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

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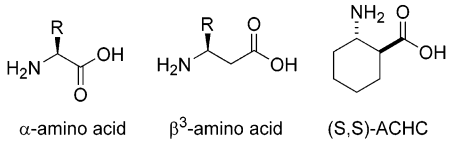
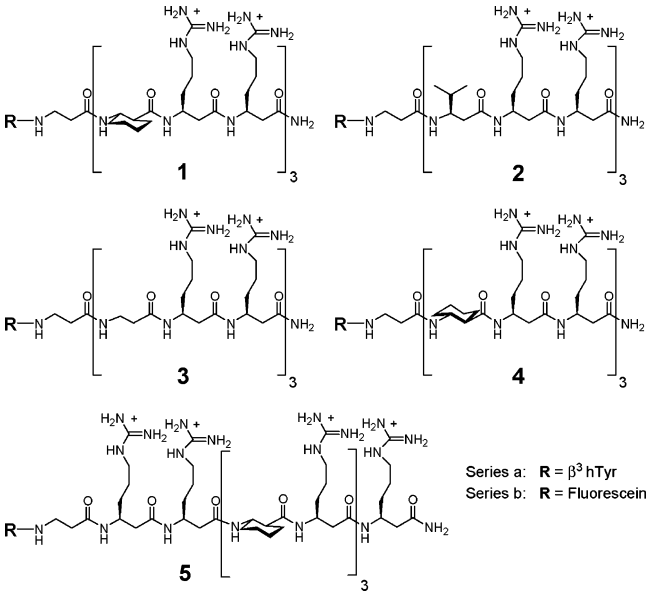
Peptides rich in Arg residues can enter the cytoplasm and ultimately the nucleus of a living cell from the external medium.[1,2] Cell entry has also been demonstrated for unnatural molecules that display multiple guanidinium groups.[2e,3] Engineered cell entry may be useful for drug delivery,[4] but 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 β^3-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 β^3-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 β^3-residues.[6] ACHC and β^3-homovaline (β^3hVal) 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-β^3hArg-β^3hArg), 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 β^3hArg residues clustered along one side (Figure 1). β-Peptide **2** (X = β^3Val) 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 β^3hVal; 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 β^3hArg 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]



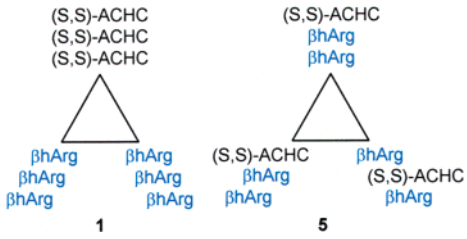


Figure 1. A 14-helical wheel diagram of **1** and **5**, showing the differential display of guanidinium residues about the helical axis.

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 NaN_3, which implies an energy-dependent uptake process.[8] Incubation of cells with **1b** in the presence of NH_4Cl 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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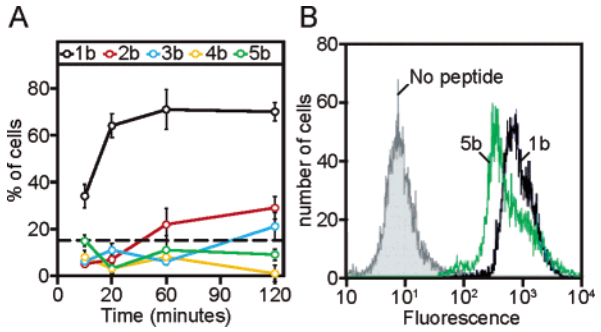

Figure 2. (A) Internalization of **1b**−**5b** over time; 8 μM β-peptide was incubated with cells for 15, 30, 60, or 120 min. The cells with nuclear staining were counted. Each data point is an average of at least four separate experiments; 70−100 cells were counted per experiment. The error bars denote 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.

Acknowledgment. This paper is dedicated to Professor Peter B. Dervan on the occasion of his 60th birthday. This work was supported by NIH Grants GM56414 to S.H.G. and GM55427 to A.K.M. Confocal images were obtained at the W.M. Keck Laboratory for Biological Imaging at the University of Wisconsin. Flow cytometry was performed at the University of Wisconsin Comprehensive Cancer Center Flow Cytometry Facility. We thank Anita Pottekat and Saulius Vainauskas for HeLa cell cultures, Adam Steinberg for preparation of figures, and Erik Puffer for helpful discussions.

