HIV Inhibition

A Peptidomimetic HIV-Entry Inhibitor Directed against the CD4 Binding Site of the Viral Glycoprotein gp120**

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The first step in the infection of a human cell with HIV is interaction between the viral envelope glycoprotein gp120 and the human protein CD4.[1] We used this molecular interaction as a template to design and synthesize a new peptidomimetic compound that has the potential to block the interaction. In HIV infection, binding is followed by a conformational change[2] after which gp120 can interact with a co-receptor (CCR5 or CXCR4). This interaction finally leads to fusion of the membrane of the virus with that of a human T-cell. The fusion process is mediated by viral gp41 and leads to infection of the human cell. Compounds that inhibit this process are referred to as entry inhibitors.[3] We present a new way to inhibit the gp120/CD4 interaction through blocking the binding site on the CD4 protein with a peptidomimetic. Other research groups have targeted the same interaction and have developed gp120- or antibody-related peptides,[4] or gp120-binding molecules.[5] In our approach, which involves the development of CD4 ligands, interference with the human immune system or viral resistance are potential problems. However, resistance of the virus to inhibitors that bind to CD4 would require large changes in the viral glycoproteins to allow the virus to utilize a different entry mechanism. Such resistance is therefore not as likely as the evasion of inhibitors of viral proteins whose binding can be circumvented by one or two mutations of the viral amino acids.

We started from the CD4-binding decapeptide NMWQKVGTPL,[6] which was derived from an X-ray structure of gp120.[2b] The peptide has a low binding affinity to CD4 ($K_D = 6$ mM, determined by the surface plasmon resonance (SPR) and saturation transfer difference (STD) NMR spectroscopy protocols described herein) but has an antiviral activity that can be detected by a virus neutralization assay. The main targets of the design phase were to lower the molecular weight of the peptide, enhance its binding affinity, and incorporate nonnatural elements to increase proteolytic stability. The docking of peptides generated by molecular modeling studies by substituting each amino acid of the lead sequence in turn with alanine (see the Supporting Information) supported the results of saturation transfer difference (STD) NMR epitope mapping:[7] The docking study results suggest that the amino acids Asn, Met, and Gln do not contribute to the binding to CD4. Therefore, these inactive subunits were removed from the lead structure to reduce the molecular weight. Interactions of the aromatic ring of Trp and the hydrophobic side chain of Leu with CD4 could be replaced by the interaction of numerous other hydrophobic side chains without losing binding energy. Only the core peptide KVGTP was considered crucial for binding. The N and C termini were modified with various hydrophobic residues connected by nonpeptidic bonds. The candidate found from the molecular modeling studies to interact best with CD4 (Figure 1) was synthesized (Scheme 1).

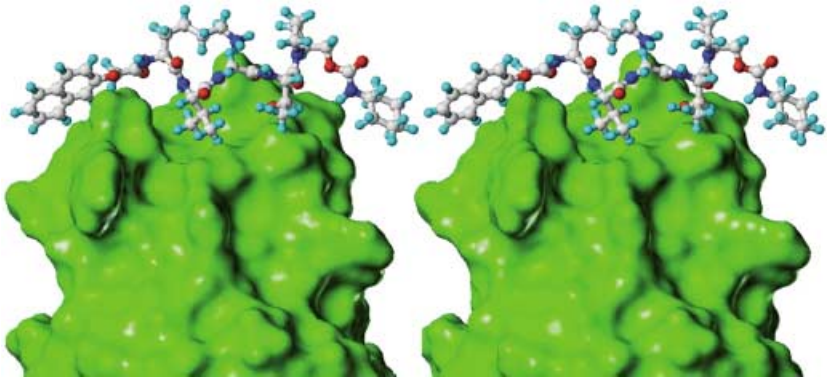

Figure 1. Stereoview of the peptidomimetic **4** (ball and stick) docked to CD4 (green surface).

We synthesized the peptidomimetic from the amino alcohol L-prolinol, which was linked to a 2′-chlorotrityl resin through the hydroxy functional group. The remaining amino acids of the sequence were coupled to the molecule by using standard peptide coupling protocols (activation by treatment with TBTU/DIPEA, Fmoc as an N-terminal protecting group, and Boc and *t*Bu as side-chain-protecting groups). Coupling of the terminal 2-naphthoxy acetic acid was accomplished in the same way through formation of a normal amide bond. The amide bond is stable to the subsequent reaction conditions and should not be cleaved by proteases. Mild acidic conditions (1% TFA in CH_2Cl_2) result in cleavage of the molecule from the resin to form the side-chain-protected free alcohol **2**. The alcohol group reacts with cyclohexylisocyanate under copper(I) catalysis to make a carbamate linkage, which results in **3**.[8] Subsequent treatment with 95% TFA gives the unprotected peptidomimetic **4**. The overall yield of the product was 5% after HPLC purification. The free intermediates and the final product were characterized by MALDI-TOF MS. The structure of the peptidomimetic was determined from its 1D and 2D NMR spectra.

