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Towards Intracellular Delivery of Peptides

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Abstract: In view of our more comprehensive understanding with respect to intracellular pathways and their regulation, a vast number of interesting drug targets appear to be in a space that can be addressed neither by small molecules nor by biologics. Especially, interference with intracellular protein–protein interactions, if successful, seems to offer considerable opportunities to expand the application of peptides as therapeutics, in particular in the field of oncology. This review focuses on requirements for the development of peptide therapeutics aiming at intracellular targets. In addition, an outlook for developments in this field based on recent examples for peptides active in cellular assays, highlighting key requirement to assess permeability, is provided.

Keywords: Intracellular delivery · Peptide synthesis · Permeability · Protein-protein interactions

Introduction

Over the past decades, research on cell-penetrating peptides (CPPs) and their conjugates has encouraged the idea of applying peptides either for delivery of drugs or to target proteins inside of the cell. Similarly, antimicrobial peptides (AMPs), involved in host innate immunity, have been investigated for their ability to kill or to attenuate growth of microorganisms due to binding to the target membrane as an initial step. AMPs consist of short sequences of cationic and hydrophobic residues, and thus, share many structural aspects of the more classical CPPs. Sequences from the CPP space have also been proposed to enable penetration of the blood brain barrier and to facilitate transdermal delivery. Recent reviews provide an excellent summary on CPPs and their mechanisms of uptake.[1–3] A selection from this vast sequence space of CPPs, illustrating a wide variety of sequence motifs thought to enable uptake, is displayed in Table 1.

More hydrophobic membrane-active sequences are not further discussed in this context, due to the difficulty to associate a particular aggregation state to their mechanism of action, and to understand the motifs supporting interaction with membranes including uptake. In the context of CPPs, tissue penetration, nasal, and oral delivery have also been described. Any of these options, if applicable to the development and finally resulting in approval as a drug, would clearly have a significant impact on the industrial application of peptides, and are not necessarily limited to pharmaceutical applications. Cell-permeable peptides seem to hold some promise for the future, since membrane penetration represents an obstacle for biologics, and targeting challenging intracellular protein–protein interactions (PPIs) is often not feasible for small molecules. So far, the problems of efficacy at lower dosing and the entrapment in endosomes/lysosomes at these lower concentrations has limited progress. Nevertheless, clinical trials using first generation of CPPs have already been initiated and it remains to be seen, whether on the basis of these more classical CPPs applied for further development, an approval can be obtained. Besides the CPPs derived mainly from natural sources, N-methylated cyclic peptides, inspired by the permeability of the natural product Cyclosporin A, have been designed. Following this concept, the Somatostatin analogue cyclo-Pro-Phe-N^{Me}-D-Trp-N^{Me}-Lys-Thr-N^{Me}-Phe, exhibiting an oral bioavailability of 10%, while largely preserving biological activity, has been identified.[9] *In silico* studies have recently culminated in the rational design of similar small permeable cyclic peptides. In line with predictions for shielding of polar groups and internalization of H-bonds, *in vivo* studies in rats resulted in an oral bioavailability for a tri-N-methylated cyclic hexapeptide (cyclo Leu-N^{Me}-D-Leu-N^{Me}-Leu-Leu-D-Pro-N^{Me}-Tyr) of 28%.[10] The preferred conformation as determined by NMR in $CDCl_3$ clearly indicates the shielding effect of the methyl groups on the amide and the formation of intramolecular H-bonds (Fig. 1).

However, modifications to render this particular structure more hydrophilic, revealed the sensitivity of the system regarding the problem to introduce polar groups and to maintain permeability.[11] Nevertheless, the principal idea to understand permeability by considerations related to energetic penalties for preferred conformations with respect to crossing the

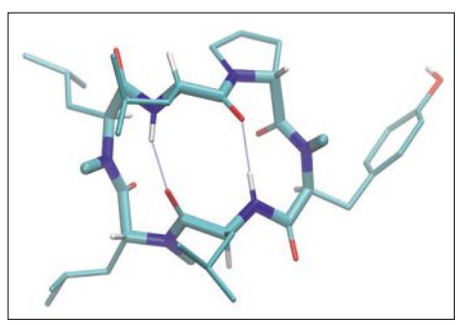

Fig. 1. Preferred conformation of cyclo Leu-N^{Me}-D-Leu-N^{Me}-Leu-Leu-D-Pro-N^{Me}-Tyr in $CDCl_3$ as determined by NMR.

