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Design and synthesis of a CD4 β-turn mimetic that inhibits human immunodeficiency virus envelope glycoprotein gp120 binding and infection of human lymphocytes

(AIDS/molecular modeling)

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ABSTRACT **Poor bioavailability, rapid degradation, antigenicity, and high cost often limit the use of proteinaceous pharmaceuticals. One goal of structural biochemistry is the reduction of complex molecules to small functional units that are amenable to high-resolution structural analysis and rapid modification. The dissection of complex proteins into small synthetic conformationally restricted components is an important step in the design of low molecular weight nonpeptides that mimic the activity of the native protein. We have developed a reverse-turn mimetic system to explore peptide and protein structure–function relationships. We now report the design and synthesis of a small molecule (M_r 810, as its trifluoroacetate salt), water soluble, proteolytically stable mimetic of residues Gln^{40}–Thr^{45} of the complementarity-determining 2-like region of CD4. This mimetic has a low micromolar K_d for human T-lymphotropic virus type IIIB gp120 and reduces syncytium formation.**

CD4 is a cell-surface glycoprotein that is found principally on T lymphocytes (1), which associates with class II major histocompatibility molecules on antigen-presenting cells (2, 3). CD4, via high-affinity binding ($K_d \approx 10^{-9}$ M) to the human immunodeficiency virus (HIV) viral envelope glycoprotein gp120, also serves as a cellular attachment site for HIV (4, 5), although other binding sites may also be important (6). Several recombinant CD4-derived candidates have been developed as anti-HIV therapeutics (7). In general, poor bioavailability, rapid degradation, and antigenicity affect the utility of proteinaceous pharmaceuticals (8, 9). The dissection of complex proteins into small synthetic conformationally restricted functional subunits may overcome these problems. Mimetics of important functional domains might possess beneficial properties in comparison to the intact proteinaceous species with regard to specificity and therapeutic potential, and they should be valuable probes for the study of molecular recognition events.

Extensive mutagenesis (10, 11) and peptide mapping experiments (12, 13) have been used to evaluate critical interactions involved in binding CD4 to gp120. There exists some deviation in pinpointing the exact range of the contact surface. However, a single stretch of amino acid residues 40–55 within the complementarity-determining 2-like region has been implicated as being essential. The x-ray structure of an N-terminal 182-residue fragment of CD4 was recently determined (14, 15). Inspection of the structure localizes residues Gln^{40}–Phe^{43} to a highly surface-exposed β-turn connecting the C′ and C″ β-strands. In addition, based on mutagenesis experiments, a significant role for binding to gp120 has been attributed to Phe-43. However, peptides derived from this loop region have failed to exhibit significant inhibitory activity (12, 16). This is presumably due to a loss of critical secondary structure elements in the peptides and highlights the necessity for developing conformationally constrained compounds (17).

Peptides are characteristically highly flexible molecules whose structure is strongly influenced by their environment (18), and their random conformation in solution may preclude their practical application for the study of molecular recognition processes (19). We have developed a method to construct conformationally restricted mimetics of peptide-chain reversals (β-turns, γ-turns, Ω-loops, etc.) (20). The surface localization of turns in proteins, and the predominance of residues containing potentially critical pharmacophoric information, has led to the belief that turns play important roles in a myriad of molecular recognition events (21). Our turn mimetic (Fig. 1*a*) possesses considerable potential for delineating structure–function relationships (20). The general system allows for the facile introduction of any natural or unnatural amino acid side-chain functionality through a modular component synthesis. The ability to alter the X-group linker provides reliable control and variation of side-chain orientations, backbone distances, and ϕ,ψ dihedral angles.

We have determined by molecular modeling that the 10-membered ring mimetic framework (X = NH) (Fig. 1*a*) closely mimics the β-turn at Gln^{40}–Phe^{43}. Based on this analysis, we have synthesized the conformationally restricted peptidomimetic **1** (Fig. 1*b*), which incorporates CD4 residues 40–45 by the protocol outlined in Fig. 2. An overlay of the mimetic structure with that of CD4 is shown in Fig. 3. This first generation small molecule mimetic (M_r 810, as its trifluoroacetate salt) abrogates the binding of human T-lymphotropic virus type IIIB gp120 to CD4$^+$ cells at low micromolar levels and reduces syncytium formation 50% at 250 μg/ml.

MATERIALS AND METHODS

The β-turn mimetic **1** was synthesized according to Fig. 2. Azetidinone **2** was prepared in six steps (22% yield) from

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Abbreviations: HIV, human immunodeficiency virus; F–gp120, fluorescein-conjugated gp120; sCD4, soluble CD4.

