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A Synthetic Heparan Sulfate-Mimetic Peptide Conjugated to a Mini CD4 Displays Very High Anti-HIV-1 Activity Independently of Coreceptor Usage

Bridgette Janine Connell,[1] Françoise Baleux,[2] Yves-Marie Coic,[2] Pascal Clayette,[3] David Bonnaffé,[4] and Hugues Lortat-Jacob[1,*]
[1]CEA, CNRS, Université Joseph Fourier – Grenoble 1, Institut de Biologie Structurale Jean-Pierre Ebel, UMR 5075, 38000 Grenoble, France
[2]Institut Pasteur, unité de Chimie des Biomolécules, URA CNRS 2128, 75015 Paris, France
[3]Laboratoire de Neurovirologie, Bertin Pharma, CEA, 92265 Fontenay aux Roses, France
[4]Laboratoire de Chimie Organique Multifonctionnelle, Equipe Glycochimie Moléculaire et Macromoléculaire, ICMMO UMR CNRS-UPS 8182, Université Paris-Sud 11, 91405 Orsay, France
*Correspondence: hugues.lortat-jacob@ibs.fr
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SUMMARY

The HIV-1 envelope gp120, which features both the virus receptor (CD4) and coreceptor (CCR5/CXCR4) binding sites, offers multiple sites for therapeutic intervention. However, the latter becomes exposed, thus vulnerable to inhibition, only transiently when the virus has already bound cellular CD4. To pierce this defense mechanism, we engineered a series of heparan sulfate mimicking tridecapeptides and showed that one of them target the gp120 coreceptor binding site with μM affinity. Covalently linked to a CD4-mimetic that binds to gp120 and renders the coreceptor binding domain available to be targeted, the conjugated tridecapeptide now displays nanomolar affinity for its target. Using solubilized coreceptors captured on top of sensorchip we show that it inhibits gp120 binding to both CCR5 and CXCR4 and in peripheral blood mononuclear cells broadly inhibits HIV-1 replication with an IC_{50} of 1 nM.

INTRODUCTION

Although tremendous progress has been made in the development of antiviral drugs to treat human immunodeficiency virus (HIV-1) infection (De Clercq, 2007) and despite the availability of some 25 approved antiretroviral compounds (most of which target HIV-1 enzymes), the virus continues to be a major concern and remains one of the leading causes of death worldwide. The rapid emergence of drug-resistant viral strains, the inability of current therapy to completely eradicate the virus, and the strong adverse side effects associated with their long-term use (Shafer and Schapiro, 2008) compromise treatment in patients benefiting from these therapies and make the development of new therapeutic options of utmost importance (Flexner, 2007). Inhibition of HIV-1 entry, a process based on the sequential interaction of the viral glycoprotein (gp120) with the cell surface CD4 (Klatzmann et al., 1984) and either one of the two chemokine receptors CCR5 or CXCR4 (Alkhatib et al., 1996; Feng et al., 1996), holds particular promise in addressing complications of current therapy and has become a compelling target for controlling viral replication (Tilton and Doms, 2010). The recent approval of maraviroc, a CCR5 antagonist (Dorr et al., 2005; Maeda et al., 2004), has validated entry inhibition as a viable approach. However, to avoid the selection of pre-existing and more pathogenic CXCR4-using HIV-1 (for which no effective antagonistic inhibitors yet exist) maraviroc has been licensed for the treatment of patients infected with viral strains using CCR5 only.

On the virus side, the gp120 constitutes the central element for all interactive events occurring during the pre-entry steps. A wealth of evidence has shown that gp120 binding to CD4 not only permits virus attachment, but also triggers extensive conformational changes of the envelope that fold and/or expose a four-stranded β sheet, known as the CD4-induced (CD4i) domain (Wu et al., 1996). Being critically involved in CCR5/CXCR4 recognition and highly conserved, this domain represents an attractive pharmacological target. Although inhibition of protein-protein interactions is clearly challenging, a striking feature of the CD4i domain is its basic nature (Kwong et al., 1998; Rizzuto et al., 1998) and, not surprisingly, many of this domain's ligands are characteristically acidic. This includes peptides selected by phage display screening (Dervillez et al., 2010), sulfated oligosaccharides from the heparan sulfate (HS) family (Crublet et al., 2008; Vivès et al., 2005), aptamers (Cohen et al., 2008), peptides derived from neutralizing antibodies (Dorfman et al., 2006), compounds issued from in silico screening of molecular libraries (Acharya et al., 2011), or peptides derived from the N-terminal sequence of CCR5 itself that comprise sulfotyrosines importantly contributing to gp120 binding (Cormier et al., 2000; Farzan et al., 2000). The cryptic nature of this CD4i surface prior to CD4 binding, however, limits its accessibility both temporally and spatially and makes it a relatively intractable pharmacological target. In that context, we recently developed a class of compounds, in which a CD4 mimetic peptide (mCD4) was linked to a HS dodecasaccharide (HS_{12}), and showed that mCD4 exposed the gp120 CD4i domain and renders it available to be blocked by the HS_{12} oligosaccharide (Baleux et al., 2009).

Here, to further develop this concept we engineered a series of tridecapeptides that mimic HS, the synthesis of which,