Table 1. Examples of cell-penetrating peptides (CPPs).

Characteristics	Name	Sequence
Anionic designed[4]	SAP(E)	VELPPPVELPPPVELPPP
Anionic[5]	P18	LSTAADMQVVTDGMASG
Cationic[6]	Tat	RKKRRQRRR
Cationic amphiphilic[7]	pVEC	LLIILRRRIRKQAHAHSK
Pro rich[8]	Pyrrhocoricin	VDKGSYLPRPTPPRPIYNRN

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membrane has been proven. This concept is thought to enable an understanding of permeability on a structural basis beyond these smaller peptides. On the other hand, a variety of delivery technologies, which are thought to enable uptake, in particular for the oral route, have been developed. The recent deal of Roche with Chiasma on an investigational oral form of Octreotide in phase III, as communicated in February 2013, exemplifies this interest. Although, oral bioavailability for this peptide using a proprietary formulation remained only around 1%, some believe it points to more significant progress along this path in the future. In principle, delivery technologies are thought to be synergistic with an optimally designed peptide regarding modifications promoting permeability. This overview, however, focuses on molecular aspects needed to achieve permeability and technologies thought to drive our understanding of cellular uptake.

Identification of Cell-permeable Peptides

In general, powerful display technologies, natural product screens, or synthetic peptide libraries are applied to discover a peptide with a particular activity on a given target. For display approaches applying a panning process for binders, the selection is driven by the intention to obtain highly potent and selective peptides. Due to the impressive development of these technologies, in particular the Random Peptide Integrated Discovery (RaPID) technology of Prof. Suga, which was commercialized by the company PeptiDream, cyclic highly modified peptides can now be produced and screened in numbers of up to 10^{13}.[12] Interestingly, the RaPID technology tolerates modifications like N-methylation, unnatural amino acids and stable ring closure, which may be used to enhance stability and/or to incorporate certain properties into a library of interest. Although permeability assessments like the parallel artificial membrane permeation assay (PAMPA) or transport through a monolayer of CACO-2 cells have been realized in quite high-throughput,[13] the selection for permeable peptides cannot be incorporated easily in any display approach. As a consequence, intracellular delivery is then achieved either by conjugation to CPPs, or an ensemble of potent sequences is tested for their ability to cross the membrane. In the context of next generation sequencing of target binder pools, in the future, in-silico property predictions are thought to help analyses for permeability.

An example for a hit follow-up using a peptide-CPP hybrid was described in a recent communication. For this work, peptides interacting with hypoxia-inducible factor (HIF), an important intracellular target in the field of oncology, were of interest.[14] In this case, the selection was performed in the intracellular milieu applying 'split intein circular ligation of peptides and proteins' technology (SICLOPPS). Out of a library of 3.2 million members screened in the intracellular reverse two-hybrid-system, the cyclic peptide CLLFVY interfering with HIF dimerization was identified. Following conjugation to the well-known CPP Tat, the construct was found to be active (IC_{50} 15–20 μM) in different cell lines.

In the synthetic space, an example for screening combinatorial peptide libraries for insertion into lipid bilayers furnished peptides, which are also able to penetrate cells, was communicated.[15] In this case, a fluorophore label was applied, and the vesicle-based screen of about 10 000 peptides revealed two major sequences, PLILLRLLRGQF and PLI*Y*LRLLRGQF. Surprisingly, Tat and other cationic CPPs present in the collection were not selected, however the claim for pure passive transport in the cellular assays has to be confirmed by additional studies.

A variety of natural peptides from different structural categories exhibit permeation properties. Activity on the intracellular rhyanodine receptor and similarities to CPPs suggested cell permeation for the scorpion toxin Maurocalcin. Penetration has been described for various cell lines, however, a comparison to other CPPs, under the conditions applied for these investigations, was not carried out.[16] Another example from the natural product group is Sanguinamide A, a novel thiazole-containing cyclic peptide isolated as a minor component from extracts of *H. sanguineus* (Fig. 2).

