruses that were first triggered with either soluble CD4 (sCD4) or NBD-556, a CD4 binding site-specific small molecule that preferentially binds to the CD4-bound conformation (31). Three viruses, ZA012 (HIV-1, Clade C), JR-FL (HIV-1, Clade B) and 7312A.V434M (HIV-2) were used for the triggering experiments (Figure 6, panel b; Supplementary Tables 2 and 3). 17b IgG was used as positive control (30, 32). Compounds **1** and **2** showed enhanced neutralization of all three viruses tested when pre-triggered with sCD4 with the most significant dose-dependent response seen with ZA012. The effect of triggering with NBD-556 was less significant both for the TYS mimetic compounds and 17b IgG. This observation is consistent with the fact that sCD4 can bind to the viral spike and induce the CD4-bound conformation. NBD-556, however, binds preferentially to the CD4-bound conformation, hence is a probe of the CD4-bound conformation but does not induce a similar conformational change.

Neutralization of engineered HIV-1 strains

The compounds were tested in two additional virological assays, each designed to maximize sensitivity for detection of activity of inhibitors that target the co-receptor interactive region of gp120. The first assay utilized a virus with a serine to alanine change (S199A) at the base of the V2 loop of gp120 that disrupts a glycosylation sequon thereby preventing N-linked glycosylation at residue 197 (33). This mutation conferred ability to enter $CD4^-$ cells and increased sensitivity to neutralization by sCD4 and various gp120-directed antibodies (33, 34). HIV-1 ADA Env-pseudovirus with the S199A mutation showed enhanced neutralization by the CD4i antibody 17b (Supplementary Figure 35) compared to the wild-type ADA virus. The efficiency of neutralization by 17b increased when the assay was performed using $CD4^-$ target cells, further enhancing sensitivity of the assay for characterization of lead stage inhibitors. We tested **1- 3** and **7** (Figure 4) for their ability to inhibit entry of ADA S199A. While **3** and **7** did not show neutralization at the concentration range tested, **1** and **2** were able to inhibit viral entry with IC_{50} values of 1.5 ± 0.5 μM and 44 ± 7 μM, respectively (Figure 6, panel c and Table 1). Toxicity assays showed that **1** and **2** were not toxic to the CF2-Luc cells up to a concentration of 250 μM (Supplementary Figure 36).

A second assay used an engineered virus, CEMAX, adapted for entry using the CCR5-(or the CXCR4-) N terminus (fused to a membrane spanning anchoring peptide) in the complete absence of coreceptor extracellular loops (35). CEMAX remained fully competent for mediating infection on either wildtype CCR5 or CXCR4. In light of CEMAX's exclusive dependence on the coreceptor N terminus for entry, we expected that this virus would be more susceptible to inhibition by compounds that target gp120 interaction with CCR5-N terminus. For the assays with CEMAX we tested compounds **2** and **7**, one from each class

shown in Figure 4. Both **2** and **7** inhibited CEMAX entry into $CCR5^+$ $CD4^+$ CF2-Luc reporter cells, with an IC_{50} of ~40 μM and showed greater than 80% inhibition at 100 μM (Figure 6, panel c). In the concentration range tested (0-100 μM) no effect was seen on entry of MuLV (Supplementary Figure 32). **2** and **7** also inhibited entry of the dual-tropic HIV-1 isolate, HIV-1/R3A and the R5-tropic viruses YU2 and SF162, albeit to a lesser extent (Supplementary Figure 33). Toxicity assays showed that **2** and **7** were not toxic to CF2-Luc cells upto a concentration of 100 μM (Supplementary Figure 34). Interestingly, the compounds not only inhibited CCR5-mediated entry, but also inhibited CEMAX entry into cells expressing CXCR4 suggesting targeting of sites common to both R5 and X4-tropic viruses.

All compounds tested (Table 1) bound to monomeric gp120 with binding affinities within 5-fold of each other, yet **1** and **2** neutralized primary isolates and the engineered virus ADA S199A to varying degrees whereas, **3** and **7** do not show neutralization of these viruses in the concentration range tested. These observations suggest isolate-specific barriers in the trimeric HIV-1 spike that further modulate activity and lead to differential activity of these compounds. When tested on CEMAX, an isolate engineered for increased dependence on the coreceptor N termini, **2** and **7** inhibit entry with similar IC_{50} values. This suggests alleviation of barriers that cause differential reactivity of the compounds against native HIV isolates.

