DISSERTATIONES TECHNOLOGIAE UNIVERSITATIS TARTUENSIS
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IMRE MÄGER

Characterization of cell-penetrating peptides: assessment of cellular internalization kinetics, mechanisms and bioactivity

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ABSTRACT

Cell-penetrating peptides (CPPs) are relatively short cationic and often amphipathic peptides capable of crossing the cell membrane and delivering their associated cargo molecules to respective intracellular targets. However, which exact pathways are of the most vital importance in this process remains under discussion to date because the comprised routes may depend for example on the CPP type, nature of the delivered cargo molecule, cell membrane constituents of a given cell line and the target cellular compartment. In this thesis, several commonly used CPPs are characterized in terms of their cytoplasmic cargo delivery kinetics and mechanisms using two different – quenched fluorescence and bioluminescence based – assays.

Because different uptake routes may compensate each other, as it has been hypothesized, the kinetic assays provide more valuable information in shedding light on the CPP mechanisms as compared to end-point studies. Indeed, we found that in some cases inhibition of an uptake route in serum-devoid environment results only in a change in internalization kinetic profile, not the total uptake level, or *vice versa*. Further it was discovered that in complete cell growth media different types of CPPs display clearly distinct concentration dependent uptake profiles, which in some cases resemble the internalization of a membrane permeable positive control. CPP uptake rate kinetic profiles vary remarkably and depend strongly on the used endocytosis inhibitors. This supports the hypothesis of several uptake pathways being active simultaneously. By using a cell permeable cytochrome c mimicking apoptosis inducing peptide, it is also demonstrated in this thesis that CPPs can be designed to have significant inherent biological activities. This strategy would in principle allow implementation of drug delivery systems in which the delivered cargo and the delivery vector can be designed to have synergistic effects – either the desired therapeutic (e.g. apoptosis inducing) or other (e.g. increased endosomal release) effects.

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LIST OF ORIGINAL PUBLICATIONS

I. **Mäger, I,** Eiríksdóttir, E, Langel, K, El Andaloussi, S, and Langel, Ü (2009). Assessing the uptake kinetics and internalization mechanisms of cell-penetrating peptides using a quenched fluorescence assay. *Biochim Biophys Acta* **1798:** 338–343.

II. Eiríksdóttir, E, **Mäger, I[*],** Lehto, T, El Andaloussi, S, and Langel, Ü (2010). Cellular internalization kinetics of (luciferin-)cell-penetrating peptide conjugates. *Bioconjug Chem* **21:** 1662–1672.

III. Jones, S, Holm, T, **Mäger, I,** Langel, Ü, and Howl, J (2010). Characterization of bioactive cell penetrating peptides from human cytochrome c: protein mimicry and the development of a novel apoptogenic agent. *Chem Biol* **17:** 735–744.

IV. **Mäger, I,** Langel, K, Eiríksdóttir, E, Lehto, T, and Langel, Ü. The role of endocytosis on the uptake of luciferin-conjugated cell-penetrating peptides. *Manuscript.*

[*] Shared first author

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In all the papers, I participated in the design and planning of the studies. My personal contribution to the articles referred to in this thesis is as follows:

Paper I synthesized and purified the peptides, performed all experiments and data analysis, wrote the paper as the corresponding author;

Paper II participated in the peptide synthesis and purification, performed uptake kinetics experiments and participated in toxicity experiments, performed data analysis, participated in the writing of the paper as the corresponding author;

Paper III synthesized and purified some of the peptides, participated in the writing of the paper;

Paper IV participated in the peptide synthesis and purification, performed uptake kinetics experiments and participated in toxicity experiments, performed data analysis, writing of the manuscript.

