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Chemistry & Drug Discovery

# Supporting Information

# Click chemistry on azidoproline: High affinity dual antagonist for HIV-1 envelope glycoprotein gp120.

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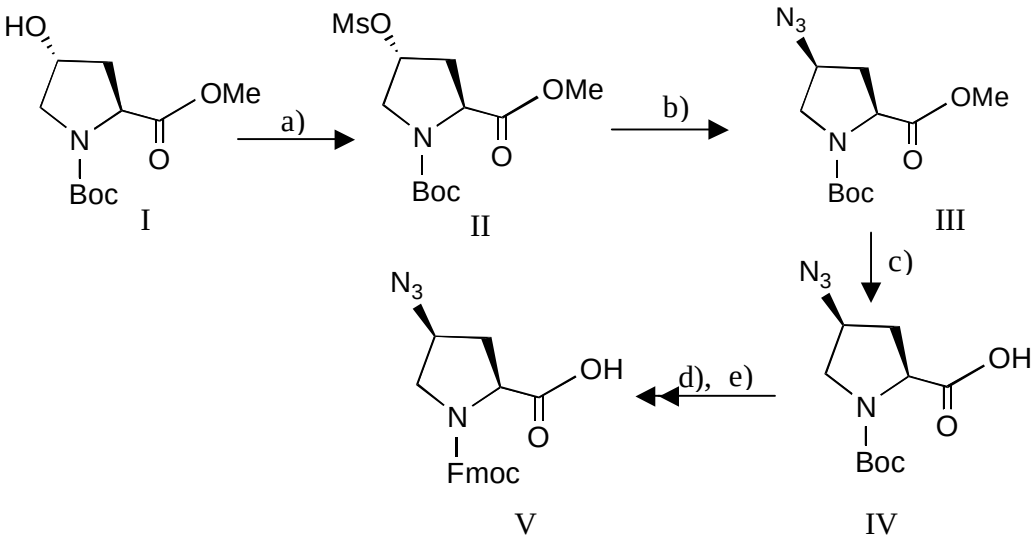
## Supporting Information

**Materials:** All Fmoc-protected amino acids, HBTU, HOBt and Hyp(OMe).HCl were purchased from Novabiochem. Fmoc- PAL-PEG-PS resin was obtained from AppliedBiosystem. Solvents and other chemicals were purchased from Aldrich or Fisher and used without further purification. Peptides were synthesized on an automated peptide synthesizer (433A Applied Biosystem) at a 0.1 mmol scale. The peptides were cleaved from the resin by using a cocktail mixture of 95:2:2:1 trifluoroacetic acid / ethylenedithiol/ water/ thioanisole. The crude peptides were purified by using $C_{18}$ column on HPLC (Beckmann Coulter) with gradient between 95:5:0.1 and 5:95:0.1 water/acetonitrile/ trifluoroacetic acid. The purified peptides were confirmed by using MALDI-TOF and used in the surface plasmon resonance experiments (Biacore 3000). The gp120 envelope protein from the CCR5-using, primary HIV-1 strain YU2 was expressed in Drosophila *Schneider 2* (S2) cells as previously described.[1] The recombinant protein was passed through an HI-Q column to separate gp120 from the serum proteins and then affinity purified on a snow-drop lectin (*Galanthus Nivalis*) affinity column using 5mM methyl-α-D-mannopyranoside to elute the bound gp120. Human monoclonal antibody 17b was expressed in human hybridoma cells [2] grown in RPMI 1640 media supplemented with 10% fetal calf serum. The antibody was purified by affinity chromatography using a protein A-linked agarose column (LTI, Gaithersburg, MD) and eluted with 100 mM citric acid (pH 3.4). sCD4 was produced by CHO cells in a hollow fiber cartridge (Fiber cell systems) with serum-free, chemically defined medium (Hyclone). It was purified by ion exchange and gel filtration chromatography.

**Experimental:**

**Fmoc-*cis*-?-azidoproline was synthesized starting from the methyl ester of Boc-L-*trans*-?-hydroxyproline as reported in the literature with modification in steps I and III to V that provides a streamlined protocol with higher yield .**[3,4]




***Scheme 1***. *Synthesis of Fmoc-azidoproline; a) MsCl/ TEA in dichloromethane 8h at room temperature. b) $NaN_3$/ DMF at 70ºC overnight. c) 1N NaOH/ MeOH 3h. at room temperature. d) TFA/ $CH_2Cl_2$ 30 min. at 0ºC. e) Fmoc-OSu/ $Na_2CO_3$/ THF.*

**Synthesis of Boc-Hyp(OMs)-OMe**: Boc-L- *trans*-?-hydroxyproline (2.45 g, 10 mmol) was dissolved in 50 mL of dry dichloromethane, cooled to 0ºC, triethylamine (1.6 mL,12 mmol) was added followed by Methanesulfonyl chloride (0.85 mL, 11 mmol). The reaction mixture was stirred at room temperature under $N_2$ for about 8 hrs and diluted with 100 mL of dichloromethane. The reaction mixture was washed with 5% HCl, % $Na_2CO_3$ and water. After the evaporation of organic solvent, *trans*-4-mesyl derivative was separated as a solid (3.1 g, 96% yield) and used directly for the next step.