NMR studies support a preferred conformation involving two intramolecular hydrogen bonds, which are protected by hydrophobic side chains from water solvation.[17] The structural features including the amide isoster are in line with the hypothesis that polar groups are shielded and H-bonds internalized. The prediction regarding a certain degree of membrane permeability was verified by an oral bioavailability for absorption in rats of 7%, despite rapid clearance (70 mL/min) and a short half-life ($t_{1/2}$ 23 min). These examples and others from the group of natural products, including Cyclosporin A, suggest permeable peptides have been optimized by nature for this particular property. Definitely, unusual types of structures like that of Sanguinamide A can inspire the design of permeable cyclic peptides and may help to identify generic modifications able to improve cellular uptake. Derivatization and optimization of CPPs and other transport-active peptides for permeability has only occasionally been carried out.[4] More work to establish a structure–transport relationship, *e.g.* in an iterative process as applied for drug discovery, using well-known sequences for benchmarking in the system of interest, would help to correlate findings from different labs. Peptides with properties like Sanguinamide A and the N-methylated cyclic hexapeptides described here, suggest a path forward to optimize for permeability in an integrated fashion without the need for conjugation to CPPs. For drug discovery of peptides, the systematic manipulation of molecular properties without compromising activity is of interest. The following chapter indicates steps in this direction on the basis of a cellular pharmaco-dynamic read-out.

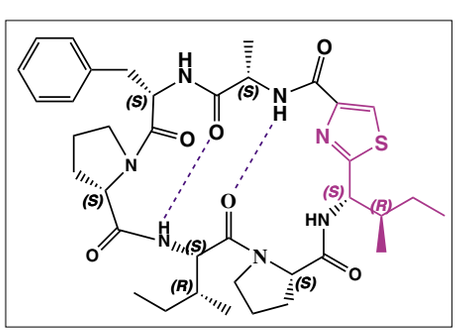

Fig. 2. Structure of Sanguinamide A (thiazole associated structure in magenta, internal H-bonds in blue).

Examples for Cellular Uptake of Peptides Targeting Intracellular PPIs

In principle, there are two possibilities for the development of peptide therapeutics that require cellular uptake. In the first approach, manipulation of side chains on the basis of a permeable scaffold can be realized to mimic known pharmacophores. Alternatively, starting from a highly potent peptide the introduction of features enhancing intracellular transport can be accomplished. The peptide cyclo-Pro-Phe-N^{Me}-D-Trp-N^{Me}-Lys-Thr-N^{Me}-Phe can be viewed as a representative of modifying a transport-active structure. More complex intrinsically transport-active peptides have been successfully modified. In the case of the scaffold Cyclotide Kalata B1, Bradykinin B1 receptor antagonist activity was integrated in this natural sequence.[18] The modified tricyclic peptide (S-S bridges) of 31 residues, carrying the antagonist motif in a loop sequence, preserved the potency of the smaller peptide and oral administration led to *in vivo* analgesic effects. In the second approach, peptides active on a given target are identified by display technologies, screening

natural products or from synthetic libraries. In addition, structure–activity relationships obtained from analogues and more detailed 3D-information from analysis of the complex with the target may be available. In this case, information on locations for modifications to manipulate properties is considered to be straight-forward. Otherwise Ala-, N-methyl-, or D-amino acid scans may reveal preferred positions for modifications towards cellular permeability.

Our interest in cell-permeable peptides, and in particular on conformationally restricted peptides, focused on addressing PPIs in the context of BCL-2 and p53. Both proteins are involved in apoptotic signalling pathways and are validated intracellular targets in oncology. Helical motifs hold frequently responsible for mediating intracellular protein-protein interaction across many biological pathways as has been demonstrated for the targets indicated above. The complex of BCL-x_L with Bim can be regarded as an example for the interaction of this category of proteins *via* helix recognition (Fig. 3).