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ABBREVIATIONS

2'-OMe RNA	2'-O-methyl-modified ribonucleic acid
Abz	2-Amino benzoic acid
ACPP	Activatable cell-penetrating peptide
C/LR	Caveolae/lipid raft dependent endocytosis
CME	Clathrin-mediated endocytosis
CPP	Cell-penetrating peptide
Cpz	Chlorpromazine
CQ	Chloroquine
CyD	Cytochalasin D
Cyt c	Human cytochrome c
DNP	Dinitrophenol
DTNB	5, 5'-Dithiobis-(2-nitrobenzoic acid)
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
Fmoc	9-Fluorenylmethyloxycarbonyl
GAG	Glycosaminoglycan
HF	Hydrofluoric acid
HKRg	Hepes-buffered Krebs-Ringer solution, supplemented with 1 mg/ml glucose
HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
LDH	Lactate dehydrogenase
mAb	Monoclonal antibody
MBHA	4-Methylbenzhydrylamine
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
MP	Macropinocytosis
MRI	Magnetic resonance imaging
MTT	3-(4,5-Dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide
naCPP	Non-amphipathic cell-penetrating peptide
Nys	Nystatin
ON	Oligonucleotide
paCPP	Primary amphipathic cell-penetrating peptide
PEG	Polyethylene glycol
PI3K	Phosphatidylinositol-3 kinase
PMO	Phosphorodiamidate morpholino oligonucleotide
PNA	Peptide nucleic acid
PSD	Sulfonamide
RNAi	RNA interference
saCPP	Secondary amphipathic cell-penetrating peptide
siRNA	Short interferring ribonucleic acid

SPPS	Solid phase peptide synthesis
t-Boc	*tert*-Butyloxycarbonyl
TFA	Trifluoroacetic acid
TNB	2-Nitro-5-thiobenzoate
TTD	Therapeutic Target Database
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

INTRODUCTION

An increasing number of novel pharmaceutical molecules require access to the cellular interior to exert their dedicated biological effects. However, more than often these molecules are incapable of entering their target cells without the aid of delivery vectors. One type of these vectors is cell-penetrating peptides (CPPs). CPPs were discovered in mid-1990s and have since been used to enable cellular entry for many types of cargo molecules. Furthermore, CPPs with intrinsic biological activities have been described showing a diverse applicability range of these vectors.

CPPs differ in their physicochemical properties and, therefore, they might not share exactly the same internalization routes into certain cellular compartments. In order to develop the most effective transporters for specific purposes, it is therefore essential to study uptake mechanisms, kinetics and biological effects of CPPs with different properties.

Kinetic assays provide many advantages over single-endpoint using methods, because different CPPs can have completely different uptake kinetic profiles, which might not be reflected in endpoint measurements. For instance properties of CPP cargo molecules, concentration and the cell line on which the experiments are conducted can affect their uptake half-life and turnover rate. The sometimes arbitrary choice of end point measurement might affect results, which in turn could lead to biased understanding on CPP uptake. Therefore, we characterized cytoplasmic uptake kinetics and mechanisms of several well-known CPPs (TP10, TP10(Cys), Tat, penetratin, pVec, MAP, M918 and EB1) using two assays, a quenched fluorescence assay and a bioluminescence based assay.

Because many CPPs are derived from naturally existing proteins, it is not a surprising idea that some CPPs can therefore have certain biological effects besides their cargo transport capability, and that CPPs with precise intrinsic biological activity could be developed. To confer this, we studied human cytochrome c (Cyt c), a mitochondria associated protein which is involved in apoptosis initiation. Cell permeable sequences from within Cyt c were predicted and their uptake efficiency and propensity to induce apoptosis were studied. In nature, pro-apoptotic signals cause small amounts of Cyt c to be released from mitochondria to cytoplasm where it can trigger calcium release from endoplasmic reticulum (ER), in turn leading to additional Cyt c release. High amount of Cyt c activates pro-apoptotic caspases which are directly responsible for the programmed cell death. The predicted cell-permeable Cyt c region was extended with a nucleoporin ligand, which lead to increased apoptogenicity and thus expansion of the Cyt c targets.

Results presented in this thesis show that kinetic assays can have a significant advantage in providing a more detailed view about cellular uptake of CPPs as compared to conventional single time point studies. Additionally, the feasibility of developing bioactive CPPs with dual functions is demonstrated. These results emphasize aspects that must be addressed when designing novel cellular transporters for biotechnological applications and for future drug development.

