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Design, Synthesis, and Biological Activity of a Cyclic Peptide: An Inhibitor of HIV-1 Tat–TAR Interactions in Human Cells

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Abstract—Replication of human immunodeficiency virus type 1 (HIV-1) requires specific interactions of Tat protein with the *trans*-activation responsive region (TAR) RNA, a 59-base stem-loop structure located at the 5′-end of all HIV mRNAs. A number of cyclic peptides are known to possess antibiotic activity and increased biological stability. Here we report the design, synthesis, and biological activity of a cyclic peptide (**2**), which inhibits transcriptional activation by Tat protein in human cells with an IC_{50} of ≈40 nM. Cyclic peptides that can target specific RNA structures provide a new class of small molecules that can be used to control cellular processes involving RNA–protein interactions in vivo.

Introduction

Protein–nucleic acid interactions are involved in many cellular functions such as transcription, RNA splicing, and translation. Small peptides with unnatural backbones that can bind with high affinity to a specific sequence or structure of nucleic acids and interfere with protein–nucleic acid interactions would provide useful tools in molecular biology and medicine. Recently, minor-groove-binding polyamide ligands have been designed for sequence-specific recognition of DNA.[1–4] In contrast to DNA, RNA molecules can fold into extensive structures containing regions of double-stranded duplex, hairpins, internal loops, bulged bases, and pseudoknotted structures.[5] The complexity of RNA structure makes it difficult to design ligands for sequence-specific RNA-recognition. Three-dimensional structures of RNA create binding sites for specific interactions with proteins. One example of such interactions is the mechanism of *trans*-activation of human immunodeficiency virus type 1 (HIV-1) gene expression that requires the interaction of Tat protein with the *trans*-activation responsive region (TAR) RNA, a 59-base stem-loop structure located at the 5′-end of all nascent HIV-1 transcripts.[6] Inhibition of Tat–TAR interactions is a potential approach for anti-HIV therapeutics. We have recently synthesized an encoded combinatorial tripeptide library of 24,389 possible members from D- and L-α amino acids on TentaGel resin.[7] Using on-bead screening we have identified a small family of mostly heterochiral tripeptides capable of structure-specific binding to the bulge loop of TAR RNA.[7] In vitro binding studies reveal stereospecific discrimination when the best tripeptide ligand is compared to diastereomeric peptide sequences. In addition, the most strongly binding tripeptide (**1**) was shown to suppress transcriptional activation by Tat protein in human cells with an IC_{50} of ∼50 nM.[7]

Efforts to develop cyclic peptide based drugs increased manifold after the antibiotic gramicidin S was found to be a cyclic decapeptide. Many antibiotics and toxins are also known to be cyclic amino acid sequences. Cyclization of amino acid sequences results in increased metabolic stability, potency, receptor selectivity and bioavailability.[8–10] Cyclic peptides have been used as synthetic immunogens,[11] transmembrane ion channels,[12] potent vaccine for diabetes,[13] antigens for Herpes Simplex Virus,[14] inhibitor against α-amylase,[15] pancreatic tripsin,[16] integrin $\alpha_v\beta_3$,[17] and as protein stabilizer.[18] Side-chain to side-chain lactamization has been utilized to improve receptor selectivity in enkephalins,[19] cholecystokinin,[20] melanotropin,[21] tachykinin,[22] RGD-dependent

Abbreviations: BOP: benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate; CAT: chloramphenicol acetyl transferase; DCC: *N*,*N*′-Dicyclohexylcarbodiimide; DIEA: diisopropylethylamine; Dmab: 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino}benzyl; DMAP: 4-Dimethylaminopyridine; DMF: *N*,*N*-dimethylformamide; Fmoc: 9-fluorenylmethoxycarbonyl; HBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBT: *N*-hydroxybenzotriazole; TFA: trifluoroacetic acid; TIS: triisopropylsilane.

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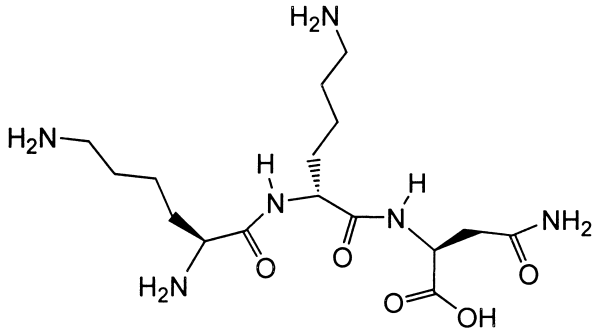
adhesion proteins,[23] and many other biological systems.[24,25] If designed carefully without causing drastic changes in the conformation of active peptides, the rigid geometry of the cyclic peptides enhances the binding affinity towards a selected target molecule compared to their linear counterparts.

Results and Discussion

Recently, we found that a tripeptide (**1**) isolated from a combinatorial library binds TAR RNA and effectively suppresses activation of HIV-1 gene expression by Tat protein[7] (Fig. 1).

To improve pharmacokinetic properties of the tripeptide (**1**), we planned to synthesize a cyclic peptide derivative (**2**) based on the tripeptide (**1**) structure. In general, homodetic cyclic peptides are made by head-to-tail, N-terminal to side-chain or side-chain to side-chain coupling methods on solid supports.[26] Our modeling studies suggested that the cyclic peptide derived from side-chain to side-chain coupling method would least affect the conformation of our linear tripeptide (**1**),



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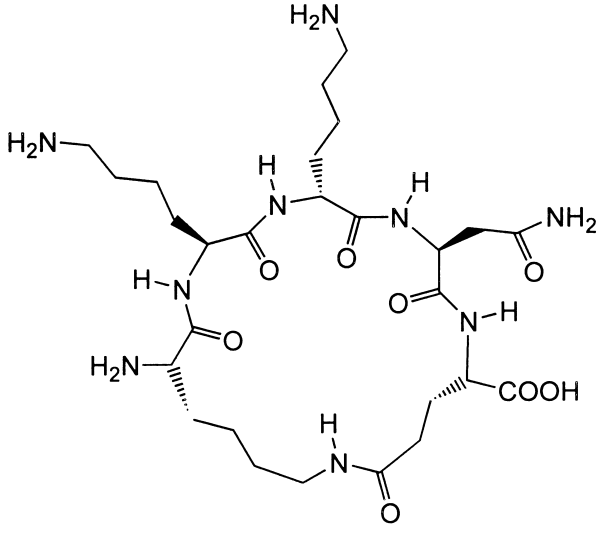

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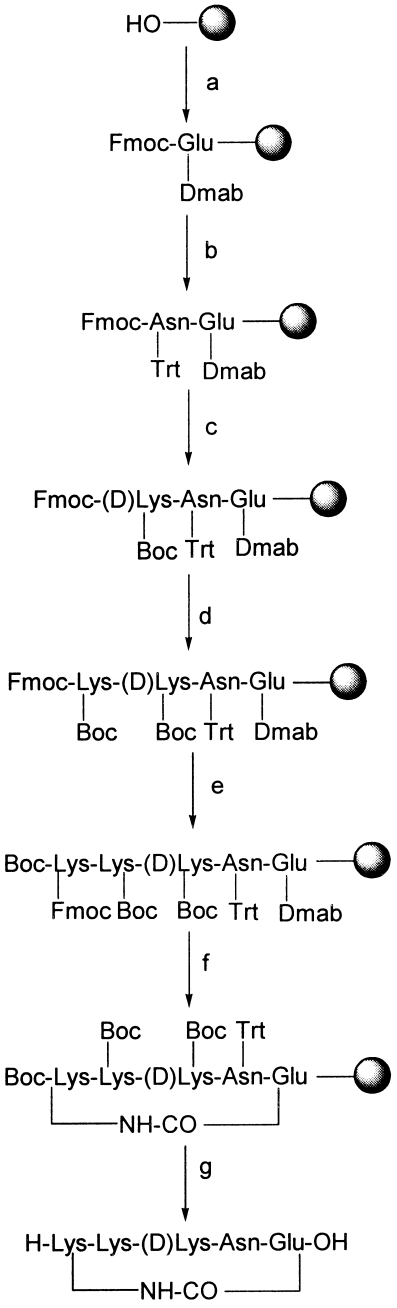
Figure 1. The structure of tripeptide (**1**) and cyclic peptide (**2**).