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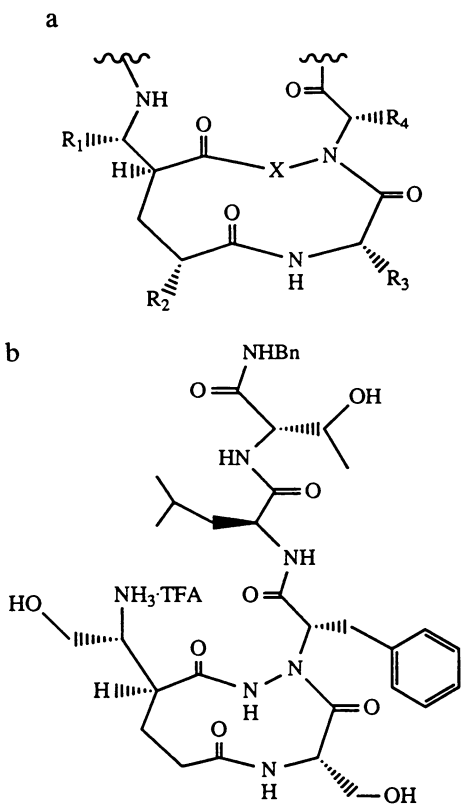


FIG. 1. (*a*) Generic structure of mimetic ring system. X = 1–5 atoms. (*b*) CD4 loop region of peptidomimetic **1**.

dimethyl-D-aspartate in a fashion analogous to procedures described by Salzmann *et al.* (23) and Williams *et al.* (24). Mixed anhydride coupling of **2** to *O*-benzyl serine benzyl ester and subsequent hydrogenolytic cleavage of the benzyl ester provided acid **4** in 88% yield. Coupling of the *Z*-protected hydrazinophenylalanine **5** (25) to **4** by the procedure of Tung *et al.* (26) proceeded smoothly. Reductive closure (27) and saponification afforded the 10-membered ring β-turn mimetic **6** in 53% yield. Dipeptide **7** was coupled to **6** by an aqueous carbodiimide protocol. Hydrogenolytic deprotection of the side-chain protecting groups and purification by reverse-phase C18 HPLC provided homogeneous material, which was characterized by 400-MHz NMR and fast atom bombardment MS. The NMR studies were carried out on a Bruker AM400 spectrometer in either C^2HCl_3 or $C^2H_3O^2H$ at 25°C.

Minimum energy conformers of the mimetic were found by a Monte Carlo search with the Macromodel program BATCHMIN (22). A modified Macromodel MM2 force field, in which the $N(sp^2)$–$N(sp^2)$ force constant was adapted from the Amber force field, was used for the calculations. There are two low-energy conformers, with the principal structural difference being reversal of the peptide bond orientation between residues 2 and 3. The energy difference is ≈0.3 kcal/mol (1 cal = 4.184 J) in vacuum; molecular dynamics simulation in a volume-based continuum solution model (28) indicates that there is no significant energy difference between the two conformers under simulated solution conditions.

Binding assays for inhibition of gp120 binding to $CD4^+$ cells, using reduction in fluorescence intensity upon binding, were performed as follows: fluorescein-conjugated gp120 (F–gp120) (F/P ratio 4.267) was incubated with the mimetics or with soluble CD4 at 22°C in binding buffer (Ca^{2+}-, Mg^{2+}-free Hanks' balanced salt solution/0.5% bovine serum albumin/0.05% sodium azide, pH 7.4, for 40 min). Approximately 300,000 $CD4^+$ cells (from a stock of 10×10^7 cells per ml) were added to tubes at 4°C in binding buffer, with a final vol of 100 μl. Samples were incubated at 4°C for 40 min, washed in binding buffer, and analyzed in a fluorescence-activated cell sorter immediately. Reduction in fluorescence intensity was used to monitor the inhibition of F–gp120 binding to $CD4^+$ cells. Data were acquired gating on live cell populations (always >90%) and were consistent whether mimetics, F–gp120, or other agents were or were not added. The β-turn mimetic CD4-1 (M_r 810, as its trifluoroacetate salt) was soluble in water at 2–4 mg/ml.

The K_d of CD4 mimetic **1** was estimated by the Cheng–Prusoff equation (29)

$$K_d\ (\text{CD4-1}) = \frac{IC_{50}\ (\text{CD4-1})}{1 + \{[\text{F–gp120}]/K_d(\text{F–gp120})\}}.$$

The K_d of F–gp120 was determined from Scatchard analysis to be 22 nM, which is consistent with the published data (30). The IC_{50} measurements were performed five times. The IC_{50} values obtained were between 6.1 and 30.6 μM.