1. LITERATURE OVERVIEW

There are 300 to 6000 unique drug targets that are currently exploited in conventional FDA approved drugs, according to different assessment methods [1]. While up to 14000 drug-targeted-proteins have been reported the number of primary drug targets is much lower. According to the Therapeutic Target Database (TTD) there are 384 successful, 292 clinical trial and 1254 research targets [2]. Although these targets are individually unique, there is one thing that is common in most successful drug targets – namely the majority of unique targets are extracellular enzymes and membrane proteins such as receptors and ion channels. Therefore the active component of most drugs mediates its effect in the extracellular environment [3, 4].

However, an ever increasing number of drug candidates and biologically interesting molecules require intracellular localization to mediate their dedicated effect, whether they are oligonucleotides (ONs) for modulating gene expression, protein-protein interaction mimicking polypeptides, small-molecule chemotherapeutic agents, or contrast agents for different biomedical imaging modalities, to name a few. Due to their physicochemical properties the aforementioned bioactive molecules are often incapable of entering cells by themselves and are thereto unable to reach their targets. Furthermore, for certain *in vivo* applications also delivery across epithelial cell layers including blood-brain-barrier is required. This feature severely limits the possibility to exploit the therapeutic potential of these compounds.

Throughout history many different types of delivery vehicles have been developed to transport bioactive molecules to their dedicated intracellular targets. Out of these the most known and thoroughly studied are liposome, micelle and polymer based systems. These versatile delivery vectors are however not free from disadvantages and for certain applications alternative transport vectors are needed.

This thesis concentrates on characterization of alternative delivery vehicles, CPPs, which aim to overcome the drawbacks of contemporary cargo delivery systems. CPPs are characterized in terms of their cellular internalization kinetics, cytosolic delivery mechanisms and bioactivity.

1.1. CPPs – universal cargo delivery vehicles

In this chapter, a general overview is provided which aims to shed light on different aspects of peptide mediated intracellular cargo delivery for various applications. Different types of CPPs are described as well as different strategies for incorporating cargo molecules. A short overview is also provided on the topic how the properties of CPPs can be enhanced by means of targeting specific tissues, activating their cellular entry only close to target sites, increasing their stability and circulation time *in vivo*. Further, bioactive CPPs which could pos-

sess inherent biological effects *per se* without any conjugated cargos are described as well.

1.1.1. Introduction to CPPs

The first cell-penetrating peptide (CPP), penetratin, was reported by Derossi et al in 1994 [5] followed by the Tat peptide by the group of Lebleu in 1997 [6]. However, it was discovered years earlier (1988) that certain full length proteins such as transcription-transactivating (Tat) protein [7] and Drosophila Antennapedia homeodomain [8] are capable of crossing biological membranes. Ever since then, the CPP field has been extensively researched and a myriad of membrane-translocating sequences have been discovered and developed.

There are many types of CPPs reported in the literature, which makes it difficult to classify them concisely and unambiguously. There are no conserved amino acid sequences that must be present to maintain the membrane translocation property and the secondary structure of CPPs in water can vary to large extent. Hence, the most intuitive way to define CPPs is to govern their common features, which are (i) they are of peptidic origin, i.e. they have a peptide backbone consisting of natural or non-natural amino acids; (ii) they carry a net-positive charge while being usually less than 30 amino acids long; and (iii) they are able to translocate one or more types of molecules over the cell membrane.

Since CPPs are peptide-based, it is relatively simple to modify their properties by attaching different chemical functional groups, for example homing/ targeting sequences. Alternatively the properties can be altered by changing some natural amino acids in CPP sequence with non-natural analogues. These modifications can have profound effects on CPPs, ranging from their cell type specificity, serum stability, interaction with cell membrane components and intracellular localization, to their biological effects, toxicity, cargo complexing ability and uptake mechanisms.

In the following chapters an overview is provided which aims to elucidate different aspects of CPPs and CPP-mediated cargo delivery.

