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Introducing metallocene into a triazole peptide conjugate reduces its off-rate and enhances its affinity and antiviral potency for HIV-1 gp120

Hosahudya Gopi[a], **Simon Cocklin**[a], **Vanessa Pirrone**[b], **Karyn McFadden**[a], **Ferit Tuzer**[a], **Isaac Zentner**[a], **Sandya Ajith**[a], **Sabine Baxter**[a], **Navneet Jawanda**[a], **Fred C. Krebs**[b], and **Irwin M. Chaiken**[a,*]

[a]Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA 19102, USA

[b]Department of Microbiology and Immunology, and Center for Molecular Therapeutics, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, PA 19102, USA

Abstract

In this work, we identified a high affinity and potency metallocene-containing triazole peptide conjugate that suppresses the interactions of HIV-1 envelope gp120 at both its CD4 and co-receptor binding sites. The ferrocene-peptide conjugate, HNG-156, was formed by an on-resin copper-catalysed [2 + 3] cycloaddition reaction. Surface plasmon resonance interaction analysis revealed that, compared to a previously reported phenyl-containing triazole conjugate HNG-105 (105), peptide 156 had a higher direct binding affinity for several subtypes of HIV-1 gp120 due mainly to the decreased dissociation rate of the conjugate-gp120 complex. The ferrocene triazole conjugate bound to gp120 of both clade A (92UG037-08) and clade B (YU-2 and SF162) virus subtypes with nanomolar K_D in direct binding and inhibited the binding of gp120 to soluble CD4 and to antibodies that bind to HIV-1_{YU-2} gp120 at both the CD4 binding site and CD4-induced binding sites. HNG-156 showed a close-to nanomolar IC_{50} for inhibiting cell infection by HIV-1_{BaL} whole virus. The dual receptor site antagonist activity and potency of HNG-156 make it a promising viral envelope inhibitor lead for developing anti-HIV-1 treatments.

Keywords

HIV-1 gp120; entry inhibitors; peptide triazoles; surface plasmon resonance; cell infection

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS), the global epidemic caused by HIV-1 infection, has created an urgent need for new classes of antiviral agents (WHO/UNAIDS, December 2006). Entry inhibitors and membrane fusion inhibitors are gaining momentum for antiretroviral therapies, especially given their potential use in microbicide formulations as well as post-infection therapy (Veazey *et al.*, 2005; Ketas *et al.*, 2007). Currently, the development of effective HIV entry inhibitors is focused mainly on natural ligands (Munk *et al.*, 2003; Gallo *et al.*, 2006), monoclonal antibodies (Zhang *et al.*, 2003; Cardoso *et al.*,

*Correspondence to: Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA 19102, USA. ichaiken@drexelmed.edu.

2005; Zhang and Dimitrov, 2007), synthetic compounds obtained by high-throughput screening of compound libraries (Ferrer and Harrison, 1999; Lin *et al.*, 2003; Zhao *et al.*, 2005) and compounds derived by structure-guided rational design to interfere with the gp120 interaction with CD4 or co-receptor interaction (Vita *et al.*, 1999; DeMarco *et al.*, 2006). The recently introduced fusion inhibitor Enfuvirtide (Fuzeon, T-20) exemplifies the potential of entry inhibitors to provide an expanded range of treatment options for anti-HIV therapy (Moore and Doms, 2003; Tsibris and Kuritzkes, 2007).

