Rational Drug Design

Molecular-Size Reduction of a Potent CXCR4-Chemokine Antagonist Using Orthogonal Combination of Conformation- and Sequence-Based Libraries**

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The information available on natural ligands provided by recent advances in genome science is exponentially increasing and amplifies the opportunities for medicinal chemists to develop novel pharmaceuticals.[1] Rational drug design of agonists/antagonists from natural ligands offers one of the most powerful methodologies for drug discovery, while development of innovative methods is required to facilitate the processes. Herein, we report a new strategy for the downsizing of bioactive peptides using two orthogonal small libraries of cyclic peptides, which allowed us to identify a novel CXCR4 antagonist equipotent to the parent peptide.[2]

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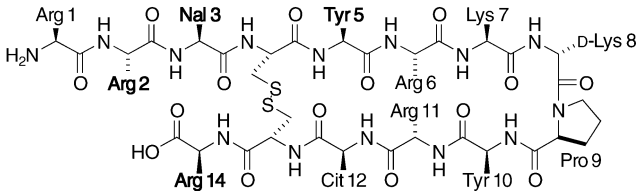
We previously reported that T140[3] is a highly potent specific antagonist of the CXCR4-chemokine receptor, which is relevant to HIV-1 infection, cancer metastasis, rheumatoid arthritis, and chronic lymphocytic B-cell leukemia.[4] Our structure–activity relationship studies on T140, which consists of 14 amino acid residues and one disulfide bridge between Cys 4 and Cys 13, have revealed that four indispensable amino acid residues, Arg 2, Nal 3, Tyr 5, and Arg 14, are responsible for its intrinsic bioactivity (Scheme 1).[3a] In addition, NMR



Scheme 1. Structure of T140; bold = indispensable residues, Nal = L-3-(2-naphthyl)alanine, Cit = L-citrulline.

and computational studies on T140 have demonstrated that it adopts an antiparallel β-sheet conformation which is connected by a type II′ β-turn with D-Lys 8-Pro 9 in the $i+1$- and $i+2$-positions. Meanwhile, the three pharmacologically significant residues, Arg 2, Nal 3, and Arg 14, are highly mobile since they are located outside the macrocycle constrained by the disulfide bridge.[3b] We chose cyclic pentapeptides as a molecular template to dispose these four requisite residues in proximity. It was our expectation that these could potentially distribute themselves in coincidence with the topology of the distal pharmacophores in the bioactive conformation of T140. Additionally, potent cyclic peptides obtained in this fashion would enable us to develop low-molecular-weight CXCR4 antagonists through rational design. A possible pentapeptide library included 192 peptides (12 sequences × 16 stereoisomers, Figure 1).[5] Two orthogonal focused libraries containing a limited number of cyclic peptides were utilized to avoid the time-consuming effort required for the synthesis of 192 cyclic peptides.

A distinctive "conformation-based" library (first library) with diverse sequences was initially designed, which included 48 cyclic pentapeptides (12 sequences × 4 stereoisomers). We expected that initial use of this library containing differential sequences could give opportunities to efficiently extract potent compounds from the 192 possible peptides, thus resulting in identification of the required sequence. On the basis of intensive research on cyclic RGD peptides by Kessler et al., in which *cyclo*(-L-Arg-Gly-L-Asp-D-Phe-L-Val-) and *cyclo*(-L-Arg-Gly-L-Asp-L-Phe-D-Val-) exhibited two different βII′/γ-turn arrangements, four well-established arrays were chosen for the library (Scheme 2): *cyclo*(-L-Xaa 1-Gly 2-L-Xaa 3-D-Xaa 4-L-Xaa 5-) **1a–12a** (group I) and *cyclo*(-L-Xaa 1-Gly 2-L-Xaa 3-L-Xaa 4-D-Xaa 5-) **1b–12b** (group II), and their enantiomers, *cyclo*(-D-Xaa 1-Gly 2-D-Xaa 3-L-Xaa 4-D-Xaa 5-) **1c–12c** (group III) and *cyclo*(-D-Xaa 1-Gly 2-D-Xaa 3-D-Xaa 4-L-Xaa 5-) **1d–12d** (group IV). Among these, the D/L-Xaa[4] residue could be in the $i+1$ position of the βII′-turn in groups I and III, while the Gly 2 residue could