Reports on the activation of apoptosis in leukemia cells and *in vivo* tumor regression by a hydrocarbon-stapled BH3 helix has triggered a great deal of interest beyond cancer research due to possibly a general utility of this novel class of cyclic peptides.[19] More stable and protease-resistant conformationally restricted peptides hold promise as chemical tools for the discovery of biological pathways, for understanding of disease mechanisms and ultimately for therapeutic intervention. While peptides have been successfully developed for extracellular targets, *in vivo* studies with peptides targeting intracellular PPIs have been hampered due to their poor cell permeability, instability of secondary structure, rapid clearance and/or proteolytic break-down. The first report of an all-hydrocarbon stapled α-helix peptide strategy by ruthenium-catalyzed ring closing metathesis (RCM) revealed an enhanced helicity and significantly increased metabolic stability for the stapled peptides.[20] This appealing concept has been further applied to claim cell-permeable peptides derived from the amphiphatic α-helical BH3 region of the BCL-2 family.[19] The authors' hypothesized helix stabilization decreases the exposure of the polar amide backbone, thereby improving membrane penetration and at the same time increasing resistance to protease cleavage due to the more rigid secondary structure. Stapled peptides for stabilizing the alpha-helix of BCL-2 domains (SAHBs), were designed to mimic the BH3 domain of the pro apoptotic BH3 only protein BID (Fig. 4).

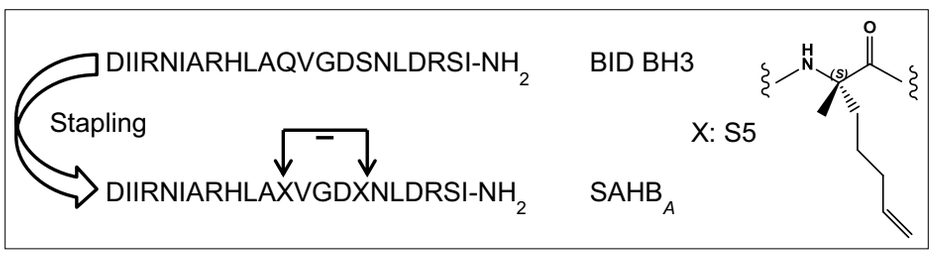

Fig. 4. Strategy for stabilizing the α-helical BH3 domain by hydrocarbon cross-linking. The stapled BID BH3 compound $SAHB_A$ is derived by substituting the i and i+4 positions Q and S in the BID sequence by X (S5, an α,α-di-substituted non-natural amino acid containing an olefin) and subsequent ruthenium-catalyzed ring closing metathesis.[19b]

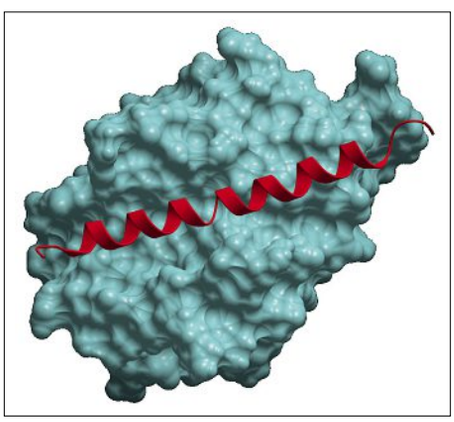

Fig. 3. Structure of BCL-x_L (mouse) with Bim (in red) as generated from 1PQ1.pdb.

These peptides were evaluated in Jurkat T leukemia cells and $SAHB_A$ inhibited the proliferation at a median inhibitory concentration of 2.2 μM in contrast to the BID BH3 wild type peptide and other mutants.[19a] Interestingly, $SAHB_A$ treatment of immunodeficient mice, bearing established human leukemia xenografts, suppressed expansion after day 3, and tumor regression was observed after day 5. Animals receiving the point mutants were not reported to show reduction of tumor volume.