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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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[S1] using a modified procedure as described by Muller, et al.[S2] (S,S)- and (R,R)-aminocyclohexanecarboxylic acid were synthesized as described by Schinnerl et al.[S3]

2. **β-Peptide Synthesis and Labeling**: All β-peptides were synthesized in parallel via manual Fmoc solid-phase synthesis on Novasyn TGR resin. Couplings were performed for 3hr using 3 equivalents of O-benzotriazole-1-yl-N-N-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^3hTyr 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^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% CO_2 gas.

5. **Confocal Microscopy**[S4]: HeLa cells grown to subconfluence on 90 mm plates were dissociated for 15 min at 37°C using Trypsin/EDTA. Cells (10^5 / 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

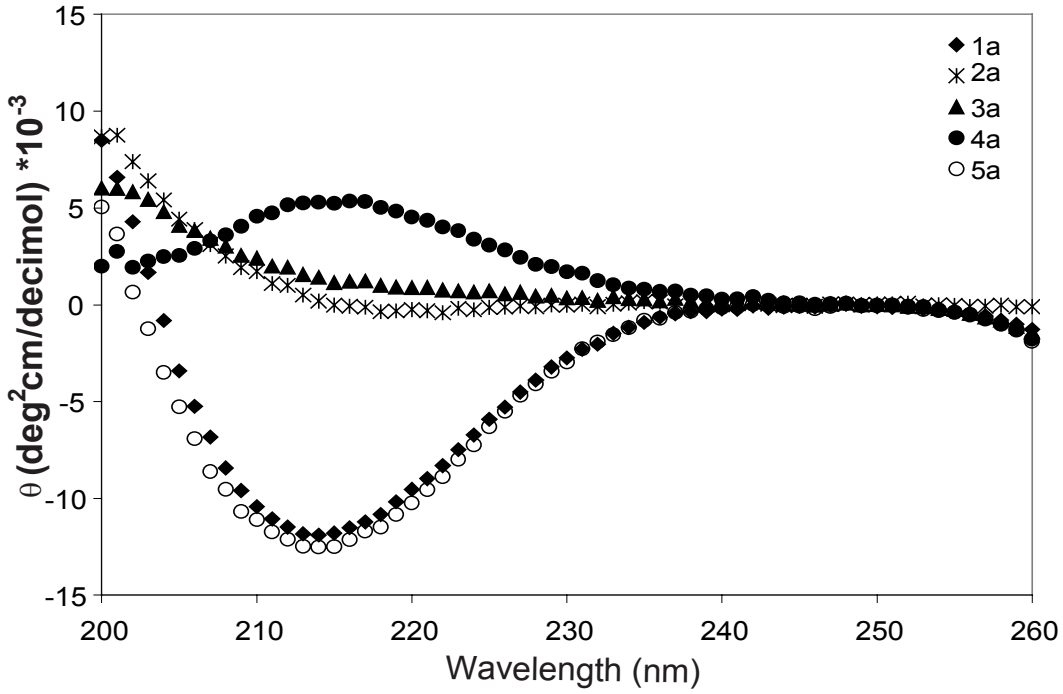
number of cells as determined by transmission images. In each case, 70-100 cells were evaluated. The results of these experiments can be seen in Figure 2. Each data point is an average of at least 4 separate experiments. Propidium iodide (PI) stained cells (<10% in most cases) were considered compromised, 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**[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_4Cl for 30 min at 37°C. β-Peptide was added, and the cells were incubated for 15 min at 37°C, washed with 3x2 ml PBS containing 50 mM NH_4Cl and viewed by confocal fluorescence microscopy. Mock-treated cells that had not been incubated with Opti-MEM containing NH_4Cl were viewed in parallel. Results can be seen in Figure S3.

