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.\(^2,3\) 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.

\(\alpha\)-Amino acid oligomers of \(\leq 20\) residues are very flexible, and it is difficult or impossible to generate sets of short \(\alpha\)-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 \(\beta\)-amino acid oligomers. \(\beta\)-Peptides containing exclusively \(\beta^3\)-residues can adopt the 14-helix secondary structure (defined by 14-membered ring hydrogen bonds between backbone groups, \(\text{C} = \text{O}(\text{i}) - \text{H} - \text{N}(\text{i+2})\)).\(^5\) For most \(\beta^3\)-sequences, however, 14-helicity is observed only in structure-promoting solvents, such as methanol, and not in aqueous solution.\(^5c\)

The preorganized \(\text{trans}-2\)-aminocyclohexancarboxylic acid (ACHC) residue has a much stronger 14-helical propensity than do \(\beta^3\)-residues,\(^6\) ACHC and \(\beta^3\)-homovaline (\(\beta^3\)-Val) have comparable net hydrophobicities,\(^6c\) so comparison of \(\beta\)-peptides in which these two residues are swapped allows one to examine the impact of 14-helix stability on other molecular properties of interest.

\(\beta\)-Peptides 1–4 contain repeating triads (\(X - \beta^3\)-Arg \(\beta^3\)-Arg), where the choice of \(X\) is intended to influence 14-helix stability. \(\beta\)-Peptide 1 is designed to form a very stable 14-helix in aqueous solution (\(X = (S,S)\)-ACHC), with the six \(\beta^3\)-Arg residues clustered along one side (Figure 1). \(\beta\)-Peptide 2 (\(X = \beta^3\)-Val) is expected to have diminished 14-helical propensity relative to that of 1.\(^5c\) In 3, the \(X\) residues are \(\beta^3\)-Gly, which is even more flexible than \(\beta^3\)-Val; 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.

\(\beta\)-Peptide 5 should form a 14-helix in which the \(\beta^3\)-Arg 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 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.\(^8\)

\(\beta\)-Peptides 1b–5b bear an N-terminal 6-carboxyfluorescein unit to allow evaluation of cell entry behavior by fluorescence microscopy. \(\beta\)-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 \(\sim 70\%\) of the HeLa cells showing nuclear staining. Entry by 1b was completely blocked in the presence of NaN\(_3\), which implies an energy-dependent uptake process.\(^8\) Incubation of cells with 1b in the presence of N\(_2\)HCl led to only endosomal uptake (no green fluorescence in the nucleus; see Supporting Information). \(\beta\)-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. \(\beta\)-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 \(\leq 15\%\) uptake as a nonspecific background effect (dashed line in Figure 2A).
Previous studies have indicated that binding to the cell surface is a prerequisite for entry by Arg-rich α-peptides and their cargo-conjugates.\textsuperscript{8,11} We used flow cytometry to probe for differences in binding to the surface of HeLa cells among 1b–5b. All five hexacationic \(\alpha\)-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\textsuperscript{26,108,111,12} 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. \(\beta\)-Peptide 1b showed extensive endocytic uptake after 15 min, while 2b did not display significant endocytic uptake until 30 min. The other three \(\beta\)-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 \(\beta\)-peptides to the nucleus. These observations do not rule out the direct entry pathray. 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 \(\beta\)-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.

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Flow cytometry was performed at the University of Wisconsin Comprehensive Cancer Center Flow Cytometry Facility. We thank Anita Pottekate and Saulius Vainauskas for HeLa cell cultures, Adam Steinberg for preparation of figures, and Erik Puffer for helpful discussions.

Supporting Information Available: Fmoc-\(\beta\)-amino acid and \(\beta\)-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.

References


(7) See data in Supporting Information.


(9) Standard error = (standard deviation)/\(n\), where \(n\) equals the number of experiments.


(12) We use the term endocytosis to describe any capture of cargo from the plasma membrane, including macropinocytosis. For a review, see: Connor, S. D.; Schmid, S. Nature 2003, 427, 37.