Prompted by these exciting results and our interest in the BCL-2 family, investigation of the stapled BID peptides in our in-house BCL-2 assay revealed weak and non-specific activity compared to ABT263 (Navitoclax) an established BCL-2 small molecule inhibitor. Furthermore, the wild type BID sequence was only weakly active relative to mutant controls, and the activity persisted in Bax/Bak deficient cells. The study revealed that reproducibility of cellular data in the absence of active controls can be difficult. Therefore, it is recommended to also apply identical compounds at the same concentration range to cell lines unspecific for the target. Our experience from a limited set of experiments is supported by a very detailed study of Okamoto *et al.*, who concluded that stabilizing the pro-apoptotic Bim BH3 helix (Bim-SAHB) does not necessarily enhance affinity and biological activity.[21] By contrast to statements from previously cited publications, indicating hydrocarbon linkages to enhance transport into cells and to improve bioavailability, the findings presented in the paper by Okamoto could not support the claims for the Bim-SAHB stapled BH3 peptide sequence. In this study, stabilized Bim-SAHB peptides, exhibited enhanced binding and cellular activity in mouse embryonic fibroblasts and Jurkat cells. In contrast to the topoisomerase II inhibitor Etoposide, this stapled peptide did not trigger cell death. Since the compounds showed significant Cytochrome c release in permeabilized cells, the authors attributed the absence of cell-killing activity to a lack of cellular uptake. Thus, under the conditions investigated, SAHBs did not enter cells in sufficient amounts to induce apoptosis even in serum free media. In the course of this study, the co-crystal structures of Bim-BH3-peptide and Bim-SAHB bound to Bcl-x_L were analyzed in detail. This effort focused on the question how the side chains of constraint peptides interact with each other and to what extent the intramolecular hydrogen-bond network influences binding affinity compared to the wild type. The analyses supported the fact that stapling does not necessarily result in higher binding affinity, and even disrupts important interactions with the target protein. Accordingly, an additional interaction between the staple and the target protein is required to enhance binding.

In a seminal contribution, on the basis of the native p53 sequence, the potency of a 16mer linear Hdm2 binding peptide was improved by stapling to arrive at a K_d of 55 nM.[22] Interestingly, the high affinity for the target could to some extent be confirmed in a viability assay on SJSA-cells (EC_{50}: 8.8 μM) and by dose-dependent caspase-3 activation (EC_{50}: 5.8 μM), respectively. In our in-house assay, potency in the biochemical FRET assay was confirmed, but cellular activity was weaker (see Table 2).

Table 2. Results from biochemical (FRET) and cellular (proliferation) p53/Hdm2 assays on the stapled peptide SAH-p53-8.[23]

Hdm2	Biochemical IC_{50} nM	Cellular IC_{50} μM
Peptide SAH-p53	**12**	**18**

Other peptides, except the most potent peptide described in the original study, did not show any effect in Hdm2 dependent cellular assays. More recently, another cellular active stapled peptide interfering with the p53/Hdm2 interaction resulted from phage display and subsequent medicinal chemistry efforts.[23] Since the biochemical and cellular measurements could not readily be compared with our own data, the most active peptide was synthesized and assayed (Fig. 5).

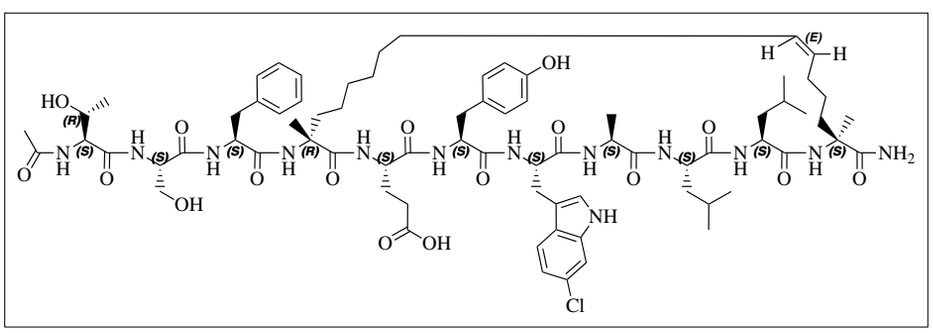

Fig. 5. Structure of the cellular active Hdm2-binding stapled peptide (*trans* isomer).[23]

The potency on the target, as determined in the biochemical binding assay, scored significantly higher; nevertheless cellular activity for the stapled version was not really improved (Table 3). In this case, similar to the experiments reported earlier, there is a difference ≥ 3 log units between biochemical potency on the target compared to a cellular read-out. On the other hand, considering assessment in both biochemical and cellular assays, stapling enhances activity only by a factor of 3. The two α-methylated building blocks may hold responsibility for this fact. These modifications induce to a certain extent a helical conformation, and as a consequence, the linear peptide is already pre-organized. Our study recapitulates the question whether this difference is sufficient to justify stapling to address potency and permeability. Clearly, this limited study did not provide evidence for an enhanced permeability due to cyclization by stapling.