Summary and implications for development of entry inhibitors that target the HIV envelope-coreceptor interaction

The co-receptor binding site, especially the region spanning the bridging sheet and the base of the V3 loop (Supplementary Figure 1) (5), is highly conserved. Antibodies that bind to this region in monomeric gp120 have broad binding specificity across different viral isolates. While the high degree of conservation is a desirable property of this potential target, this region forms only upon CD4 engagement of the HIV-1 spike. After CD4 binds, the proximity of the viral and cell membranes renders the co-receptor binding site sterically inaccessible to molecules as large as antibodies (36), though antibody fragments and single chain antibody-variants retain access (37). Small molecules, therefore, may present a viable strategy for intervention at the site of co-receptor binding on HIV-1.

We have used structural information for *in silico* screening and coupled this to experimental screening to identify small molecule inhibitors of HIV-1 binding to its co-receptor. This led to the identification of a novel phenylsulfonate-linker-aromatic motif with HIV entry inhibition properties. Since information from ligands (CCR5-N terminus and 412d antibody) that specifically target CCR5-using viruses was used to screen for small molecule mimetics, we expected these mimetics to be active only against CCR5-mediated entry, but found that they inhibited entry independent of whether it occurred with CCR5 or CXCR4 (Figure 6, panel a). While somewhat unexpected, one potential explanation is the high degree of conservation of the tyrosine-sulfate binding regions on gp120 across all HIV isolates, irrespective of tropism.

HIV uses a number of mechanisms to evade antibody-mediated neutralization. These include variable loop occlusion (38), glycan shielding (39), and conformational masking (40). Comparison of the properties of the TYS mimetic small molecules determined in this study with other known HIV-1 neutralizing molecules (Table 2 and Supplemental Table 4) show that their properties strongly correlate with those of molecules that target the HIV co-receptor binding site. First, 15-40 fold tighter binding of the TYS mimetic small molecules was observed to gp120 fixed in the CD4-bound conformation relative to gp120 that was unrestrained. Second, they competed with antibodies that bind to the CD4-induced epitope of gp120. Third, neutralization of primary isolates was enhanced by pre-triggering with

sCD4. Fourth, viruses with impaired conformational masking (e.g. ADA S199A) were neutralized with greater potency than native HIV-1 isolates. These results and the strong correlation of properties with molecules targeting the CD4i epitope of gp120 (Table 2) reveal that the mechanism of action of these tyrosine-sulfate mimetics involves binding to the conformationally malleable co-receptor binding region of gp120, specifically the CD4-induced site. Conformational masking appears to hinder the ability of these small molecules to neutralize primary HIV-1 isolates. Our studies indicate that these tyrosine-sulfate mimetics may work best in synergy with molecules that induce the CD4-bound state of gp120 such as sCD4 or CD4-mimetic peptides (27-29).

In summary, we report the structure-guided identification and characterization of a new class of tyrosine-sulfate mimicking small molecules that bind to the HIV-1 gp120 envelope glycoprotein and inhibit entry. Recognition of and inhibition by these tyrosine-sulfate mimetics provide an example for how the *O*-sulfation post-translational modification can be exploited therapeutically. Analysis of the neutralization mechanism of these compounds indicates that the conformational barriers evolved by HIV-1 to protect the highly conserved co-receptor binding region from antibody-mediated neutralization also restricts neutralization by small molecules that target to this region. Despite this evasion tactic, the ability of antibody fragments to potently neutralize HIV-1 (37) provides proof-of-principle that – with sufficient affinity – effective neutralization by this class of tyrosine-sulfate mimetics can be achieved. It will be interesting to see if improved mimicry increases neutralization potency, for example, with small molecules that contain tyrosine-sulfate as opposed to tyrosine-sulfonate. Moreover, compounds **1** and **2** neutralized primary HIV-1 isolates far better than compounds **3** and **7**, despite less than 5-fold difference in K_D; suggesting that small changes in affinity may have more dramatic effects on neutralization potency.