The CD4-1 mimetic was used for inhibition of syncytia formation. SupT1 cells were used as target cells for infection (6). Dilutions (1:2) of soluble CD4 (sCD4), CD4-1 mimetic, or the CD4 hexapeptide (amino acids 40–45) were made in 96-well plates in RPMI 1640 medium containing 10% fetal calf serum. H9/IIIB cells were then plated at a density of $\approx 10^4$ cells per well. SupT1 target cells were added (5×10^5 cells per well) and syncytium formation was qualitatively and quantitatively determined after a 3-day incubation period.

RESULTS AND DISCUSSION

Mimetic **1** was assayed for its ability to inhibit binding of fluoresceinated human T-lymphotropic virus type IIIB gp120 (F–gp120) (30) to $CD4^+$ Jurkat and SupT1 human cells (Fig. 4; see ref. 8). The compound competitively inhibits F–gp120 binding to CD4 in a concentration-dependent manner with an apparent K_d in the low micromolar range.** In contrast, our previously described reovirus receptor-specific 10-membered ring reverse-turn mimetic (YSGSS) (20) and the linear hexapeptide Gln^{40}–Thr^{45} had no effect on the binding of F–gp120. The failure of the reovirus receptor mimetic to inhibit gp120 binding stresses the importance of side-chain orientation in mediating binding, since the two frameworks are identical. These studies illustrate the critical role that the β-turn framework plays in appositely positioning pharmacophoric information in a manner comparable to its presentation by the intact CD4 molecule.

Syncytium formation is effected by the interaction of gp120, expressed in the membranes of infected cells, with the CD4 receptor on neighboring cells (31). We examined the ability of the CD4-1 mimetic to inhibit gp120–CD4-dependent fusion processes in a cocultivation model. At a concentration of 250 μg/ml, there is an ≈50% decrease in the number of syncytia formed (Fig. 5).

It has been suggested that binding of sCD4 to F–gp120 is temperature dependent, and that the affinity of sCD4 for F–gp120 at 4°C decreases by 1 order of magnitude when compared to binding at 37°C (32). We found that the CD4-1 mimetic exhibits similar behavior and most efficiently inhibited F–gp120 binding to cells when preincubated at 37°C (data not shown). It is interesting that the mimetic of the comple-

The K_d for the CD4 mimetic **1 was estimated to be between 4 and 20 μM based on the Cheng–Prusoff equation. These values were corroborated by a standard commercially available ELISA (American Biotechnologies, Cambridge, MA).