a. Fmoc-Glu(Dmab)-OH, DCC, DMAP

b. 1. 20% piperidine/DMF; 2. Fmoc-Asn(Trt)-OH, HBTU/HOBT/DMF-DIEA

c. 1. 20% piperidine/DMF; 2. Fmoc-(D)Lys(Boc)-OH, HBTU/HOBT/DMF-DIEA

d. 1. 20% piperidine/DMF; 2. Fmoc-Lys(Boc)-OH, HBTU/HOBT/DMF-DIEA

e. 1. 20% piperidine/DMF; 2. Boc-Lys(Fmoc)-OH, HBTU/HOBT/DMF-DIEA

f. 1. 20% piperidine/DMF; 2. 2% hydrazine/NMP; 3. BOP/NMP-DIEA

g. TFA:TIS:Water

Scheme 1. The synthesis of cyclic peptide **2**.

therefore, we designed a synthetic method based on this strategy. In previous studies, most of the cyclic peptides have been prepared either totally in solution,[27,28] or by assembling the linear peptide on solid support followed by cleavage and cyclization in solution. We synthesized our peptide and performed cyclization on the solid support. The advantages of performing peptide cyclizations on solid phase over solution phase include favored intramolecular cyclization, completion of the reaction driven by large excess of reagents and removal of unreacted reagents by filtration and washing.[26] Fmoc peptide chemistry was used to synthesize the cyclic peptide utilizing the strategy of selective deprotection of glutamic acid and lysine side chains. Placing the trimer peptide (**1**) in the middle, glutamic acid at the C-terminal and lysine at the N-terminal were incorporated as cyclization handles. Thus, the pentapeptide, Boc-Lys(Fmoc)-Lys(Boc)-(D)Lys(Boc)-Asn(Trt)-Glu(Dmab) was synthesized on Wang resin by the standard Fmoc chemistry (Scheme 1).

As shown in Scheme 1, intramolecular cyclization was accomplished through the N-terminal Lys and C-terminal Glu side chains. Therefore, it was necessary to selectively remove protecting groups from the N-terminal Lys and C-terminal Glu while keeping the other functional groups protected. We achieved selective deprotection by using the N^{α}-Boc and N^{ϵ}-Fmoc protected N-terminal Lys and Dmab protected Glu. The Fmoc group was removed by 20% piperidine in DMF followed

