Communication

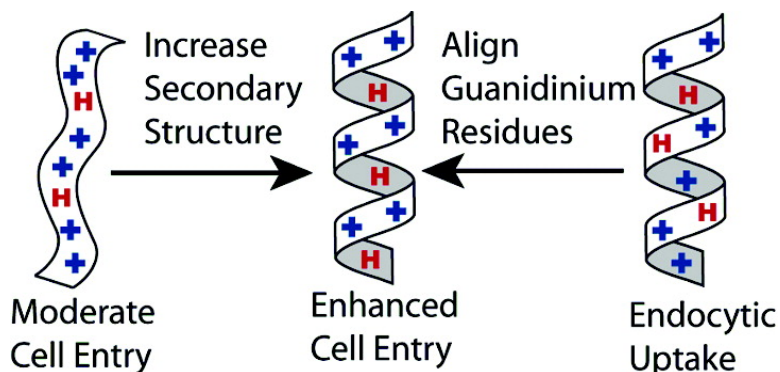# Effects of Conformational Stability and Geometry of Guanidinium Display on Cell Entry by β-Peptides

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# Effects of Conformational Stability and Geometry of Guanidinium Display on Cell Entry by β-Peptides

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Peptides rich in Arg residues can enter the cytoplasm and ultimately the nucleus of a living cell from the external medium.[1,2] Cell entry has also been demonstrated for unnatural molecules that display multiple guanidinium groups.[2e,3] Engineered cell entry may be useful for drug delivery,[4] but the mechanism is not yet clear and may vary as a function of entry agent, cell type, and/or other factors. As a step toward understanding how molecular structure influences cell entry activity, we have explored the effects of conformational stability and geometry of guanidinium display on this behavior.

α-Amino acid oligomers of ≤20 residues are very flexible, and it is difficult or impossible to generate sets of short α-peptides that manifest a wide range of conformational stabilities while being comparable in other characteristics. In contrast, conformational stability can be easily varied among short β-amino acid oligomers. β-Peptides containing exclusively β³-residues can adopt the 14-helix secondary structure (defined by 14-membered ring hydrogen bonds between backbone groups, C=O(i)- -H−N(i-2)).[5] For most β³-sequences, however, 14-helicity is observed only in structure-promoting solvents, such as methanol, and not in aqueous solution.[5c] The preorganized *trans*-2-aminocyclohexanecarboxylic acid (ACHC) residue has a much stronger 14-helical propensity than do β³-residues.[6] ACHC and β³-homovaline (β³hVal) have comparable net hydrophobicities,[6c] so comparison of β-peptides in which these two residues are swapped allows one to examine the impact of 14-helix stability on other molecular properties of interest.
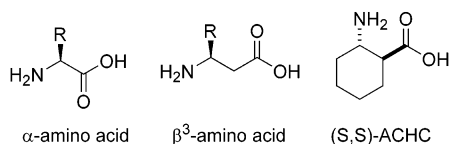
β-Peptides 1−4 contain repeating triads (X-β³hArg-β³hArg), where the choice of X is intended to influence 14-helix stability. β-Peptide 1 is designed to form a very stable 14-helix in aqueous solution (X = (S,S)-ACHC), with the six β³hArg residues clustered along one side (Figure 1). β-Peptide 2 (X = β³Val) is expected to have diminished 14-helical propensity relative to that of 1.[6c] In 3, the X residues are βhGly, which is even more flexible than β³hVal; 14-helical folding is therefore unlikely for 3. The configurational switch of the ACHC residues in 4 (X = (R,R)-ACHC) relative to those of diastereomer 1 (X = (S,S)-ACHC) should prevent 14-helix formation for 4. Overall, the likelihood of 14-helix formation should decrease dramatically from 1 to 4.

β-Peptide 5 should form a 14-helix in which the β³hArg residues are distributed around the periphery rather than segregated along one side, as in sequence isomer 1 (Figure 1). For both 1 and 5, the 14-helix conformation is expected to be highly populated in aqueous

Series a: R = β³ hTyr
Series b: R = Fluorescein
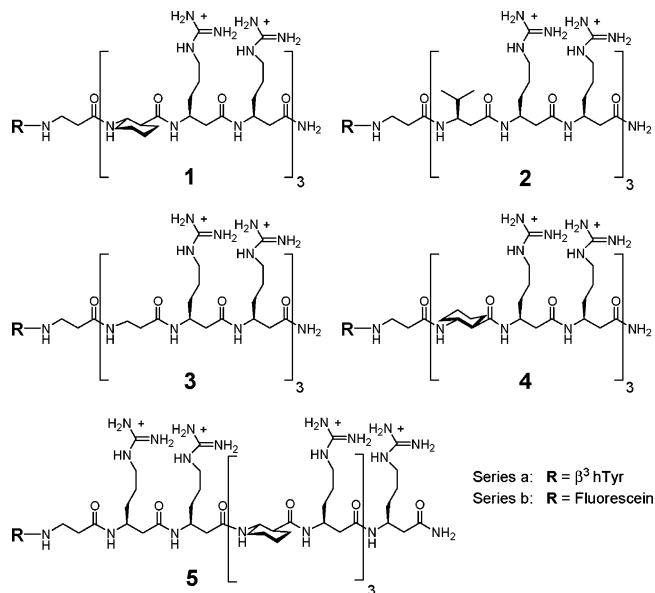
solution. Thus, comparing 1 and 5 should indicate whether cell entry activity is affected by the spatial arrangement of guanidinium groups. Circular dichroism (CD) data indicate that 1a−5a display the expected folding behavior.[7]