Methods

Additional details for each section are available in Supporting Information.

Virtual Screening

gp120-bound coordinates of 412d and CCR5-N terminus were used for virtual screening. ROCS (21) searches were done on the ZINC database and hits were rescored and docked with Gold (22). In a complimentary approach the druglike subset of a database of commercially available compounds was docked to the protein models using Glide v4.5 (Schrödinger, LLC).

ELISA-based screening assay

The ELISA-based screening was performed as described earlier (25) using 500 μM compound concentration (unless otherwise indicated).

Surface Plasmon Resonance

Different compound concentrations were injected onto a gp120-coated surface in absence or presence of M48-U1. For competition assays, 412d and C11 IgG were immobilized on different flow cells. A gp120-M48-U1 complex with or without compound was injected and initial slopes were calculated for the on-phase.

HIV entry inhibition assays

The ability of the compounds to inhibit entry of CEMAX (35) was evaluated on canine thymocyte cells (CF2-luc) stably expressing a luciferase reporter under control of an HIV-1 LTR (kindly provided by D. Gabuzda, Harvard).

All other viral entry inhibition assays were performed as previously described (41), except assays with ADA S199A, where target cells were canine thymocytes (Cf2Th) stably expressing human CCR5 or both CD4 and CCR5 (42). The triggering assays were performed as described earlier (32).

Target cell viability was determined in a format similar to the neutralization assays using a luminescence-based assay measuring cellular expression of adenosine triphosphate (CellTiter-Glo Luminescent Cell Viability Assay, Promega Corp.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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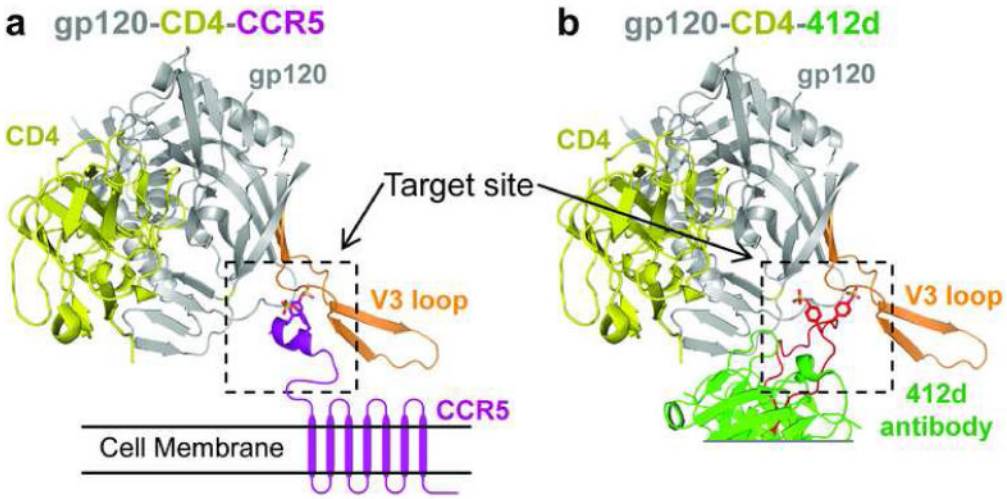


Figure 1.

Target site. A critical step of HIV entry involves interaction with sulfated tyrosine residues in the CCR5 co-receptor, which the antibody 412d mimics to neutralize HIV-1. a) gp120-CCR5-N terminus interaction. HIV-1 gp120 (grey) interacts with its cell surface receptor CD4 (yellow) and co-receptor CCR5 (magenta). CCR5-N terminus binds in an α-helical conformation at the base of the V3 loop (orange) of gp120. Two sulfated tyrosine residues (stick representation) are critical for binding of the CCR5-N terminus to gp120. b) Mimicry of gp120-CCR5-N terminus interaction by CDR H3 loop of 412d antibody. The CDR H3 loop (red) of antibody 412d (green) is a functional mimic of the CCR5-N terminus with two sulfated tyrosine residues (sticks) dominating its interaction with gp120.