1.1.2. Primary, secondary and non-amphipathic CPPs

While it is possible to divide CPPs into families based on their origin, i.e. protein derived (Figure 1A), chimeric (Figure 1B) and synthetic/designed (Figure 1C) CPPs [9], this classification does not inherently contain information regarding differences in their properties. Therefore, when aspects of CPP uptake mechanisms are being studied, this classification does not help to categorize the data systematically. It does however reflect the range of sources and evolution of CPPs. Shortly, the first CPPs (penetratin and Tat) are derived, as stated above, from protein domains [5, 6]. Later chimeric CPPs were introduced which were derived, at least partly, from the respective parts of naturally occurring

peptides or proteins (e.g. TP10, MPG, Pep-1) [10–12]; after which, purely synthetic peptides such as polyarginine [13, 14], MAP [15], YTA-2 and YTA-4 [16], and CADY [17] were described.

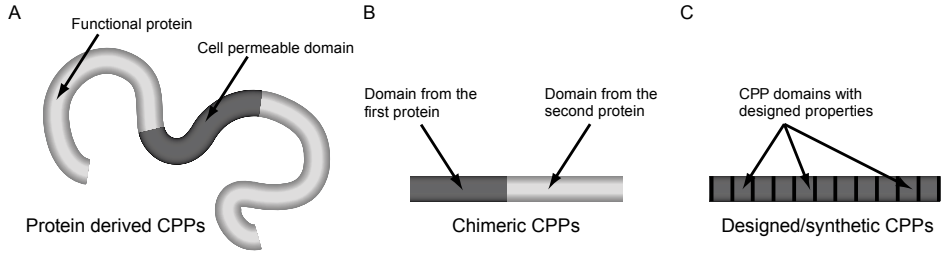

Figure 1. CPP classification based on their origin. CPPs can be derived from a single protein (A), chimeric CPPs can be developed consisting of two functional domains (B), and purely synthetic CPPs can be designed as well (C).

A more intuitive and perhaps an expressive way would be to divide CPPs into subgroups based on their structural properties. This yields in the following classification: (i) primary amphipathic CPPs (paCPPs), (ii) secondary amphipathic CPPs (saCPPs), and (iii) non-amphipathic or polycationic CPPs (naCPPs) [18], see Figure 2.

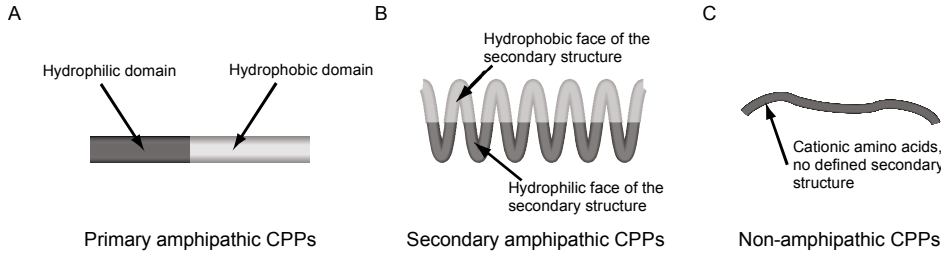

Figure 2. CPP classification based on their structural properties. Primary amphipathic CPPs consist of a hydrophilic and a hydrophobic domain (A), the secondary structure of secondary amphipathic CPPs has a hydrophobic and hydrophilic face in 3D space (B), and cationic/non-amphipathic CPPs do not possess any amphipathicity (C).

Primary amphipathic CPPs (paCPPs)

paCPPs contain hydrophobic and cationic domains within their primary sequence (Figure 2A). These peptides are usually more than 20 amino acids long which exceeds the length of cell membrane bilayer hydrophobic core [19, 20]. paCPPs bind predominantly to membranes via hydrophobic interactions indicated by their affinity both to neutral and anionic lipid membranes [21–26]. These peptides insert into model membranes and induce leakage in a concen-

tration dependent manner and often lyse bacteria without affecting eukaryotic membranes [27]. This suggests that paCPP interactions with phospholipids depend strongly on the membrane composition. Additionally, the transmembrane potential is important factor for interaction between paCPPs and cell membrane [28]. However, it should be noted that most of these properties apply when naked or small molecule labeled peptides are used and it is reasonable to assume that the presence of large bioactive cargoes, e.g. proteins or oligonucleotides (ONs), can affect the binding and membrane interactions considerably.

