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Important Considerations Related to Permeability of Peptides

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Abstract: This review on intracellular delivery and oral bioavailability of peptides reflects a number of principal investigations at Novartis. Our studies were aimed at either understanding features enabling peptides to interfere with intracellular protein–protein interactions, or to achieve a more patient-friendly delivery by the oral route. In the light of these objectives, we have also spent some effort on assay development to come up with alternative methods for monitoring cellular peptide uptake. This summary of our insights is intended to help in the assessment and development of peptide therapeutics requiring membrane transition

Keywords: Intracellular delivery · Metabolism · Oral bioavailability · Permeability · Pharmacokinetics

1. Introduction

This contribution focuses on lessons learnt regarding intracellular transport of peptides and systemic exposure upon oral uptake. Publications relevant for the first topic including cell-penetrating peptides (CPPs), their conjugates, and antimicrobial peptides (AMPs) have been summarized previously.[1] Meanwhile, more recent reviews on CPPs are available and various mechanisms for cellular uptake were discussed in the context of drug discovery.[2,3] Thus, the focus with respect to intracellular delivery is on our detailed study of two stapled peptides and on assay development to enable quantification of intracellular delivery. In the second part, our insights regarding improvements to increase oral bioavailability of peptides are presented.

2. Cellular Uptake of Peptides

Our own studies focused on a more detailed understanding of two stapled peptides inhibiting the p53/HDM2 protein–protein interaction relevant for the treatment of solid tumours.[4] Two peptides from previous publications of two independent groups were resynthesized and assessed, in particular since biochemical and cellular assay data could not be readily compared. In our biochemical assay, both stapled peptides exhibited sub nM potency in the same range, while the cellular effects were considerably weaker (low μM activity). Interestingly, an enhanced cellular activity (about 10-fold) resulted for the longer peptide. The discrepancy in terms of cellular potency between the two closely related stapled peptides has not been resolved. According to unpublished profiling in MDCK cells, measurements of the shorter peptide in the B-A direction, by contrast to the longer peptide, suggested it to represent a substrate for efflux pumps, which may explain the lower cellular activity of the shorter peptide. According to image analysis, a dye-labelled analogue was able to reach the nucleus (site of target). The structure of the stapled peptide in the complex with its target reveals an interesting feature. (Fig. 1) The Glu side chain, supporting solubility of the peptide, can interact with polar groups at the N-terminal thereby shielding polarity and enhancing permeability.

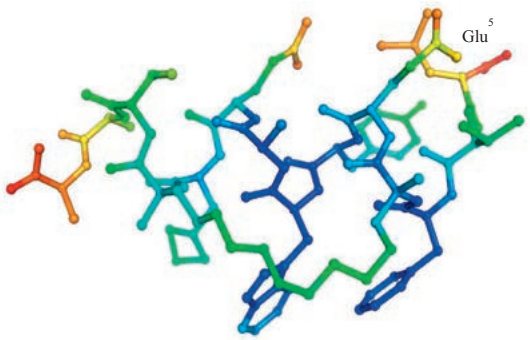

Fig. 1. Structure of the cellular active HDM2-binding stapled peptide 2 Ac-LTFR_8*EYWAQ-Cba-S_5*SAA-NH_2 (*cis* isomer, Cba: cyclobutyl-Ala).[4] Blue and green regions are less flexible and especially the dark blue side chains interact with the target. Yellow and red areas are more flexible.

So far, the sequence of the stapled peptide undergoing clinical development is not in the public domain. Therefore, questions related to putative property improvements and how to close the gap between biochemical and cellular potency remain unanswered. In addition, the mechanism of entry (active or passive) has not been investigated in detail. In general, current assay technologies are not suited to discriminate active from passive transport, and thus, do not allow for a dedicated optimization of import. Furthermore, most of these cannot clearly discriminate between endosomal/lysosomal and cytosolic localization. In addition, especially in the case of active uptake, a quantification of the cytosolic concentration, *e.g.* to follow and optimize for escape from endosomes/lysosomes, is required. Therefore, there was an interest to improve the measurement and quantitative visualization for cellular delivery of peptides. Several attempts have already been published, and in particular, contributions by the group of Pellois and the chloroalkane-assay by the lab of Kritzer are worthwhile mentioning.[5,6] Since methods for bioorthogonal chemistry have been optimized to enable reactions in the cellular environment, a fluorophore on a permeable molecule was applied to study the behavior of MDM2-p53 inhibitors in a disease-relevant cellular model.[7] Out of a validated set of bioorthogonal probes, a maximum signal for the trans cycloocten (TCO) modified inhibitor was demonstrated, when reacted in the cytosol with BODIPY and TAMRA dyes functionalized with tetrazine (Tz). This assay can be applied in the competition mode, and using automated image

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analysis, information related to intracellular localization, target binding and target upregulation is generated.