Recently, we reported a peptide inhibitor generated from the combination of peptide and click chemistry (Gopi *et al.*, 2006). Click-derived triazoles were constructed from both aryl and alkyl acetylenes on an internally incorporated azidoproline, present at position 6, within a variant of the previously reported peptide 12p1 (RINNIPWSEAMM) (Ferrer and Harrison, 1999; Biorn *et al.*, 2004). Using this strategy, we identified a conjugated peptide HNG-105 (**105**), through reaction with phenylacetylene, that exhibited a sub-micromolar affinity to gp120 that was two orders of magnitude greater than the affinity of the initial 12p1 (Gopi *et al.*, 2006). Peptide **105** showed broad spectrum inhibition of the molecular interactions of CD4 with gp120s derived from viruses of clades A, B, C, D and CRF07_BC (Cocklin *et al.*, 2007). In addition, **105** inhibited infection of recombinant luciferase-containing viruses pseudotyped with envelopes from clades A, B and C (Cocklin *et al.*, 2007). From this starting point, we examined the structure–activity relationships between structural variation of the substituted triazole and affinity for gp120 (Gopi *et al.*, 2008). This has led us to a ferrocene-conjugated peptide derivative that has strikingly potent affinity and inhibition activity against HIV-1 gp120. We report here the characteristics of the gp120 antagonist activity of this inhibitor and in particular its ability to inhibit HIV-1 Env interactions with multiple ligands of the gp120 molecule.

MATERIALS AND METHODS

Materials

All Fmoc-protected amino acids, HBTU, HOBt, and Hyp(OMe)·HCl were purchased from Novabiochem. Rink amide resin was obtained from Applied Biosystems. Solvents and other chemicals were purchased from Aldrich or Fisher and used without further purification. Fmoc-*cis*-4-azidoproline was synthesized starting with commercially available Hyp(OMe)·HCl. HIV-1 strain BaL (catalogue no. 510) was obtained from the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID). This strain of HIV-1, which was prepared using primary human cells of monocytic origin, uses CCR5 as its co-receptor.

Peptide synthesis and click conjugation

Peptides were synthesized by manual solid phase synthesis using Fmoc chemistry on Rink amide resin at 0.1 mmol scale. The [3 + 2] cycloaddition reaction of azide and terminal alkynes was carried out by an on-resin method (Gopi *et al.*, 2006). The peptides were cleaved from the resin by using a cocktail mixture of 95:2:2:1 trifluoroacetic acid/1,2-ethanedithiol/water/thioanisole. Crude peptides were purified by using a C_4 preparative column by HPLC (Beckmann Coulter) with gradient between 95:5:0.1 and 5:95:0.1 water/acetonitrile/trifluoroacetic acid. Peptide purity and mass were confirmed by using a C_{18} analytical column (HPLC) and by MALDI-TOF, respectively.

Protein reagents

HIV-1_{YU2} gp120 was produced as described previously in Drosophila S2 cells (Biorn *et al.*, 2004; Pancera *et al.*, 2005). Cells were spun down and supernatant sterile filtered. Supernatant was purified over an F105-antibody column (NHS-activated Sepharose, Amersham; mAb F105 coupled according to manufacturer's instructions). HIV-1_{YU2} was

eluted from the column with glycine buffer, pH 2.4, dialysed against PBS and frozen at −80°C. sCD4 was expressed in CHO cells in a hollow fibre bioreactor. Supernatant from the hollow fibre bioreactor was purified with an SP-column and bound fractions were then run over a Q-column. Unbound material was concentrated and analysed by SDS-PAGE. The gp120 proteins from HIV-1_{SF162} and HIV-$1_{92UG037-08}$ were used in previous inhibitor binding studies (Cocklin *et al.*, 2007); HIV-1_{SF162} gp120 was obtained through NIH AIDS Research and Reference Reagent Program from DAIDS and NIAID, while HIV-$1_{92UG037-08}$ gp120 was a gift from Dr James Arthos as reported in Cocklin *et al.* (2007). The following monoclonal antibodies were obtained through the NIH AIDS Research and Reference Reagent Program: 2G12 from Dr Hermann Katinger; F105 from Dr Marshall Posner and Dr Lisa Cavacini and b12 from Dr Dennis Burton and Carlos Barbas.