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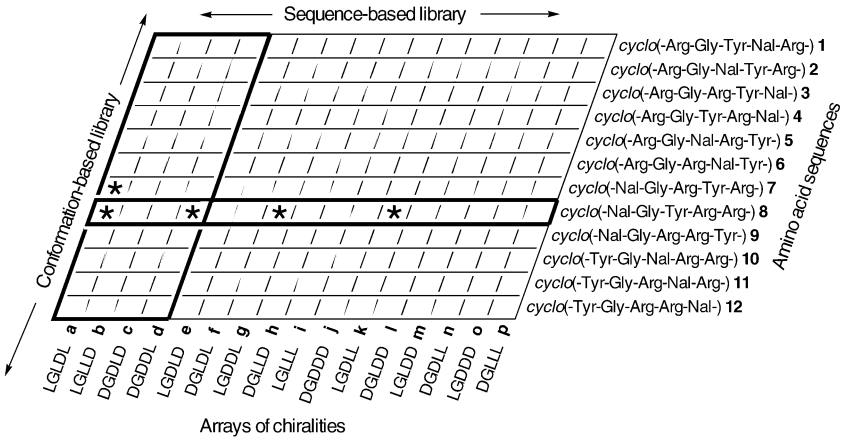
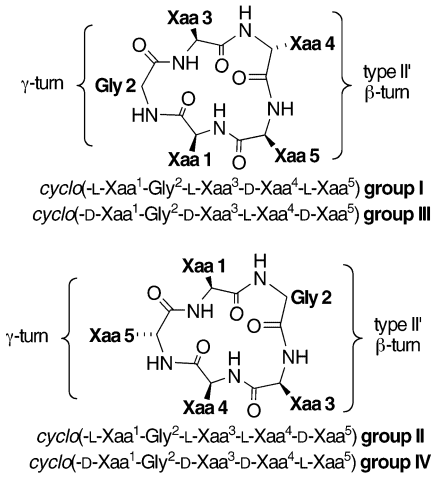


Figure 1. Design of two orthogonal libraries of cyclic peptides; L = L-amino acids, D = D-amino acids, G = glycine.



Scheme 2. Structures of the peptides used in the "conformation-based" library.

occupy the same position in groups II and IV. Parallel synthesis of 48 cyclic peptides followed by evaluation of their biological activities were performed.[6] As a result, we found three compounds, *cyclo*(-L-Nal 1-Gly 2-L-Arg 3-D-Tyr 4-L-Arg 5-) (**7a**), *cyclo*(-L-Nal 1-Gly 2-L-Tyr 3-D-Arg 4-L-Arg 5-) (**8a**), and *cyclo*(-D-Nal 1-Gly 2-D-Tyr 3-D-Arg 4-L-Arg 5-) (**8d**), with moderate CXCR4-antagonistic activity and anti-HIV activity from the "conformation-based" library (see the Supporting Information).[7]

Next, we addressed the two cyclic peptides **8a** and **8d** resulting from the first library that possessed two amino acids with common chirality and functionality in the 4- and 5-positions. This similarity indicated that potent compounds would be obtained from *cyclo*(-D-Nal 1-Gly 2-L-Tyr 3-D-Arg 4-L-Arg 5-) (**8f**) and/or *cyclo*(-L-Nal 1-Gly 2-D-Tyr 3-D-Arg 4-L-Arg 5-) (**8g**), which were Nal 1 and Tyr 3 epimers of **8a** and **8d**. In fact, **8g** was more potent than any other compounds evaluated up to this point (IC_{50} value for CXCR4 = 0.008 μM, EC_{50} value for HIV replication = 0.11 μM). Hence, we designed a "sequence-based" library (second library), wherein diverse chirality arrays of the common amino acid sequence, *cyclo*(-L/D-Nal 1-Gly 2-L/D-Tyr 3-L/D-Arg 4-L/D-Arg 5-), were employed (1 sequence × 16 stereoisomers).[8] In this library *cyclo*(-L-Nal 1-Gly 2-D-Tyr 3-L-Arg 4-L-Arg 5-) (**8k**), which is an epimer of **8g** at Arg 4, exhibited the most potent CXCR4-antagonistic activity (IC_{50} = 0.004 μM) and anti-HIV activity (EC_{50} = 0.038 μM) among all the cyclic pentapeptides (Table 1).

A [^{125}I]SDF-1–CXCR4 (SDF = stromal cell-derived factor) binding inhibition assay showed that the binding affinities of cyclic peptides **8d**, **8g**, and **8k** paralleled their anti-HIV activity. This supported the conclusion that the anti-HIV activities of these cyclic peptides were based on a similar CXCR4 inhibition as that of T140. Peptides, such as **8k**, which have an affinity comparable to that of T140 can be used to evaluate the bioactive conformations of T140. Moreover, these peptides can act as valuable tools to understand the essential pharmacophore topology to CXCR4 antagonism, and thus may facilitate the design of novel nonpeptidic CXCR4 antagonists.

Table 1: Biological activities of T140 and cyclic peptides.