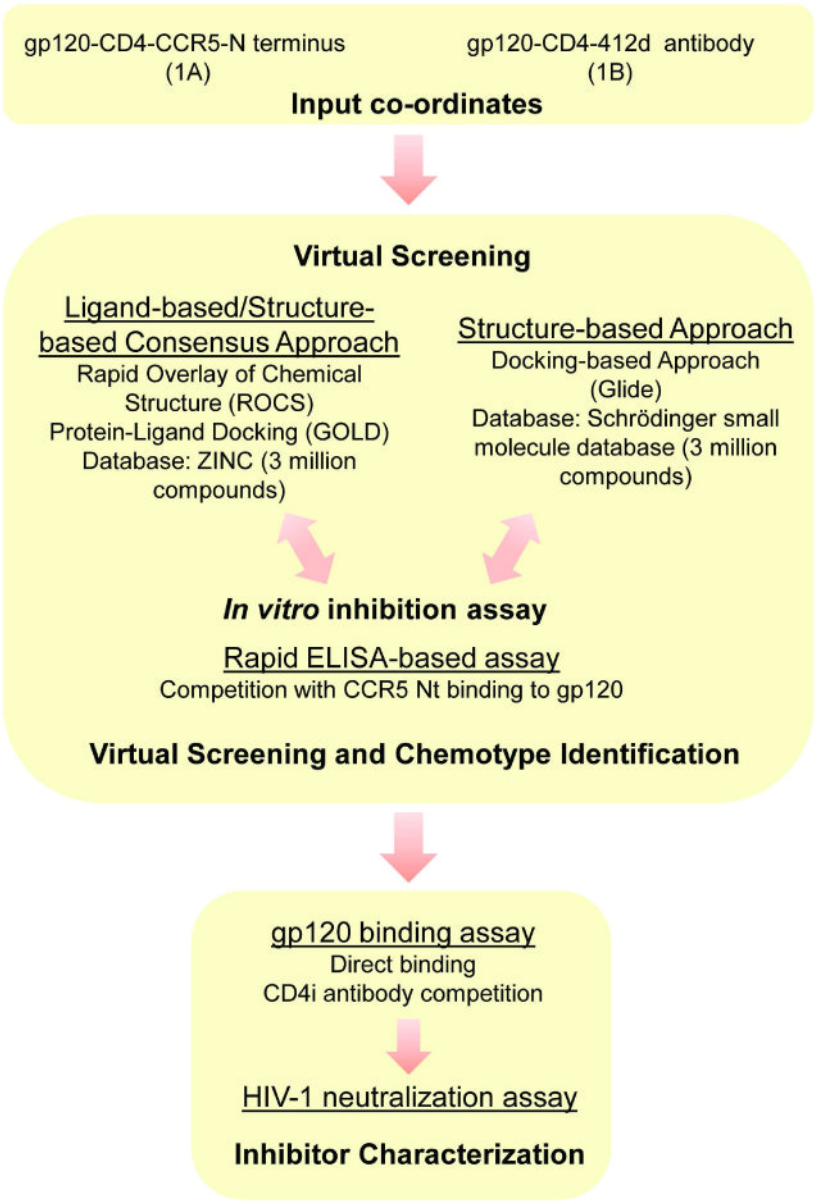


Figure 2.

Inhibitor identification flowchart. Starting from atomic level co-ordinates of gp120-CD4-CCR5-N terminus and gp120-CD4-412d Fab, two methods of in silico screening were used in combination with an ELISA-based screen to identify binding chemotypes. Selected compounds were characterized further for their gp120 binding and HIV-1 neutralization properties.