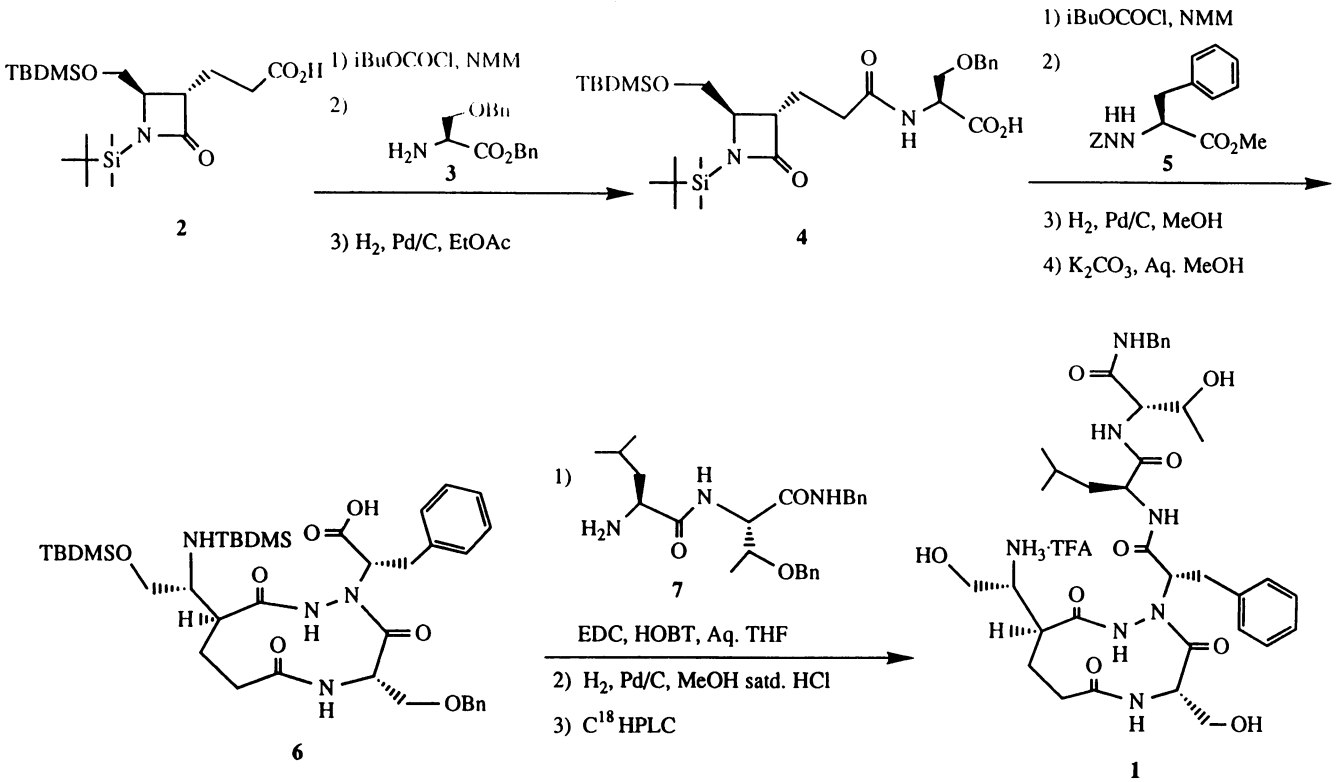


FIG. 2. Synthesis of β-turn mimetic **1**. TBDMS, tertiary butyl dimethyl silyl; TFA, trifluoroacetic acid; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; HOBT, 1-hydroxybenzotriazole hydrate; THF, tetrahydrofuran.

mentarity-determining region 2 loop from CD4 behaves in a manner consistent with the behavior of the intact molecule, in both its binding and kinetic properties. sCD4 is highly sensitive to proteolytic degradation. In contrast, the binding of peptidomimetic CD4-1, like the reovirus mimetic YSGSS (20), is minimally affected by treatment with serum, trypsin, or chymotrypsin (data not shown).

In summary, a small conformationally restricted β-turn mimetic of CD4 residues 40–45 has been designed and synthesized. It effectively inhibits binding of F–gp120 to cell-surface CD4 at low micromolar levels. Similar strategies are being used to explore other important regions of CD4 (33).

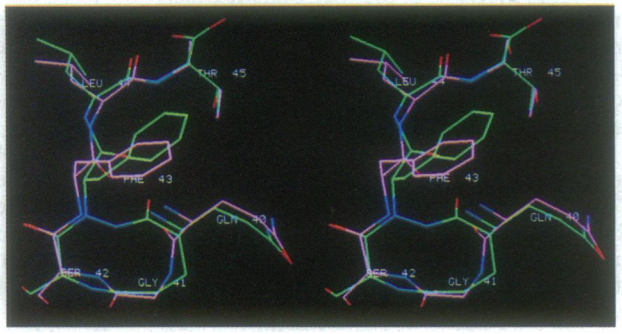


FIG. 3. Stereoview of CD4 residues 40–43 mimetic matched to CD4 residues Gln^{40}–Phe^{43}, with mimetic in green and CD4 in magenta. There are two low-energy conformers, with the principal structural difference being reversal of the peptide-bond orientation between residues 2 and 3. The energy difference is ≈0.3 kcal/mol in vacuum; molecular dynamics simulation in a volume-based continuum solution model (22) indicates that there is no significant energy difference between the two conformers under simulated solution conditions. The conformer equivalent to the II′ turn found in CD4 is shown here. The six-atom rms deviation between the CD4 atoms $C_\alpha^{40}C(=O)^{40}C_\alpha^{41}C_\alpha^{42}N^{43}C_\alpha^{43}$ and the corresponding mimetic positions is 0.44 Å.

The ability to readily synthesize a family of conformationally restricted β-turn mimetics that accurately replicate critical elements of peptide secondary structure should prove invaluable for studying a wide range of molecular recognition events.