Optical biosensor binding assays

All surface plasmon resonance (SPR) experiments were performed on a Biacore 3000 optical biosensor (Biacore, Inc., Uppsala, Sweden). A CM5 sensor chip was derivatized by amine coupling by using *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxy-succinimide (Ishino *et al.*, 2006) with either HIV-1_{YU2} gp120, soluble CD4, mAb 17b Fab, or as a control surface mAb 2B6R (antibody to human IL-5 receptor α). For direct binding experiments, HIV-1_{YU2} gp120 was immobilized on the surface (~4000 RU); peptide analytes in PBS buffer were passed over the surface at a flow rate of 50 μL/min. with 5 min. association phase and 5 min dissociation phase. The gp120 proteins from HIV-1_{SF162} and HIV-$1_{92UG037-08}$ were immobilized on the sensor chip surface and direct binding analyses carried out, similarly as described above for HIV-1_{YU2} gp120. For competition experiments, ligands (sCD4, 17b mAb, b12, and F105) were immobilized on a surface with a density of approximately 2000 RU. The indicated analytes were passed over the surfaces at a flow rate of 50 μL/min with 2.5 min association phase and 2.5 min dissociation phase. Surfaces were regenerated by using 35 mM NaOH and 1.3 M NaCl for sCD4 and HIV-1_{YU2} gp120 surfaces, and 10 mM HCl for 17b surface.

Data analysis was performed using BIAEvaluation® 4.0 software (GE Healthcare, Uppsala, Sweden). The responses of a buffer injection and responses from the control surface to which the mAb 2B6R was immobilized, were subtracted to account for nonspecific binding. Experimental data were fitted to a simple 1:1 Langmuir binding model with a parameter included for mass transport. The average kinetic parameters (association [k_a] and dissociation [k_d] rates) generated from a minimum of four datasets were used to define equilibrium association (K_A) and dissociation constants (K_D).

Inhibition of HIV-1 infection using whole virus assay

P4-CCR5 MAGI cells (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID) were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS), sodium bicarbonate (0.05%), antibiotics (penicillin, streptomycin and kanamycin, 40 μg/mL each), and puromycin (1 μg/mL) (Charneau *et al.*, 1994). P4-CCR5 cells were seeded at a density of 1.2×10^4 cells/well in a 96-well plate approximately 18 h prior to experiment. The cells were then incubated for 2 h with HIV-1_{BaL} (2.4 ng/mL final concentration) in the presence of HNG-156 (**156**), or dextran sulphate as a positive control. After the 2 h incubation, cells were washed, cultured for an additional 46 h, and subsequently assayed for HIV-1 infection using the Galacto-Star®-Galactosidase Reporter Gene Assay System for Mammalian Cells as per manufacturer's instructions (Applied Biosystems, Bedford, MA). Infectivity remaining is expressed relative to mock-treated, HIV-1-infected cells. Data were fit to a sigmoidal inhibition model using Prism GraphPad software to yield values for IC_{50}, the concentration at which exposure to the compound resulted in a 50% decrease in infectivity relative to mock-treated, HIV-1-

infected cells. The above assay design was similar to that used in prior studies (Krebs *et al.*, 1999).

In vitro cytotoxicity

P4-CCR5 cells were seeded at a density of 4×10^4 cells/well in a 96 well plate approximately 18 h prior to experiment. Cells were then exposed to the indicated concentrations of **156** and dextran sulphate for 2 h. The cells were subsequently washed and assessed for viability using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay of viability (previously described in Krebs *et al.* (1999). Concentrations were tested in triplicate in two independent assays.

RESULTS AND DISCUSSION

Identification of the high affinity HIV-1 gp120 synthetic peptide inhibitor HNG-156