Secondary amphipathic CPPs (saCPPs)

The second type of amphipathicity arises from the secondary structure of certain peptides. saCPPs, such as penetratin [5], MAP [15] and pVec [29], are often structurally unstable in solution (i.e. appear in random coil conformation) but these peptides adapt an α-helical or in some cases a β-sheet structure upon interaction with membrane components, such as lipids and glycosaminoglycans (GAGs). The projections of their secondary structures reveal the origin of their amphipathicity, when hydrophobic and charged residues become separated into different regions in space [18] (Figure 2B). Contrary to paCPPs, saCPPs bind predominantly to membranes which contain at least 20% of anionic lipids and their interaction with neutrally charged membranes remains weak [24, 30] and membrane perturbation is generally not induced, [31]. However, there might be exceptions, as suggested by the relatively high toxicity of some saCPPs (e.g. MAP).

Non-amphipathic CPPs (naCPPs)

naCPPs, e.g. Tat [6] and polyarginine transporters [13, 32], do not possess any amphipathic properties neither in their primary nor secondary structure. Instead they have high charge density and in some cases they contain only positively charged amino acids (Figure 2C). These polycationic peptides do not seem to interact with membrane lipids but require anionic counterparts such as GAGs for interaction [18, 24]. Their structure nevertheless remains unordered in any environment [33] and it appears that greater structural diversity of these molecules could lead to greater uptake [34]. naCPPs are often shorter than peptides from the other groups. For example polyarginine transporters are most effective when they consist of 6–9 arginine residues and shorter sequences tend to lose their activity while longer sequences can be considerably toxic [34–36].

1.1.3. Cargo conjugation strategies – covalent conjugation and non-covalent complexation

In principle there are two different strategies how to exploit CPPs for cargo delivery. One way is to conjugate a cargo covalently to a CPP [37–39] (Figure

3A), while the second approach is to form non-covalent CPP/cargo complexes simply by mixing these two components [38, 40] (Figure 3B).

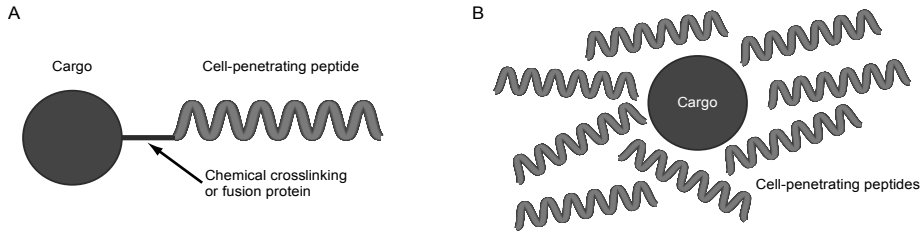

Figure 3. Cargo incorporation strategies. CPPs and cargo molecules can be covalently linked to CPPs either by using chemical crosslinking or expressing CPP-cargo fusion proteins (A). Alternatively, cargo and CPPs can be complexed non-covalently via hydrophobic and electrostatic interactions (B).

These strategies have found successful use in numerous pre-clinical and clinical delivery-tasks [41–45] but both methods have clear advantages and disadvantages. Therefore the delivery strategy must be carefully considered for each application. In this thesis covalent CPP-cargo conjugates are used and therefore a more thorough introduction is provided for this strategy.

Covalent conjugates

CPP and cargo covalent conjugates can be achieved by chemically cross-linking the two molecules, e.g. via a disulfide bridge, by using suitable linkers, or by cloning and expressing of a CPP fusion protein [46], which leads to chemically well-defined molecules. Different covalent conjugation strategies have been extensively used in studies concentrating on intracellular delivery of small molecules (e.g. in the delivery of cytotoxic agents doxorubicin, methotrexate and taxol) [44], therapeutic peptides/proteins [47–49] and ONs [50–52] to treat certain types of tumors.

