Bioorganic & Medicinal Chemistry Letters 27 (2017) 5378–5381

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# Structure-activity relationship study of Aib-containing amphipathic helical peptide-cyclic RGD conjugates as carriers for siRNA delivery

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ARTICLE INFO

*Article history:*
Received 23 August 2017
Accepted 8 November 2017
Available online 10 November 2017

*Keywords:*
Amphipathic helical peptide
α-Aminoisobutyric acid (Aib)
Cell-penetrating peptide (CPP)
RGD
siRNA

ABSTRACT

The conjugation of Aib-containing amphipathic helical peptide with cyclo(-Arg-Gly-Asp-D-Phe-Cys-) (cRGDfC) at the C-terminus of the helix peptide (**PI**) has been reported to be useful for constructing a carrier for targeted siRNA delivery into cells. In order to explore structure–activity relationships for the development of potential carriers for siRNA delivery, we synthesized conjugates of Aib-containing amphipathic helical peptide with cRGDfC at the N-terminus (**PII**) and both the N- and C-termini (**PIII**) of the helical peptide. Furthermore, to examine the influence of **PI** helical chain length on siRNA delivery, truncated peptides containing 16 (**PIV**), 12 (**PV**), and 8 (**PVI**) amino acid residues at the N-terminus of the helical chain were synthesized. **PII** and **PIII**, as well as **PI**, could deliver anti-luciferase siRNA into cells to induce the knockdown of luciferase stably expressed in cells. In contrast, all of the truncated peptides were unlikely to transport siRNA into cells.



α-Aminoisobutyric acid (Aib) is a non-coded amino acid whose chemical structure differs from that of alanine in that the α-hydrogen of alanine is substituted with a methyl group, i.e., Aib has an α, α-dimethyl group. The naturally occurring Aib-containing peptide, peptaibol, is an antibiotic peptide isolated from fungus and its structure and biological activity have been studied in a variety of fields.[1] Aib-containing peptides prefer to adopt α- and $3_{10}$-helical structures due to the steric hindrance of the α,α-dimethyl groups of Aib.[2] Furthermore, the incorporation of Aib into a peptide enhances the interaction of the peptide with cell membrane,[3] the permeability of the peptide into cells,[4] the resistance of the peptide to proteolytic enzymes,[5] and antibiotic activity of the peptide.[6]

We have been engrossed in the development of carriers of oligonucleotides, such as small interfering RNA (siRNA), for nucleic acid therapeutics by using Aib-containing cell-penetrating peptide (CPP). siRNA regulates gene expression by inhibiting specific messenger RNA (mRNA) translation. This process, known as RNA interference (RNAi), is a promising therapeutic tool for infections, cancers, and genetic disorders.[7] To achieve the targeted delivery of siRNA, five conjugates of Aib-containing amphipathic helical peptides based on the sequence of MAP(Aib) with mono-, di-, and trivalent cyclo(-Arg-Gly-Asp-D-Phe-Cys-) (cRGDfC), which is known to bind to $\alpha_v\beta_3$ integrin, at several positions of the helical peptide have been designed and synthesized.[8] Among those conjugates, the monovalent cRGDfC conjugating at the C-terminus of the helical peptide (**PI**, Table 1) enhances the targeted delivery of siRNA into A549 cells. In order to construct more potential carriers, we conducted structure-activity relationship studies based on the structure of **PI** and synthesized the conjugates of the Aib-containing helical chain with cRGDfC at the N-terminus (**PII**) and both the N- and C-termini (**PIII**) of the helical peptide. Moreover, to examine the influence of **PI** helical chain length on siRNA delivery, we synthesized truncated peptides **PIV**, **PV**, and **PVI** at the N-terminus of **PI**, in which 4, 8, and 12 amino acid residues corresponding nearly one, two, and three turns of the α-helix were removed, respectively. We describe herein the syntheses of these Aib-containing peptide-cRGDfC conjugates and the cellular uptake of the peptide-RGD conjugate/siRNA complexes, followed by the knockdown effects, in comparison with those of **PI**.

The peptide-cRGDfC conjugates were synthesized according to our previously reported method.[8,9] The Aib-containing helical chains of **PII** and **PIII** and the truncated chains of **PI** (**PIV**–**PVI**) were synthesized by Fmoc-based solid-phase synthesis, and the Cys residue was inserted into the N-terminus (**PII**), the C-terminus (**PIV**–**PVI**), or both the N- and C-termini (**PIII**). The conjugation of the Aib-containing helical chain with cRGDfC was accomplished through the formation of a disulfide linkage. The linear peptide chain harboring a thiol group was reacted with 3-nitro-2-pyri-

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https://doi.org/10.1016/j.bmcl.2017.11.018
0960-894X/

**Table 1**
Amino acid sequences of cRGDfC-conjugated Aib-containing peptides.[a]

| Peptide | Sequence[b] |
|---|---|
| **PI** | acetyl-KLULKLULKULKAULKLUG***C(cRGDfC)***-$NH_2$ |
| **PII** | acetyl-***C(cRGDfC)***KLULKLULKULKAULKLUG-$NH_2$ |
| **PIII** | acetyl-***C(cRGDfC)***KLULKLULKULKAULKLUG***C(cRGDfC)***-$NH_2$ |
| **PIV** | acetyl-KLULKULKAULKLUG***C(cRGDfC)***-$NH_2$ |
| **PV** | acetyl-KULKAULKLUG***C(cRGDfC)***-$NH_2$ |
| **PVI** | acetyl-AULKLUG***C(cRGDfC)***-$NH_2$ |

[a] Aib-containing peptides and cRGDfC [cyclo(-Arg-Gly-Asp-D-Phe-Cys-)] were conjugated through the formation of a disulfide bond between the thiol group of Cys in the helical chains and that of Cys in cRGDfC.
[b] Letter U in the sequence: α-aminoisobutyric acid (Aib).

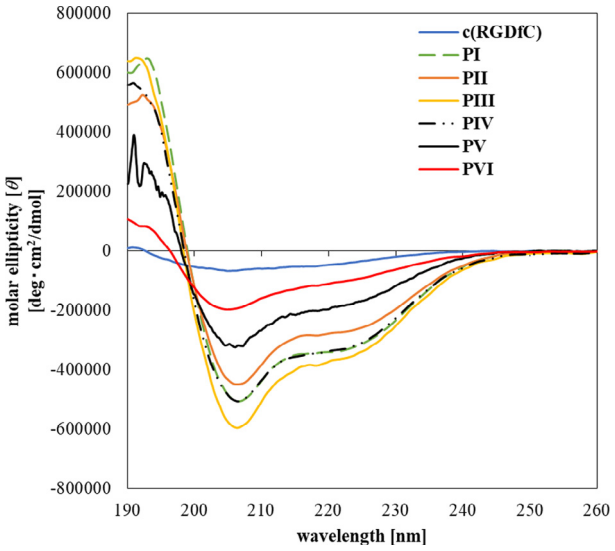


**Fig. 1.** CD spectra of cRGDfC-conjugated Aib-containing helical peptides in 2,2,2-trifluoroethanol.

dylthio-activated cRGDfC to give peptide-cRGDfC conjugates (**PII**–**PVI**) in 40–60% yields.

It has been recently reported that helical peptide structures facilitate the passage of peptides through the cell membrane.[10] **PI** in 2,2,2-trifluoroethanol solution also forms mainly an α-helical structure.[8] Firstly, we analyzed the secondary structures of **PII**–**PVI** on the basis of their CD spectra. Fig. 1 shows the CD spectra of the cRGDfC-conjugated Aib-containing peptides. All of the peptides gave rise to double minimum bands around 208 and 222 nm (Fig. 1), suggesting that their preferred secondary structures are right-handed helices, and even **PVI** containing 8 amino acid residues in the peptide chain forms a helix structure. Analyses of the CD curves of **PI**, **PII**, and **PIII** having long chains revealed that the molar ellipticity of those peptides slightly differed according to the position and the number of cRGDfCs in the helical chains. In contrast, the molar ellipticity decreased as the number of amino acid residues in the peptide chain decreased (**PIV**, **PV**, and **PVI**).

Next, to view directly the cellular uptake of the peptide/AlexaFluor488-labeled siRNA complexes, confocal laser-scanning microscopy (CLSM) experiment was performed on viable human lung carcinoma A549 cells. siRNA used in all experiments consists of 21 nt and targets the luciferase gene.[11] The peptide/siRNA

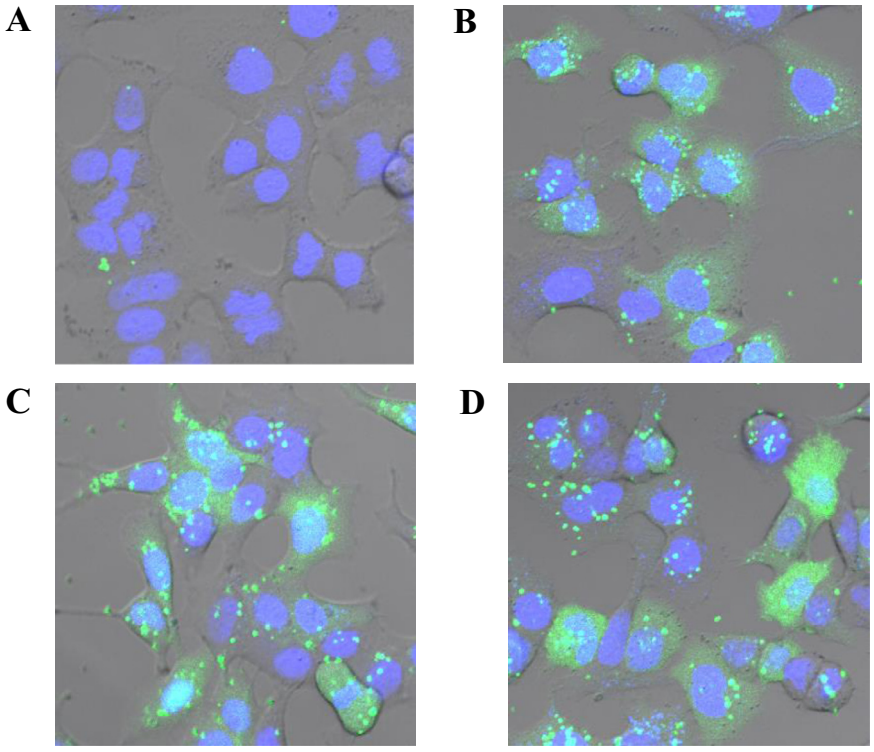


**Fig. 2.** Cellular uptake of peptide-cRGDfC/siRNA complexes into A549 cells. A549 cells were incubated with AlexaFluor488-labeled siRNA alone (A), and peptide/AlexaFluor488-labeled siRNA complexes (B: **PI**; C: **PII**; D: **PIII**) at the concentrations of 2.5 μM/25 nM for 6 h. Fluorescence images were merged with differential interference contrast images. Nuclei were stained with Hoechst 33342. Green and blue colors show fluorescence-labeled siRNA and nuclei, respectively.