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Topochemical exploration of potent compounds using retro-enantiomer libraries of cyclic pentapeptides†

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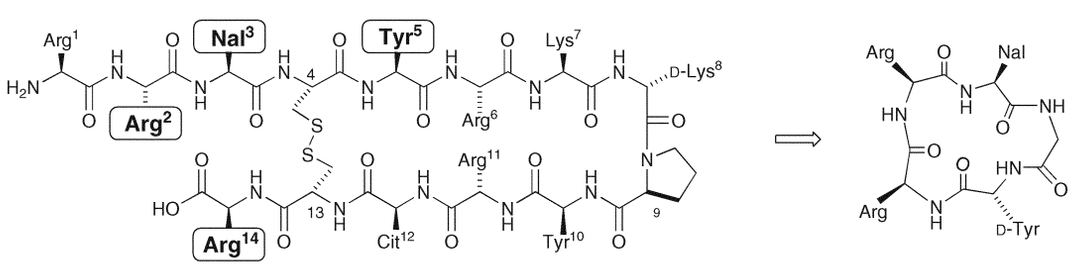
Cyclic pentapeptides have been adopted as conformationally restricted peptide templates to dispose pharmacophores of bioactive peptides. In our recent study, use of two orthogonal cyclic pentapeptide libraries involving conformation-based and sequence-based libraries containing critical residues of a bioactive peptide led to the discovery of potent downsized peptides that possess activity comparable to that of the parent peptide. The present study demonstrates that a third library consisting of retro-enantiomers (*retro-inverso* peptides) that possess not only all residues with the opposite configuration to those in the corresponding original peptide but also amino acid sequences with reversed arrangement, is important as an alternative library for rationally finding active compounds.

Introduction

Development of intelligent general methodologies based on natural peptides and proteins is an important area in drug discovery. To date, there have been ample reports on cyclic peptapeptides having biological activity,[1–6] suggesting that these are useful templates for pharmacophores of natural ligands. Recently, we reported an efficient methodology for the molecular-size reduction of bioactive peptides to cyclic pentapeptides utilizing orthogonal conformation-based and sequence-based libraries.[7] T140 is a 14-mer peptide possessing a disulfide bridge, which specifically antagonizes a chemokine receptor, CXCR4,[8,9] which is involved in cell progression and metastasis of several types of cancer,[10,11] HIV-1 entry[12] and rheumatoid arthritis.[13] Arg^2, L-3-(2-naphthyl)alanine (Nal)^3, Tyr^5 and Arg^{14} were characterized to be the critical residues of T140 (Fig. 1).[14] We selected a cyclic pentapeptide library involving two L/D-Arg, L/D-Tyr, L/D-Nal and Gly to dispose the critical side-chain functionality of T140 in proximity. Although this complete library would consist of 192 peptides, two focused libraries (total 60 peptides) involving orthogonal conformation-based and sequence-based libraries were utilized to reduce the number of synthetic peptides (Fig. 2).[7] Use of the conformation-based library (the first library) containing cyclic pentapeptides, which are expected to form βII′/γ-turn intra-structures based on Kessler's research on cyclic RGD peptides,[2,4,5] led to the discovery of lead compounds having moderate CXCR4-antagonistic activity. Subsequently, based on the amino acid sequence of the obtained leads, the sequence-based library (the second library), having diverse chirality arrays of the common amino acid sequence, cyclo(-L/D-Nal-Gly-L/D-Tyr-L/D-Arg-L/D-Arg-), was adopted to refine the discovery of potent leads, such as FC131 (Fig. 1). In terms of general topochemical consideration, the enantiomer of peptide A, peptide B, in which all amino acid residues have the opposite configuration to those in peptide A, is the antipode of peptide A. The retro-enantiomer (*retro-inverso*) of peptide A, peptide C, in which all residues have the opposite configuration to those in peptide A combined with reversed arrangement of the amino acid sequence, is a topochemical analog of peptide A: peptides A and C have very similar topology, differing by the reversed direction of amide bonds.[15–20] Peptide C has an orientation of the side-chains similar to peptide A and retains the biological activity of peptide A in some cases. This prompted us to utilize the third library consisting of retro-enantiomers of cyclic pentapeptides, which

† Electronic supplementary information (ESI) available: experimental procedures, procedures of biological assays, Table S1: characterization data of novel synthetic compounds and HPLC chromatograms for **10E**,**10K** and **10L**. See http://www.rsc.org/suppdata/ob/b4/b401485p/



T140 **FC131**

Fig. 1 Reduction of the molecular size of T140 using conformation-based and sequence-based cyclic pentapeptide libraries. Circled residues are the indispensable residues of T140.

