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The effect on membrane translocation and perturbation by modulating the helicity of cell-penetrating peptides

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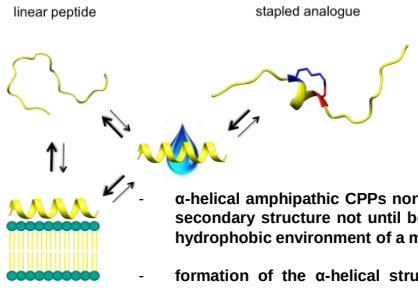
Biological Background

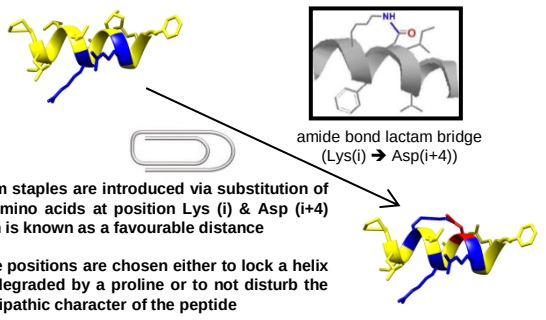
Known as the most effective α-helix nucleators, lactam stapled peptides confer greatest α-helicity compared to other stapling techniques, but all success achieved beyond *in vitro* characterisation, the biological targets have been only extracellular or membrane-bound. Although lactamisation is a well-known stapling technique there is much less evidence in literature compared to e.g. hydrocarbon-stapling that shows thereby an improvement in cell permeability.[1,2] Especially in the case of potent cell-penetrating peptides, whose cargos aim on intracellular targets, this issue of optimizing membrane translocation plays a major role. Our goal is to analyze the membrane binding properties and conformational features of lactam stapled analogues of α-helical amphipathic CPP, and to determine their uptake efficiency and toxic effect on different types of membranes. Circular dichroism (CD) is used to determine the success of peptide stapling by evaluating the degree of induced α-helical stabilization in the aqueous phase via comparison with the linear analogues. The most potent candidates are used to investigate their uptake efficiency and their toxicity on different bacteria as well as on Jurkat, HEK293T, and red blood cells. In addition, we started *in vitro* experiments to investigate translocation in lipid vesicles and leakage.

The Idea



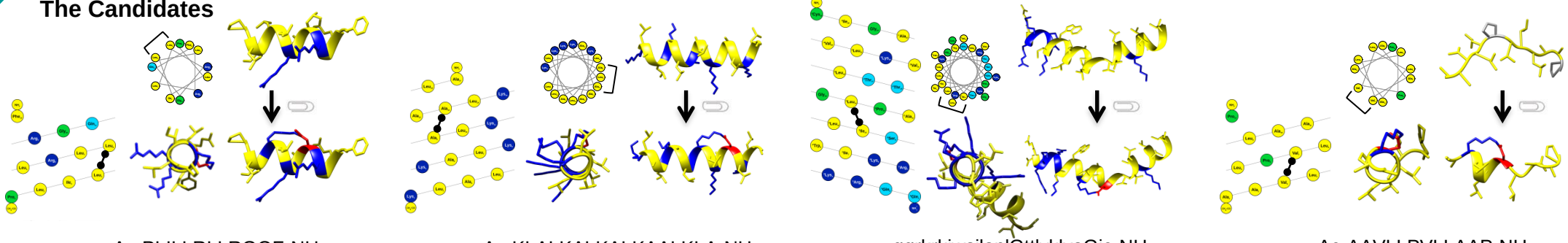
- **α-helical amphipathic CPPs normally adopt their secondary structure not until being close to the hydrophobic environment of a membrane**
- **formation of the α-helical structure already in solution should facilitate the binding properties and therefore improve the translocation process**

The lactam stapling



- **lactam staples are introduced via substitution of two amino acids at position Lys (i) & Asp (i+4) which is known as a favourable distance**
- **single positions are chosen either to lock a helix turn degraded by a proline or to not disturb the amphipathic character of the peptide**