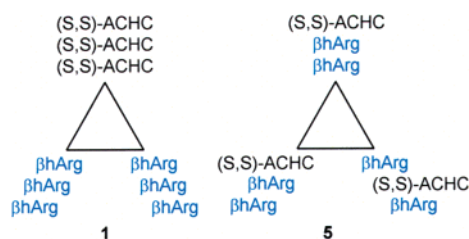
Figure 1. A 14-helical wheel diagram of 1 and 5, showing the differential display of guanidinium residues about the helical axis.

β-Peptides 1b−5b bear an N-terminal 6-carboxyfluorescein unit to allow evaluation of cell entry behavior by fluorescence microscopy. β-Peptide 1b entered cells to a greater extent than did 2b−5b (Figure 2A). Cell entry by 1b seemed to peak within 60 min, with ∼70% of the HeLa cells showing nuclear staining. Entry by 1b was completely blocked in the presence of NaN₃, which implies an energy-dependent uptake process.[8] Incubation of cells with 1b in the presence of NH₄Cl led to only endosomal uptake (no green fluorescence in the nucleus; see Supporting Information). β-Peptide 2b displayed modest cell entry, but only after 60 min, whereas the less structured 3b did not result in significant nuclear staining until 120 min. β-Peptides 4b−5b appeared to enter ca. 12−18% of the cells, although the behavior varied between 15 and 120 min. Because of this erratic variation, we regard ≤15% uptake as a nonspecific background effect (dashed line in Figure 2A).
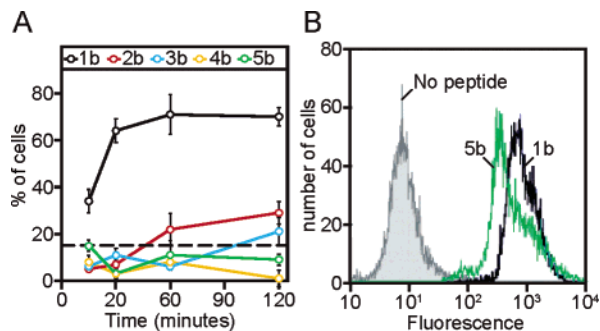
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Figure 2. (A) Internalization of **1b**−**5b** over time; 8 µM β-peptide was incubated with cells for 15, 30, 60, or 120 min. The cells with nuclear staining were counted. Each data point is an average of at least four separate experiments; 70−100 cells were counted per experiment. The error bars denote standard error.[9] Data below the dashed line (15%) are considered insignificant as variations between 0 and 15% occur over time. (B) Surface binding comparison of β-peptides **1b** and **5b** by flow cytometry. Cells treated with NaN$_3$ were incubated with 8 µM peptide for 10 min at 37 °C, washed, and analyzed by flow cytometry. β-Peptides **2b**−**4b** show similar histograms to that of **5b** (see Supporting Information).

Previous studies have indicated that binding to the cell surface is a prerequisite for entry by Arg-rich α-peptides[10] and their cargo-conjugates.[4c,11] We used flow cytometry to probe for differences in binding to the surface of HeLa cells among **1b**−**5b**. All five hexacationic β-peptides bind to the cell surface, with ca. 2-fold higher binding for **1b** relative to that of **2b**−**5b** (Figure 2B and Supporting Information). Because this difference is small, we conclude that the observed variations in cell entry are *not* primarily caused by differences in cell surface binding.

The role of endocytosis[2e,f,10b,11,12] in cell entry by Arg-rich peptides and their cargo-conjugates is a topic of ongoing debate. We monitored uptake of **1b**−**5b** via microscopy, looking for the punctate pattern of internal fluorescence that indicates endosomal distribution. β-Peptide **1b** showed extensive endocytic uptake after 15 min, while **2b** did not display significant endocytic uptake until 30 min. The other three β-peptides showed endocytic uptake only after 60 min. These differences parallel the variations in extent of cell entry observed across the series **1b**−**5b**, which is consistent with the hypothesis that endocytic uptake is necessary for access of these β-peptides to the nucleus. These observations do not rule out the direct entry pathway. In addition, these observations suggest that endocytic uptake does not guarantee access to the cytoplasm or nucleus.

The results reported here show that both the spatial arrangement of guanidinium groups (**1b** vs **5b**) and the rigidity of the molecular scaffold that displays the guanidinium groups (**1b** vs **2b**−**4b**) affect the entry of an oligocation into live cells. Our ability to examine the influence of these structural features on cell entry depends on the unique control of helix stability offered by β-peptides. The molecular designs we have introduced should be useful for exploring the mechanism(s) of cell entry by guanidinium-rich compounds, which ultimately could allow us to design cationic oligomers with improved cargo delivery ability.

Supporting Information Available: Fmoc-β-amino acid and β-peptide synthesis, circular dichroism, and biological assay procedures. Figures of circular dichroism, ammonium chloride treatment, and flow cytometry data. This material is available free of charge via the Internet at http://pubs.acs.org.

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