Kritzer, J.A. Stapled peptides: How to be quick on the uptake. *Nat. Chem. Biol.* **2016** *12*(10), 764-765. doi:10.1038/nchembio.2183.

Subject Title: Stapled peptides

Title: How to be quick on the uptake

Joshua A. Kritzer

Stapled helices are promising compounds for inhibiting intracellular protein-protein interactions, but discovering peptides with the key property of cellular uptake has been largely trial-and-error. A new study defines physicochemical parameters for designing hydrocarbon-stapled helices with greater likelihood of cellular uptake.

Various covalent bonds, including disulfides and lactams, have been used to enforce a helical structure by covalently linking two side chains of a short peptide.[1,2] In 2004, it was revealed that “stapling” a helix using an all-hydrocarbon cross-link can not only stabilize helical structure, but can produce molecules cell-penetrant enough to allow *in vivo* activity.[3] Despite intense interest, the reasons why some of these unique molecules can penetrate cells have remained elusive. In this issue, Bird et al take a great step in addressing this question by quantitating the cellular uptake of a large collection of stapled helices.[4] In doing so, they reveal the physicochemical parameters that promote cellular uptake, providing useful guidelines for developing stapled helices as cellular probes and potential therapeutics.

The seemingly general ability to make cell-penetrant, helical peptides sent a shock wave through academic science and drug development. How could these peptides access the cytoplasm? It became clear that stapled helices could not simply slip through the plasma membrane (they were not passively cell-permeable). Rather, they were being taken up via an energy-dependent endocytosis pathway.[5,6] The process starts with membrane binding, in a manner that alters membrane thickness and/or curvature. At some point, membrane alterations prompt the endocytosis machinery to take over, initiating the budding and internalization of endosomes containing the peptides. The enclosure of peptides within endosomes is typically referred to as cellular uptake. The next step is poorly understood, but the peptides eventually

escape from endosomes into the cytoplasm. Thus, for stapled helices, the current working model for cell penetration involves discrete steps of membrane binding, endocytosis, and endosomal escape (Fig. 1).[5,6]

While the use of all-hydrocarbon staples seemed to be modular, it was clear even from the earliest applications that cell penetration was dependent on several key factors such as incubation time, dosage, presence of serum in culture media, and precise molecular composition. Early discrepancies have been resolved based on one or more of these factors, and many independent groups have produced cell-penetrant stapled helices that engage cytoplasmic targets.[6] These targets range from viral targets to signaling proteins to transcription and translation factors. Despite these varied modes of action, the precise physical properties that promote cellular uptake remained poorly defined. As a result, twelve years of research in this area has relied almost exclusively on trial-and-error to discover cell-penetrant stapled helices.

In this issue, Bird et al take a systematic and quantitative approach aimed at improving stapled helix design[5]. First, they devised a high-throughput, high-content fluorescence microscopy protocol that uniformly measures the cellular uptake of fluorescein-conjugated peptides. The image processing and fluorescence quantitation is conservative and careful, ensuring only intracellular fluorescence is measured. The authors characterize the dependence of uptake on time, dosage, and serum content, but the most systematic part of the paper deals with effects of peptide composition. Three different collections of stapled helices were compared. The first varies staple position within the BIM-BH3 helix, the second includes diverse point mutants of a cell-penetrant stapled BIM-BH3 helix, and the third varies staple position within an unrelated helical sequence derived from SOS1. The authors quantitate the total internalized fluorescence intensity (TIFI) of 46 peptides in all, enabling an apples-to-apples comparison of their cellular uptake. Rather than drawing subjective conclusions, the authors apply unbiased statistical methods to identify the critical variables and “sweet spots” for cellular uptake. They found that uptake was maximal for stapled helices with high, but not excessive, hydrophobicity and high, but not complete, α-helicity. The propensity of stapled helices to disrupt membranes and kill cells (cell-lytic activity) was also carefully quantitated and analyzed, revealing that lytic effects can be minimized by maintaining a moderately basic pI and moderate hydrophobicity. Finally, they confirmed that staple position matters. Helices with staples adjacent to the hydrophobic binding interface had greater cellular uptake, leading to the conclusion that a large, contiguous hydrophobic surface is a key requirement for cell penetration.

This work provides long-sought-after, practical guidelines for the design of stapled helices. These guidelines may be further applicable to non-helical peptides and peptides cross-linked with different chemistries. In fact, a growing body of work is beginning to define a route for cell penetration for structured, hydrophobic peptides that is complementary to that of polycationic peptides.[5–9] One limitation of the present study is the lack of distinction between endocytic and cytoplasmic localization – the current results speak only to cellular uptake, and only indirectly to cell penetration. An implicit assumption is that endosomal escape is roughly proportional to cellular uptake, and in key cases (most notable, BIM-BH3 helices) this assumption is largely borne out by biological activity. Still, more work remains to be done to explain how endocytosed material escapes into the cytoplasm, and to uncover molecular properties that promote endosomal escape. In the meantime, the results from Bird et al provide valuable parameters for the design of more cell-penetrant stapled helices.