**Boc- L-cis-4-azidoproline:** *Trans*-4-mesyl proline derivative (1.61 g, 5 mmol) from the above step was dissolved in dry DMF. $NaN_3$ (1.3 g, 20 mmol) was added. The reaction mixture was stirred overnight under $N_2$. The reaction mixture was poured in to a 50 mL of water and extracted with ethyl acetate (3 x 50 mL). The organic solvent was washed with water and dried over $Na_2SO_4$. After the evaporation of ethyl acetate under reduced pressure, methyl ester of Boc- L-*cis*-4-azidoproline separated as slightly yellowish oil (1.2 g, 90%). The methyl ester was subjected to base hydrolysis.

Methyl ester of Boc- L-*cis*-4-azidoproline (1.08 g, 4 mmol) was dissolved in 10 mL of MeOH and 2 mL of 1N NaOH was added. The reaction mixture was stirred for 2hrs at room temperature and diluted with 50 mL of water. The MeOH was removed under reduced pressure and the aqueous layer extracted with ether (3x 20 mL). The aqueous layer was acidified to pH 3 by using % 5 HCl and extracted with ethyl acetate (3x50 mL). After the aqueous work-up, the organic solvent was evaporated under reduced pressure to yield 0.97 g (95%) of Boc- L-*cis*-4-azidoproline.

**Fmoc- L-cis-4-azidoproline:** Boc- L-*cis*-4-azidoproline (0.76 g, 3 mmol) was dissolved in 5 mL of dichloromethane, cooled to 0ºC. 5mL of trifluoroacetic acid was added and stirred for 30 min. The solvent was evaporated, the residue was dissolved in 50 mL of water and the pH was adjusted 8 by adding solid $Na_2CO_3$. Fmoc-OSu (1 g, 3 mmol) was dissolved in 10 mL of THF and added to the reaction mixture. After completion of the reaction, the reaction mixture was extracted with ether (3 x 50 mL). The aqueous layer was acidified to pH 2. The separated white precipitate was extracted to ethyl acetate (3 x50 mL). After the aqueous work-up and evaporation of organic solvent, the yield was 0.98 g (87%) of Fmoc- L-*cis*-4-azidoproline. A pure sample was obtained after recrystallization from ethyl acetate / n-heptane. The pure Fmoc-L-*cis*-?-azidoproline was directly used in peptide synthesis.

**[3+2] Cycloaddition reaction on resin[5]:** The resin of protected peptide **3** (0.1 mmol), with L-*cis*-4-azidoproline group, was suspended in 5 mL of acetonitrile/ water/ DIEA/ pyridine (4:4:1:0.5) mixture. Phenylacetylene (109.8 µL, 1 mmol) was added, followed by a catalytic amount of $Cu^{I}I$. The reaction was stirred overnight at room temperature; the

solution was filtered and washed with 5% HCl, an excess of DMF and dichloromethane. The peptide **5** was cleaved from the resin by using a cocktail mixture of 95:2:2:1 trifluoroacetic acid / ethylenedithiol/ water/ thioanisole and purified by HPLC using a $C_{18}$ column. The peptide was confirmed by MALDI-TOF. $Mass_{Calc}$= 1604 Da. $Mass_{Obt}$ =1603.83Da.

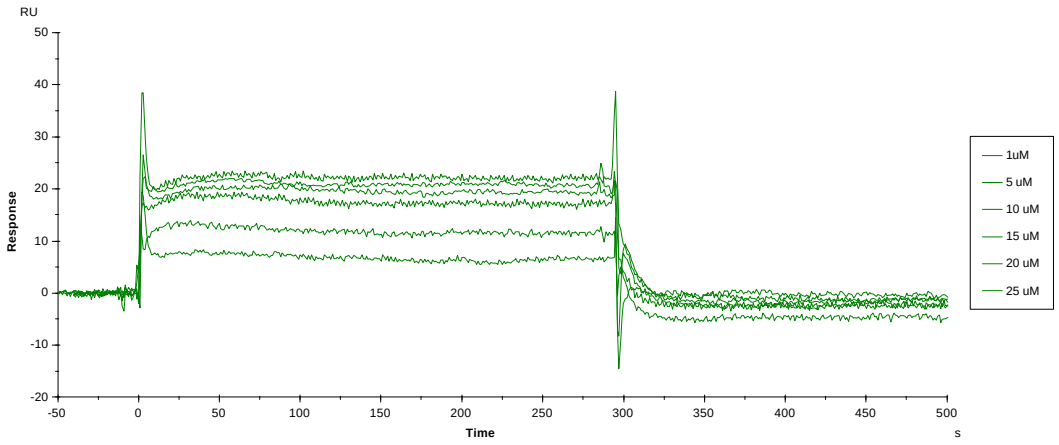
The same procedure was used in all other [3+2] cycloaddition reactions. The purified peptides were confirmed by mass using MALDI-TOF.

**Conversion of azide to amine and on resin[6]:** The resin of fully protected (including N-terminus by Boc-/ Fmoc-) peptide **3** with L-*cis*-4-azidoproline (0.1 mmol) was suspended in 5 mL of a 1:1 mixture of dioxane /water. Neat trimethylphosphine 0.2 mL (2 mmol) was added to the reaction mixture and stirred for about 30 min. The resin was washed several times with DMF and dichloromethane. The peptide was cleaved from the resin and purified. The peptide was confirmed by MALDI- TOF. $Mass_{Calc}$= 1475 Da. $Mass_{Obt}$ =1475.99 Da.