The MS and HPLC analyses indicate the presence of just one compound, but the NMR spectra revealed two signals for

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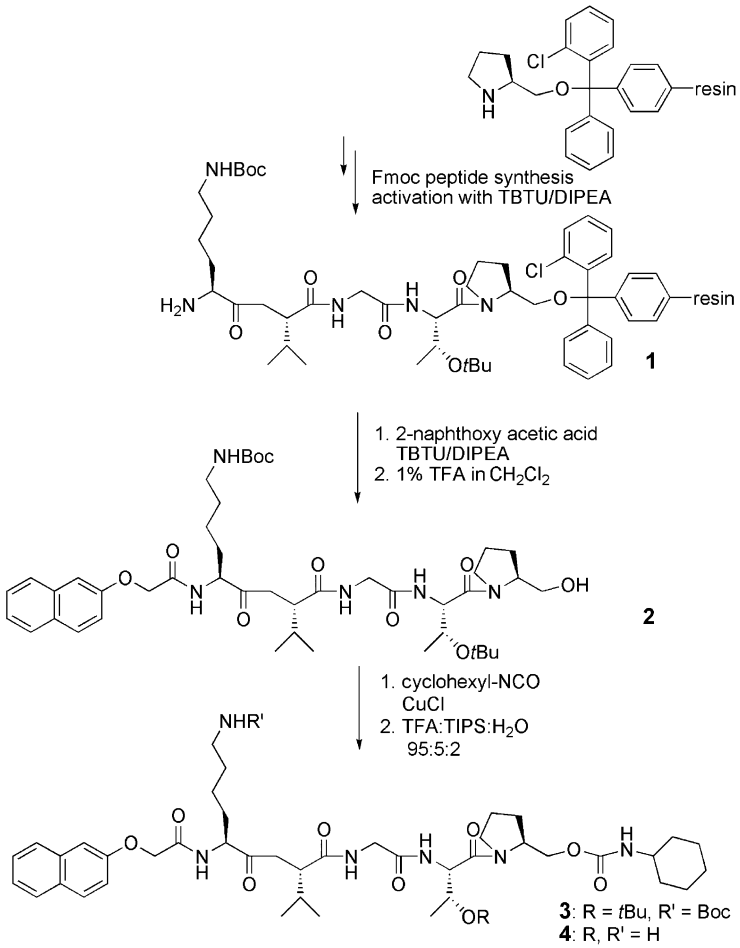
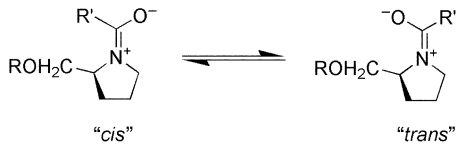


Scheme 1. Synthesis of the CD4-binding peptidomimetic **4**. The peptide and the 2-naphthoxy acetic acid residue were coupled on a solid support. Treatment with a mild acid cleaves **2** from the resin, while the side-chain-protecting groups are retained. Coupling of the isocyanate produces the carbamate **3**. The side-chain-protecting groups were finally removed by treatment with 95% TFA to yield **4**. Fmoc = 9-fluorenylmethoxycarbonyl, Boc = *tert*-butoxycarbonyl, TBTU = *O*-benzotriazol-1-yl-*N*-tetramethyluronium tetrafluoroborate, DIPEA = diisopropylethylamine, TFA = trifluoroacetic acid, TIPS = triisopropylsilyl.



Scheme 2. *cis* and *trans* rotamers of the Thr–prolinol amide bond. About 70% *trans* and 30% *cis* rotamer was detected by ^{1}H NMR spectroscopy in H_2O/D_2O (9:1) at pH 3 and 320 K.

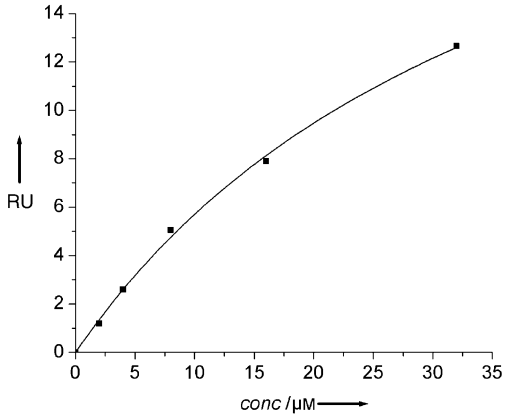


Figure 2. Determination of the binding properties of **4** by surface plasmon resonance on a Biacore 3000 instrument. CD4 was immobilized on a dextrane-coated chip. Ligand **4** in 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffered saline (150 mM, pH 7.4) was passed over the chip. The association curve is concentration-dependent and calculations with these data result in a dissociation constant K_D of 39 μM. RU = response units from the Biacore instrument.