Table 3. Results from biochemical (FRET) and cellular (downstream target induction) p53/Hdm2 assays of the linear and the stapled peptide (*trans* isomer) as indicated in Fig. 5.

Hdm2	Biochemical IC_{50} [nM]	Cellular IC_{50} [μM]
Linear peptide	**0.5**	**~ 30**
Stapled peptide (trans)	**0.15**	**8**

In summary, technologies for hit finding and optimization usually deliver highly potent and selective binders for stable intracellular proteins, but the path forward to design in permeability remains to be defined. However, stapled peptides have been expedient in many drug discovery projects providing tools for the elucidation of targets, and finally, to understand the biology.[24] This intriguing concept is being further developed and more studies are performed to validate their potential to arrive at superior therapeutic peptides for intracellular targets. Other categories of conformationally constraint peptides exhibiting distinct and stable secondary structures are also expected to be cellular permeable, if size is limited and polar groups are well shielded within the molecule.

In addition to the fast progress in display technologies, new methodologies for producing small and medium-sized cyclic peptides are supported by innovative new chemistries, *e.g.* ligation methods.[25] This expansion beyond classical peptide know-how will further enrich the chemical repertoire. Most importantly, this progress is believed to broaden synthesis in a way to access structures complementary to those obtained from natural peptide products and to improve on cyclic peptide formats applied for display technologies. Following an expansion of chemistry to enable routine production of a diverse ensemble of highly modified peptides, synthetic methodologies may be applied not only for lead optimization, but also for primary hit finding.

Conclusions

Definitely, innovation in peptide chemistry will encourage the ability to apply more versatile molecular features required to engineer in the desired properties. Modifications to internalize H-bonds or to apply amide isosters instead of the natural backbone are regarded as relevant examples for derivatives considered to represent generic requirements for enhancement of cellular uptake. Thus, all known and upcoming alterations can be viewed to expand the 'permeability tool box'. This collection of validated transport enhancing groups and chemistries for their incorporation is thought to support a variety of different applications. In general, the capability to cross a membrane, at least in part, is reflected in particular properties of the molecule. In the context of this perspective, any modifications or pro-drug approaches supporting intracellular delivery of peptides, most likely also have an impact on molecules in need to cross the blood–brain barrier, to enhance tissue penetration, or to address oral bioavailability. In addition to functional and structural modifications, a recent publication on the effect of counter ions may as well contribute to the 'permeability tool box' enabling the design of cellular active peptides without compromising on activity or selectivity.[26] Polyion-counterion complexes are reported as a universal motif to generate and modulate function in biomembranes in the broadest sense. Examples include transport, biosensing, aptamer sensing as well as cellular uptake of DNA, RNA and CPPs.

Although progress has been achieved regarding intracellular delivery of peptides, penetration at concentrations relevant for therapeutic dosing has hardly been demonstrated at the cellular level. On the other hand, most of the time, benchmarking by reference peptides either active on the target or from the area of the CPPs is not considered. This standardization is desperately needed, since peptides are applied in a variety of projects and quite a number of cell lines, requiring different conditions for assaying, are studied. For this reason, data from publications on the ability of various peptides to enter cells cannot be readily compared. Moreover, semi-quantitative results obtained from labelled molecules do not offer much help in ranking certain peptides, or modifications contained within a particular sequence, for the potential to promote intracellular uptake. First of all the tag may influence the penetration properties in one way or the other. In addition, the amount of peptide captured in membranes and organelles versus the concentration freely accessible to the target localized in the cytosolic com-

partment remains to be determined. Thus, a pharmaco-dynamic read-out on the basis of a validated standardized cellular assay seems to represent the basis for a systematic investigation.