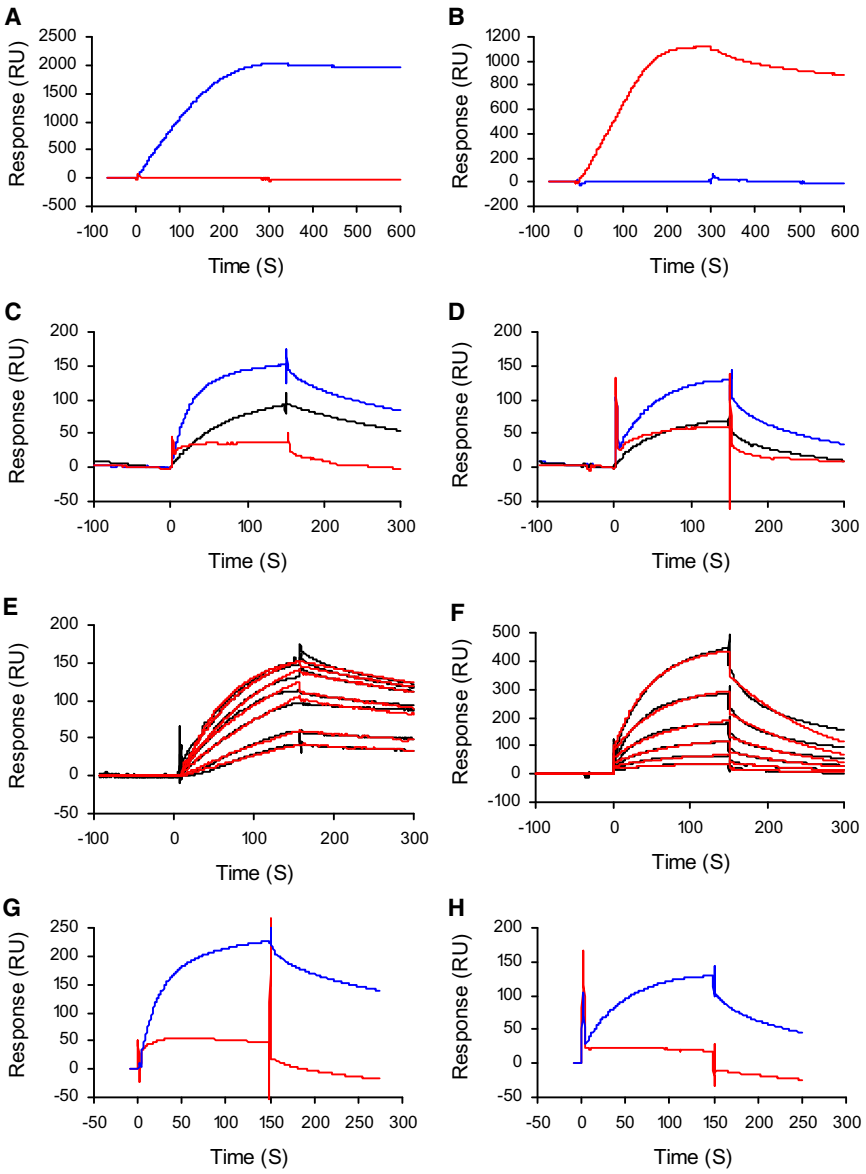


Figure 1. Ligand Binding to CCR5 and CXCR4 Immobilized Sensorchips

Carboxy-terminal C9 tagged CCR5 or CXCR4 were solubilized from Cf2Th cells and captured on top of a mAb 1D4 activated CM4 sensorchip. CCR5 (left) and CXCR4 (right) ligands were injected over the coreceptor surfaces, and the binding responses (in RU) were recorded as a function of time (in S). Binding of 25 nM of mAb 2D7 (blue) and mAb 12G5 (red) to CCR5 (A) and CXCR4 (B). Binding of YU2 gp120 (black), YU2/mCD4 (blue), or YU2/mCD4/maraviroc (red) to CCR5 (C) or MN gp120 (black), MN/mCD4 (blue), or MN/mCD4/AMD3100 (red) to CXCR4 (D). Binding of the equimolar complex of YU2/mCD4 at (from top to bottom) 100, 66, 44, 29, 19, and 12.5 nM to CCR5 (E) or equimolar complex of MN/mCD4 at (from top to bottom) 225, 150, 100, 66, 44, and 29 nM to CXCR4 (F). The black traces correspond to the experimental data, and the red traces correspond to the fitted data using a 1:1 langmuir model. Binding of YU2 or MN gp120 (100 nM) preincubated with 100 nM of mCD4 (blue) or mCD4-HS_{12} (red) to CCR5 (G) or CXCR4 (H).

although amenable to large-scale production, remains extraordinary complex (Dilhas et al., 2008). We then set up a binding assay in which detergent solubilized CCR5 and CXCR4 were both functionally captured on top of sensorchips and used them to show that, conjugated to a mini CD4, a HS mimicking peptide efficiently targets the CD4i domain of gp120 and blocks its interaction with the coreceptors. This compound displays antiviral activity against LAI and Ba-L HIV strains with an IC_{50} as low as 1 nM, two to four orders of magnitude lower than the above-described anionic compounds. To our knowledge, this is the most potent gp120 targeting molecule, with the unique property to simultaneously block two critical and conserved regions of gp120. Importantly it inhibits CCR5 and CXCR4 using viruses equally well, and is also highly active against a number of viral primary clinical isolates. These results should have strong implications for the development of a new anti-HIV-1 therapy.

RESULTS

HIV-1 Coreceptors Immobilization and gp120 Binding

Assessing the ability of molecules to target the coreceptor binding site of gp120 would strongly benefit from a direct coreceptor-gp120 interaction assay. To that end, both HIV-1 coreceptors were solubilized from Cf2Th cells, recombinantly expressing either CCR5 or CXCR4, using a specific cocktail of lipids and detergents that was adapted from that previously described (Navratilova et al., 2005). Solubilized coreceptors, which feature a C-terminal C9 tag (Babcock et al., 2001; Mirzabekov et al., 1999) allowing their oriented capture with the cognate 1D4 antibody, were immobilized on top of a sensorchip to a level of ~4,000 resonance units (RU). To verify whether the coreceptors remained functional, we first investigated their binding capacity with the conformationally sensitive mAb 2D7 for CCR5 (Khurana et al., 2005; Lee et al., 1999) and 12G5 for CXCR4 (Baribaud et al., 2001). As shown in Figures 1A and 1B, injection of these mAbs over the CCR5 and CXCR4 functionalized surfaces gave rise to strong and coreceptor-specific binding signals, indicating both the presence of the coreceptor on the surface and the integrity of the corresponding epitopes.