A Biacore® 3000 SPR optical biosensor was used to assess both the direct interactions of peptide conjugates with various subtypes of gp120 and their inhibitory effects on interactions of gp120 at its CD4 and co-receptor binding sites (Zhang *et al.*, 1999; Dowd *et al.*, 2002; Biorn *et al.*, 2004; Cocklin *et al.*, 2007). We evaluated synthetic conjugate variants of the recently reported **105**, focusing on more bulky hydrophobic groups to replace the phenyl group substituent on the triazole (Gopi *et al.*, 2008). The binding and dual inhibition activities of the peptide conjugate with the bulky ferrocene triazole (Figure 1), herein denoted **156**, were improved strongly over those of **105**. To test for conjugate binding, gp120 was covalently immobilized on a CM5 biosensor chip (5000 RU) using standard amine coupling. Interactions were measured by injecting various concentrations of **156** over the immobilized gp120 sensor chip in PBS buffer. **156** peptide was found to bind to a number of HIV-1 gp120s including those from several subtypes. The most striking improvement in affinity observed to date was for the gp120 derived from the clade A virus HIV-1 $_{92UG037-08}$. Here, the K_D was approximately two orders of magnitude lower (greater affinity) than that for **105**. Representative sensorgrams depicting the direct binding of **156** to HIV-$1_{92UG037-08}$ gp120 binding are shown in Figure 2 (top left). Shown in Figure 2 (top right) is the comparison of the off-rate kinetic analysis for binding of both **156** and **105** to HIV-1 $_{92UG037-08}$ gp120. Similar data are shown in Figure 2 (bottom) for HIV-1_{SF162} gp120. SPR-derived kinetic interaction parameters, determined for the set of HIV-1 gp120 variants tested, are given in Table 1. The SPR binding data reveal that the high binding affinity of **156** versus **105** is due largely to a slower off-rate. This was observed with several subtypes of HIV-1 gp120. Interestingly, a recent study correlating the kinetics of SIV gp120 interaction to monoclonal antibodies with neutralization efficiencies found that a slower dissociation rate correlated with enhanced neutralization capacity of a given mAb in viral assays (Steckbeck *et al.*, 2005). As such, improving this feature both in this derivative, and future peptide conjugates and their truncates, could be advantageous for antagonist design.

Suppression of binding at multiple interaction sites of HIV-1 gp120 by HNG-156

Competition SPR analysis was used to measure the inhibitory effects of **156** on the binding of gp120 to CD4, to the gp120 CD4-binding site (CD4bs) monoclonal antibodies (Yuan *et al.*, 2006) F105 and IgG b12, and to the CD4-induced (CD4i) mAb 17b (Kwong *et al.*, 1998), which shares its epitope with the co-receptor binding site of gp120. The analyte HIV-1_{YU2} gp120 (100 nM) was passed over the immobilized protein ligands sCD4, mAb 17b, mAb b12, mAb F105 and control mAb 2B6R in the absence or presence of increasing amounts of **156**. In control experiments, **156** exhibited no direct binding to any of the immobilized protein ligands (data not shown). Figure 3 shows that increasing the concentration of **156** suppressed gp120 binding to all of the protein ligands tested that

recognize receptor and co-receptor sites. In contrast, **156** did not show any effect on the binding of another broadly neutralizing antibody, 2G12 (Trkola *et al.*, 1996), which binds to a discontinuous epitope involving glycosylation sites in the envelope protein (data not shown).

Inhibition of HIV-1 cell infection by whole virus

In vitro experiments were conducted to measure the anti-HIV-1 activity of **156**. This assay was carried out using the subtype B strain HIV-1_{BaL} (R5 phenotype), as this was the strain used previously to measure antiviral activity for the parent peptide 12p1 and derivatives (McFadden *et al.*, 2007; Gopi *et al.*, 2008). We found (Figure 4) that **156** exhibited an IC_{50} 96 nM. This potency was close to three orders of magnitude more than that (48 μM) measured previously for 12p1 (Gopi *et al.*, 2008; McFadden *et al.*, 2007) and 15-fold more than that (1.43 μM) measured for **105** (Gopi *et al.*, 2008). Importantly, **156** had no effect on P4-CCR5 cell viability when assessed at concentrations as high as 0.1 mg/mL (59 μM). The strong potency in the HIV-1_{BaL} infection assay argues for follow-up studies, currently under way, to evaluate the breadth of antiviral potency with both whole virus and pseudoviral cell infection assays.

Implications of improved interaction kinetics for HIV-1 gp120 targeted drug discovery and affinity capture

Results with the new metallocene-conjugated peptide **156** argue for its potential usefulness as a starting point to develop entry inhibitor leads for AIDS treatments. This is suggested by its low nM affinity for HIV-1gp120 and substantial potency in inhibiting cell infection. Furthermore, the binding data reported here hint at the possibility that HNG-156 may have a broad HIV-1 subtype specificity, a property that is currently being evaluated. The presence of the triazole-indole side chain cluster at positions 6 and 7 suggests the potential to focus on this locus in developing smaller molecular weight derivatives. While the structural nature for the latter cannot be predicted at present, it could include the triazole grouping itself, which is chemically stable (Rostovtsev *et al.*, 2002), or other structural elements that recapitulate the specific binding properties of the triazole grouping. The binding kinetics of **156**, taken with the multi-clade specificity of this peptide, also suggest the potential to utilize this peptide conjugate as a molecular tool for developing broad-specificity affinity capture ligands for HIV-1 gp120. The slow dissociation rate of the gp120–**156** noncovalent complex suggests that immobilized forms of **156** could retain gp120 strongly. We have recently found (unpublished results) that we can form the peptide HNG-105C, containing the **105** sequence with the C-terminal extension Gly-Gly-γOrn-Cys(SH)-CONH2. The HNG-105C derivative, when covalently attached via the Cys-SH to an SPR sensor chip surface, retained high binding affinity for gp120 analyte. This preliminary result opens up the possibility to use the homologous HNG-156C as a low-cost immobilized ligand in chromatographic and other affinity surfaces for separation and sensing of HIV-1 envelope protein and envelope complexes.

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Abbreviations used

12p1	12-residue peptide #1

CD4	cluster of differentiation-4 (receptor on leukocytes)
CHO	Chinese hamster ovary
CM	carboxy-methyl
CRF07_BC	circulating subtype B-C recombinant form of HIV-1
Env	envelope protein
Fab	fragment antigen binding
Fmoc$^-$	9-fluorenylmethyl-oxycarbonyl
gp120	glycoprotein 120
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HIV-1	human immunodeficiency virus type 1
HNG-156	the peptide Hosahudya N Gopi −156
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
Hyp	trans-4R-hydroxy-L-proline
IC_{50}	50% inhibitory concentration
IL-5	interleukin 5
mAb	monoclonal antibody
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
NHS	N-hydroxy-succinimde
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
PBS	phosphate buffer saline
Q	quaternary amino
RU	response units
sCD4	soluble CD4
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIV	simian immunodeficiency virus
SP	sulphopropyl
SPR	surface plasmon resonance

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HNG-105: H-Arg-Ile-Asn-Asn-Ile-X_1-Trp-Ser-Glu-Ala-Met-Met-CO-NH_2

HNG-156: H-Arg-Ile-Asn-Asn-Ile-X_2-Trp-Ser-Glu-Ala-Met-Met-CO-NH_2

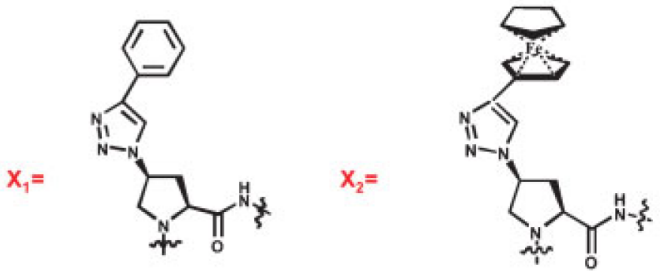

Figure 1.

Sequences of conjugate peptides **105** and **156**. X_1 = (2S, 4S)-4-(4-phenyl-1H-1, 2, 3-triazol-1-yl) pyrrolidine-2-carboxylic acid and X_2 = (2S, 4S)-4-(4-ferrocenyl-1H-1, 2, 3-triazol-1-yl) pyrrolidine-2-carboxylic acid. The general procedures for synthesis of triazole conjugates of 12p1 have been reported (Gopi *et al.*, 2006, 2008). The specific synthesis protocol for **156** is given in the text.

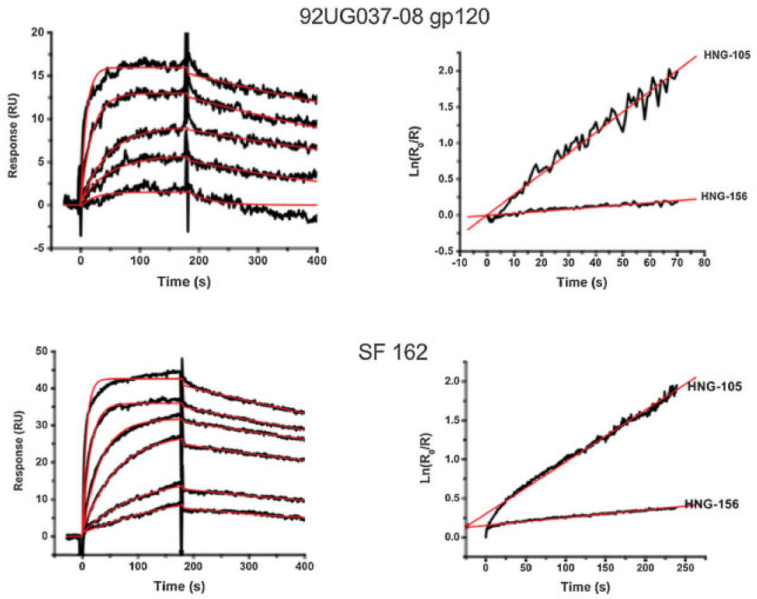

Figure 2.

Direct binding of **156** to surface-immobilized HIV-$1_{92UG037-08}$ gp120 (top) and HIV-1_{SF162} gp120 (bottom). Left: Sensorgrams depicting the interaction of **156** with gp120 at **156** concentrations of 0.125, 0.25, 0.5, 1, 2, and 4 μM. Black lines indicate experimental data, whereas red lines indicate fitting to a 1:1 Langmuir binding model with a parameter included for mass transport. Right: comparison of the linearized dissociation rates of **105** and **156**.

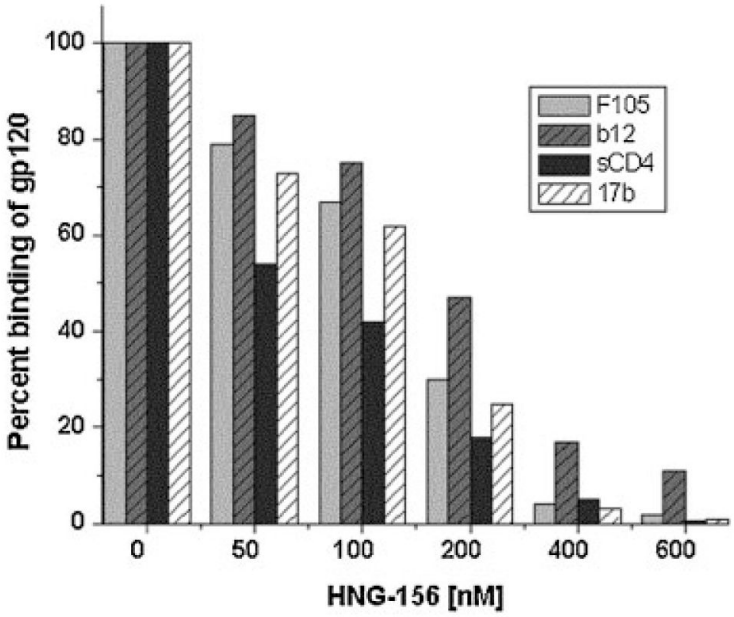

Figure 3.

Inhibition by **156** of the binding of HIV-1_{YU2} gp120 to CD4bs and CD4i antibodies and to soluble CD4. mAb F105, IgG b12, sCD4 and mAb 17b were immobilized on the surface of biosensor CM5 chip. A constant concentration of HIV-1_{YU2} gp120 was passed over these immobilized ligands in the presence of increasing concentrations (0–600 nM) of **156**. The per cent (%) binding of gp120 to immobilized antibodies is plotted against the concentration of the peptide. **156** inhibited the binding of HIV-1_{YU2} gp120 to F105, b12, CD4 and 17b at IC_{50} values of 131 (±30), 200 (±42), 94 (±38) and 137 (±39) nM, respectively.

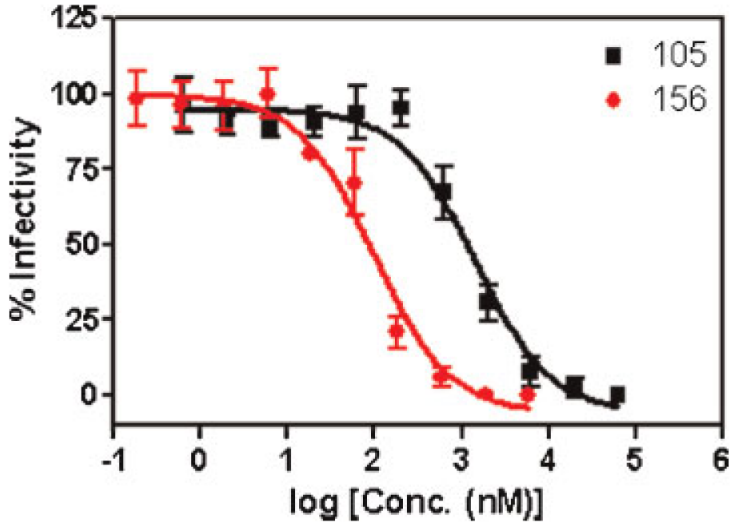

Figure 4.

Analysis of activity of **156** in inhibiting infection of P4-CCR5 cells by HIV-1_{BaL} whole virus. The data points for **156** and **105** were fit to a simple sigmoidal inhibition model using Prism GraphPad software to derive the best-fit lines (solid lines) and IC_{50} values. **156** IC_{50} = 96 ±0.1 nM; **105** IC_{50} = 1430 ±100 nM.

Table 1

Kinetic parameters derived by SPR for the interactions of **156** to different gp120s determined by direct interaction SPR analysis, with comparison to those results obtained for **105**

Peptide	HIV-1 Env	k_a(1/Ms)	k_d (1/s)	K_D (M × 10^{-9})
12p1	YU2 (B)	1.36×10^{4}	0.072	5400
105	YU2 (B)	$3.39\pm0.22\text{x } 10^{5}$	$7.79\pm3.2\times10^{-3}$	22.9
156	YU2(B)	$1.29\pm0.2\times10^{5}$	$9.55\pm0.15\times10^{-4}$	7. 4
105	92UG037-08 (A)	$1.63\pm0.8\times10^{4}$	$3.0\pm2\times10^{-2}$	1840
156	92UG037-08 (A)	$1.06\pm0.2\text{x}10^{5}$	$1.73\pm0.8\times10^{-3}$	16.3
105	SF162 (B)	$1.05\pm0.3\text{x}10^{5}$	$7.4\pm0.18\times10^{-3}$	70.5
156	SF162(B)	$1.22\pm0.3\times10^{5}$	$1.1\pm0.4\times10^{-3}$	9.0

These data are taken from Gopi *et al.* (2008). Designations in parentheses are clades of HIV-1 from which gp120 subtypes were derived. The peptide denoted “12p1” is the parent peptide (RINNIPWSEAMM) (Ferrer and Harrison 1999; Biorn *et al.*, 2004), from which peptides HNG-105 and HNG-156 were derived using the click conjugation.