However, optimization on the target becomes tricky due to endocytosis favored in particular at lower concentration, which leads to entrapment of the active peptide in endosomes/lysosomes. As a consequence of these various endocytotic processes, and due to unspecific protein binding outside of endosomes/lysosomes, the free concentration in the vicinity of the target is largely unknown. Since similar mechanisms for active transport, *e.g.* for uptake of antibody–drug conjugates by the corresponding receptors, result in related obstacles, the question arises how to avoid entrapment in these compartments in the first place and/or how to achieve an efficient release. Hopefully, delivery technologies like pH sensitive liposomes can help to overcome the challenge of release from endosomes/lysosomes.[27] Some other ideas how to overcome these obstacles have been reported, but a systematic study of sequences or preferred conformations encouraging escape have not been presented so far. It remains to be seen whether sequences or structural motifs emerge that enable transport directly to the cytosol or facilitate an efficient release from endosomes/lysosomes. An alternative concept for delivery to the cytosol was recently proposed.[28] Heat-shock protein (HSP) mediated endocytosis was shown to be responsible for internalization of a peptide developed as a cancer vaccine. Furthermore, due to the overexpression of HSPs in tumor cells, this approach suggests opportunities for targeting cancer cells. However, similar to other contributions, this paper did not indicate the proportion of the peptide captured in endosomes/lysosomes. For a judgment on the transport efficiency to the cytosol, a reliable determination of the cytosolic concentration for peptides is desperately needed. So far, a generic methodology to measure intracellular concentrations is lacking, however the sensor technology developed by Kai Johnsson may find some application in this context.[29] This intracellular biosensor consists of SNAP-tag, a fluorescent protein, and a metabolite-binding protein. The SNAP-tag is specifically labeled with a synthetic molecule containing a ligand of the metabolite-binding protein and an additional fluorophore. In the labeled sensor, only a cell-permeable metabolite of interest can displace the intra-molecular ligand from the binding protein to generate a read-out based on ratiometric fluorescent. Once developed for the assessment of cytosolic peptide concentrations, this semisynthetic fluorescent sensor proteins based on the SNAP-tag could become an important option for optimization of permeability. In principle, subcellular fractionation and analysis by mass spectrometry including stable isotopes would represent another option to absolutely determine the free concentration in the cytosol. So far, sensitive peptide detection and very accurate protein/peptide quantitation was combined to assess the intracellular concentration of labeled cell-penetrating compounds on the basis of MALDI-TOF mass spectroscopy.[30] This method relies on a UV-light-absorbing tag on the peptide of interest plus a neutral matrix, and in addition, on the stable isotope labeling of the tagged compounds as reference standards for quantification. Although label-free methods are in general preferred, this technology can be regarded as a starting point for quantification of intracellular concentrations. The application of analytical methods for reliable determination of the cytosolic concentration combined with a robust cellular assay will hopefully enable to define a rational path forward towards the optimization of cellular potency. Once systematic studies in this direction have been initiated, more accurate *in silico* analyses for examination of large hit lists, as indicated in the section on 'Identification of Cell-permeable Peptides', may be generated. For smaller peptides, *in silico* design and analyses tools have already been reported and are thought to help in identification of the best candidates for cellular transport.[10] In an iterative process, feeding more accurate data on larger peptides and enhancing analyses tools, improved computational approaches are thought to emanate. These techniques will not only support the ranking of peptides from hit lists with respect to permeability, but also help to rationalize findings from a larger ensemble of experimental data, and thus, will catalyze optimization of molecular properties. Finally, progress in computational chemistry will help to translate our predictions and structural assessments usually carried out on the basis of a 2-dimensional representation into a 3D-perspective.

As a consequence, progress in the various technologies is thought to encourage fine-tuning of the peptide's properties at the molecular level, to render it more drug-like, and enable intracellular transport without loss of activity or selectivity. The delicate task is to efficiently manipulate groups not in contact with the target, bearing in mind that these sites can also affect the conformational preferences, and thus have an influence on activity. In the long run, besides tailored formulation and potency and selectivity improvements by molecular design, targeting to cells, tissues or organs to reduce side-effects remain the challenges to be mastered. For this reason, targeting peptide therapeutics more efficiently only to the diseased tissue or organ will be of prime importance in the future.

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