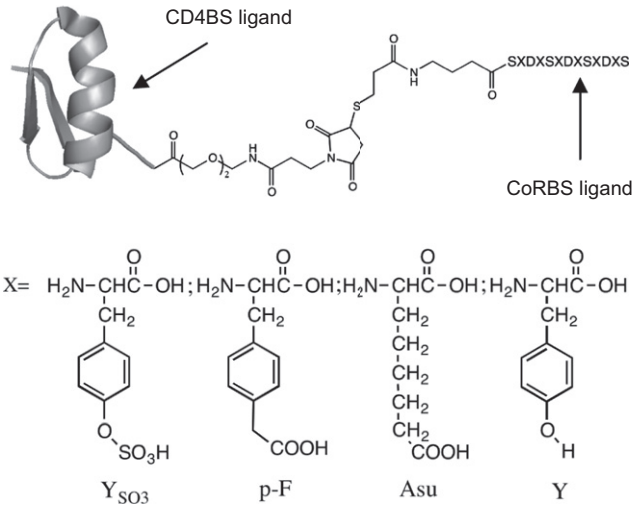
Following this, we analyzed whether the immobilized coreceptors bound gp120, in a CD4-dependent manner. For that purpose, 100 nM of either YU2 or MN (R5 and X4 envelopes respectively), in the absence or presence of mCD4, a CD4 mimetic peptide that was previously found to bind gp120 and induce the conformational change that lead to the folding/exposure of the coreceptor binding site (Baleux et al., 2009),

was injected over the coreceptor surfaces. Both envelopes interacted with their coreceptors, presumably because the CD4i epitope is transiently exposed on the dynamic structure of gp120, as already observed with anti-CD4i antibodies (Thali et al., 1993). The binding responses, however, were significantly enhanced by the presence of mCD4 and efficiently inhibited by 1 μM of maraviroc or AMD3100 (Figures 1C and 1D), two compounds targeting CCR5 and CXCR4 respectively, and having anti-HIV-1 activity (Tilton and Doms, 2010). Next, dose-response experiments were performed with mCD4:gp120 ratios fixed at 1:1 and injected over the immobilized CCR5 or CXCR4 surfaces. Sensorgrams were obtained for both envelopes (Figures 1E and 1F), which evaluations (see Supplemental Experimental Procedures) returned estimated affinities of 11.5 ± 2.9 nM and 154 ± 68 nM for CCR5 and CXCR4, respectively. These values were identical to that reported by a similar technique (Navratilova et al., 2005) or radioligand binding assay with cell membrane-embedded CCR5 (Doranz et al., 1999) as to that reported for CXCR4, using proteoliposome embedded coreceptors and radiolabeled gp120 (Babcock et al., 2001).

We previously reported that the gp120 CD4i epitope can be targeted by HS (Crublet et al., 2008; Vivès et al., 2005), and that a HS dodecasaccharide covalently linked to mCD4 (mCD4-HS_{12}) binds gp120 and blocks its subsequent interaction with mAb 17b (Baleux et al., 2009). mAb 17b belongs to a group known as "anti-CD4i" antibodies, which recognizes a conserved element of gp120, induced by CD4 and partially overlapping the coreceptor binding site (Xiang et al., 2002). We thus made use of the coreceptor binding assay described above to investigate whether mCD4-HS_{12} would also inhibit gp120 binding to CCR5 and CXCR4. As shown in Figures 1G and 1H, both YU2 and MN gp120 in complex with mCD4-HS_{12} featured a strongly reduced ability to recognize CCR5 or CXCR4 compared to that of gp120 in complex with mCD4 alone. This suggests that such molecules could serve as lead compounds for the future development of a new class of entry inhibitors.

Chemical Synthesis of mCD4 Linked HS Mimetic Peptides

HSs are, however, notoriously difficult to synthesize. In addition, their inherent sequence heterogeneity, in terms of sulfation pattern and saccharide composition, would currently make the preparation of a dodecamer series out of reach. Thus, based on the mCD4-HS_{12} template, we tested the hypothesis that the HS moiety could be mimicked by peptides, the chemical synthesis of which is more straightforward, and more easily amenable to sequence-activity relationship investigation. To display the functional hydroxyl, carboxyl, and sulfate groups that characterize HS, peptides comprising Ser, Asp, and Tyr, the latter being possibly sulfated, were considered. This strategy is supported by the observation that a SYDY tetrapeptide binds to the HS binding domain of the vascular endothelial growth factor (Maynard and Hubbell, 2005) and that phage display screenings against the CD4i epitope of gp120 returned sequences enriched in YD motifs (Dervillez et al., 2010). It is also worth noting that a number of antibodies against the gp120 coreceptor binding domains feature sulfotyrosines in their paratope, as does the N terminus of both CCR5 and CXCR4 (Choe et al., 2003).



X	S(XDXS)$_3$ name
YSO_3	P3YSO_3
pF	P3pF
Asu	P3Asu
Y	P3Y

Figure 2. mCD4-S(XDXS)$_3$ Constructs

A miniCD4 was used as a CD4 binding site (CD4BS) ligand and covalently conjugated through an appropriate linker to S(XDXS)$_3$ peptides investigated as potential coreceptor binding site (CoRBS) ligands. S and D are serine and aspartic acid residues respectively and X is either a sulfotyrosine (Y_{SO3}), a p-carboxymethyl phenylalanine (pF) an aminosuberic acid (Asu) or a tyrosine (Y). See also Figures S1 and S2 and Table S1.

Building of a S(XDXS)$_n$ sequence (were X stands for different possible amino acids; see below) using the peptide builder of Hyperchem 5, showed that a 13 amino acid peptide (n = 3), in its extended configuration (φ, ψ, and ω angles set to 180°) would have a length equivalent to the HS 12 mer (data not shown). Thus, a tridecapeptide, alternating OH/COO^- and OH/SO_3^- groups, having the sequence: $SY_{SO3}DY_{SO3}SY_{SO3}DY_{SO3}SY_{SO3}DY_{SO3}S$ (X being in this case a sulfotyrosine; Y_{SO3}) was first synthesized (P3YSO_3). The nonsulfated equivalent (P3Y) was also prepared along with a number of other peptides in which X was replaced by p-carboxymethyl phenylalanine (P3pF) or aminosuberic acid (P3Asu), two residues that have been shown to functionally mimic sulfotyrosine in cholecystokinin type B receptor ligand CCK8 (McCort-Tranchepain et al., 1992) and sulfakinins (Nachman et al., 2005). A tridecaglutamate (displaying 13 carboxylic groups) was also prepared (E13) as a nonspecific poly anionic peptide (Figure 2). In order to maintain an appropriate distance between mCD4 and these peptides, enabling the final molecule to reach both the CD4 and coreceptor binding sites, a γ-aminobutyric acid (γ-Abu) was introduced on their N terminus. These peptides were derivatized with S-acetylthiopropionic acid to allow the coupling to Lys^5 of a maleimide-activated mCD4. All compounds were purified to a level of 95% by RP-HPLC (see Table S1 and Figures S1 and S2 available online), controlled by mass spectrometry and