each spin system, with large differences in the chemical shifts of some of the NH and Hα protons (see the Supporting Information). Although the prolinol signals could not be individually assigned, we observed that the biggest differences in chemical shift occurred at the isocyanate residue and the shift differences decreased along the sequence. No differences could be measured at the naphthyl residue. An equilibrium between *cis* and *trans* rotamers at the Thr–prolinol amide bond is the likely explanation (Scheme 2). The ratio of the *trans*:*cis* isomers at 320 K is 2.3:1.

The binding specificity of compound **4** was determined by SPR studies on a Biacore instrument and by STD NMR spectroscopy. Compound **4** was passed over CD4 that had been immobilized on a Biacore CM5 chip. About 11 fmol CD4 was active after immobilization. A concentration-dependent binding assay was performed with 2, 4, 8, 16, and 32 μM **4** in buffer. The results of the binding assays are shown in Figure 2. A regression analysis with a one-site binding model gave a binding constant of $K_D = 39$ μM.

STD NMR experiments (Figure 3) can also be used to determine dissociation constants.[7d] An 8.8 μM solution of soluble CD4 in buffered D_2O was used in the presence of various concentrations of the peptidomimetic. Analysis of the dependence of the STD amplification factor on the concentration of **4** gives the K_D value (Figure 4). This analysis shows that the binding model fits the data very well. The resulting K_D values are 18 μM (Val-Hγ), 29 μM (cyclohexyl-H3), and 45 μM (naphthyl-H6/7), respectively. The binding epitope for the interaction of the peptidomimetic **4** with CD4 has almost identical interacting groups to those found in the original lead structure (Scheme 3).[6] Digestion of the peptidomimetic **4** and NMWQKVGTPL with pronase shows that **4** has a half life ($t_{1/2}$) 4.5-times longer than that of the lead peptide.

We have developed a CD4-binding peptidomimetic that has the potential to block HIV infection. The mimetic has a 170-fold higher K_D value (K_D = ca. 35 μM) than the lead peptide (K_D = ca. 6 mM). The peptidomimetic is also four to five times more stable to proteolysis. The biological activity of the lead structure was determined in earlier experiments. The similarity of the binding epitope of **4** to that of the lead peptide suggests that further optimization of the binding properties of **4** is possible. It remains to be shown in future experiments whether the increased binding affinity of the ligand is also operative in competition with the CD4/gp120 interaction in vivo.

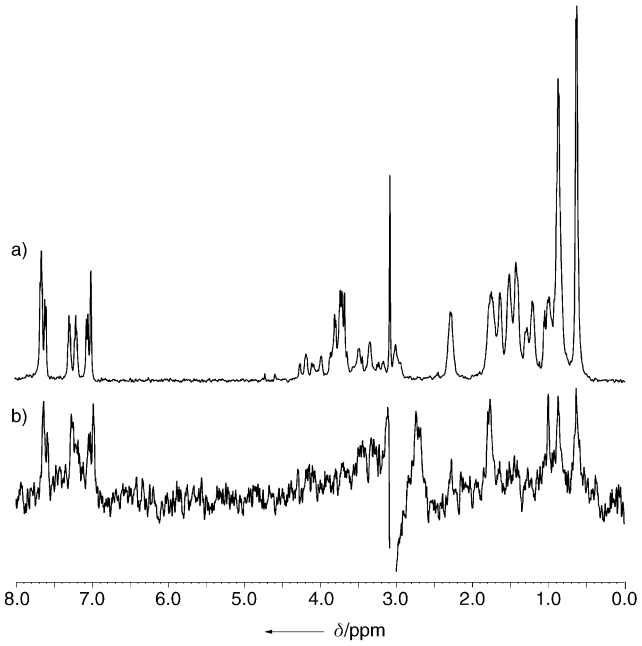


Figure 3. a) ^{1}H NMR spectrum of the peptidomimetic **4**. b) ^{1}H STD NMR spectrum of the peptidomimetic **4** (156 μM, 17.6-fold excess over soluble CD4, 15000 scans) in phosphate-buffered saline (150 mM) in D_2O solvent at pH 7.4. The ^{1}H STD spectrum shows very different relative intensities compared to those in the spectrum shown in (a). This information can be used to define the binding epitope of ligand **4** (see Scheme 3).