The quest for a more sensitive method prompted a development based on bioluminescence.[8] In this case, the reaction between an intracellularly liberated D-Cys and a benzothiazole analogue (CBT) forms a substrate for luciferase. This 'Split Luciferin Peptide' assay (SLP) is more sensitive, and lowers the background. Concentration-dependent studies showed for the octa-D-Arg peptide, carrying N-terminally the D-Cys modification attached *via* an S-S bridge to an L-Cys in the peptide sequence, a dose-dependent uptake in the range from 25 nM to 200 nM. This peptide was superior to other CPPs with respect to total uptake after 10 min. In addition, an *in vivo* study in mice showed about a 3-fold enhanced signal of the octa-D-Arg derivative, when compared to a non-permeable tetra-Glu control peptide. The ability to use this method real-time *in vitro* and *in vivo* may help to gain more insights regarding cellular delivery of peptides. Unfortunately, the free amino- and carboxyl-group of the D-Cys moiety may have, dependent on the overall polarity of the peptide, a significant influence on the properties. For this reason, a further development, *e.g.* to transiently mask this additional polarity, would be beneficial to preserve physico-chemical properties, and in particular permeability.

Another approach to measure cytosolic concentrations, based on an intracellular FRET system, has been demonstrated to work for detection of p53/HDM2 inhibitors.[9] This sensor, exquisitely designed by GGS-spacers and poly-Pro residues to optimally arrange for a FRET system, contains the target and a moderately active binding peptide to enable interaction in an intramolecular fashion (Fig. 2).

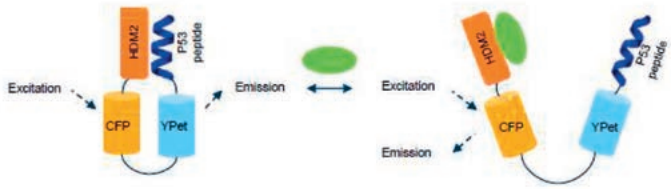

Fig. 2. Design of the label-free p53-based sensor. The fusion protein consists of a p53 peptide on YPet connected through a flexible Pro-linker to CFP plus the target HDM2.[9] The free analyte in solution (green ellipsoid) displaces the p53-peptide intramolecular ligand and shifts the sensor to the open conformation as a result reducing the FRET efficiency.

This construct opens up upon binding of a small molecule or a peptide eliciting a fluorescent signal. The isolated fusion protein can be studied *in vitro* to assess affinity for the corresponding inhibitors. Alternatively, the biosensor is transiently expressed intracellularly as a fusion protein and upon addition of p53/HDM2 inhibitors to the cell, intracellular fluorescence can be monitored with spatiotemporal resolution. This method may be able to discriminate different uptake pathways based on the observed kinetics of inhibition. Furthermore, by targeting the fusion protein to different cellular compartments, this assay enables the local concentration to be measured, and thus, provides a technology to optimize molecules for their ability to reach the desired intracellular space.

3. Oral Bioavailability of Peptides

Although the requirement for oral bioavailability to reduce or to shield polarity for optimizing passive permeability are common denominators for both avenues – intracellular delivery and oral uptake – it seems there is a more pronounced size limitation for the latter. For instance, the cell-permeable stapled peptides, as described above, did not show a significant systemic exposure upon oral administration. For a long time, Cyclosporin A (CsA) has been regarded as the absolute exception with respect to oral uptake of peptides. Studies on other peptides resulted in a much more modest outcome and even sophisticated formulations did not seem to be beneficial for significantly improving systemic concentrations upon oral delivery. Unfortunately, for interesting studies like the effects of oral administration of a Cyclotide kalata B analogue in a multiple sclerosis mouse model, pharmacokinetic details were not made available.[10] Our interest for more systematic investigations arose from a key paper by the Lokey group, which clearly demonstrated the effects of *N*-methylation, shielding of polar atoms, and the involvement of intramolecular H-bonds to achieve quite high exposures upon oral administration.[11] We could absolutely confirm the results obtained on the tri-*N*-methylated cyclic hexapeptide (cyclo Leu-N^{Me}-D-Leu-N^{Me}-Leu-Leu-D-Pro-N^{Me}-Tyr) (oral bioavailablility (BAV): 28%).[12] Results from NMR studies in different solvents and an X-ray following crystallization from MeOH confirmed the intramolecular H-bonding network and indicated quite a rigid scaffold, by contrast to the chameleonic properties as claimed essential for CsA. In the course of this study, following MD-simulations we developed an algorithm to calculate the solvent accessible 3D polar surface area (SAPSA) and showed the correlation to *in vitro* permeability assays. Due to the quite limited solubility (30 µM) of the peptide indicated above, we investigated further variants in order to find out whether reduction of grease by replacing the aromatic Tyr with aliphatic side chains, and two instead of three *N*-methylation sites could improve the solubility while maintaining permeability properties. Accordingly, the N^{Me}-Tyr position, as indicated in Fig. 3, was exchanged for Ala and Abu and the corresponding peptides showed an excellent solubility of 0.6 and > 1 mM, respectively.[13]

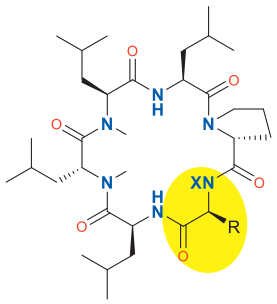

Fig. 3. Generic model of the cyclic hexapeptides studied in the context of oral bioavailability (X = H or Me).

