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Conformational Study of a Highly Specific CXCR4 Inhibitor, T140, Disclosing the Close Proximity of Its Intrinsic Pharmacophores Associated with Strong Anti-HIV Activity

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Abstract—We report the solution structure of T140, a truncated polyphemusin peptide analogue that efficiently inhibits infection of target cells by T-cell line-tropic strains of HIV-1 through its specific binding to a chemokine receptor, CXCR4. Nuclear magnetic resonance analysis and molecular dynamic calculations revealed that T140 has a rigidly structured conformation constituted by an antiparallel β-sheet and a type II′ β-turn. A protuberance is formed on one side of the β-sheet by the side-chain functional groups of the three amino acid residues (L-3-(2-naphthyl)alanine[3], Tyr[5] and Arg[14]), each of which is indispensable for strong anti-HIV activity. These findings provide a rationale to dissect the structural basis for the ability of this compound to block the interaction between CXCR4 and envelope glycoproteins from T-tropic strains of HIV-1.

A perfect therapy has not yet been established for HIV-1 infection. Although "highly active anti-retroviral therapy (HAART)", which involves a combination of reverse transcriptase/protease inhibitors, has dramatically improved the clinical treatment of individuals with AIDS, there are several serious problems with this regimen.[1] These include the emergence of viral strains with multi-drug resistance, significant side effects and a high cost. An ideal therapeutic approach would block an early stage of HIV-infection, such as viral entry. The discovery that a subset of chemokine receptors function as coreceptors for membrane fusion mediated by the envelope glycoprotein and the identification of CCR5 and CXCR4 as the front line coreceptors for entry by macrophage (M-) and T-cell line (T-) tropic strains of HIV-1 provide molecular targets for such a strategy.[2–5] This approach is supported by the relative resistance to infection of commonly transmitted forms of HIV-1 by individuals carrying a protective allele that encodes a non-functional form of CCR5.[3f] We have previously demonstrated that a peptide with anti-HIV-1 activity, T22 ([Tyr[5,12], Lys[7]]-polyphemusin II), is an inhibitor of CXCR4 that blocks T-tropic HIV-1 entry mediated by this coreceptor. T22 is an 18-residue peptide amide, which was previously found by us based on analysis of the structure–activity relationships (SARs) of tachyplesins and polyphemusins, which function as autoprotective peptides of horseshoe crabs.[6] Determination of the solution structure of T22 by proton nuclear magnetic resonance (NMR) spectroscopy revealed that it assumes an antiparallel β-sheet conformation connected by a type II β-turn that is maintained by two disulfide bridges.[7] A downsized analogue, TW70 (des-[Cys[8,13],Tyr[9,12]]-[D-Lys[10],Pro[11]]-T22), which is a 14-residue peptide amide with one intrachain disulfide bond, was also found to have an antiparallel β-sheet structure with a type II′ β-turn by NMR.[8] SAR studies of TW70 enabled us to discover additional analogues, T134 ([L-citrulline (Cit)[12]]-TW70 lacking the C-terminal amide) and T140 {[L-3-(2-naphthyl)alanine (Nal)[3]]-T134}, which have increased anti-HIV-1 activity and diminished cytotoxicity, in comparison with

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T22 and TW70.[9,10] T140, shown in Figure 1, which is a 14-residue peptide with a single disulfide bridge, has the highest level of HIV-1 inhibition (50% effective concentration {EC_{50}}, 3.5 nM) and antagonism of target cell entry by T-tropic strains of HIV-1 (EC_{50}, 0.18 nM) among all antagonists of CXCR4 that have been reported to date. The solution structure of T140 has been determined to elucidate the structural basis for the anti-HIV-1 activity of this CXCR4 antagonist. Our previous analysis of circular dichroism of this compound suggested that it adopts a β-sheet structure.[10] In this report, we determined the structure of T140 in solution by ^{1}H NMR spectroscopy and distance geometry calculation using QUANTA/CHARMm. The finding that T140 has a β-sheet/type II′ β-turn architecture in which side chains of critical amino acid residues are oriented to form a protrusion on one aspect provides insight into the structural basis for the activity of this compound as a CXCR4 antagonist.