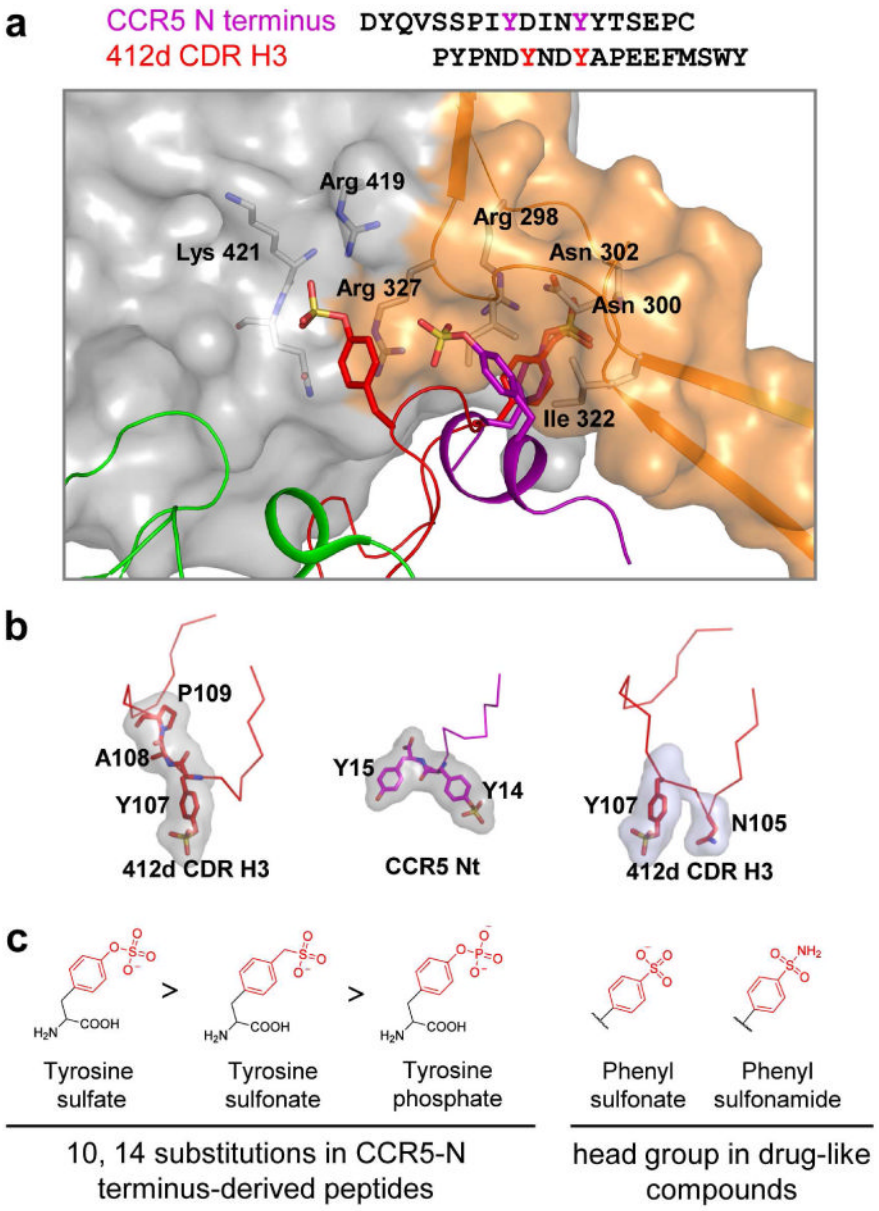


Figure 3.

Tyrosine-sulfate mimicry in inhibitor identification. The critical role of two N terminal tyrosine-sulfate residues in CCR5 binding and their unique chemistry suggested the utility of the tyrosine-sulfate binding sites as drug targets. a) Tyrosine-sulfate binding sites on gp120. Sequences of CCR5-N terminus and 412d CDR H3 loop are shown above with the tyrosine residues that are sulfated and critical for binding colored red. The CCR5-N terminus (magenta) and the CDR H3 loop (red) of 412d antibody interact with the base of the V3 loop (orange) of gp120 (grey). The region surrounding the tyrosine-sulfate residues was targeted for small molecule screening. b) Ligand-based probes for *in silico* screening. Structure-based probes (shown as sticks) centered around the tyrosine-sulfate moieties were used for shape based screening of the ZINC small molecule library. c) Tyrosine-sulfate analogs. The rank order of binding affinity of CCR5 peptides containing modified tyrosines at positions

10 and 14 are shown. Compounds containing phenyl sulfonate and phenyl sulfonamide groups were screened in this study.