The Candidates



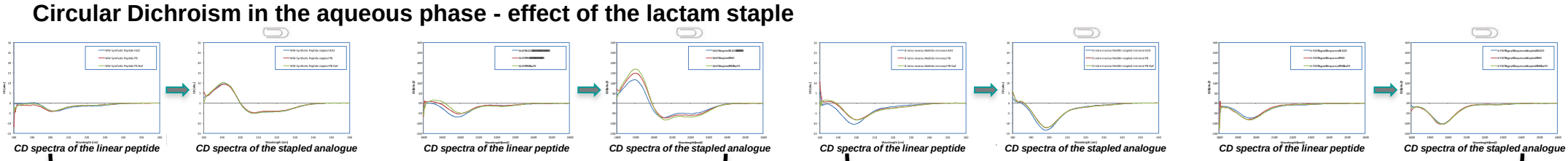
Ac-PLILLRLLRGQF-NH_2
WW synthetic peptide

Ac-KLALKALKALKAALKLA-NH_2
MAP

qqrkrkiwsilaplGttlvklvaGic-NH_2
D-retro-inverso Melittin

Ac-AAVLLPVLLAAP-NH_2
K-FGF Signal Sequence

Circular Dichroism in the aqueous phase - effect of the lactam staple



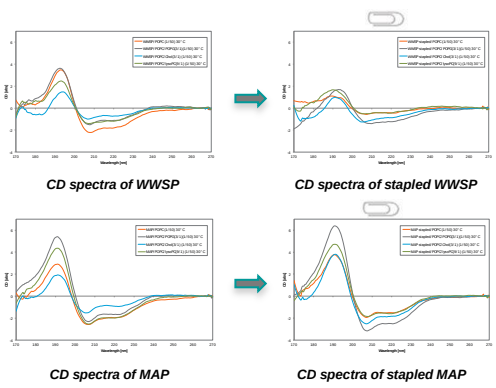
CD spectra of the linear peptide | *CD spectra of the stapled analogue* | *CD spectra of the linear peptide* | *CD spectra of the stapled analogue* | *CD spectra of the linear peptide* | *CD spectra of the stapled analogue* | *CD spectra of the linear peptide* | *CD spectra of the stapled analogue*

formation of an α-helical secondary structure

no structural change

In aqueous solution the lactam stapling shows the strongest effect of an α-helical formation and stabilization for „WW synthetic peptide“ and „MAP“

Circular Dichroism in membrane mimicking lipid vesicles

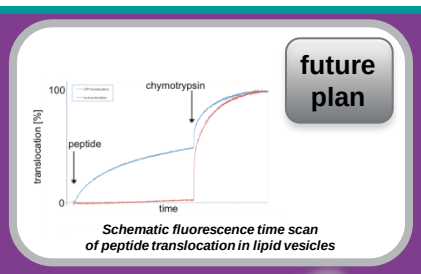


CD spectra of WWSP | *CD spectra of stapled WWSP*

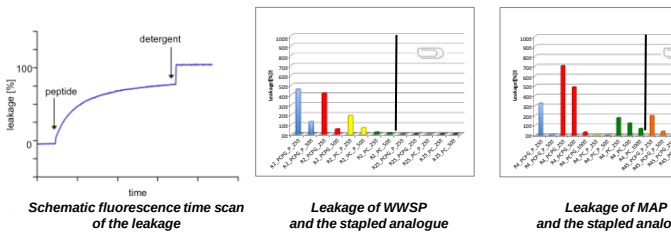
CD spectra of MAP | *CD spectra of stapled MAP*

Lactam stapling restrains the natural α-helical formation of the peptides in lipid environments

Secondary Structure ? Uptake/Translocation Toxicity/Leakage



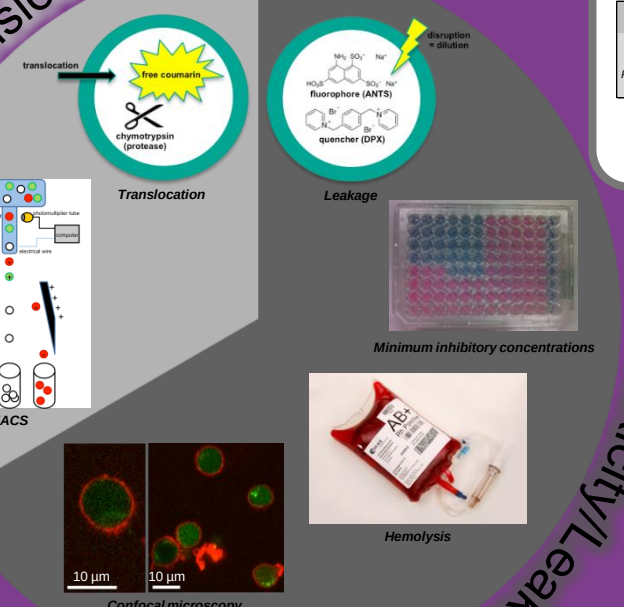
Schematic fluorescence time scan of peptide translocation in lipid vesicles