The CD4 glycoprotein is a member of the immunoglobulin supergene family. Poliovirus and rhinovirus, both members of the picornavirus family, utilize immunoglobulin superfamily molecules as their respective receptors (34–36). Information obtained from this study may provide a general approach to the design of competitive inhibitors of viral receptors belonging to the immunoglobulin superfamily (37). Furthermore, the natural role of immunoglobulin superfamily molecules in growth, cell adhesion, and major histocompatibility

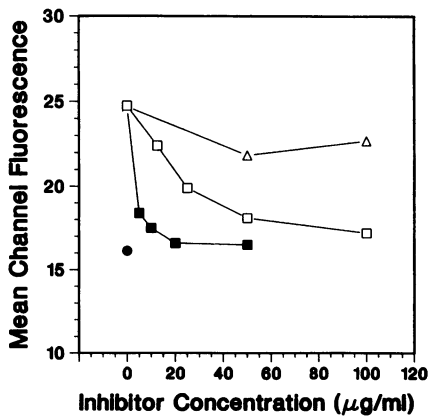
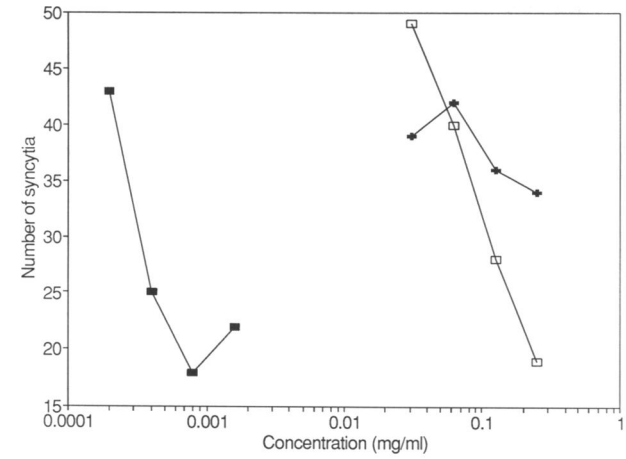


FIG. 4. Inhibition of F–gp120 binding to $CD4^+$ cells, as monitored by reduction in fluorescence intensity. □, CD4-1 mimetic; △, 87.1 reovirus mimetic; ●, autofluorescence; ■, sCD4. Inhibition by the linear peptide of CD4 residues 40–45 was equivalent to that of the 87.1 reovirus mimetic (data not shown).



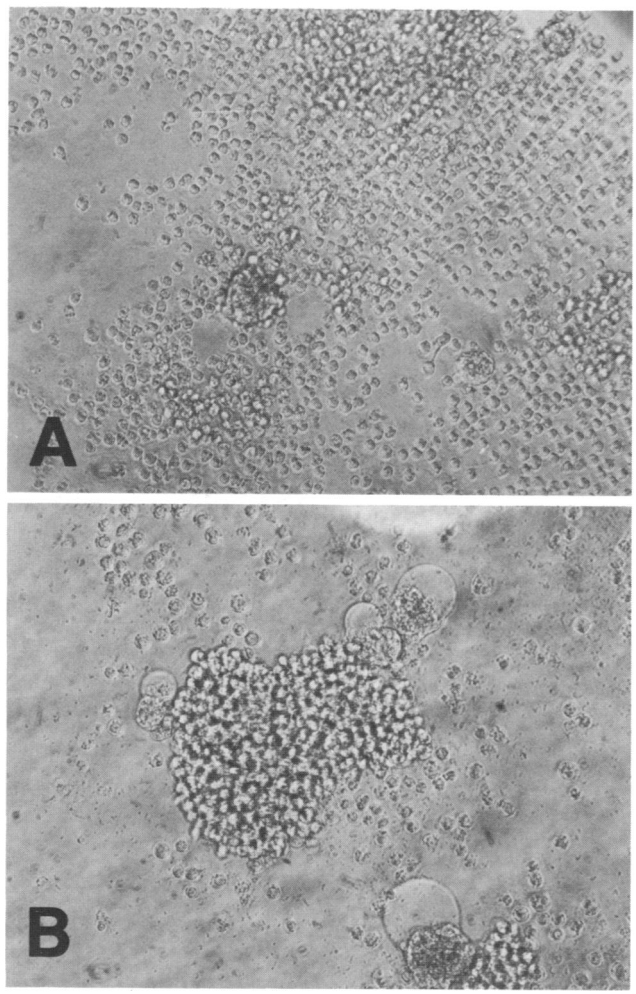


FIG. 5. Inhibition of syncytia formation by CD4-1 mimetic. (*Upper*) Number of syncytia per well counted on visual inspection as a function of concentration for CD4-1 mimetic (□), hexapeptide CD4 residues 40–45 (+), and sCD4 (■). (*Lower*) Photos show syncytia of SupT1 cells cultured with CD4-1 mimetic at 250 μg/ml (*A*) and CD4 (amino acids 40–45) hexapeptide at 250 μg/ml (*B*).

complex restricted antigen presentation (38) could be investigated by this approach.

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