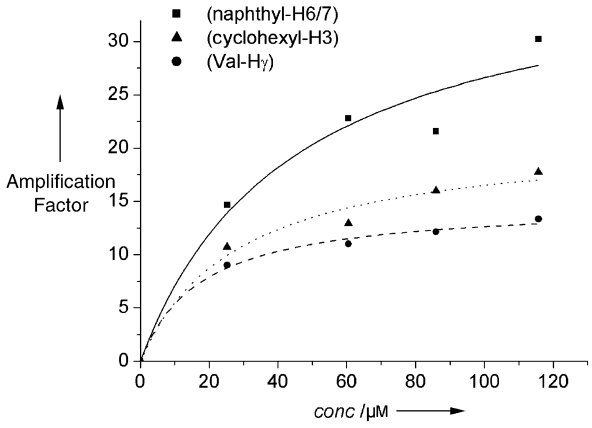
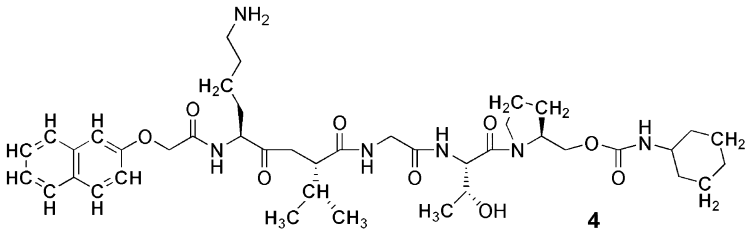


Figure 4. Determination of the binding properties from STD NMR titration data. The titration curves of three protons from different parts of the molecule (see Scheme 3b) are shown. The K_D values were determined from the STD NMR titrations by using a mathematical model of one-site binding. The resulting K_D value for **4** is 31 ± 13 μM.

Experimental Section

The solid-phase synthesis was performed on an ACT 496-Ω robot system. 2′-Chlorotrityl resin (55 mg) bearing L-prolinol (50 μmol; ACT) was shaken for 2 h with Fmoc-Thr(*t*Bu)-OH (4 equiv) and DIPEA/TBTU (5 equiv) in DMF. After washing the resin, this procedure was repeated twice. The resin was treated twice with 10% Ac_2O in DMF for 15 min. Cleavage of the Fmoc group was achieved by treatment with 20% piperidine in DMF for 2 × 10 min. The next amino acids and 2-naphthoxy acetic acid were coupled by using the same protocol. The resin was transferred to a glass frit and was shaken



Scheme 3. STD NMR epitope mapping of the peptidomimetic **4**. The protons in bold are in close contact to CD4.

with a freshly prepared solution of dichloromethane (1880 μL), TFA (20 μL), and TIPS (100 μL) for one hour. The mixture was filtered and the resin was washed with the same solution (1 equiv) then three times with dichloromethane. The combined solutions were washed three times with a 5% aqueous sodium acetate solution. The aqueous phase was re-extracted with dichloromethane and the combined organic phases were shaken with Amberlyst A-21 for one hour. The ion-exchange resin was filtrated and washed with dichloromethane, the organic phases were dried over $MgSO_4$, and the dichloromethane was evaporated to give crude **2**. The alcohol **2** was dissolved in dry DMF and then a freshly prepared suspension of CuCl (50 μmol, 5 mg) in dry DMF (250 μL) and cyclohexylisocyanate (50 μmol, 6.3 mg) added. This mixture was shaken for one hour and then diluted with a 5% aqueous $(NH_4)HCO_3$ solution and extracted three times with dichloromethane. The organic phase was dried over $MgSO_4$ and the solvents were evaporated to give the protected carbamate **3**. This compound was dissolved in TFA (1.9 mL), TIPS (100 μL), and water (40 μL) and the solution was shaken for one hour. The solvents were evaporated and the product was purified by HPLC (solvent A: water/acetonitrile (95:5); solvent B: water/acetonitrile (5:95); both solvents contained 0.1% TFA; 80% A for 1 column volume (CV), then gradients of 1 CV to 50% A and 5 CV to 0% A; product elutes at 38.6% A on a preparative C18 substituted silica column). The pure product was lyophylized. Yield: 2 mg (2.5 μmol) white solid **4**; overall yield: 5%.

2: MALDI-TOF MS: *m/z* 827 ($[M+H]^+$), 849 ($[M+Na]^+$), 865 ($[M+K]^+$); **3**: MALDI-TOF MS: *m/z* 952 ($[M+H]^+$), 974 ($[M+Na]^+$), 990 ($[M+K]^+$); **4**: MALDI-TOF MS: *m/z* 796 ($[M+H]^+$), 818 ($[M+Na]^+$), 834 ($[M+K]^+$); NMR data for **4** are given in the Supporting Information.

The SPR protocol and details of the docking procedures can be found in the Supporting Information.

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