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Supporting Information for ‘Effects of Conformational Stability and Geometry of Guanidinium Display on Cell Entry by β-Peptides’

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1. **Fmoc β-amino acid syntheses**: Fmoc-β-Arg(PMC)-OH and Fmoc-β-Val-OH were synthesized via Arndt-Eistert homologation\textsuperscript{S1} using a modified procedure as described by Muller, et al.\textsuperscript{S2} (S,S)- and (R,R)-aminocyclohexanecarboxylic acid were synthesized as described by Schinnerl et al.\textsuperscript{S3}

2. **β-Peptide Synthesis and Labeling**: All β-peptides were synthesized in parallel via manual Fmoc solid-phase synthesis on Novasynt TGR resin. Couplings were performed for 3hr using 3 equivalents of O-benzotriazole-1-yl-N-N'-tetramethyluronium hexafluorophosphate (HBTU), hydroxybenzotriazole (HOBT) and 6 equivalents of DIEA in DMF. Deprotections were performed in 20% piperidine/DMF. Double couplings were employed for the second and third (S,S)-ACHC residues in both 1 and 5. A β-homoglycine (βhGly) linker was conjugated to the N-terminus of each peptide. Half of the resin was then coupled to 6-carboxyfluorescein (3 equivalents HBTU/HOBT, 9 equivalents DIEA) for 12 hr. The other half of the resin was coupled to Fmoc-β\textsuperscript{3}hTyr followed by Fmoc deprotection. β-Peptides were cleaved from resin using 92.5% TFA with 5% thioanisole and 2.5% ethanedithiol for 5 hr. All β-peptides were purified by preparative reverse-phase high-pressure liquid chromatography (RP-HPLC) on a Vydac C4 silica column using a non-linear gradient of water/acetonitrile containing 0.1% v/v TFA. β-Peptide identity was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis on a Bruker Reflex II instrument. The purified β-peptides were lyophilized and redissolved in water. Concentrations were determined by UV-visible spectroscopy at 494 nm (fluoresceinated β-peptides) or 275 nm (β\textsuperscript{3}-homotyrosine-containing β-peptides).
3. **Circular Dichroism**: Measurements were taken on an Aviv 62A Circular Dichroism Spectrometer using quartz 0.1 cm cuvettes with 5 s averaging times. The concentration of each sample was determined by tyrosine absorbance at 275 nm. β-Peptides 1a-5a were dissolved in 350 µl of phosphate buffered saline (PBS), MeOH or 5 mM dodecylphosphocholine (DPC) micellar in phosphate buffer to a final concentration of 100 µM (PBS and MeOH) or 50 µM (DPC micelles). Results can be seen in Figure S1.

4. **Cell Culture**: HeLa cells were cultured in Dulbecco’s modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 µg/ml) in a humidified incubator containing 5% CO₂ gas.

5. **Confocal Microscopy**: HeLa cells grown to subconfluence on 90 mm plates were dissociated for 15 min at 37°C using Trypsin/EDTA. Cells (10⁵ / well) were plated onto 35 mm glass-bottom culture dishes (MatTek) and cultured overnight in DMEM. The medium was removed after 24 hr, and the cells washed with PBS. Opti-MEM (1 ml) containing 8 µM β-peptide was then added, and the cells were incubated for 15-120 min at 37°C. The cells were then washed with 3x2 ml PBS, each containing 4 µL propidium iodide for viability determination. Cells were incubated for 5 min at 37°C between wash steps. The cells were then viewed by confocal microscopy using a BioRad MRC 1024 laser scanning confocal microscope with excitation at 488 nm for fluorescein and excitation at 568 nm for propidium iodide. Emission filter sets for 522 (+/-17) nm for fluorescein and 605 (+/-16) nm for propidium iodide were employed. Quantification of uptake by 1a-5a was determined via cell counting. The number of cells showing nuclear uptake of fluorescein-labeled peptide was compared to the total
number of cells as determined by transmission images. In each case, 70-100 cells were evaluated. The results of these experiments can be seen in Figure 2. Each data point is an average of at least 4 separate experiments. Propidium iodide (PI) stained cells (<10% in most cases) were considered compromised, and were not included in the cell counting data (i.e., there were no PI-stained cells among the 70-100 evaluated in a given experiment). Examples of confocal images used for cell counting can be seen in Figure S2.