**Surface plasmon resonance (SPR) kinetics interaction analyses:** All surface plasmon resonance experiments (SPR) were performed on a BIA3000 optical biosensor (Biocore, Inc., Uppsala, Sweden). A CM5 sensor chip was derivatized by amine coupling by using EDC.HCl/ HOSu with either YU2 gp120, soluble CD4, mAb 17b Fab, or as a control surface mAb 2B6R Fab (from antibody to IL-5 receptor a). For direct binding experiments, YU2 gp120 was immobilized on the surface (5000RU); peptide analytes in PBS buffer were passed over the surface at a flow rate of 5 µL/min. with 5min. association phase and 5min. dissociation phase. For competition experiments, ligands were immobilized on a surface with a density of approximately 2000RU. 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 1.3M NaCl for sCD4 and YU2 gp120 surfaces, and 10 mM HCl for 17b surface. Buffer injections and control surface binding were subtracted in all of the experiments.

**Peptide 1 direct binding to YU2 gp120 (5000 RU)**

**(a)**




**(b)**

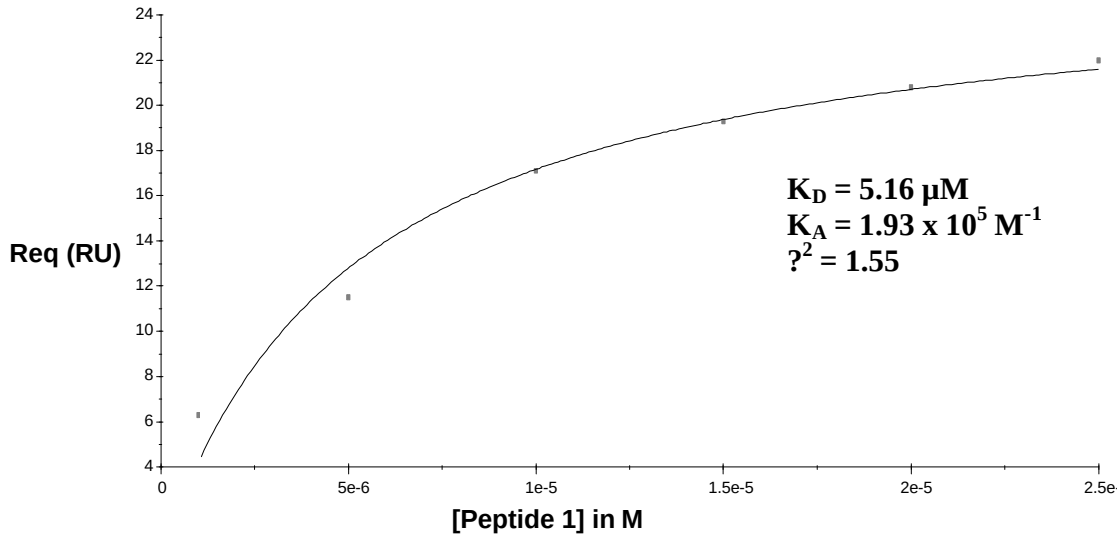
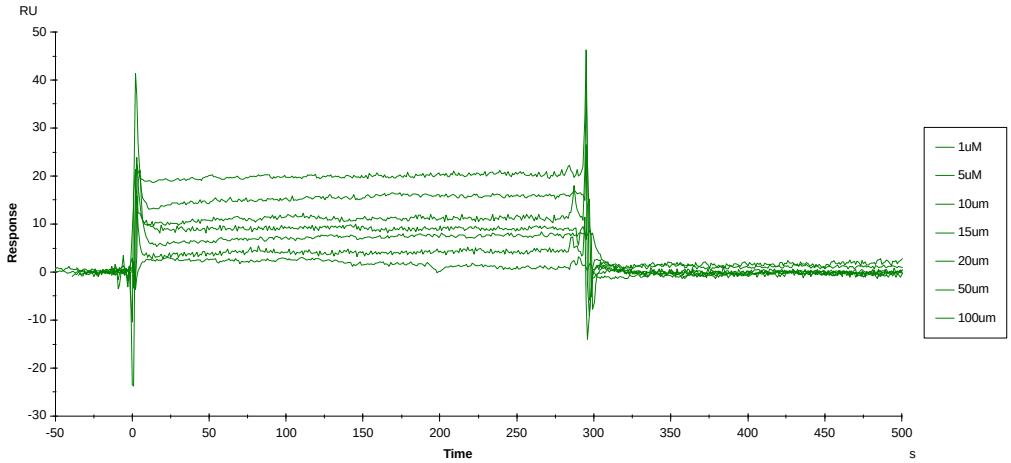



**Figure 1**. Direct binding of peptide **1** to immobilized gp120. (a) Response curves for increasing concentrations of peptide **1** binding to the immobilized gp120. (b) Fit of direct binding data to a steady state affinity 1:1 binding model. Req was calculated from 205-285s from each curve in (a) and plotted against the concentration of peptide **1**.