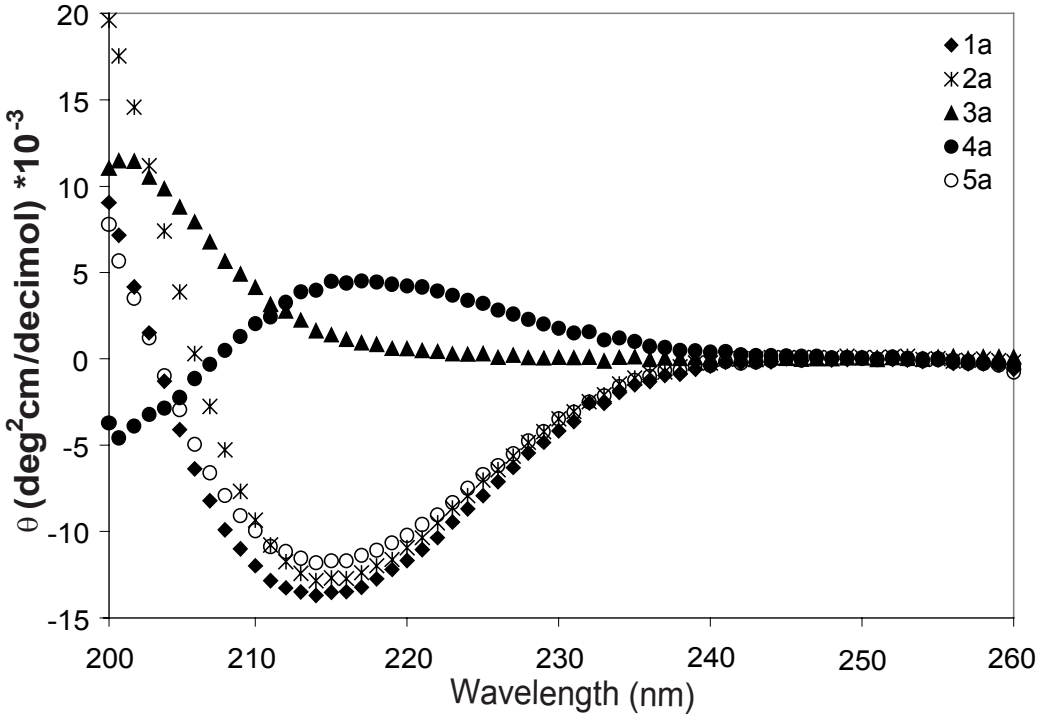
7. **Flow Cytometry**: HeLa cells grown to subconfluence on 90 mm plates were dissociated for 1 hr at 37°C using non-enzymatic dissociation medium containing EDTA. The cells were spun at 1800 rpm for 7 min and resuspended in glucose-free Hepes buffered saline (HBS) containing 50 mM 2-D-deoxyglucose and 10 mM sodium azide (NaN_3). $2x10^5$ cells were aliquoted per falcon tube, and incubated for 30 min at 37°C. β-Peptide was added from a stock solution to give 8 μM β-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 β-peptides are shown in Figure S4.

Figure S1:

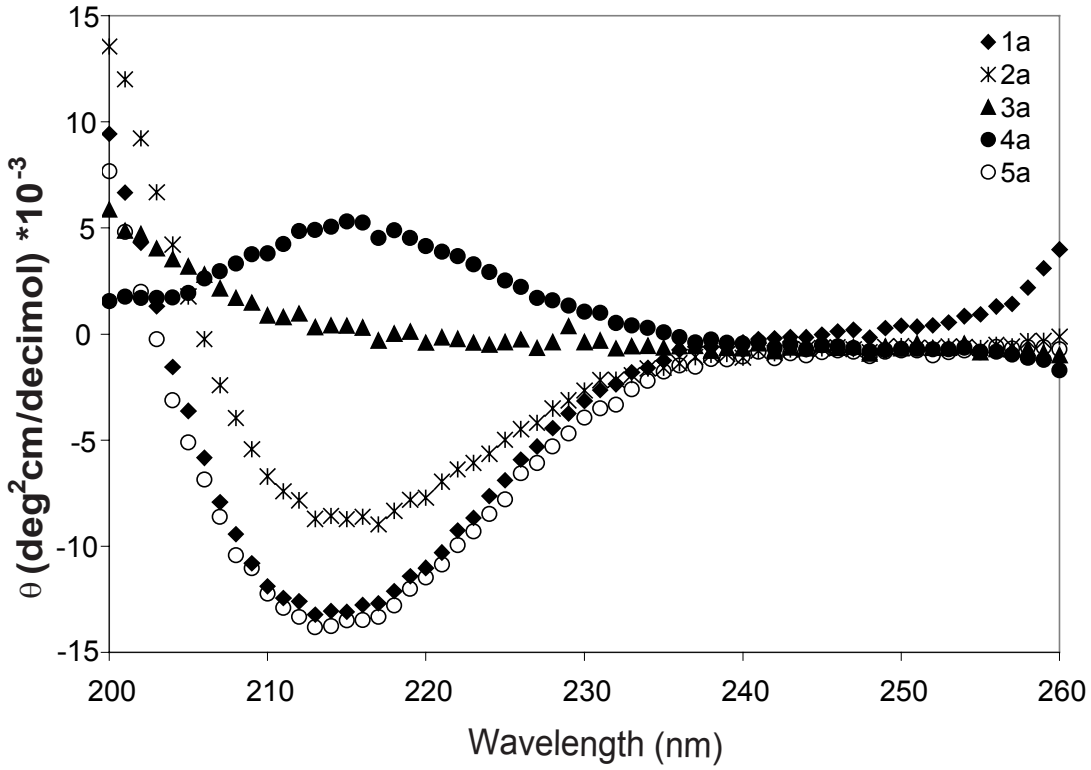
A. Circular Dichroism Data in PBS, pH 7.4 (100 μM β-peptide)