NMR Spectroscopy of T140

T140 was dissolved in a mixture of H_2O and D_2O (9:1) at a concentration of 6.8 mM. The pH of the solution was approximately 5. The NMR spectra were recorded at 25 °C on Varian UNITY 500 and Bruker DMX-750 spectrometers at 500 MHz and 750 MHz ^{1}H frequencies, respectively. Chemical shifts were quoted relative to the signal of H_2O as 4.67 ppm. WETGCOSY[11] was recorded in the absolute value mode. WETTNTOCSY,[11] TOCSY and WETNOESY[11] were all recorded in the phase-sensitive mode. The large water resonance was suppressed using the water suppression enhanced through the T_1 effects (WET) method.[12] The two-dimensional data for WETGCOSY, WETTNTOCSY, TOCSY and WETNOESY were recorded on 2 K×2048 W, 2 K×1024 W, 4 K×512 W and 2 K×1024 W matrix points, respectively. The mixing time for the NOESY experiments was set at 500 ms.

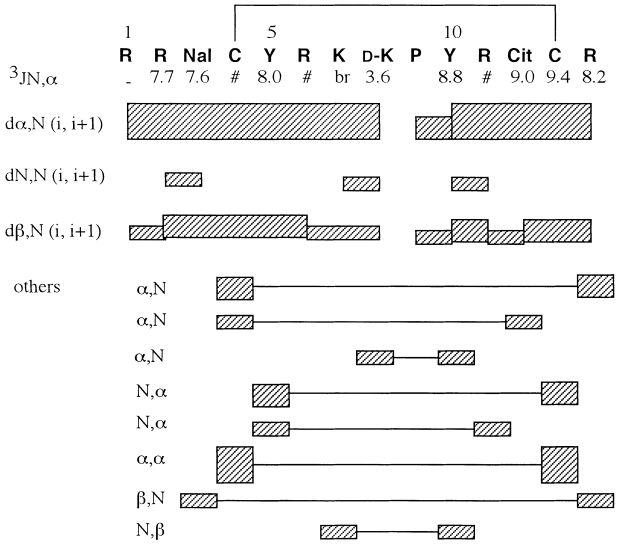

Figure 1. Amino acid sequence of T140 and main experimental data. A disulfide linkage is shown by a solid line. Shade bars show that NOEs were observed. Height is correlated to the relative intensity of cross-peaks. *J*-couplings are shown in Hz. #: $^3J_{\alpha,N}$ could not be determined due to overlapping of signals.

Molecular Dynamic Simulations

Molecular dynamic simulations were made using the QUANTA/CHARMm program (QUANTA98, Molecular Simulation Inc., California, USA) on a Silicon Graphics Workstation. CHARMm functions as an interpreter of a program language adapted for restrained molecular dynamics. The initial structure of the backbone of T140 was manually modeled as an antiparallel β-sheet connected by a type II′ β-turn with D-Lys^8-Pro^9 at the (i + 1) and (i + 2) positions and linked by a disulfide bond to satisfy the NOE data from the NMR experiment. The NOE data were also used for modeling of side-chain conformations. The NOEs of several side chains were not observed and the initial coordinates of those residues were modeled as an extend form. The dynamics calculations were carried out with NOE restrictions on the modeled T140 structure that was completely immersed in a 30 Å periodic boundary box of water molecules. One of the simulated structures, which had the lowest energy, was subsequently refined.

Results and Discussion

Spin system identification was achieved by performing WETGCOSY, WETTNTOCSY and TOCSY experiments. The results of the subsequent sequential assignment made using the WETNOESY spectra are summarized in Table 1. The sequential NOE connectivities and spin coupling constants between the NH and the α proton resonances ($^3J_{NH,\alpha H}$) are shown in Figure 1. The strong $d_{\alpha,N}$ connectivities associated with a small number of $d_{N,N}$ connectivities suggest the presence of an antiparallel, pleated β-sheet structure extending from residue 3 to 14 with a β-turn formed by Lys^7 (i), D-Lys^8 (i + 1), Pro^9 (i + 2) and Tyr^{10} (i + 3). This structure is in good agreement with the values of spin coupling constants, $^3J_{NH,\alpha H}$, where all the coupling constants are more than 7.6 Hz, except those of D-Lys^8 (i + 1) and Pro^9 (i + 2). The presence of the β-sheet structure was further confirmed by the detection of long range NOEs between the protons of (Cys^4 Hα, Arg^{14} NH), (Cys^4 Hα, Cit^{12} NH), (D-Lys^8 Hα, Tyr^{10} NH), (Tyr^5 NH, Cys^{13} Hα), (Tyr^5 NH, Arg^{11} Hα), (Cys^4 Hα, Cys^{13} Hα), (Nal^3 Hβ, Arg^{14} NH) and (Lys^7 NH, Tyr^{10} Hβ), as shown in Figures 1 and 2. These NOEs are characteristic of those protons that are located on the opposite β strands of β-sheets and oriented towards the interior (Fig. 2). These results were consistent with the previous studies on TW70.[8] In Figure 2, the peptide backbone was constructed in accordance with these NOEs in the way hydrogen bonds were formed between opposite β strands. As an inevitable consequence, it is suggested that the β-turn of T140 is type II′. Therefore, the initial structure of the backbone of T140 for the following simulations was modeled as an antiparallel β-sheet connected by a type II′ β-turn with D-Lys^8-Pro^9 at the (i + 1) and (i + 2) positions and linked by a disulfide bond.