**Peptide 2 direct binding to YU2gp120**

**(a)**




**(b)**

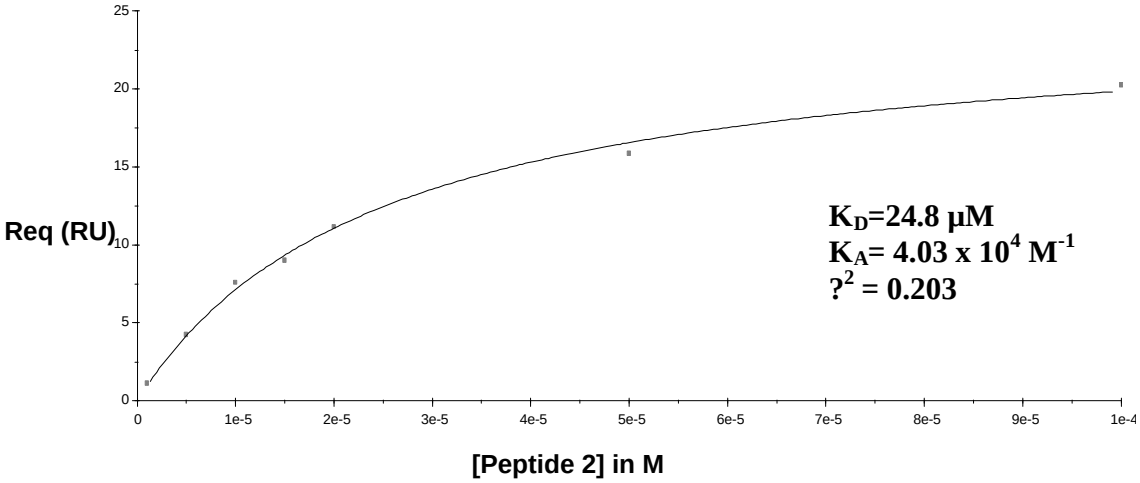



**Figure 2**. Direct binding of peptide **2** to immobilized gp120. (a) Response curves for increasing concentrations of peptide **2** binding to the immobilized gp120. (b) Fit of direct binding data to a steady state affinity 1:1 binding model. Req was calculated from 205-285s from each curve in (a) and plotted against the concentration of peptide **2**.