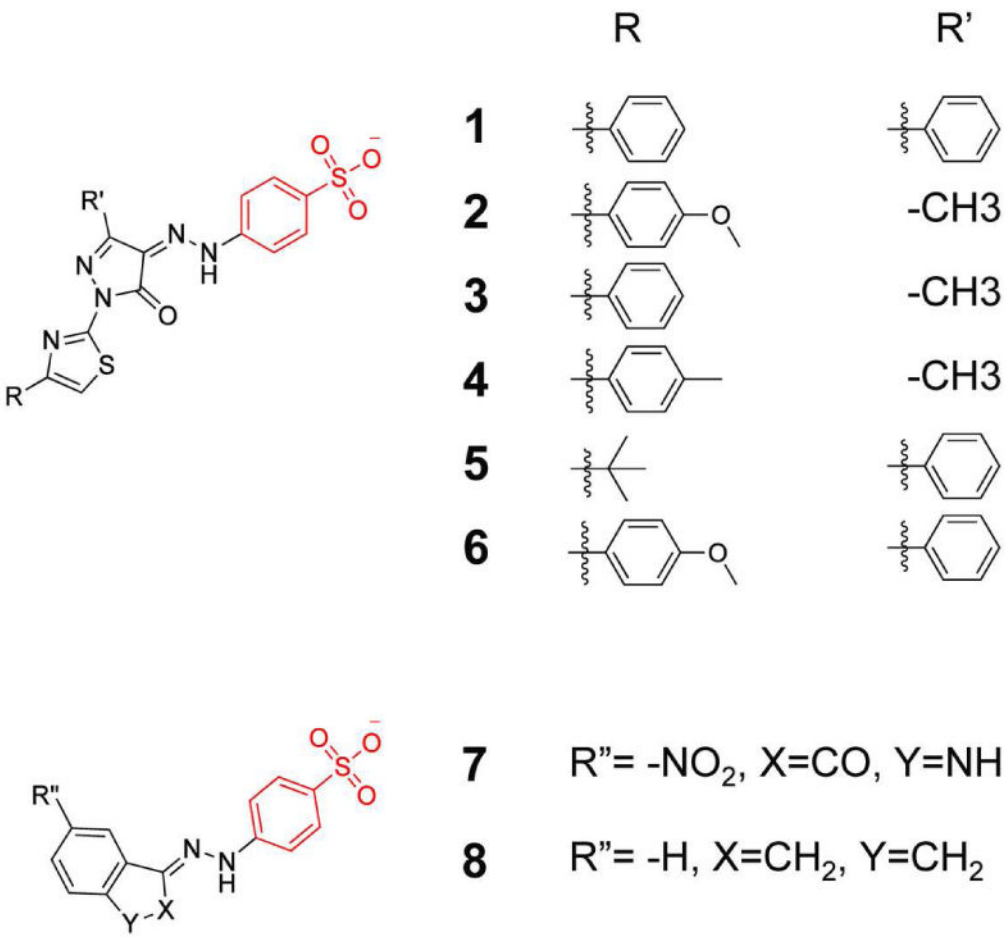


Figure 4.
Small molecule inhibitors of CCR5-N terminus binding to gp120. Small molecules were selected on the basis of their ability to inhibit binding of gp120 to a CCR5-N terminus peptide analog. All inhibitors contained a phenyl sulfonate moiety, which retains the anionic and aromatic properties of tyrosine-sulfate. The two classes of compounds shown were selected for further analyses.

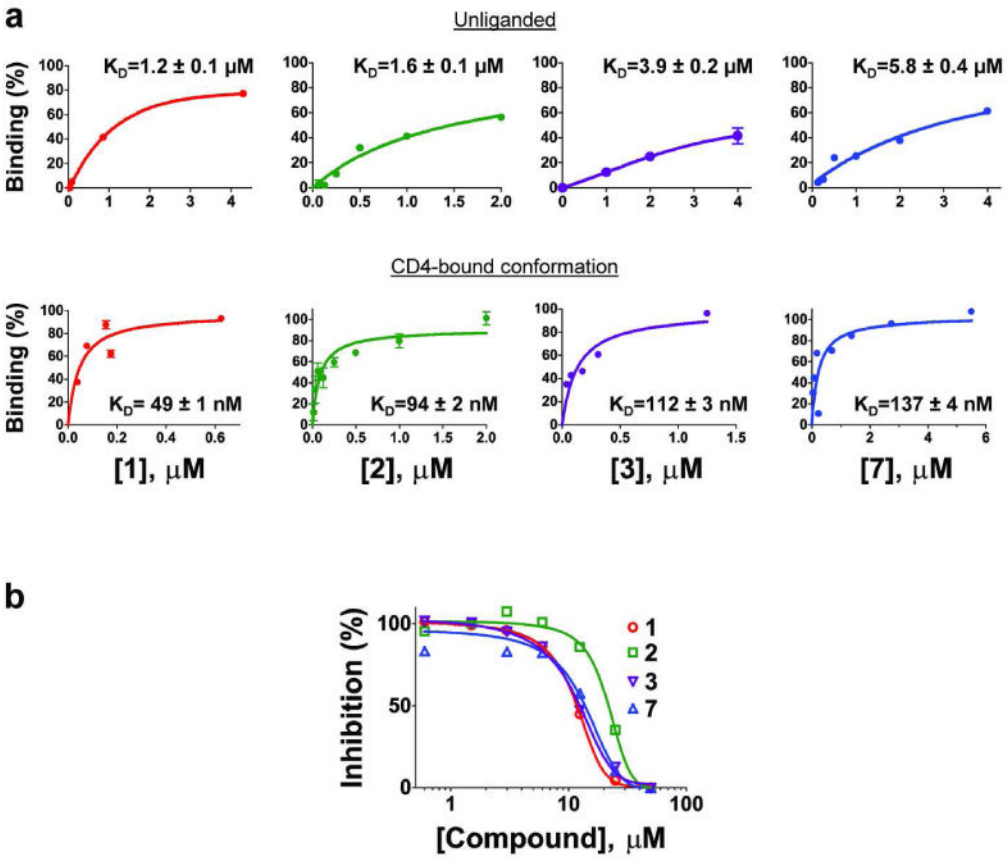


Figure 5.

gp120 binding. a) Direct binding to gp120. Binding of compounds **1, 2, 3** and **7** to gp120 in the unliganded (top panel) and in the CD4-bound conformation (bottom panel) was measured by SPR and affinity calculated by steady state analysis. b) Inhibition of binding of CD4i antibody 412d to gp120 in the CD4-bound conformation by the compounds.