Joshua Kritzer is at the Department of Chemistry, Tufts University, Medford, Massachusetts, USA.
email: joshua.kritzer@tufts.edu

References

1. Andrews, M. J. I. & Tabor, A. B. Forming stable helical peptides using natural and artificial amino acids. *Tetrahedron* **55,** 11711–11743 (1999).
2. Harrison, R. S. *et al.* Downsizing human, bacterial, and viral proteins to short water-stable alpha helices that maintain biological potency. *Proc. Natl. Acad. Sci.* **107,** 11686–11691 (2010).
3. Walensky, L. D. *et al.* Activation of Apoptosis in Vivo by a Hydrocarbon-Stapled BH3 Helix. *Science* **305,** 1466–1470 (2004).
4. Bird, G. H.
5. Chu, Q. *et al.* Towards understanding cell penetration by stapled peptides. *Medchemcomm* **6,** 111–119 (2015).

6. Walensky, L. D. & Bird, G. H. Hydrocarbon-Stapled Peptides: Principles, Practice, and Progress. *J. Med. Chem.* **57,** 6275–6288 (2014).
7. Bock, J. E., Gavenonis, J. & Kritzer, J. A. Getting in Shape: Controlling Peptide Bioactivity and Bioavailability Using Conformational Constraints. *ACS Chem. Biol.* **8,** 488–499 (2013).
8. Qian, Z. *et al.* Discovery and Mechanism of Highly Efficient Cyclic Cell-Penetrating Peptides. *Biochemistry (Mosc.)* **55,** 2601–2612 (2016).
9. Henriques, S. T. *et al.* The Prototypic Cyclotide Kalata B1 Has a Unique Mechanism of Entering Cells. *Chem. Biol.* **22,** 1087–1097 (2015).

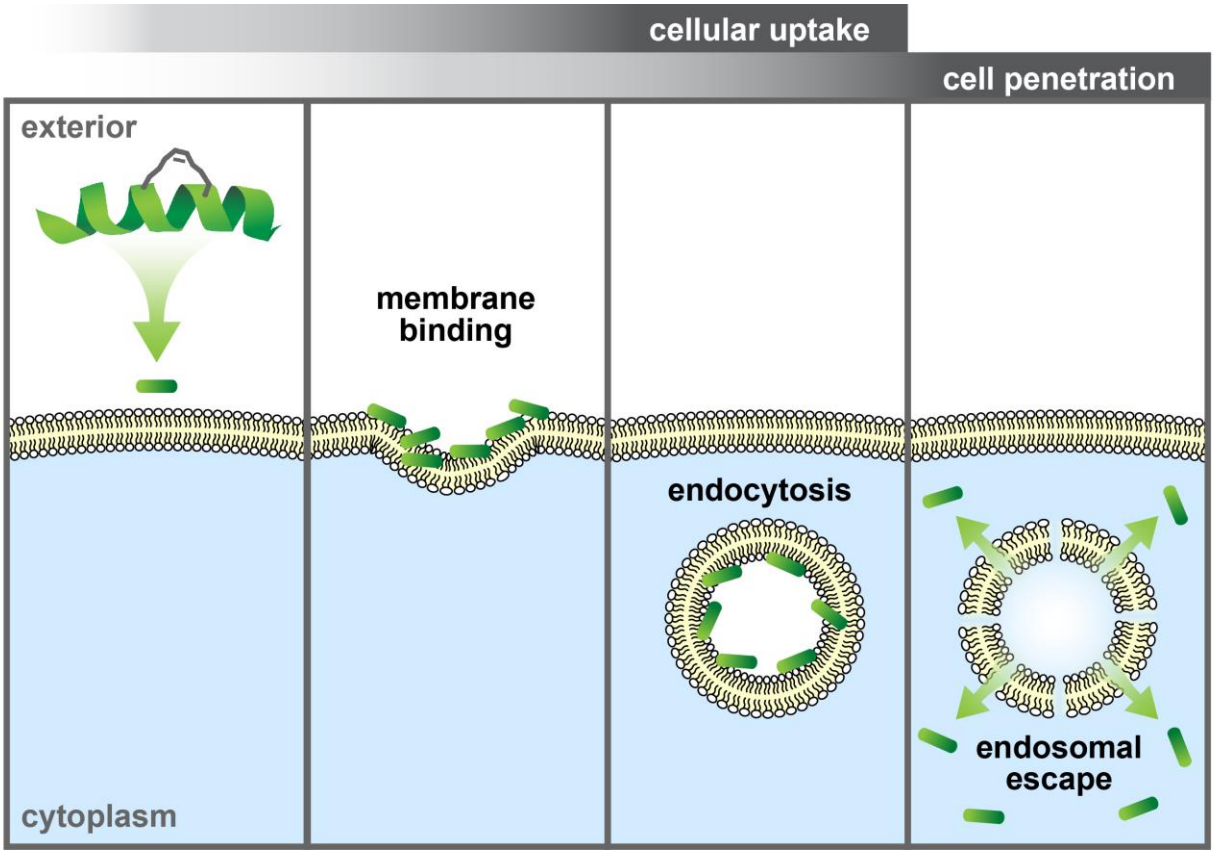

Figure 1. Cellular uptake and cell penetration of stapled helices. Bird et al quantitated the cellular uptake of a large collection of stapled helices, revealing useful design rules for maximizing cellular uptake and cell penetration.