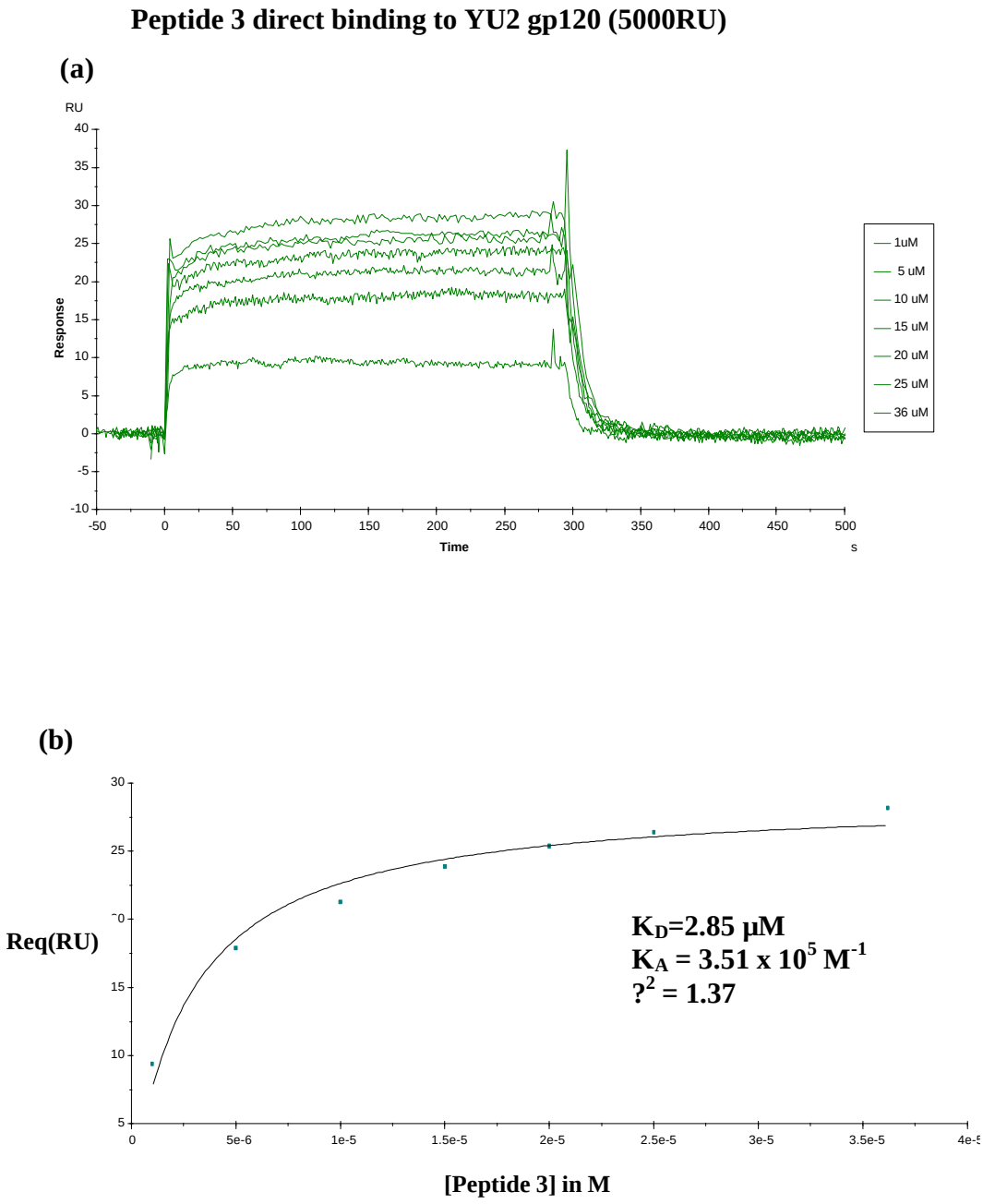



**Figure 3**. Direct binding of peptide **3** to immobilized gp120. (a) Response curves for increasing concentrations of peptide **3** binding to the immobilized gp120. (b) Fit of direct binding data to a steady state affinity 1:1 binding model. Req was calculated from 205-285s from each curve in (a) and plotted against the concentration of peptide **3**.

**Peptide 5 competition with sCD4 on immobilized YU2 gp120 with reverse orientation**

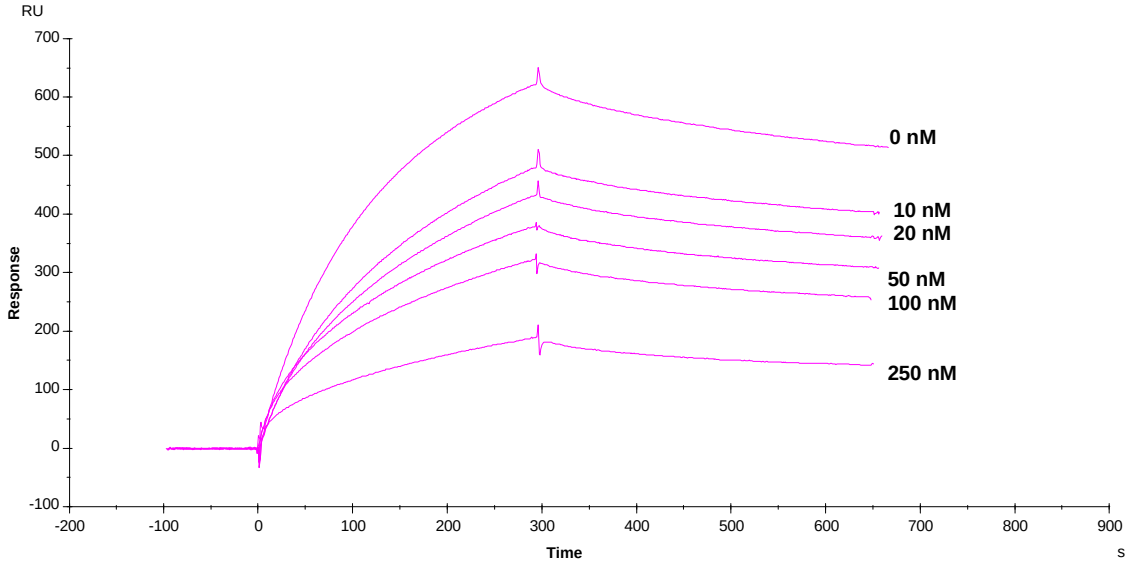
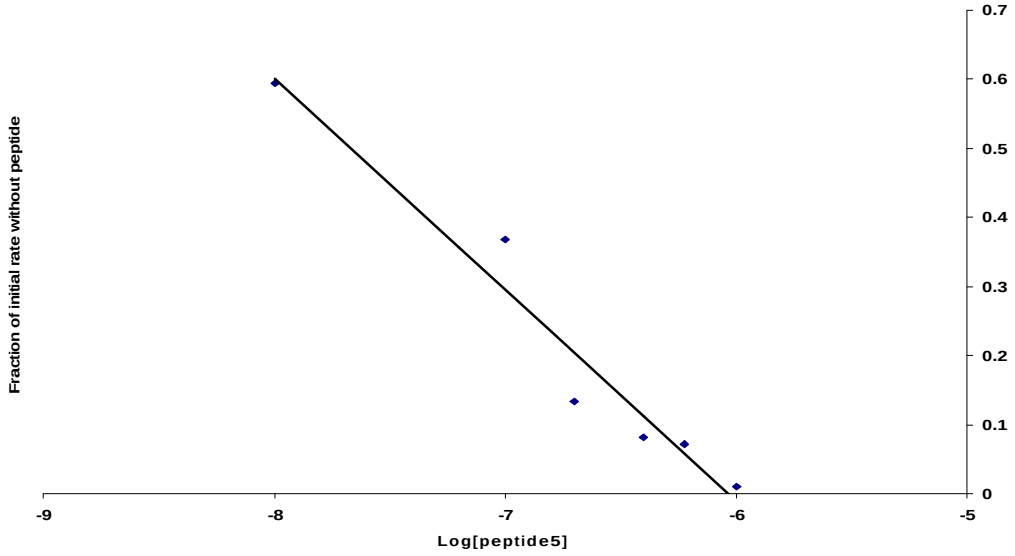



**Figure 4**: Peptide **5** competition with CD4 for binding to immobilized gp120. 50 nM of CD4 was passed over high-density YU2 gp120 surface (5000RU) at a rate of 5 µL/min. with increasing concentration of peptide **5**.