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 β^3homotyrosine 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.[S5] Both **2a** (X = β^3hVal) 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,[S5b] the CD signature of **2a** (X = β^3hVal) 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 = β^3hVal) 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.

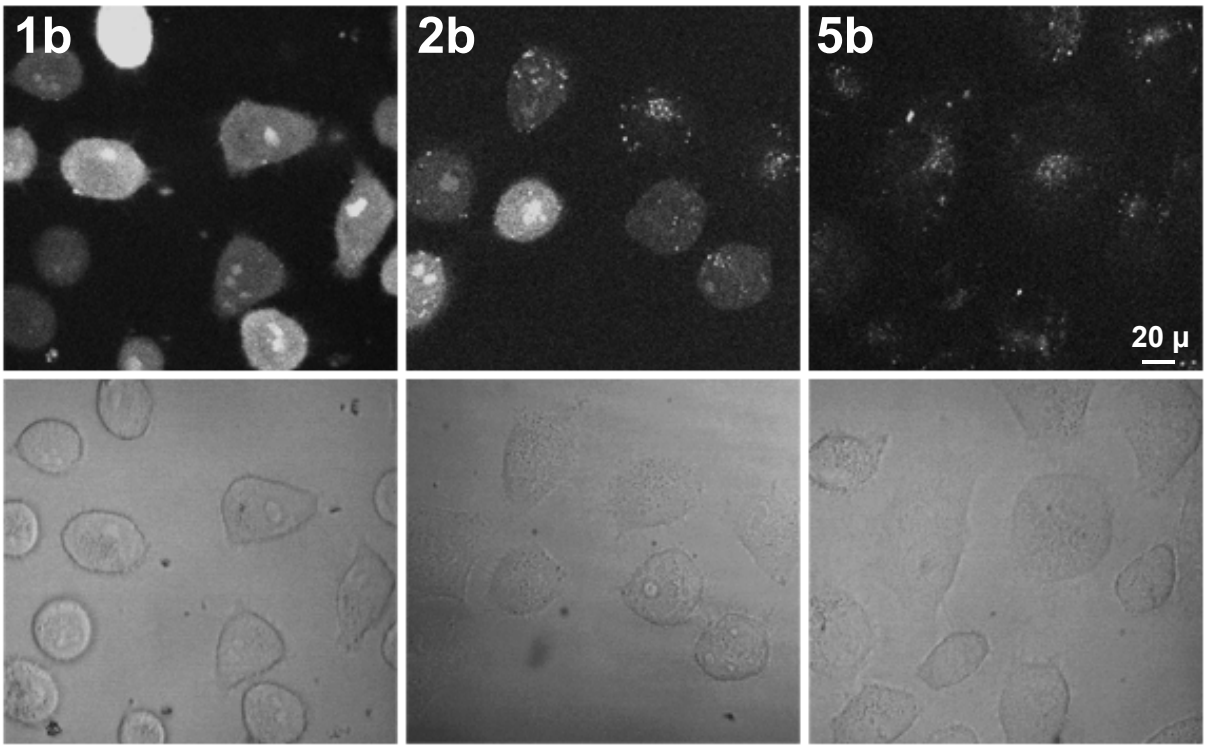


Figure S3: Ammonium Chloride Treatment
Uptake of β-peptide **1b** in the absence and presence of NH_4Cl.