Peptide	IC_{50} [μM][a]	EC_{50} [μM][b]	Peptide	IC_{50} [μM][a]	EC_{50} [μM][b]
T140	0.004	0.060			
8a	0.1–1.0	4.3	**8j**	1.0–10	17
8d	0.016	0.28	**8k**	0.004	0.038
8e	0.1–1.0	20	**8l**	1.0–10	> 200
8f	0.1–1.0	> 200	**8m**	0.1–1.0	11
8g	0.008	0.11	**8n**	0.1–1.0	0.76
8h	> 10	27	**8o**	0.1–1.0	8.2
8i	0.14	2.4	**8p**	0.1–1.0	4.4

[a] IC_{50} values for the cyclic pentapeptides are based on the inhibition of [^{125}I]SDF-1 binding to CXCR4 transfectants of Chinese hamster ovary (CHO) cells. [b] EC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells.

To confirm the importance of the ring structure as well as the sequence of the cyclic peptides **8d**, **8g**, and **8k** for the anti-HIV activity, the linear peptides **13a–e**, **14a–e**, and **15a–e**, which corresponded to linear products containing the five peptide residues, were synthesized and evaluated (see the Supporting Information). The remarkably reduced activity of the linear peptides (less than 10^{-2} times the activity of the corresponding cyclic congeners) showed that an appropriate ring structure was essential for bioactivity.

The solution conformation of **8k** was estimated by measurement of the ^{1}H NMR spectra in DMSO, according to the previous reports on various cyclic pentapeptides, to estimate its solution conformation.[2] No low temperature coefficients were observed for the chemical shifts of the amide protons. This observation could potentially exclude a reduction in the solvent accessibility as a result of the formation of internal hydrogen bonds. In some cases, pronounced cross-peaks could be observed in NOESY spectra between two amide protons or an amide proton and

an α-proton of the respective forward residues. Cross-peaks between Gly2 H^N/D-Tyr3 H^N, Arg4 H^N/Arg5 H^N, and Arg5 H^N/Nal1 H^N indicate that these pairs of amide hydrogen atoms may be oriented in the same directions. On the other hand, the strong NOE interactions of Nal1 H^α/Gly2 H^N and D-Tyr3 H^α/Arg4 H^N show that these pairs of protons are in proximity across the peptide bonds. We performed simulated annealing molecular dynamics/energy minimization by using dihedral and distance constraints derived from ^{1}H NMR measurements (Figure 2). The peptide backbones in

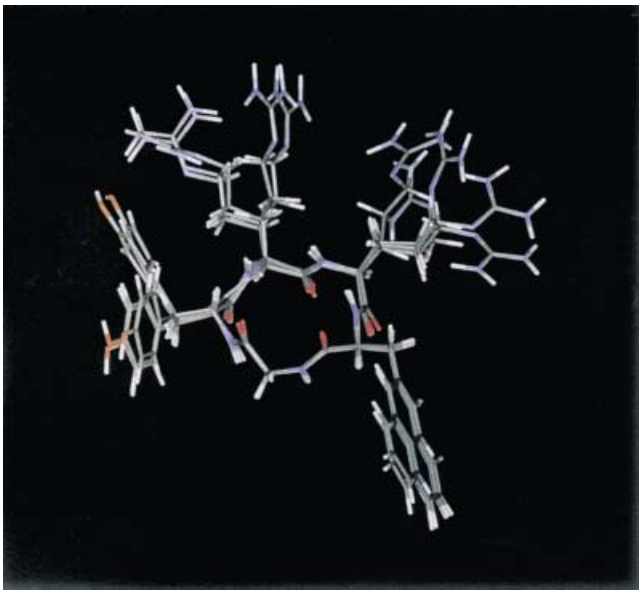


Figure 2. Overlay of five low-energy structures of **8k**.

five energy-minimized structures exhibited nearly symmetrical pentagonal shapes. The carbonyl oxygen atoms in four amide bonds, excluding that between Nal1 and Gly2, were oriented away from the side chains of the respective following residues. This result was consistent with the structures expected from the NOESY spectra. While the exact correlation of the spatial dispositions of each pharmacophore of **8k** with those of T140 are not clear, the calculated structures can provide insight that is applicable to the structure–based design of nonpeptide CXCR4 antagonists.

In summary, we have designed two orthogonal cyclic peptide libraries, which consist of a "conformation-based" library and a "sequence-based" library. The distinctive sequential use of these libraries led to the efficient discovery of new active cyclic peptides **8d**, **8g**, and **8k** that possess bioactivities comparable to T140. This orthogonal strategy may be widely useful for finding new lead compounds from natural/unnatural bioactive peptides and proteins.

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Keywords: antagonists · antiviral agents · drug design · high-throughput screening · peptides

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[6] The synthesis and evaluation of cyclic peptides are described in detail in the Supporting Information.

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