Distance geometry calculations by QUANTA/CHARMm were made by using all of the sequential and long range distance NOEs, which were classified based on their intensity. The best structure, which had the lowest energy among all those preliminarily calculated, was refined to predict a final three-dimensional architecture (Fig. 3). The result strongly correlates with the same type of backbone of a right-twisted β-sheet and a type II′ β-turn formed by Lys^7 (i), D-Lys^8 (i+1), Pro^9 (i+2) and Tyr^{10} (i+3). Thus, T140 is deduced to assume a conformation with a rigid antiparallel, pleated β-sheet structure and a flexible NH_2-terminal tail.

T140 has been shown to be a highly specific CXCR4 antagonist derived from the T22 and TW70 lead compounds without changing the secondary structure or the overall architecture. We previously determined the NMR coordinates of T22 and TW70 without involving distance geometry calculations for conformational analysis. In the present study, distance geometry calculations were incorporated into the analysis of the NMR data to establish the backbone structure and the orientation of amino acid side chains of the T140 molecule in the solution. Our previous analysis of T140 SAR by Ala-scanning mutagenesis clearly established that Arg^2, Nal^3, Tyr^5 and Arg^{14} are critical to the strong anti-HIV activity.[13] These four residues are close to each other in the T140 hairpin structure formed by the β-sheet and type II′ β-turn (Figs 2 and 3). Furthermore, our conformational analysis of T140 predicted that the side chains of Nal^3, Tyr^5 and Arg^{14} extend in the same orientation from the plane of the β-sheet to form a protrusion opposed to the side chain of Arg^2, which is in a vicinal region of the flexible NH_2-terminal segment. The three residues on the restricted backbone (Nal^3, Tyr^5 and Arg^{14}) and the single residue in the flexible region (Arg^2) form the intrinsic pharmacophore of T140. The pharmacophore-guided approach based on this structure could lead to the rational design and synthesis of novel small molecule inhibitors of CXCR4.

Table 1. ^1H chemical shift of T140 at 25 °C

Residue	Chemical shift (ppm)[a]			
	NH	$C^{\alpha}H$	$C^{\beta}H$	Others
Arg^1	—	3.88	1.70, 1.61	$C^{\gamma}H$ 1.30, 1.30 $C^{\delta}H$ 2.88, 2.81 $N^{\varepsilon}H$ 6.84
Arg^2	8.65	4.52	1.58, 1.58	$C^{\gamma}H$ 1.45, 1.39 $C^{\delta}H$ 2.94, 2.94 H $N^{\varepsilon}H$ 6.95
Nal^3	8.38	4.81	3.10, 3.10	C2H 7.24 C3H 7.64 C5H 7.69 C6H 7.32 C7H 7.30 C8H 7.64 C10H 7.52
Cys^4	8.35	5.36	2.83, 2.50	
Tyr^5	8.75	4.56	2.76, 2.65	C2, 6H 6.68 C3, 5H 6.44
Arg^6	8.35	4.66	1.67, 1.56	$C^{\gamma}H$ 1.43, 1.32 $C^{\delta}H$ 2.96, 2.96 $N^{\varepsilon}H$[b]
Lys^7	8.45	4.38	1.72, 1.46	$C^{\gamma}H$ 1.34, 1.25 $C^{\delta}H$ 1.62, 1.62 $C^{\varepsilon}H$ 2.88, 2.88 $H^{\zeta}H$ 7.44
D-Lys^8	8.63	4.31	1.60, 1.60	$C^{\gamma}H$ 1.39, 1.24 $C^{\delta}H$ 1.54, 1.54 $C^{\varepsilon}H$ 2.83, 2.83 $H^{\zeta}H$ 7.40
Pro^9		4.28	1.87, 1.45	$C^{\gamma}H$ 1.60, 0.83 $C^{\delta}H$ 3.59, 3.40
Tyr^{10}	7.50	4.54	2.95, 277	C2, 6 H 7.04 C3, 5H 6.70
Arg^{11}	8.35	4.50	1.58, 1.41	$C^{\gamma}H$ 1.26, 1.21 $C^{\delta}H$ 2.93, 2.93 $N^{\varepsilon}H$[b]
Cit^{12}	8.46	4.34	1.32, 0.98	$C^{\gamma}H$ 1.20, 1.16 $C^{\delta}H$ 2.88, 2.88[c]
Cys^{13}	8.39	5.20	2.77, 2.61	
Arg^{14}	8.60	4.39	1.65, 1.33	$C^{\gamma}H$ 1.42, 1.35 $C^{\delta}H$ 2.99, 2.99 $N^{\varepsilon}H$[b]