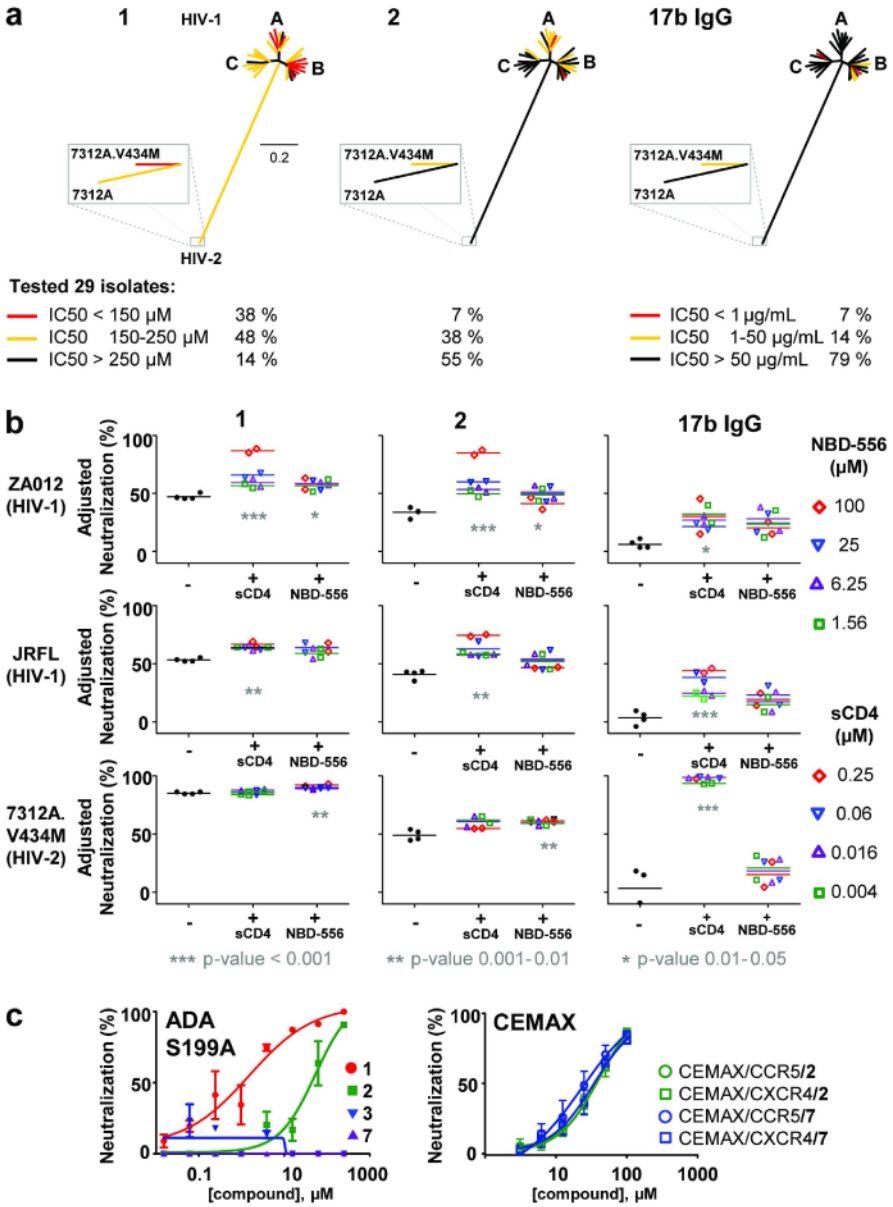
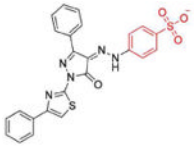
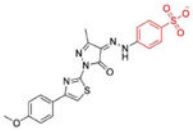
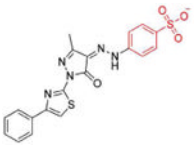
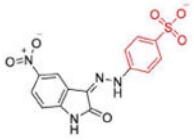


Figure 6.

Inhibition of HIV entry. a) Neutralization profiles of compounds **1, 2** and monoclonal antibody 17b depicted with 29-isolate neutralization dendrograms. SIV and MuLV were used as controls. IC_{50} values are listed in Supplementary Table 1. Insets show neutralization of two HIV-2 isolates tested. b) Inhibition of entry in presence of two-domain CD4 (sCD4) or the small molecule NBD-556. The p-values indicate statistical significance for effect of triggerring with sCD4 or NBD-556 compared to untreated virus calculated using ANOVA. c) Left: inhibition of entry of HIV-1 ADA S199A, and right, inhibition of CCR5-mediated entry (circles) and of CXCR4-mediated entry (squares) of CEMAX.

Table 1

Binding and entry inhibition

	Compound	K_D (unliganded gp120) (μM)	K_D (gp120 in CD4-bound conformation) (μM)	Geometric IC_{50} mean of primary HIV-1 neutralization (μM)
1		1.2 ± 0.1	0.049 ± 0.001	157.6*
2		1.6 ± 0.1	0.094 ± 0.002	123.7*
3		3.9 ± 0.2	0.112 ± 0.003	>250
7		5.8 ± 0.4	0.181 ± 0.004	>250

*Geometric means were calculated for viruses that neutralized with IC_{50} value < 250 μM

Table 2

Significance of agreement between phenotypes of molecules targeting different neutralization sites on HIV-1. Numbers in table are p-values from Fisher's exact test. Supplementary Table 2 lists phenotypes of neutralizing molecules.

	HIV-1 neutralizing molecules					
	CD4-induced site targeting	**CD4-binding site targeting**	**glycan targeting**	**gp41 targeting**	**V3 targeting**	**Quaternary site targeting**
TYS mimetic small molecules	**0.003**	*1*	*0.467*	*1*	**0.048**	*0.524*
CD4-induced site targeting		*0.464*	*0.464*	*1*	**0.048**	*1*
CD4-binding site targeting			*0.067*	*0.196*	*0.52*	*0.464*
Glycan targeting				*0.464*	*0.167*	*1*

p-value < **0.05**: significant, p-value > *0.05*: not significant

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