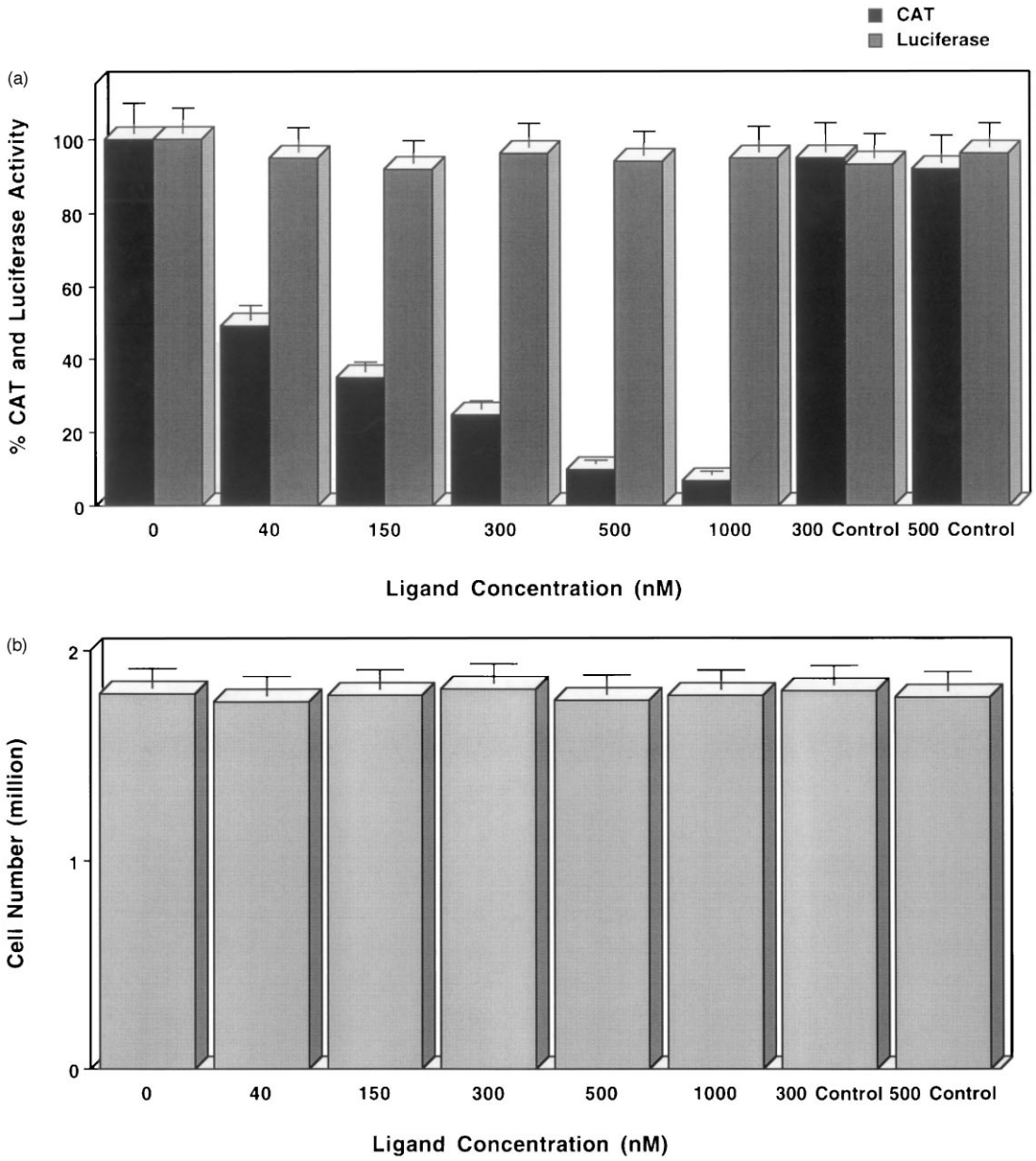


Figure 2. Inhibition of Tat *trans*-activation by the cyclic peptide (**2**) in vivo. CAT activity expressed from the integrated HIV-1 LTR of HL3T1 cells with increasing amounts of the cyclic peptide (**2**) is shown. Monitoring luciferase activity was a control experiment to quantify the transfection efficiency and non-specific inhibition of gene expression by the addition of the cyclic peptide (**2**). Transfection and enzymatic activity (CAT and Luciferase) assays were performed as described previously .[30,31] CAT and luciferase activities were measured from three experiments and normalized to 100%.

by the removal of Dmab by treatment with 2% hydrazine. Side-chain to side-chain cyclization was carried out by activating the glutamic acid carboxyl group with BOP in the presence of DIEA. After overnight reaction at room temperature, the resin gave a negative Kaiser test indicating the completion of the cyclization. Cleavage from the resin and deprotection was effected with a mixture containing TFA:TIS:water (95:2.5:2.5). After ether precipitation, the crude peptide was purified by reverse-phase HPLC using semi-preparative C-8 column. The mass of fully deprotected and purified peptide was confirmed by MALDI/TOF mass spectrometry. Calculated mass is 628.76 $[M+H]^+$, observed 628.72 $[M+H]^+$.

To determine whether the cyclic peptide (**2**) could be used to control HIV-1 gene expression in vivo, we used HL3T1 cells, a HeLa cell line derivative containing an integrated HIV-1 LTR promoter and CAT reporter gene.[29] We added different amounts of the cyclic peptide (**2**) during transfection of pSV2-Tat[30] and pAL[31] plasmids into HL3T1 cells. Plasmids pSV2Tat and pAL express the first exon of Tat protein and luciferase enzyme, respectively. Luciferase reporter gene provides an internal control. Transfection of HeLa cells with pSV2Tat enhanced transcription as determined by CAT activity. As shown in Figure 2, increasing amounts of the cyclic peptide (**2**) resulted in a decrease of CAT activity while luciferase activity was not affected. In the presence of 500 nM concentrations of cyclic peptide (**2**), more than 90% of Tat *trans*-activation was inhibited. To rule out the possibility that the observed inhibition of *trans*-activation could be due to some nonspecific toxicity of cyclic peptide (**2**) or reduction of the pSV2Tat plasmid uptake, transcription of luciferase gene was monitored (Fig. 2). Transcription of luciferase gene was not affected by cyclic peptide (**2**). Further control experiments showed that a scrambled tripeptide had no inhibitory effect on Tat transactivation. Cell viability assays showed that cyclic peptide (**2**) treatment was not toxic to the cells (Fig. 2b). These results demonstrate that the cyclic peptide (**2**) inhibits Tat-TAR interactions in vivo. We have described the design and synthesis of a cyclic peptide that can be used to design small molecules for controlling gene expression in vivo. These results also establish an example of the application of cyclic peptides as artificial regulators of cellular processes involving RNA–protein interactions in vivo.

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