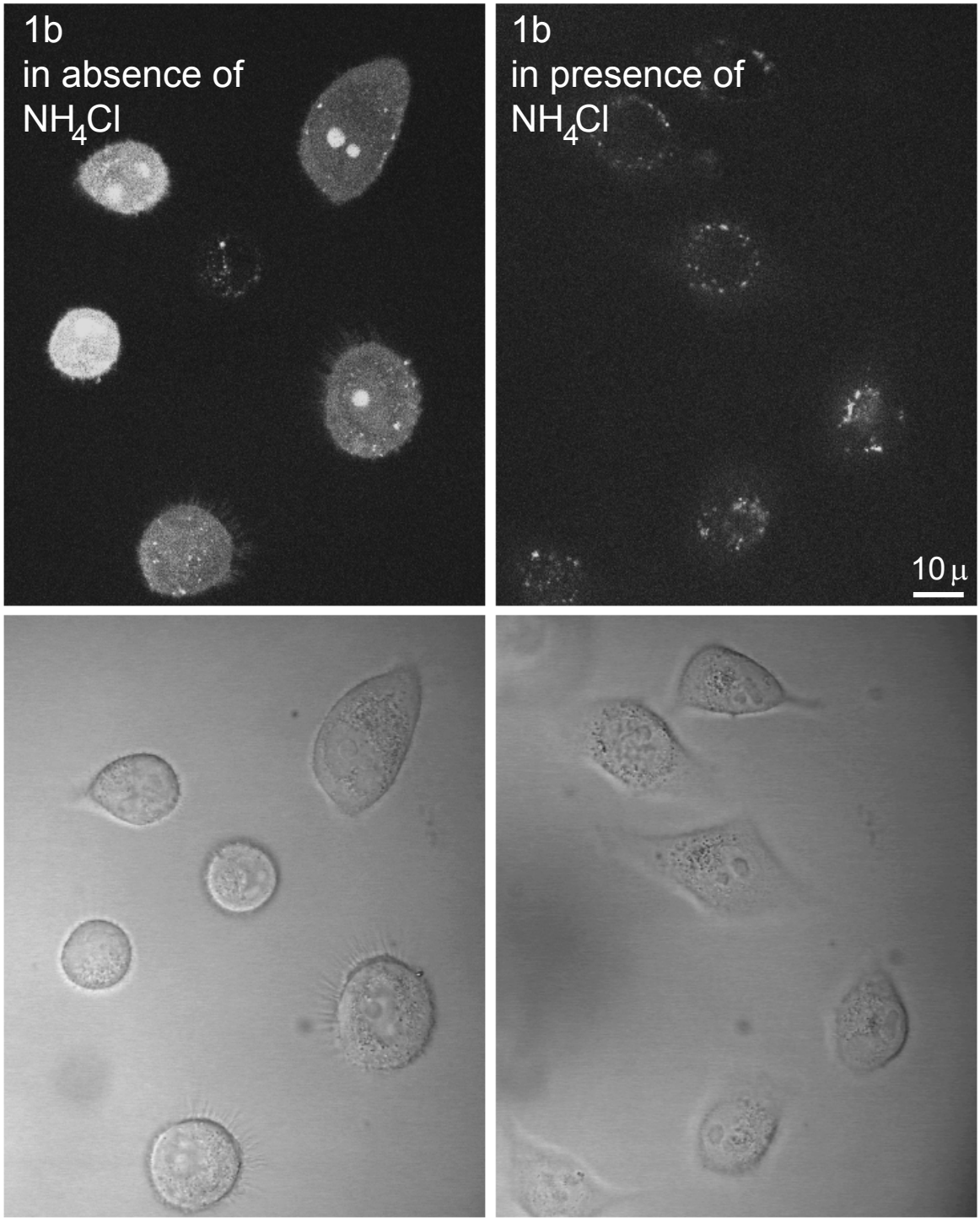
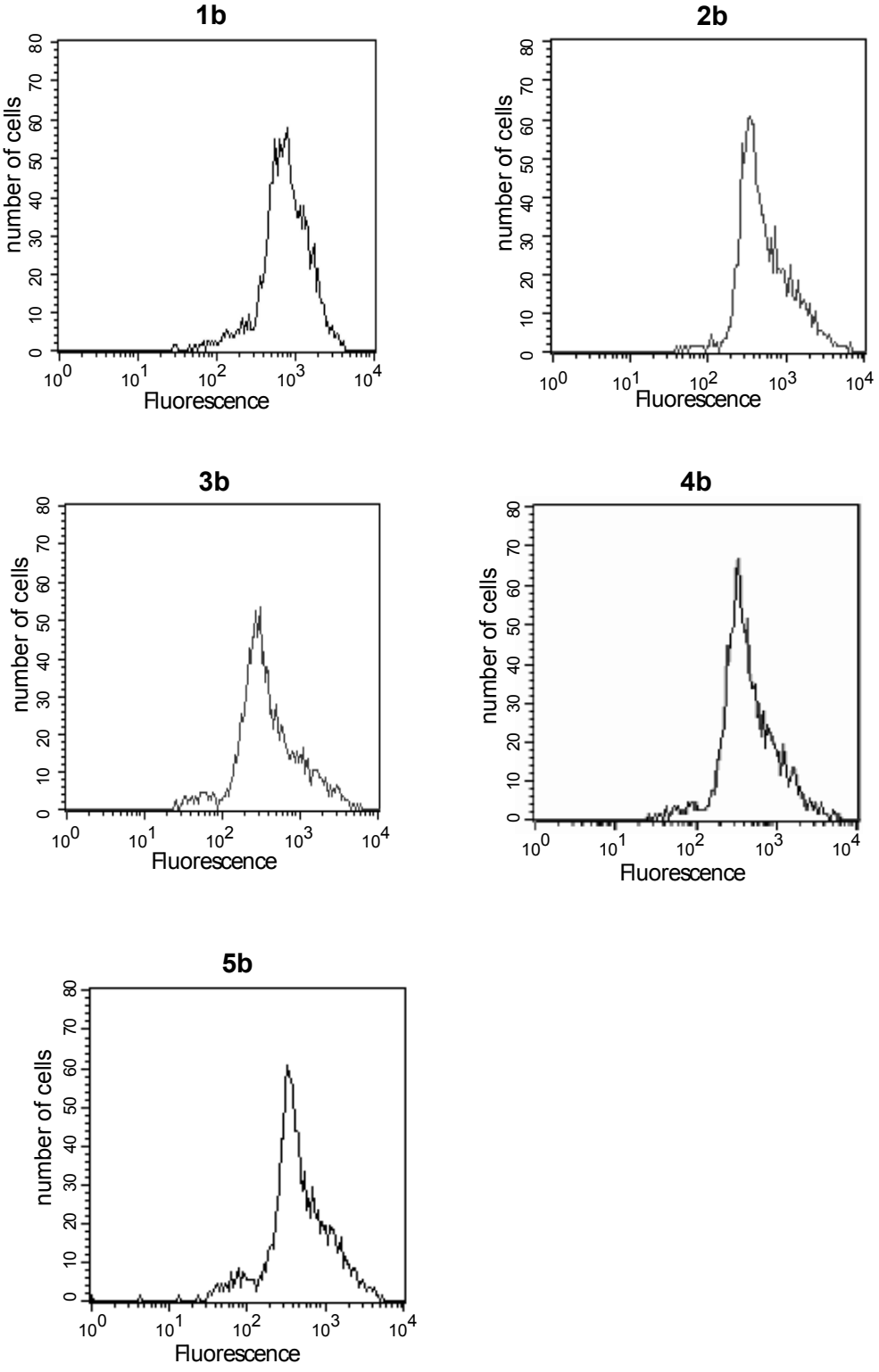


Figure S4: Surface Binding by Flow Cytometry
8 μM β-peptide was added to cells pretreated with 10 mM NaN_3 and 50 mM 2-D-deoxyglucose, washed with PBS containing NaN_3 and analyzed by flow cytometry.



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