**Log plot for determining $IC_{50}$ values for peptide 5 inhibition of binding of gp120 to sCD4 and 17b.**

**(a) $IC_{50}$ value for inhibition of binding of gp120 to CD4 is 22 nM**



**(b) $IC_{50}$ value for inhibition of binding of gp120 to 17b is 29 nM**

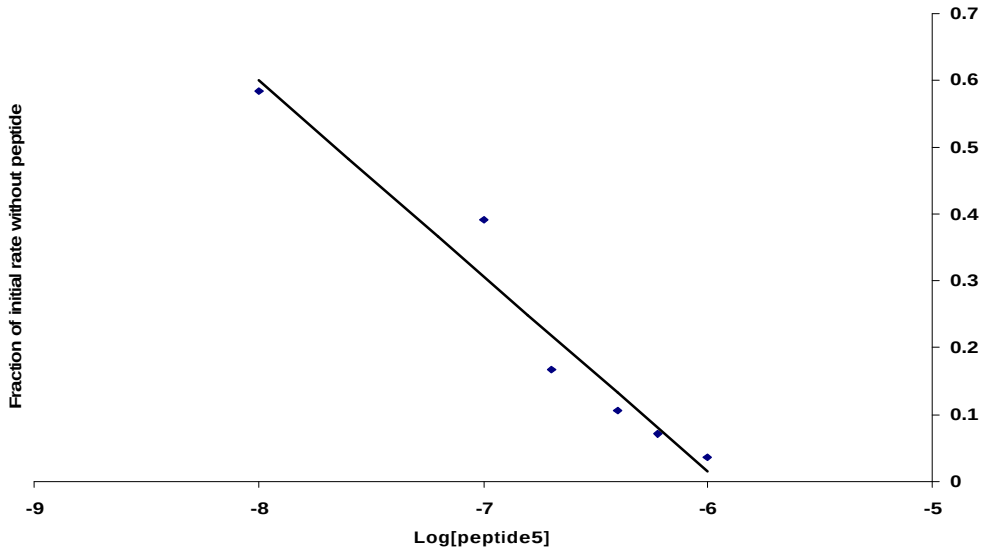



**Figure 5**: The initial rate (*v*i) of binding of gp120 to either CD4 (a) or 17b (b) in the presence or absence of peptide **5** was determined during the period of the association phase from 6 to 20s. From the slope of that line (RU/s), *v*i at each peptide concentration was calculated using Biaevaluation 3000 software. The fraction of the *v*i of gp120 binding in the presence or absence of the peptide was plotted against the log of peptide concentration. The peptide concentration at which the *v*i of gp120 binding was half of that without peptide was designated as $IC_{50}$.

**Direct binding of peptide 6 to Yu2 gp120**

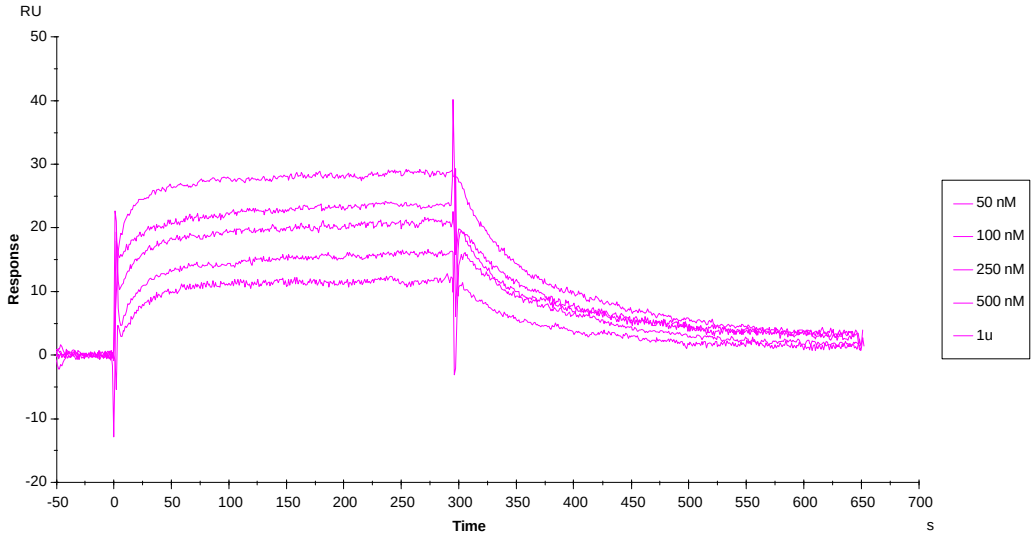



**Figure 6**. Direct binding of peptide **6** to immobilized gp120. Response curves for increasing concentrations of peptide **6** binding to the immobilized gp120. The kinetic constants $k_{(on)}$, $k_{(off)}$ and $K_D$ values are calculated from global fit (1:1 Langmuir model) of the curves by using Biaevaluation software 3000.

**Direct binding of peptide 13 to Yu2 gp120**

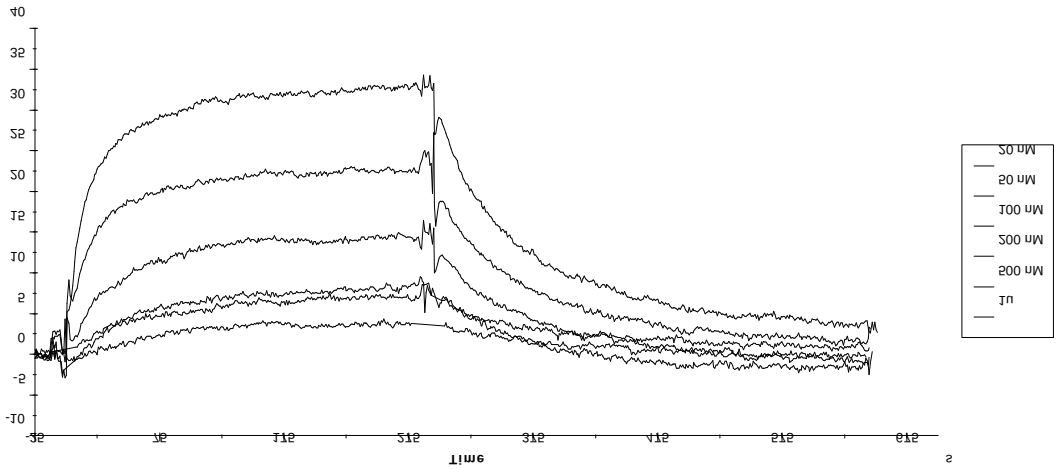


**Figure 7**. Direct binding of peptide **13** to immobilized gp120. Response curves for increasing concentrations of peptide **13** binding to the immobilized gp120. The kinetic constants $k_{(on)}$, $k_{(off)}$ and $K_D$ values are calculated from global fit (1:1 Langmuir model) of the curves by using Biaevaluation software 3000.

**Direct binding of peptide 14 to Yu2 gp120**

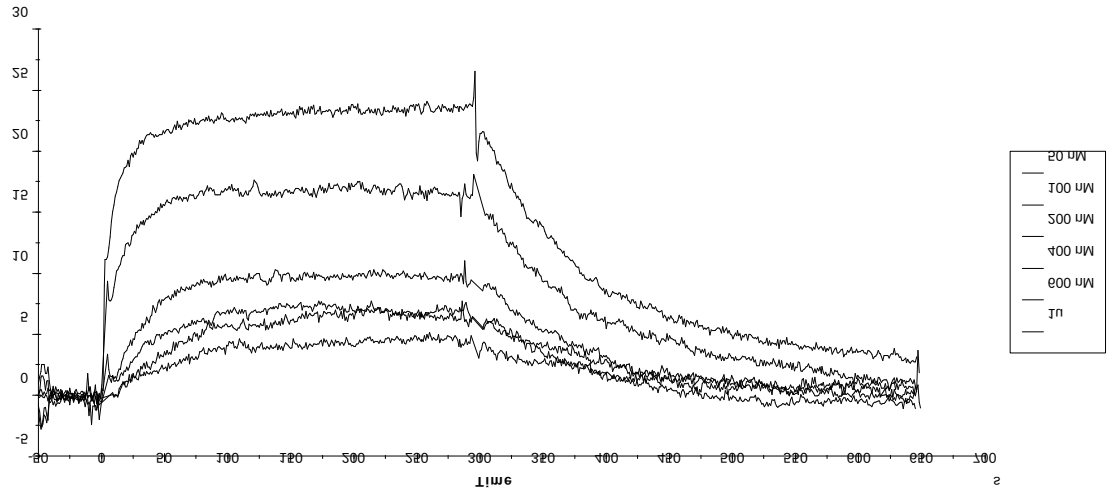


**Figure 8**. Direct binding of peptide **14** to immobilized gp120. Response curves for increasing concentrations of peptide **14** binding to the immobilized gp120.The kinetic constants $k_{(on)}$, $k_{(off)}$ and $K_D$ values are calculated from global fit (1:1 Langmuir model) of the curves by using Biaevaluation software 3000.

**Peptide 9 direct binding to YU2 gp120 (5000RU)**

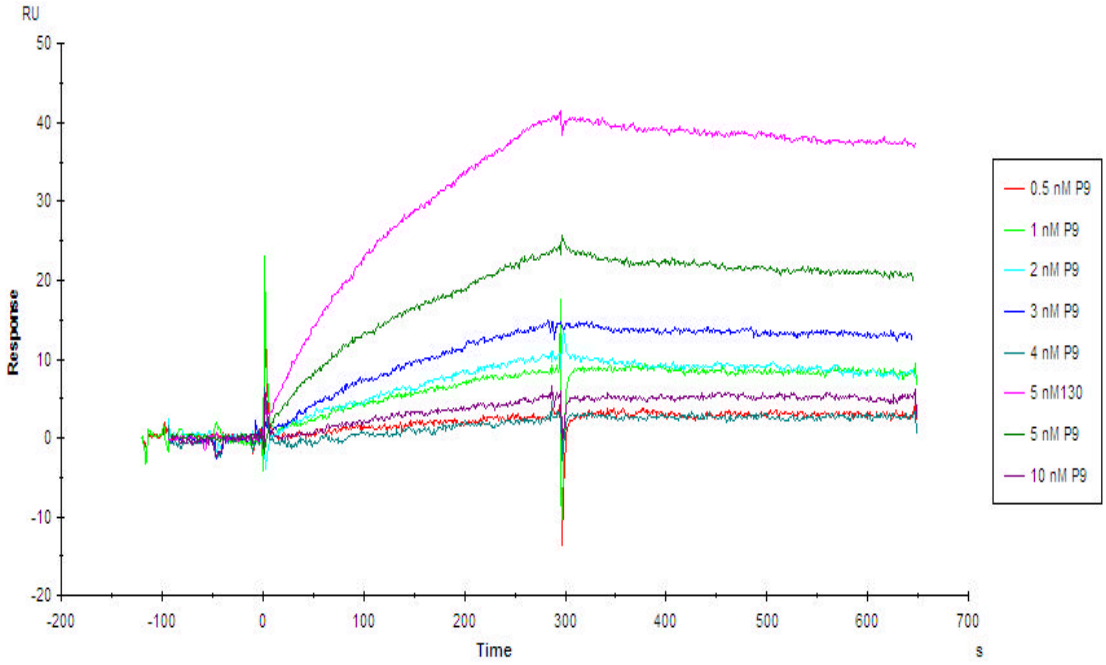
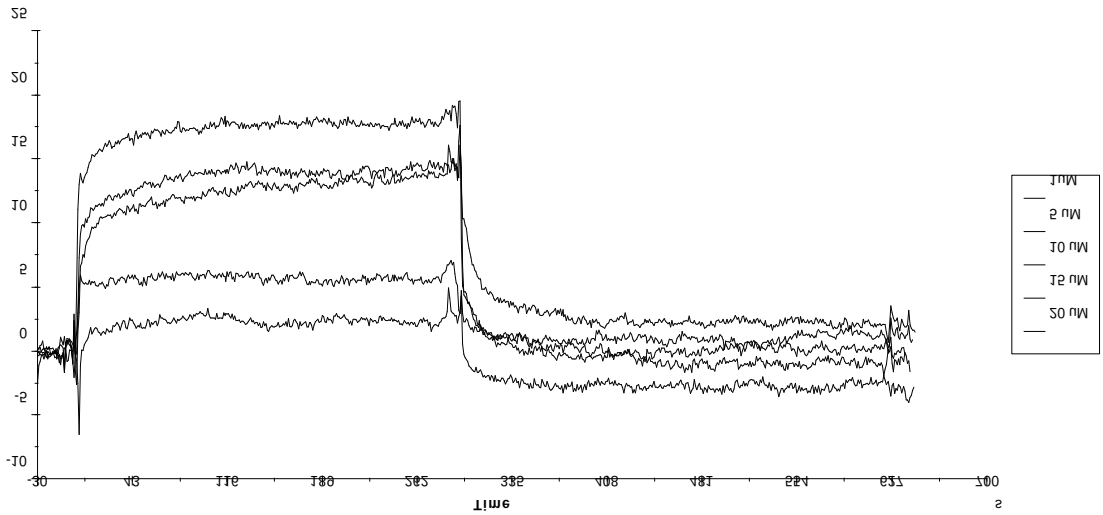


**Figure 9**. Direct binding of peptide **9** to immobilized gp120. Response curves for increasing concentrations of peptide **9** binding to the immobilized gp120. The sensorgrams are shown in the above figure are not concentration dependent.

**Direct binding of peptide 25 to Yu2 gp120**

**(a)**



**(b)**

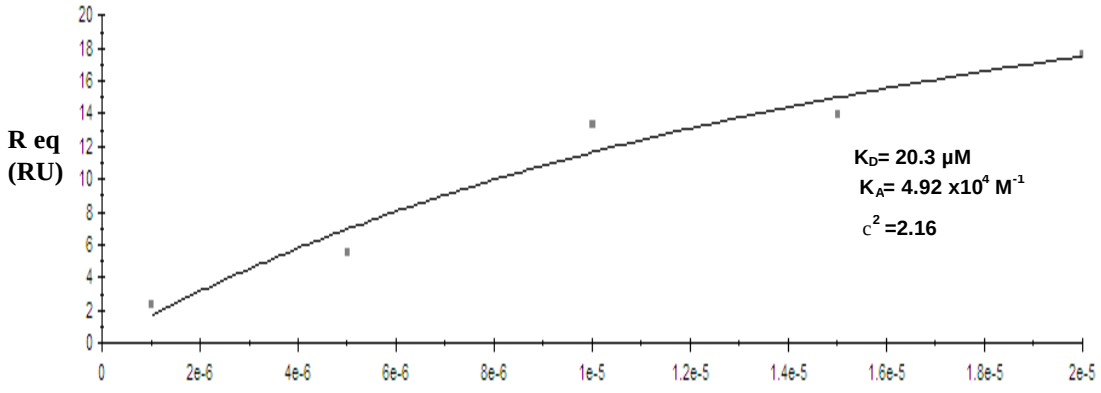



**Figure 10**. Direct binding of peptide **25** to immobilized gp120. (a) Response curves for increasing concentrations of peptide **25** binding to the immobilized gp120. (b) Fit of direct binding data to a steady state affinity 1:1 binding model. Req was calculated from 205- 285s from each curve in (a) and plotted against the concentration of peptide **25.**

**References**: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