Schematic fluorescence time scan of the leakage

Leakage of WWSP and the stapled analogue

Leakage of MAP and the stapled analogue



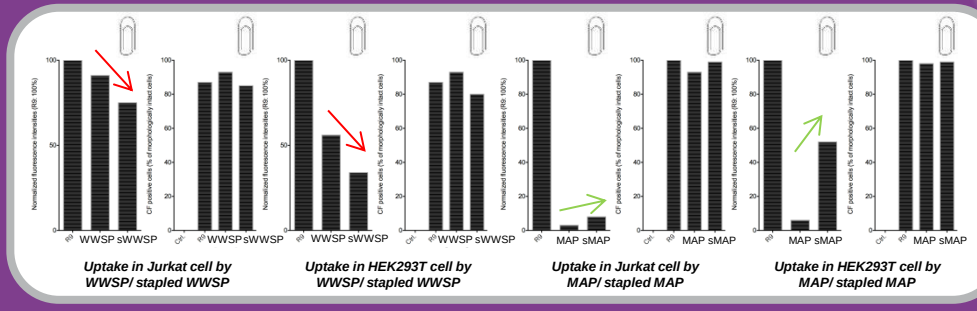
Translocation | *Leakage* | *FACS* | *Confocal microscopy*

	WW signal sequence	stapled WW signal sequence	MAP	stapled MAP
E. coli	256 µg/mL	-	16 µg/mL	64 µg/mL
S. aureus	16 µg/mL	-	16 µg/mL	256 µg/mL
P. aeruginosa	-	-	256 µg/mL	-
E. faecalis	64 µg/mL	-	256 µg/mL	-

Minimum inhibitory concentrations of WWSP, MAP and the stapled analogues

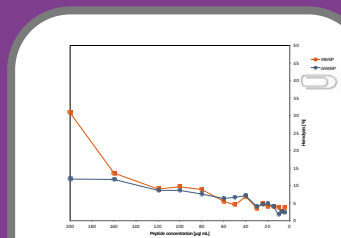
Minimum inhibitory concentrations

Hemolysis

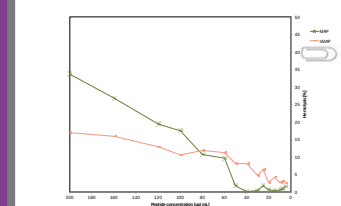


Uptake in Jurkat cell by WWSP/ stapled WWSP | *Uptake in HEK293T cell by WWSP/ stapled WWSP* | *Uptake in Jurkat cell by MAP/ stapled MAP* | *Uptake in HEK293T cell by MAP/ stapled MAP*

Viability of Jurkat cell after treatment with WWSP/ stapled WWSP | *Viability of HEK293T cell after treatment with WWSP/ stapled WWSP* | *Viability of Jurkat cell after treatment with MAP/ stapled MAP* | *Viability of HEK293T cell after treatment with MAP/ stapled MAP*



Hemolysis of erythrocytes after treatment with WWSP/ stapled WWSP



Hemolysis of erythrocytes after treatment with MAP/ stapled MAP

Conclusion

The idea of simply staple a peptide to improve in general its biofunctional properties is recently heavily discussed. Supporters of peptide stapling often defend studies showing no improvement in cell permeability with the lack of evidence that the introduced staple has really increased helicity. Furthermore, stabilizing a peptide may lower the energy barrier for binding via reducing entropic efforts, but it is making them not necessarily cell-penetrant. We could show for our successful stapled peptide that even with an induced α-helical formation in solution that there is no general improvement in cellular uptake and it even seems the stapling is disturbing the natural formation of the helix in a membrane environment. We could also demonstrate in divers experiments that membrane attraction by the stapled analogues seems to be degraded. Nevertheless, stapled peptides have shown potential as therapeutic agents and therefore it is worth investigating further in this field of peptide science.

References

1) de Araujo, A. D. et al. Comparative alpha-helicity of cyclic pentapeptides in water. *Angew Chem Int Ed Engl* **53**, 6965-6969 (2014).
2) Lau, Y. H., de Andrade, P., Wu, Y. & Spring, D. R. Peptide stapling techniques based on different macrocyclisation chemistries. *Chem Soc Rev* (2014)

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