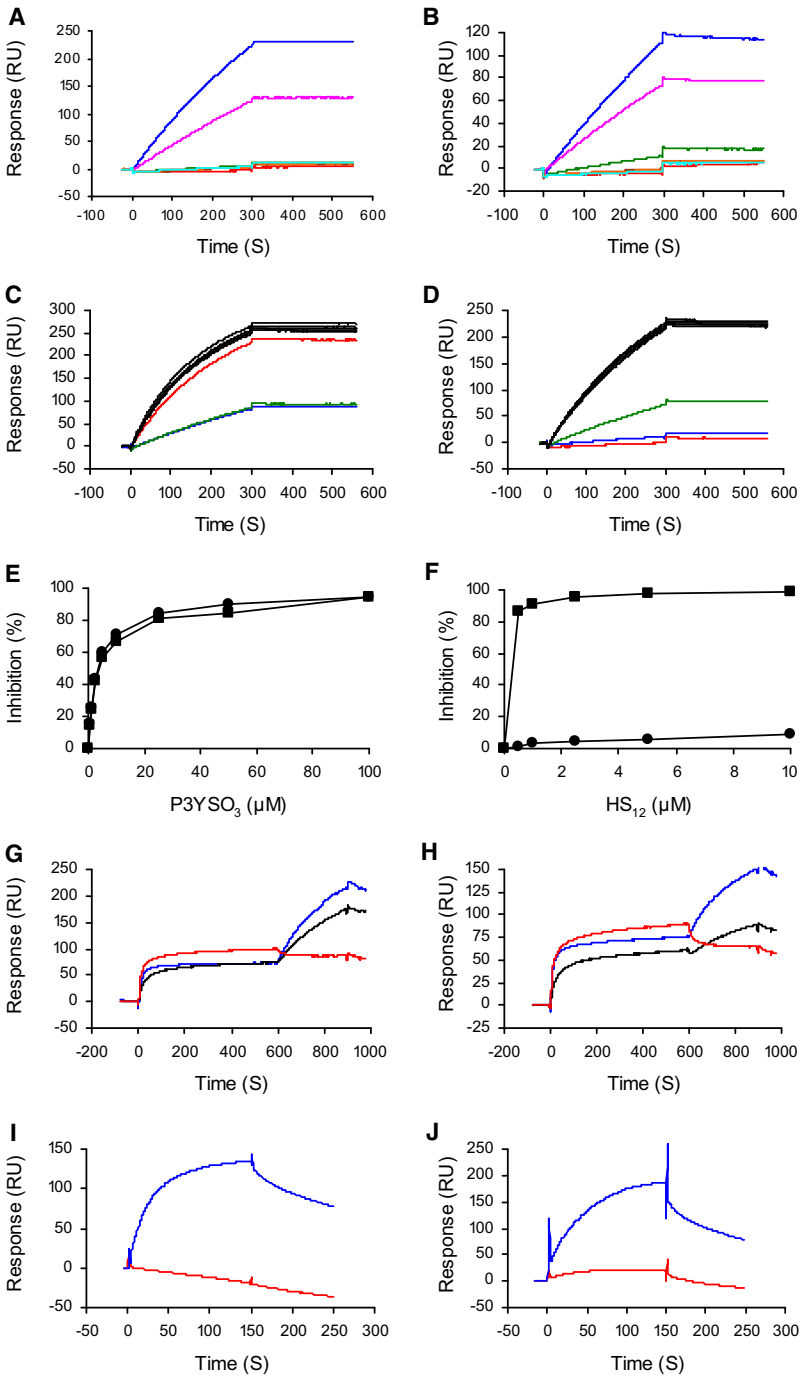


Figure 3. The S(XDXS)$_3$ HS Mimetic Peptides Coupled to mCD4 Inhibit gp120-CD4, gp120-mAb 17b, and gp120-Coreceptor Interactions through Binding to the CD4 and the Coreceptor Binding Sites of gp120

Binding responses measured when YU2 (A) or MN (B) gp120 at 100 nM, either alone (blue) or preincubated with 100 nM of mCD4 (pink), mCD4-P3Y (green), mCD4-E13 (turquoise), mCD4-P3pF (orange), mCD4-P3Asu (brown), or mCD4-P3YSO$_3$ (red) were injected over a CD4 activated surface. YU2-mCD4 (C) or MN-mCD4 (D) complexes (25 nM) were preincubated with 5 μM of HS$_{12}$ (red), P3YS0$_3$ (green), or the other HS mimetic peptides (none, P3Y, E13, P3pF, and P3Asu; all in black) and injected over a mAb 17b activated surface. The blue trace shows the binding of gp120 to mAb 17b in the absence of mCD4. The P3YSO$_3$ peptide (E) or HS$_{12}$ (F) at different concentrations were coincubated with YU2-mCD4 (circle) or MN-mCD4 (square) and injected over a mAb 17b surface. The binding response (mean of triplicate experiment) recorded at the end of the injection phase was plotted versus the concentration of the inhibitors in μM. Overlay of sensorgrams showing the injection of 100 nM of mCD4 (blue), mCD4-P3Y (black) or mCD4-P3YSO$_3$ (red), from 0 to 600 s, over immobilized YU2 (G) or MN (H) gp120, after which 15 μg/ml of mAb 17b was injected from 600 to 900 s. Binding of YU2 or MN gp120 (100 nM) preincubated with 100 nM of mCD4 (blue) or mCD4-P3YSO$_3$ (red) to CCR5 (I) or CXCR4 (J). In all graphs, binding signals were recorded in RU as a function of time (S).

quantified by amino acid analysis as described in the Supplemental Experimental Procedures.

mCD4 Linked HS Mimetic Peptides Inhibit Binding of gp120 to CD4, mAb 17b, and Coreceptors

To verify that peptide conjugation did not prevent the ability of mCD4 to interact with gp120, a competition assay was performed, in which YU2 or MN were incubated with the different mCD4 conjugates and injected over a CD4 functionalized surface. Results showed that the mCD4 conjugates all very efficiently prevent gp120-CD4 interaction, with greater potency than that of unconjugated mCD4 (Figures 3A and 3B). Next, the capacity of the anionic peptides to target the gp120 CD4i epitope was investigated by analyzing their ability to prevent gp120 binding to mAb 17b, in the presence of soluble mCD4. While unliganded gp120 was not (MN) or only poorly (YU2) recognized by mAb 17b (Figures 3C and 3D; blue trace), preincubation with mCD4 strongly promoted binding (black trace). When the gp120-mCD4 complexes were further incubated with 5 μM of the above-described tridecapeptides, strong inhibition was observed for P3YSO$_3$ (green trace). The tridecaglutamate (E13) was devoid of activity, indicating that the anionic character of the peptide is not sufficient to provide binding, as were the unsulfated P3Y or the sequence in which the sulfotyrosine mimetics (pF and Asu) were introduced (Figures 3C and 3D, black traces). HS$_{12}$ (red trace) also fully blocked mAb17b binding to MN-, but not to YU2-gp120. Together, this showed that among the different peptides investigated only the SY$_{SO3}$DY$_{SO3}$ motif competes with mAb 17b to interact with the gp120 CD4i domain. To better quantify the inhibitory activity of this peptide, the same assay was run, with a range of P3YSO$_3$ concentrations, and compared with HS$_{12}$. A similar concentration dependency was observed on both R5 (YU2) and X4 (MN) envelopes, with IC$_{50}$ of 2.9 and 3.1 μM, respectively, indicating that, interestingly, P3YSO$_3$ interacts with gp120 independently

of coreceptor tropism. In contrast, HS_{12} strongly inhibited the interaction between MN and mAb 17b (with a concentration as low as 0.5 μM) but was ineffective toward YU2, at concentrations up to 10 μM (Figures 3E and 3F). Next, to determine the binding mechanism of the mCD4-$S(XDXS)_3$ constructs, X4- and R5-gp120 were immobilized on a sensorchip and first allowed to bind to mCD4, mCD4-P3Y, or mCD4-$P3YSO_3$. The resulting complexes were then probed with mAb 17b, the binding of which being a marker of the coreceptor binding site accessibility. As expected, mCD4 binding to gp120 renders the coreceptor binding site accessible, a point that was also observed, although with a lower efficiency, with mCD4-P3Y. These data indicate that while mCD4-P3Y bound to gp120, the unsulfated peptide did not sufficiently interact with the newly available surface to block mAb 17b recognition. In contrast, when mCD4-$P3YSO_3$ was used instead of mCD4 or mCD4-P3Y, the mAb 17b was no longer able to interact with the complex. Altogether, these data thus support the view that mCD4 first binds to gp120 and exposes the coreceptor binding site, with which the $P3YSO_3$ moiety then interacts strongly enough to prevent antibody binding (Figures 3G and 3H). Finally, using the direct gp120-coreceptor interaction assay described in Figure 1, we also demonstrated that mCD4-$P3YSO_3$ very potently inhibits gp120 binding to both CCR5 and CXCR4 (Figures 3I and 3J). This suggests that this compound could be a coreceptor independent HIV-1 entry inhibitor.

mCD4 Linked $P3YSO_3$ Peptides Display Strong Antiviral Activity

Having characterized the binding mechanism of these compounds, we investigated whether these anionic peptides, either conjugated or not to mCD4 displayed anti-HIV-1 activity. This was performed using an assay in which viral replication was measured (reverse transcriptase quantification) in the supernatant of blasted peripheral blood mononuclear cells (PBMCs) isolated from three to four donors and infected by either of the HIV-1 reference strains R5 (Ba-L) or X4 (LAI). When used alone, none of the peptides demonstrated antiviral activity at the highest concentration tested (500 nM; data not shown). However, when conjugated to mCD4, they displayed inhibitory activity against the LAI strain, with effective doses giving 50% inhibition (ED_{50}) as low as 0.5 nM for mCD4-$P3YSO_3$, which compares well to 1.4 nM for mCD4-HS_{12}. Consistently with the biochemical data, the importance of the sulfate groups was shown by the large increase of ED_{50} (98 nM) that characterized mCD4-P3Y, whereas the other anionic peptides (mCD4-P3pF, mCD4-P3Asu, and mCD4-E13) displayed 8.2–30 nM ED_{50} (Figure 4A). The Ba-L strain was also very strongly inhibited by mCD4-$P3YSO_3$, with an ED_{50} of 1.3 nM versus 18 nM for mCD4-HS_{12}. None of the other conjugates displayed significant antiviral activity (Figure 4B). AZT, used as a reference anti-HIV molecule in the same assay returned ED_{50} of 8.7 and 11 nM for R5 and X4 viruses, respectively (Figures 4A and 4B).

We also observed that mCD4-$P3YSO_3$ does not need to be preincubated with the virus to be active. Indeed, addition of the molecule either to the cells prior to the viral challenge or to the virus prior to the cell infection return identical results (Table S2). This is consistent with the high affinity this molecule displays for the viral envelope, presumably enabling a fast binding to its target, and also suggests a potential use of this kind of compounds as a microbicide, a condition in which inhibitors are present within the host tissues, before viral infection.

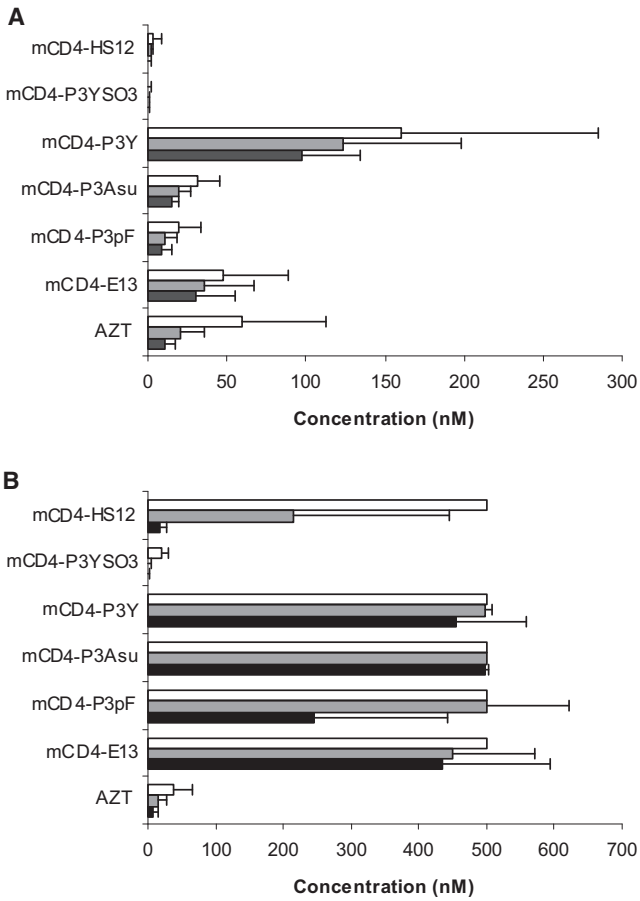


Figure 4. Antiviral Activity of mCD4 Linked to Either HS_{12} or $S(XDXS)_3$ HS Mimetic Peptides

PHA-P-activated PBMCs were infected with either (A) LAI (X4 tropic) or (B) Ba-L (R5 tropic) HIV-1 strains, preincubated with each of the drugs under investigation (1:5 dilutions between 500 nM and 320 pM). Molecules and viruses were maintained throughout the culture, and cell supernatants were collected at day 7 postinfection. Reverse transcriptase activity was quantified from which 50 (black), 70 (gray), and 90% (white) effective doses (ED) were calculated. In the absence of the inhibitory compounds, the RT level was in the range of 10,000–25,000 and 6,500–10,000 pg/ml (depending on the donor) for LAI and Ba-L strains respectively. Data are represented as mean of triplicate experiments (±SEM) performed on PBMCs from three to four donors. See also Table S2.

Having established that mCD4-$P3YSO_3$ displayed very strong antiviral activity against LAI and Ba-L HIV-1 strains, used as model systems, we extended our investigations to using a series of more clinically relevant primary strains, including 92UG029, SF162, 92US723, 96USHIPS4, 92HT599, and 98IN017. As shown in Table 1, mCD4-$P3YSO_3$ displayed a high level of antiviral activity, characterized by ED_{50} in the range of 0.2–1.2 nM for five of them and 29 nM for HIV-1 98IN017. As for the LAI and Ba-L strains, the mCD4 or $P3YSO_3$ were only poorly active or inactive, further supporting the very strong synergistic effect induced by the coupling strategy. None of the molecules showed cytotoxicity at up to 1 μM (data not shown).

Table 1. Anti-HIV-1 activity of AZT, mCD4-P3YSO$_3$, P3YSO$_3$, and mCD4 against Clinical HIV-1 Isolates

Viral Strain Clade-Tropism		92UG029 A-X4	SF162 B-R5	92US723 B-R5/X4	96USHIPS4 B-R5/X4	92HT599 B-X4	98IN017 C-X4
AZT	ED$_{50}$	7 ± 0	8 ± 7	8 ± 0.1	19 ± 9	9 ± 4	8 ± 3
	ED$_{70}$	16 ± 3	13 ± 8	17 ± 1	27 ± 11	22 ± 5	19 ± 5
	ED$_{90}$	61 ± 17	31 ± 3	59 ± 19	56 ± 15	110 ± 13	108 ± 25
mCD4-P3YSO$_3$	ED$_{50}$	0.2 ± 0.0	0.3 ± 0.2	0.3 ± 0.1	1.2 ± 1	0.5 ± 0.2	29 ± 18
	ED$_{70}$	0.3 ± 0.1	0.4 ± 0.3	0.35 ± 0.2	1.6 ± 1.2	1.3 ± 0.9	147 ± 9
	ED$_{90}$	0.8 ± 0.3	0.9 ± 0.2	0.45 ± 0.2	3 ± 1.4	3.5 ± 0.0	>500
P3YSO$_3$	ED$_{50}$	>500	>500	>500	>500	>500	>500
	ED$_{70}$	>500	>500	>500	>500	>500	>500
	ED$_{90}$	>500	>500	>500	>500	>500	>500
mCD4	ED$_{50}$	403 ± 76	245 ± 155	23 ± 1	>500	355 ± 155	>500
	ED$_{70}$	>500	352 ± 105	34 ± 10	>500	>500	>500
	ED$_{90}$	>500	>500	52 ± 22	>500	>500	>500

The table shows the effective dose (ED, mean of triplicate determination), in nM (±SD) required to inhibit 50%, 70%, and 90% of HIV-1 replication.

DISCUSSION

Targeting gp120 for HIV-1 inhibition is both attractive (because the protein engages multiple interactions key to viral entry, thus offering multiple sites for inhibition) and challenging (in the entry complex, the buried surface to block comprises both the gp120-CD4 and gp120-coreceptor interfaces). Although protein-protein interfaces are often relatively featureless and devoid of traditional cavities into which a small molecule can dock, the realization that the gp120 coreceptor binding site displays a restricted number of functionally important basic residues has very recently attracted the attention of many studies. Many of them reported that anionic molecules target the CD4i epitope, as shown by their ability to competitively inhibit mAb 17b binding with IC$_{50}$ in the 1–100 μM range (Acharya et al., 2011; Brower et al., 2009; Cohen et al., 2008; Cormier et al., 2000; Crublet et al., 2008; Dervillez et al., 2010; Farzan et al., 2000; Kwong et al., 2011; Seitz et al., 2010). HS belongs to this class of CD4i domain targeting molecules (Crublet et al., 2008), and a highly sulfated and regular sequence comprising 12 monosaccharide units has been recently prepared. Conjugated to mCD4, it displays strong anti-HIV-1 activity (Baleux et al., 2009). However, HS is extraordinary complex and heterogeneous in sequence (Esko and Lindahl, 2001). Based on the 48 different units that the polymer theoretically comprises, a 12 mer library would reach 10^{10} molecules. Although the reality is less (all the combinations are not possible), it remains much more than can be realistically synthesized for structure-activity relationship studies. Thus, to further develop this kind of molecule we attempted to design HS mimetic peptides, with the general sequence S(XDXS)$_3$ and showed that, when X was a sulfotyrosine, it binds to the CD4i epitope, blocking mAb 17b with IC$_{50}$ of 3 μM, thus comparing very well with the above-mentioned molecules. Interestingly, this peptide interacts equally well with R5 and X4 gp120, whereas HS especially binds to the X4 envelope (Figures 3E and 3F). More importantly, the conjugation of this peptide to mCD4 dramatically enhances its binding activity, the conjugated molecule being able to fully prevent the gp120/mAb 17b interaction at low nM concentration, showing that the covalent linkage induced a strong synergistic effect. This is consistent with the view that high-affinity mCD4 binding takes place initially, inducing the exposure of the mAb 17b epitope to which the sulfated peptide can then bind. As such, this molecule is distinct from other mAb 17b blocking peptides that suppress CD4 binding and subsequent coreceptor binding site exposure through an allosteric inhibitory effect rather than competitive inhibition (Biorn et al., 2004).

Although widely used as a CCR5 or CXCR4 surrogate, mAb 17b, however, only imperfectly defines the gp120 coreceptor binding site which, in addition to the CD4 induced bridging sheet, is also constituted by the V3 loop in particular (Dragic, 2001). Thus, to better assess the blocking efficiency of molecules targeting the gp120-coreceptor interaction, and taking into account domains outside the CD4i epitope itself, CCR5 and CXCR4 were solubilized and functionally captured on top of biacore sensorchips. Binding of gp120 to CCR5 and CXCR4 proved to be both CD4 and concentration dependent and inhibited by specific antagonists. Fitting of the binding data was expectedly complicated by several parameters, such as the complexity of the buffer system used, the reversible nature of both the 1D4-coreceptor and mCD4-gp120 complexes and the conformational flexibility of gp120, thus the calculated affinity values reported should probably be considered as estimates only. Nevertheless, we report K$_D$s of 10 and 150 nM for the YU2-CCR5 and MN-CXCR4 interactions respectively, comparable to those obtained with cellular systems in which the coreceptors remained in their natural cell membrane environment (Babcock et al., 2001; Doranz et al., 1999). This assay provides a useful, label-free method, to identify both binding capacity of envelopes and inhibitory activity of potential drugs. This was especially true in the framework of this study investigating sulfated/polyanionic compounds to target the gp120 coreceptor binding site. Although tyrosine sulfation of coreceptors has been shown to play a less significant role in CXCR4- than in CCR5-dependent HIV-1 entry (Farzan et al., 2002), we found that when conjugated to mCD4 the sulfated P3YSO$_3$ displays very strong binding activity toward both R5- and X4- gp120. Using this assay, we indeed report that gp120 binding to both CCR5 and CXCR4

was fully inhibited by 1:1 stoichiometric condition of mCD4-P3YSO$_3$. The overall positive charge of the V3 loop, which is much higher in X4- than in R5-gp120 (Moulard et al., 2000) strongly influences the electrostatic potential of the coreceptor binding region of the protein. In the case of CXCR4-using viruses, electrostatic interactions between the sulfated peptide and the V3 loop may thus also participate in the blocking mechanism. This view is consistent with the fact that the V3 loop (which importantly contributes to coreceptor binding) is located close to the CD4i bridging sheet and with its known capacity to interact with polyanions (Moulard et al., 2000). This is further supported by the observation that all the anionic peptides prepared during the course of this study (mCD4-P3Asu, mCD4-P3pF, mCD4-E13, and mCD4-P3Y) also display some level of antiviral activity against X4- but not against R5- viruses. This also suggests that, in engineering such compounds, it should be advantageous to use sulfated peptides with only modest specificity so that they can broadly target distinct envelopes, the high specificity of the conjugated bivalent compound being brought by the mCD4 moiety. Structural studies of mCD4-P3YSO$_3$, in complex with different gp120 would be interesting approaches to further define these aspects. In this regard, it can be noted that sulfated peptides would represent an advantage over HS, the crystallography of which, in complex with proteins appearing to be specially challenging (Imberty et al., 2007).

Although relatively limited in molecular mass (5,500 Da) the mCD4-P3YSO$_3$ molecule has the remarkable property to target two critical and conserved regions of gp120, and thus to simultaneously block two large protein surfaces (i.e., the CD4 and the coreceptor binding site). In complete agreement with the biochemical data, it displays 1 nM ED$_{50}$ anti-HIV-1 activity, for both CXCR4 and CCR5 using model viruses in a cellular assay. Importantly, we also found that this compound had a broad neutralizing activity and was very effective against a number of HIV-1 clinical isolates, strongly suggesting that this approach deserves further investigation toward in vivo evaluation. No effective antagonistic inhibitors yet exist for CXCR4. This compound, which at 1 μM is devoid of toxicity, could be a valuable weapon against the more aggressive CXCR4-tropic HIV-1 strains or for patients featuring a mixed HIV-1 population for which CCR5 antagonist cannot be used.

SIGNIFICANCE

While very significant progress has been made in the development of anti-HIV-1 drugs, the emergence of drug-resistant viruses, the inability of current therapy to be curative, and its adverse side effects have led to an urgent need for new blocking strategies. As a target, gp120 that features the coreceptor binding site is particularly attractive. However, its cryptic nature makes it a difficult target that up to now has resisted attacks.

Here, we covalently linked a sulfotyrosine containing tridecapeptide that targets the gp120 coreceptor binding site, to a CD4 mimetic (mCD4). We showed that the mCD4, in interacting with gp120, induces conformational changes that expose the coreceptor binding site and renders it available to be blocked by the sulfated peptide. In cellular assays, this compound, which successfully targets two critical domains of gp120, displays strong antiviral activities and neutralizes HIV-1 with 1 nM IC$_{50}$.

The conjugate was much more effective than a mixture of mCD4 and tridecapeptide alone, indicating that the covalent linkage is essential to produce a synergistic effect. To our knowledge, this compound establishes a new type of inhibitor and suggests a concept by which a relatively low specific molecule (the sulfated peptide), coupled to a highly specific compound (the mCD4) can reach very high affinities for its target. Combining these two characteristics may enable the molecule to accommodate mutations that invariably characterize acquired viral resistance.

These results should have strong implications for the development of a new class of anti-HIV-1 therapy: the mCD4-conjugate simultaneously blocks the attachment and entry domains of gp120 and thus inhibits viral replication at a very early stage of the viral life cycle. Most importantly, it has the remarkable and unique property to neutralize both CCR5- and CXCR4-tropic HIV-1. This is definitively a strong advantage since HIV-1 may escape from CCR5 antagonists through selection of CXCR4-using variants.