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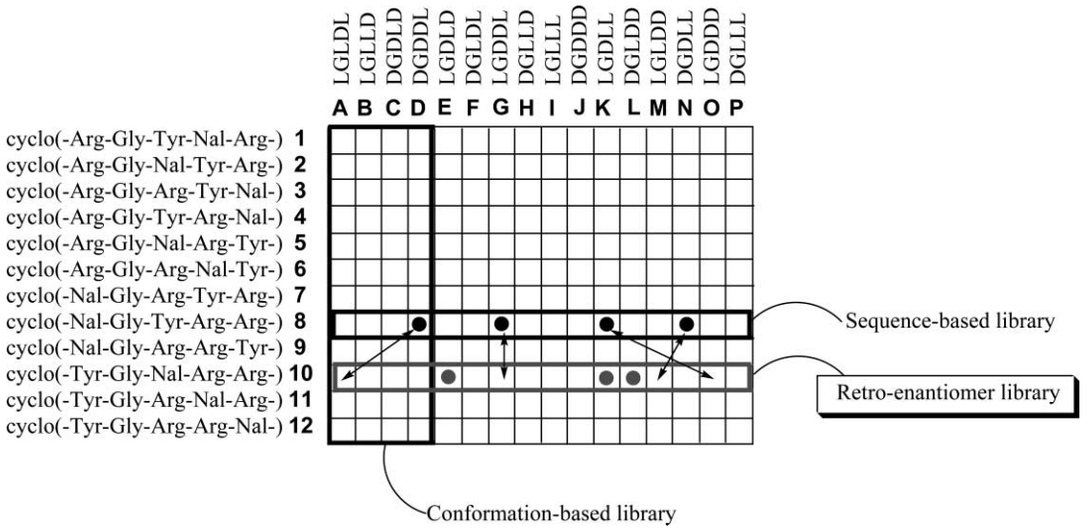

Fig. 2 A retro-enantiomer library of cyclic pentapeptides following conformation-based and sequence-based libraries. L = L-amino acid, D = D-amino acid, G = glycine. Dots indicate hit compounds with $EC_{50} < 3$ μM.

belong to the sequence-based library, to fully explore topochemical space to find other potent leads (Fig. 2). Our aim was to evaluate whether the retro-enantiomers of particular peptides have the same or different topologies, and what effect these topological differences have on their biologies. Essentially, we constructed a "reversed sequence" version of the second library (Fig. 2, line 8). The unique fact that all chirality combinations are represented in the sequence-based library, resulted in its reversed sequence version (Fig. 2, line 10) which also contains all chirality combinations. Thus, the retro-enantiomer (*retro-inverso*) analog of each second library member can be found somewhere in the reversed sequence version, which eventually forms the retro-enantiomer library. The comparative biological study on the sequence-based library and the retro-enantiomer library was also performed.

Chemistry

The retro-enantiomers of cyclic peptides, **8A**, **8B**, **8C**, **8D**, **8E**, **8F**, **8G**, **8H**, **8I**, **8J**, **8K**, **8L**, **8M**, **8N**, **8O** and **8P**, are **10D**, **10C**,**10B**, **10A**, **10E**, **10F**, **10G**, **10H**, **10J**, **10I**, **10O**, **10P**, **10N**, **10M**, **10K** and **10L**, respectively (Fig. 2). These peptides were synthesized by Fmoc-based solid-phase synthesis on a hydrazino resin followed by cleavage from the resin, cyclization with the azide procedure and deprotection, as reported previously[7] (all characterization data are shown in the ESI †).

Biological results and discussion

Since anti-HIV activity based on the inhibition of HIV-1-induced cytopathogenicity in MT-4 cells can be evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method by high throughput screening, the anti-HIV activity of synthetic compounds was determined as a representative of several biological activities against CXCR4 (described in the ESI †).[21] The anti-HIV activity and cytotoxicity of cyclic pentapeptides, **8A**, **8B**, **8C**, **8D**, **8E**, **8F**, **8G**, **8H**, **8I**, **8J**, **8K**, **8L**, **8M**, **8N**, **8O** and **8P**, and their corresponding retro-enantiomers, **10D**, **10C**, **10B**, **10A**, **10E**, **10F**, **10G**, **10H**, **10J**, **10I**, **10O**, **10P**, **10N**, **10M**, **10K** and **10L**, respectively, are shown in the left-right alignment in Table 1. Structural and spatial relationships of a representative pair of retro-enantiomers, **8P** and **10L**, are shown in Fig. 3. These side-chains are thought to be superimposed barring introduction of other complications in the topology/configuration of the structures. **8D**, **8G** and **8K** showed potent anti-HIV activity, as reported previously.[7] **10O**, the retro-enantiomer of **8K**, exhibited moderate activity while **10A** and **10G**, the retro-enantiomers of **8D** and **8G**, showed no detectable and slight activities, respectively. On the other hand, **10E**, the retro-enantiomer of **8E** that has no detectable activity, showed potent anti-HIV activity. **10K** and **10L**, the retro-enantiomers of **8O** and **8P** that have moderate activity, also showed potent activity. The retro-enantiomer library contains several potent and slightly active compounds, as well as the sequence-based library. However, retro-enantiomers of potent compounds did not always exhibit potent activity. The following two reasons can account for these results: (1) a couple of topological analogs (a cyclic pentapeptide and its retro-enantiomer) might have different spatial dispositions of the side-chain functional groups in contradiction to a general idea that retro-enantiomers are very similar topologically, differing by the reversed direction of amide bonds.[15–20] Due to the amide bond reversal, the possible intra-molecular hydrogen bond pattern of the retro-enantiomer peptide is shifted and its spatial structure differs from its parent peptide structure, according to Kessler's research on cyclo(-L-Arg-Gly-L-Asp-D-Phe-L-Val-) and its *retro-inverso* analog.[4] In cyclic pentapeptides, a retro-enantiomer is not topologically identical to the parent compound.[22] (2) The amide groups (CO–NH and NH–CO) might contribute to the anti-HIV activity through their hydrogen bonds to amino acid residues of CXCR4. The reversed direction of amide bonds seems to impair suitable interaction with CXCR4. To elucidate the details, conformational analyses will be required in the future.