[a]Chemical shifts are measured ±0.01 ppm at 500 MHz frequency.
[b]$N^{\varepsilon}H$ protons could not be determined due to overlapping of signals between 7.04 and 6.98 ppm.
[c]Other side-chain protons could not be determined due to overlapping of signals.

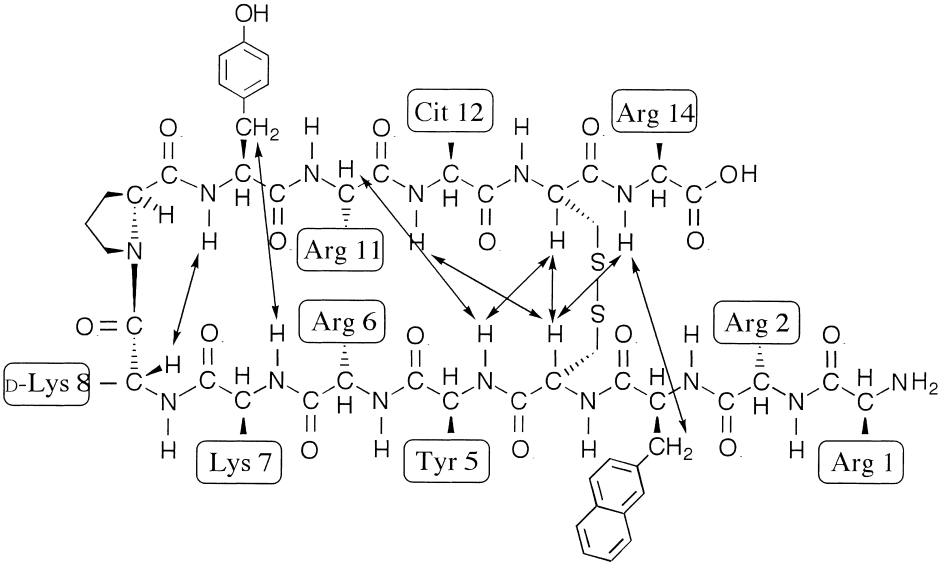

Figure 2. Schematic representation of the structure of T140 deduced from NMR analysis. The observed inter-strand NOE connectivities between NH and $C^{\alpha}H$ protons, $C^{\alpha}H$ and $C^{\alpha}H$ protons or NH and $C^{\beta}H$ protons are shown by arrows.

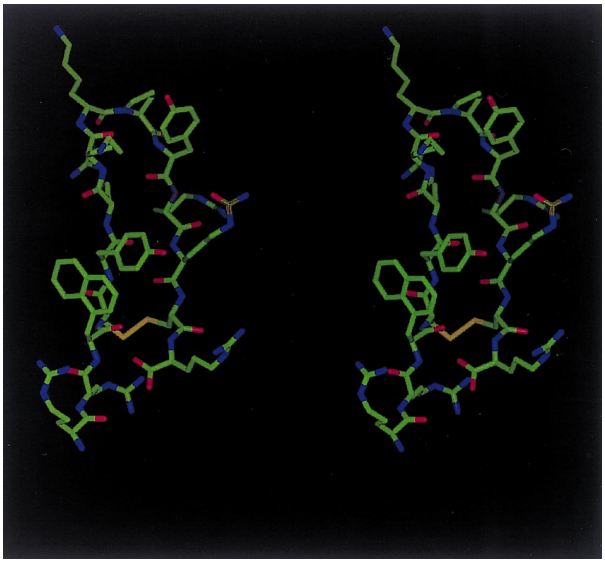

Figure 3. Stereoview of the three-dimensional structure of T140 calculated by Quanta/CHARMm.

In addition, these results are useful for clarification of the precise nature of the interaction of this family of inhibitors with CXCR4 and provide insight into the mechanism for their ability to block its fusogenic activity, which is critical to the entry of T-tropic strains of HIV-1.

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