However, permeability was reduced as compared with the Phe analogue and the parent compound (N^{Me}-Tyr). Thus, the exposures upon oral administration in rats were considerably lower for the aliphatic residues, and the low oral BAV for the Phe analogue was attributed to the limited solubility. Interestingly, an extremely high clearance for the Ala-analogue was observed. For this reason, the first pass effect of the Ala- and the Abu-peptide was studied in a rat portal vein study.[13] Surprisingly, the Ala-peptide experienced a considerable reduction when comparing concentrations in the portal versus the jugular vein (77% liver first pass effect). In parallel, we desired to achieve a more efficient shielding by pyridyl-Ala modifications while still improving on solubility.[14] Especially, the 2-pyridyl-Ala (2-Pal) modification was considered beneficial for permeability, since in this case, the pyridyl-nitrogen is able to form an H-bond to the NH of the same residue (Fig. 4). Following *in vitro* profiling, the beneficial effects of the 2-Pal residue with respect to solubility and permeability were clearly demonstrated. In addition, ^{1}H-NMR in chloroform showed the presence of an H-bond from the pyridyl nitrogen to the backbone.

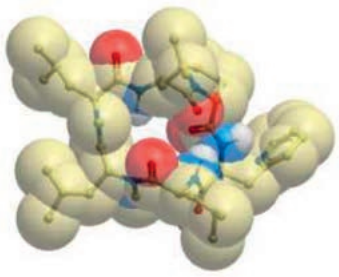

Fig. 4. Structure of the di-*N*-methylated 2-Pal cyclic hexapeptide analogue obtained from MD-simulations, indicating an H-bond between the pyridyl-nitrogen and the NH of the backbone.[14]

However, the 2-Pal peptide experienced a high clearance, and for this reason, the higher permeability did not pan out for enhanced systemic exposure upon oral administration. Nevertheless, the introduction of N^{Me}-2-Pal, carrying a total of 3 *N*-methylation sites (Fig. 3), showed a 10-fold enhanced solubility compared to the parent, and achieved an 88% oral bioavailability at excellent exposures. Based on these results, we concluded that metabolism including a first pass effect may profoundly limit systemic exposures even though permeability is fine, and peptides are proteolytically stable. In order to find out whether a highly metabolized peptide can be rescued, if hotspots are addressed, the cyclic hexapeptide (cyclo Leu-N^{Me}-D-Leu-N^{Me}-Leu-Leu-D-Pro-Ala) was investigated.[15] Surprisingly, the Ala side chain did not present a major problem for metabolic instability. Instead, two particular Leu side chains were shown to become hydroxylated, and to overcome this liability the cyclopropyl-Ala (Cpa) was inserted into these positions. However, a metabolic switching to other Leu side chains and finally to the Pro was observed. In the course of this work, all Leu side chains were finally changed to Cpa and instead of the D-Pro, 4,4 difluoro-Pro (DiF-D-Pro) was inserted. Interestingly, the *in vitro* assays showed a decrease in permeability upon introduction of each Cpa residue, probably due to restriction of side chain flexibility limiting shielding of polar atoms. Although, the original suboptimal side chain Ala was kept throughout the process, the cyclic hexapeptide (cyclo Cpa-N^{Me}-D-Cpa-N^{Me}-Cpa-Cpa-DiF-D-Pro-Ala) still maintained a good solubility (0.39 mM) and exhibited an oral BAV of 96% at an excellent exposure. As a result of this study, stable isosteres for Leu and Pro were identified and removing metabolic hotspots tremendously increased systemic exposure upon oral administration albeit properties were not significantly altered.

4. Conclusions

As indicated above, a more in-depth understanding of cellular uptake and lysosomal release including further development of new assay technologies are mandatory to enable a smooth drug discovery process and to limit side effects, if intracellular peptide delivery is required. In a first step, the tool box to engineer passive permeability into macromolecules needs to be enhanced, while principal insights into systems enabling active transport have to be improved. Furthermore, a pharmaco-dynamic read-out plus cross-validated and standardized cellular assays represent prerequisites to achieve progress. So far improvements for oral bioavailability have mainly focused on passive permeability, in a sense to reduce polar surface area. Some more options for internalization of H-bonds have been indicated earlier.[16] Other modifications, a better understanding regarding the conformational and dynamic requirements for membrane transition, or pro-drug approaches may improve peptide delivery. Site-selected modification on a given scaffold can help to quickly fine-tune properties in addition to target recognition. This late-stage functionalization concept was recently demonstrated on the basis of Negishi cross-coupling reactions in the cyclic hexapeptide context.[17] As exemplified in this contribution, in a more comprehensive review on oral delivery of peptides, low permeability and high metabolism are still regarded as the major barriers to development.[18] In addition to the review by Brayden *et al.*,[18] indicating formulations and alternative intestinal uptake pathways, peptide products and other routes of administration were recently highlighted from a patient-centric perspective.[19]

Acknowledgements

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