EXPERIMENTAL PROCEDURES

Materials

A BIAcore 3000 machine, CM4 sensorchip, amine coupling kit and HBS-P (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20 [pH 7.4]) were from GE-Healthcare. Streptavidin and Piperidin were from Sigma. MN and YU2 gp120 were from Immunodiagnostic. Soluble CD4, mAb 17b and Cf2Th coreceptor expressing cells were obtained through the NIH AIDS Research and Reference Reagent Program. The antibodies 12G5 and 2D7 were purchased from R&D systems and BD Pharmingen, respectively. The HIV-1 entry inhibitors AMD3100 and Maraviroc were from Fernando Arenzana (Pasteur Institute, Paris). The1D4 antibody was from Flint Box, University of British Columbia. Synthetic phospholipid blend 1,2-dioleoyl-sn-glycero-3-phosphocholine/1,2-dioleoyl-sn-glycero-3-phospho-L-serine formulation (DOPC/DOPS; 7:3, w/w), the Mini-Extruder kit, filter supports and polycarbonate filters with defined pore diameter (100 nm) were purchased from Avanti Polar Lipids. Detergents, n-dodecyl-β-D-maltopyranoside (DOM), 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate/N,N-Dimethyl-3-sulfo-N-[3-[[3α,5β,7α,12α)-3,7,12-trihydroxy-24-oxocholan-24-yl] amino]propyl]-1-propanaminium (Chaps) and Cholesteryl hemisuccinate tris salt (CHS) were purchased from Anatrace. Complete, EDTA-free protease inhibitor tablets were from Roche Diagnostics. Polyethylene glycol 8,000 50% w/v solution was purchased from Hampton research. Resins for peptide synthesis were purchased from RAPP Polymere GmbH and Fmoc AAs, HATU, NMP, DMF, and TFA were from Applied Biosystems. Fmoc-Tyr (SO3.NnBu4)-OH and Fmoc-γ-Aminobutyric-OH (γ-Abu) were from Novabiochem, (S)-Fmoc-2-amino-octanedioc acid-8-ter-butyl ester (Asu) from Polypeptides, and Fmoc -L-4 (O-tButylcarboxymethyl)-Phe-OH (pF) from Anaspec. HPLC grade triethylamine acetate buffer was from GlenResearch. *N*-succinimidyl-S-acetylthiopropionate (SATP) was from Pierce.

CCR5/CXCR4 Solubilization

The human receptors CCR5 and CXCR4, featuring a C-terminal C9 tag (TETSQVAPA), were expressed in Cf2Th canine thymocyte cells as described previously (Mirzabekov et al., 1999). The CCR5 and CXCR4 solubilization protocol was adapted from a described procedure (Navratilova et al., 2005). Briefly Cf2Th.CCR5- or CXCR4-expressing cells (5–8 × 10^6) were solubilized in 1 ml buffer consisting of 100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 15% PEG 8000, protease inhibitors, CHS (0.2%), DOM (1.5%), CHAPS (1.5%), and 0.33 mM DOPC:DOPS liposomes (see detailed buffer preparation in the Supplemental Experimental Procedures). The cell suspension was sonicated (6 × 1 s pulses) and placed on a rotating wheel

at 4°C for 3 hr. The solutions containing the solubilized coreceptors were centrifuged at 14,000 rpm for 30 min at 4°C and the supernatants were either used directly in SPR analysis or stored at −80°C until further use.

Surface Plasmon Resonance-Based Binding Platform

The interactions between gp120 and its ligands (CD4, mAb 17b, CCR5, and CXCR4) were analyzed by surface plasmon resonance (SPR) technology. For that purpose, N-ethyl-N′-(diethylaminopropyl)-carbodiimide (EDC)/N-hydroxy-succimide (NHS) activated CM4 sensorchips were functionalized with either 1,200 RU of soluble CD4, 700 RU of mAb 17b, or 7,000 RU of mAb 1D4 and blocked with pH 8.5 1 M ethanolamine. The C9-tagged CCR5 or CXCR4 were captured onto the 1D4 mAb to a level of ~4,000 RU. In some cases, gp120 were also immobilized onto CM4 sensorchip. For this, MN (50 μg/ml in 5 mM maleate buffer [pH 6]) or YU2 (50 μg/ml in 10 mM acetate buffer [pH 4.8]) were injected at 5 μl/min over an EDC/NHS activated flow cell until levels of 4,500 RU was obtained. Molecules under investigation were injected over the different surfaces and the binding responses were recorded as a function of time (see Supplemental Experimental Procedures).

Peptide Synthesis and Purification

Peptides were prepared by solid-phase peptide synthesis on H-Ser(tBu)-2-ClTrt-PS-resin using Fmoc chemistry excepted for the E13 peptide which was prepared on Fmoc-Glu(tBu)-PHB-PS-resin. Fmoc-Tyr-(SO3.NnBu4)-OH was used to synthesize the sulfotyrosines containing peptide. SATP was used to introduce a protected sulfhydryl groups at the N terminus of each purified peptide, which were then conjugated in presence of hydroxylamine to a K^5 maleimide-activated mCD4, the synthesis of which has been reported elsewhere (Baleux et al., 2009) to yield the desired conjugates mCD4-P3YSO_3, mCD4-P3Y, mCD4-P3pF, mCD4-P3Asu and mCD4-E13. All compounds were purified by RP-HPLC. Analytical procedures, characterization, and quantification of these materials are described in the Supplemental Information.

Antiviral Assay

Phytohemagglutinin (PHA)-P-activated PBMCs were infected either with the reference lymphotropic HIV-1/LAI strain (Barré-Sinoussi et al., 1983) or with the reference macrophage-tropic HIV-1/Ba-L strain (Gartner et al., 1986). These viruses were amplified in vitro with PHA-P-activated PBMCs, and 50% tissue culture infectious doses ($TCID_{50}$) were calculated using Kärber's formula (Kärber, 1931). Viruses (125 $TCID_{50}$) were incubated for 30 min with five concentrations (1:5 dilutions between 500 nM and 320 pM) of each of the molecules to be tested and added to 150,000 PBMCs (moi ~0.001). Cell supernatants were collected at day 7 postinfection and stored at −20°C. In some cases, the compounds were added to the cells prior to viral challenge. Viral replication was measured by quantifying reverse transcriptase (RT) activity in the cell culture supernatants using the Lenti RT Activity Kit (Cavisi) and AZT was used as reference anti-HIV-1 molecule. In parallel, cytotoxicity was evaluated on day 7 in uninfected PHA-P-activated PBMC using a colorimetric methyl-tetrazolium salt (MTS/PMS) assay (Promega). Experiments were performed in triplicate and 50, 70 and 90% effective doses (ED) were calculated using SoftMaxPro software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.12.009.

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