6. **Ammonium Chloride Treatment**\(^\text{S4}\): Cells were plated to a density of \(10^5\)/well in glass-bottom plates as described above. The medium was replaced after 24 hr with 1 ml of Opti-MEM containing 50 mM NH\(_4\)Cl for 30 min at 37°C. \(\beta\)-Peptide was added, and the cells were incubated for 15 min at 37°C, washed with 3x2 ml PBS containing 50 mM NH\(_4\)Cl and viewed by confocal fluorescence microscopy. Mock-treated cells that had not been incubated with Opti-MEM containing NH\(_4\)Cl were viewed in parallel. Results can be seen in Figure S3.

7. **Flow Cytometry**: HeLa cells grown to subconfluence on 90 mm plates were dissociated for 1 hr at 37°C using non-enzymatic dissociation medium containing EDTA. The cells were spun at 1800 rpm for 7 min and resuspended in glucose-free Hepes buffered saline (HBS) containing 50 mM 2-D-deoxyglucose and 10 mM sodium azide (NaN\(_3\)). 2x10\(^5\) cells were aliquoted per falcon tube, and incubated for 30 min at 37°C. \(\beta\)-Peptide was added from a stock solution to give 8 \(\mu\)M \(\beta\)-peptide / tube. The solutions were incubated for 10 min at 37°C and then centrifuged at 1800 rpm for 7 min, resuspended in NaN\(_3\) buffer, vortexed, and centrifuged again. The pellets were resuspended in FACS buffer, and placed on ice. Analysis was performed on a FACSCan benchtop cytometer. The data for all five \(\beta\)-peptides are shown in Figure S4.
Figure S1:

A. Circular Dichroism Data in PBS, pH 7.4 (100 µM β-peptide)

B. Circular Dichroism Data in MeOH (100 µM β-peptide)
C. Circular Dichroism data in 5 mM DPC micelles (100 µM β-peptide)

Discussion of circular dichroism results: Circular dichroism (CD) data indicate that 1a-5a display the expected folding behavior. (These β-peptides contain an N-terminal β3-homotyrosine residue to allow concentration determination via UV absorbance.) In aqueous buffer, only the (S,S)-ACHC-containing β-peptides, 1a and 5a, display a strong minimum at ca. 214 nm (Figure S1A). Correlations between two-dimensional NMR and CD data obtained for related β-peptides have previously shown that this signature is characteristic of 14-helical folding.\textsuperscript{55} Both 2a (X = β3hVal) and 3a (X = βhGly) appear to be completely unfolded in water, while 4a shows a weak maximum at ca. 214 nm, presumably a reflection of the local conformational bias of the (R,R)-ACHC residues. In methanol, a helix-promoting solvent for β-peptides,\textsuperscript{55b} the CD signature of 2a (X = β3hVal) displays the most significant change relative to aqueous solution (Figure S1B), going from unfolded in water to highly 14-helical in methanol. The lack of significant CD
change for 1a and 5a between water and methanol suggests that these β-peptides may be fully 14-helical even in water. The minimal solvent dependence seen for 3a and 4a indicates that these β-peptides have little or no propensity to form the 14-helix. We also examined the effect of dodecylphosphocholine (DPC) micelles on the CD spectra of 1a-5a in aqueous solution (Figure S1C). DPC has a zwitterionic headgroup, as is commonly found among the lipids on the outer surface of eukaryotic cells. The effect of DPC micelles on 1a-5a is very similar to the effect of switching from aqueous to methanolic solution: only 2a (X = β3hVal) displays a significant change, which probably results from 14-helix induction by the micelles.
Figure S2: β-Peptide Uptake by Confocal Microscopy.
Uptake of β-peptides 1b, 2b, and 5b. Cells were incubated with 8 µM β-peptide for 2 hr at 37°C, washed to reduce background fluorescence and imaged live by fluorescence confocal microscopy.
Figure S3: Ammonium Chloride Treatment

Uptake of β-peptide 1b in the absence and presence of NH₄Cl.
Figure S4: Surface Binding by Flow Cytometry
8 μM β-peptide was added to cells pretreated with 10 mM NaN₃ and 50 mM 2-D-deoxyglucose, washed with PBS containing NaN₃ and analyzed by flow cytometry.
References:


