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A preorganized β-amino acid bearing a guanidinium side chain and its use in cell-penetrating peptides

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A cyclic β-amino acid (APCα+) bearing a side-chain guanidinium group has been developed. The APCα+ residue was incorporated into an α/β-peptide based on the Tat(47-57) fragment, leading to an oligomer with substantial helicity in methanol that enters HeLa cells much more readily than does the corresponding Tat α-peptide.

Cell-penetrating peptides (CPPs) can deliver various molecules, including drugs, proteins, and DNA, into cells.1-5 To date, a variety of CPPs has been developed, many of which are rich in arginine, including oligo-arginines (Arg)n and the HIV Tat(47-57) peptide.6-11 The guanidino moiety of Arg appears to play a crucial role in the cell entry process,6-9 and the internalization of those CPPs occurs via endocytosis as one of the major routes of the endocytic uptake. However, in the presence of counterions such as pyrenebutyrate, the CPPs such as (Arg)n were internalized by direct translocation through plasma membranes.12 Indeed, many unnatural oligomers that present multiple guanidino side chains, including peptoids,13 α-peptides,14 β-peptides,15-18 and oligocarbamates19 can enter cells. Previously it was shown that the efficiency of cell entry by β-peptides can be enhanced by increasing helicity.20,21 This conformational stabilization was achieved by using cyclic β residues. Here we extend this concept to oligomers with a heterogeneous backbone, containing both α- and β-amino acid residues (*α/β-peptides*).

α/β-Peptides containing 25-33% β residues that are evenly distributed among the α residues have been shown to adopt a helical conformation that is very similar to the well-known α-helix formed by pure α-peptide backbones.21-24 This type of *α/β*-peptide helix can be stabilized by use of β residues with a five-membered ring constraint and *trans* disposition of the amino and carboxyl groups. Two specific examples of this type of constrained β residue have been widely explored, *trans*-2-aminocyclopentane-1-carboxylic acid (ACPC) and *trans*-4-amino pyrrolidine-3-carboxylic acid (APC). The pyrrolidine ring amino group of APC can be used to attach other peripheral units.25 Here we introduce a new derivative of APC in which the pyrrolidine nitrogen forms part of a guanidino group (APCα+). We describe the preparation of a protected β-amino acid that can be used to incorporate APCα+ residues via solid-phase synthesis. In addition, we evaluate the cell-penetrating ability of an APCα+ containing *α/β*-peptide based on Tat (47-57),26,27 and we show that the helicity provided by the new β residue correlates with enhanced activity.

Fig. 1 Chemical structures of (S,S)-APC and (S,S)-APCα+

The synthetic route to Fmoc-(S,S)-APCα+-OH (3) is shown in Scheme 1. Fmoc-(S,S)-APCβc-OH (1) was prepared by the reported method.28 The side-chain Boc group was removed under acidic conditions to give Fmoc-(S,S)-APC-OH (2) in quantitative yield. Guanidinylation of the side-chain amino group in 2 was accomplished by treatment with 3 equivalents of N,N′-di-Boc-N,N′-triflylguanidine under basic conditions.29

Scheme 1 Synthesis of Fmoc-(S,S)-APCα+-OH (3).

Building block 3 was used to prepare an α/β-peptide analogue of Tat(47-57) containing APCα+ residues at positions 3, 7 and 10, to generate Tat-1. Two analogous α/β-peptides were prepared as well, Tat-2, which has β-Arg at positions 3, 7 and 10, and Tat-3, which has APC at these three positions. Two versions of the α-peptide Tat and the three α/β-peptides were synthesized, one set bearing N-terminal fluorescein (6FAM).
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having (responsible for entry in this case. which suggests that ma
amiloride inhibited cell entry by pathways other than the three we inhibited. In contrast,
suggests that cell entry by these two molecules involves endocytosis,
inhibitors on entry of *p<0.05, **p<0.01.
Fig. 3
We examined the abilities of F-Tat and the three analogous α/β-peptides F-Tat-1-3 to enter HeLa cells. After incubation of cells with each compound for 2 h at 37 °C, the cells were lysed, and the fluorescence intensity of the lysate was measured. The α/β-peptides entered cells more efficiently than the α-peptide F-Tat. In particular, F-Tat-1, containing (S,S)-APC\(^\text{C}^{\text{19}}\) (Z\(^3\)) residues, showed a 65-fold higher extent of entry relative to F-Tat (Figure 3).

![Fig. 2 Sequences of Tat derivatives Tat, Tat-1-3.](image)

![Fig. 3 Cellular uptake of F-Tat, F-Tat-1-3 at concentrations of 1 µM (incubation time: 2 hr). Error bars represent standard deviation, n = 5. *p<0.05, **p<0.01.](image)

Figure 4 shows the effects of three endocytosis pathway inhibitors on entry of F-Tat and F-Tat-1-3 into HeLa cells.\(^{31-34}\) Amiloride is an inhibitor of macropinocytosis,\(^{33,34}\) chlorpromazine is an inhibitor of clathrin-mediated endocytosis,\(^32,34\) and filipin is an inhibitor of caveolae-mediated endocytosis.\(^32,34\) None of these agents hindered the uptake of α-peptide F-Tat or F-Tat-2 (β residues = β\(^1\)-hArg) which suggests that cell entry by these two molecules involves pathways other than the three we inhibited. In contrast, amiloride inhibited cell entry by F-Tat-3 (β residues = APC), which suggests that macropinocytosis is at least partially responsible for entry in this case. The uptake level of F-Tat-1 having (β residues = APC\(^\text{C}^{\text{20}}\)) was inhibited by both amiloride and chlorpromazine, which suggests that both macropinocytosis and clathrin-mediated endocytosis contribute to cell entry in this case. Low temperature (4°C) incubation almost completely inhibited the internalization of F-Tat and F-Tat-1-3 (Figure S2), suggesting that those peptides were mainly internalized into the cells via endocytosis.

![Fig. 4 Effects of inhibitors on internalization of F-Tat, F-Tat-1-3 at concentrations of 1 µM (incubation time: 2 hr). Error bars represent standard deviation, n = 5. *p<0.05, **p<0.01.](image)
Fig. 5 Intracellular distribution of F-Tat, F-Tat-1-3 (green) at concentrations of 1 µM (incubation time: 2 hr). The acidic late endosomes/lysosomes were stained with LysoTracker Red (red) and the nuclei were stained with Hoechst 33342 (blue). (A) CLSM images of HeLa cells treated with Tat peptide derivatives. The scale bars represent 20 µm. (B) Quantification of Tat peptide derivatives colocalized with LysoTracker Red. Error bars in the graph represent standard deviation, n = 15. **p<0.01.

The conformations of Ac-Tat and Ac-Tat-1-3 were analyzed via circular dichroism (CD) in MeOH (Figure 6). The α/β-peptides containing cyclic β residues (Ac-Tat-1 and Ac-Tat-3) showed a minimum at around 207 nm, which is similar to the minima observed in CD spectra of α/β-peptides that form right-handed (P) helices (Figure 6). Furthermore, addition of 10 mM SDS has little effect on the helical structures of Tat-1 and Tat-3 (Figure S3). On the other hand, Ac-Tat and Ac-Tat-2, which contain exclusively acyclic residues, show weaker CD signatures that suggest little or no folding.

Conclusions

Tat-1 with (S,S)-APC^Cu (Z), Tat-2 with β^3-Arg (R), and Tat-3 with (S,S)-APC (Z) showed higher cell penetrating activities than the α-peptide Tat in HeLa cells. In particular, Tat-1 showed a 65-fold higher permeability than Tat. These results indicate that stabilization of a helical secondary structure is synergistic with the presence of guanidino groups in promoting cell entry. It is noteworthy that Tat-3 which contains (S,S)-APC residues and only three guanidino groups (on the three Arg residues) enters cells relatively efficiently. The new cyclic β-amino acid residue APC, which combines the advantages of conformational preorganization and a side-chain guanidino group should prove to be a useful tool for further development of biologically active β-peptides and α/β-peptides.

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Notes and references


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