We confirmed that the novel potent compounds derived from the retro-enantiomer library are CXCR4 inhibitors. **10E**, **10K** and **10L** showed significant activity in an inhibition assay of binding of stromal cell-derived factor-1 (SDF-1) to CXCR4: 26%, 40% and 37% inhibition at 1 μM of inhibitors, respectively (described in the ESI †).[23]

Conclusions

In conclusion, the third library involving retro-enantiomers that possess inversion of chiralities of all residues and amino acid sequences has proven to be important for rational discovery of active compounds. Since retro-enantiomers have a different backbone conformation and side-chain topology compared to the corresponding parent cyclic pentapeptides and the reversed direction of amide bonds, retro-enantiomers of hit compounds might not be highly active compounds. On the other hand, there possibly exist potent compounds among retro-enantiomers of poorly active compounds. Thus, in the case that hit compounds are found in the conformation-based and sequence-based libraries, it is desirable to prepare all compounds that belong to the retro-enantiomer library to discover

Table 1 Anti-HIV activity and cytotoxicity of the synthetic compounds

Compound no.[a]	EC_{50}/μM[b]	CC_{50}/μM[c]	Compound no.[a]	EC_{50}/μM[b]	CC_{50}/μM[c]
8A	11	>100	**10D**	>100	>100
8B	>100	>100	**10C**	>100	>100
8C	>100	>100	**10B**	>100	>100
8D	1.0	62	**10A**	>100	>100
8E	>100	48	**10E**	2.9	>100
8F	>100	48	**10F**	11	80
8G	0.34	>100	**10G**	75	>100
8H	>100	>100	**10H**	7.9	>100
8I	3.5	>100	**10J**	9.6	>100
8J	66	>100	**10I**	65	>100
8K	0.088	>100	**10O**	9.6	>100
8L	>100	>100	**10P**	63	>100
8M	12	>100	**10N**	10	>100
8N	1.7	>100	**10M**	9.1	>100
8O	9.8	>100	**10K**	2.0	>100
8P	11	>100	**10L**	1.7	>100
T140	0.026	>10			
AZT	0.014	260			

[a] Each retro-enantiomer pair is shown (*e.g.* **8A** and **10D**). [b] EC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. [c] CC_{50} values are based on the reduction of the viability of mock-infected MT-4 cells. Since the cytotoxicity of T140 was previously evaluated as CC_{50} > 40 μM, further estimation at high concentrations was omitted in this study. All data are the mean values for at least three independent experiments.

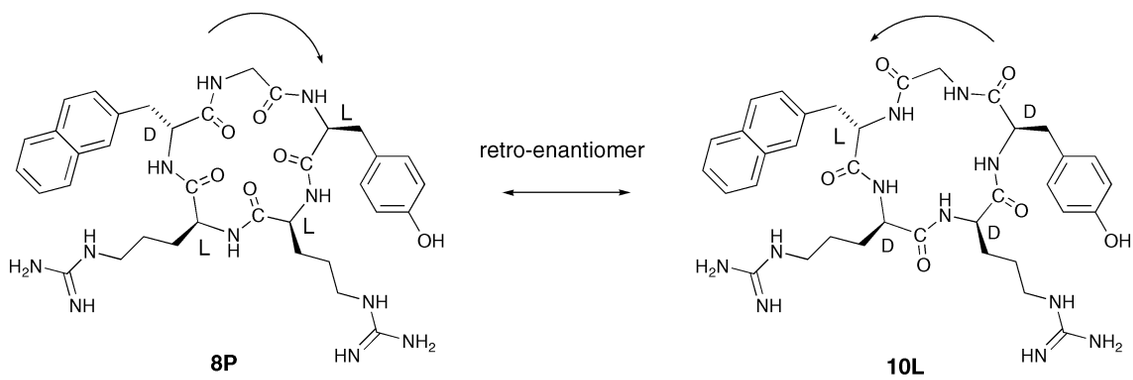

Fig. 3 Structures of a representative pair of retro-enantiomers, **8P** and **10L**. Arrows indicate the direction of peptide chains (N to C).

other potent compounds. A combined use of conformation-based, sequence-based and retro-enantiomer libraries will be a useful strategy for rapid characterization of hit compounds.

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