Besides in tumor related research, covalently conjugated ONs and their analogs have found use in many other areas. For example, peptide nucleic acid (PNA) conjugated to transportan has been used for modifying pain transmission *in vivo* [42], for modulating gene expression and splicing via steric block antisense mechanism [53], and for regulation of microRNA (miRNA) mediated gene expression effects [54, 55]. For similar applications many other types of ONs, such as phosphorodiamidate morpholino oligiomers (PMO) and 2'-O-methyl-modified RNAs (2'-OMe ONs) have been exploited [56]. There have even been attempts to form covalent conjugates between CPPs and short interfering RNA (siRNA). This strategy has found prosperous use in some studies [57–59], but it has not been successful in all performed investigations [60, 61]. The divergent results add some controversy to the field but the differences might be explained with methodological dissimilarities. In the successful

siRNA delivery studies the covalent siRNA-CPP conjugates have not been purified after chemically cross-linking these two molecules together, but the unsuccessful reports used the purified constructs [56].

It has been suggested that free unconjugated ONs can aggregate unwillingly in the presence of CPPs, and negatively charged ONs can, as a side effect, inactivate cationic CPPs (the negative charge of a siRNA molecule is generally much higher than the positive charge of a CPP). In support of this hypothesis, high covalent conjugate concentration is usually required to achieve observable knockdown [60]. However, both of the aforementioned properties can be exploited in other types of cargo transport applications where targeted delivery or stable biologically active CPP/cargo non-covalent complexes are required. This will be described in chapter 1.2.

Non-covalent complexes

There are several reports on CPPs, mostly involving paCPPs and saCPPs, which are capable of forming non-covalent complexes with cargo molecules [46]. Generally non-covalent complexation protocols are simple, as they often require mere mixing of the CPP and cargo. The simple method makes it unnecessary to fine-tune individual peptide and cargo synthesis schemes. Also, in non-covalent settings the CPP might be expected to affect the bioactivity of the cargo molecule less. Despite these advantages with non-covalent conjugation, particle aggregation, complexation affinity and other factors may in some cases hamper the applicability of this strategy and might lead to physiochemically ambiguous and ill-defined systems.

Originally the non-covalent delivery strategies were developed for ONs [46]. Indeed, the most successful reports using this strategy are on gene delivery and delivery of splice correcting oligonucleotides (SCOs) and siRNA [62–68] although some attempts have also been made to deliver proteins by means of this method [12].

Because only covalent CPP-cargo conjugates are used in this thesis, to get a detailed overview of the non-covalent complexation method, the reader is referred to one of the following reviews [39, 46, 56, 69, 70].

1.2. Enhancing the biological properties of CPPs

1.2.1. Targeting strategies

It seems that traditional CPPs are capable of delivering their cargos into almost any type of cells with only minimal differences in efficacy. Of course, there are some cell lines, e.g. primary cells and suspension cells, which are considered to be “difficult-to-transfect” by conventional means and CPPs may face difficulties here, but nevertheless there are successful reports about cargo delivery even in these settings [66, 71]. This indiscriminate property can be advantageous for some applications such as *in vitro* screening/assessment of delivery

systems and comparison of therapeutic leads but it can be a major drawback when targeted delivery is required e.g. when delivery into specific organs or enhanced tumor retention is needed, especially in the case of drug resistance [45].

Since CPP technology is based on solid phase synthesis, it is relatively simple to modify these delivery vehicles by adding specific functional units or targeting ligands into their sequence (Figure 4A). This might help to overcome the mentioned specificity problem, which indeed has been demonstrated in numerous studies. For example, several tumor homing sequences have been identified from phage display experiments [72–78] or chosen based on their known interaction partners on cell membranes such as certain angiogenesis related cell adhesion integrins [79–82] or cell surface gangliosides [83]. Other commonly exploited strategies involve using ligands for membrane bound receptors, such as transferrin and growth-factor receptors, which are highly expressed in some tumor cells [84]. These ligands have been used both for the CPP-mediated delivery of radioactive markers and magnetic resonance imaging (MRI) contrast agents into tumors for imaging purposes as well as for targeted and increased transport of doxorubicin, paclitaxel and other anticancer agents [84].

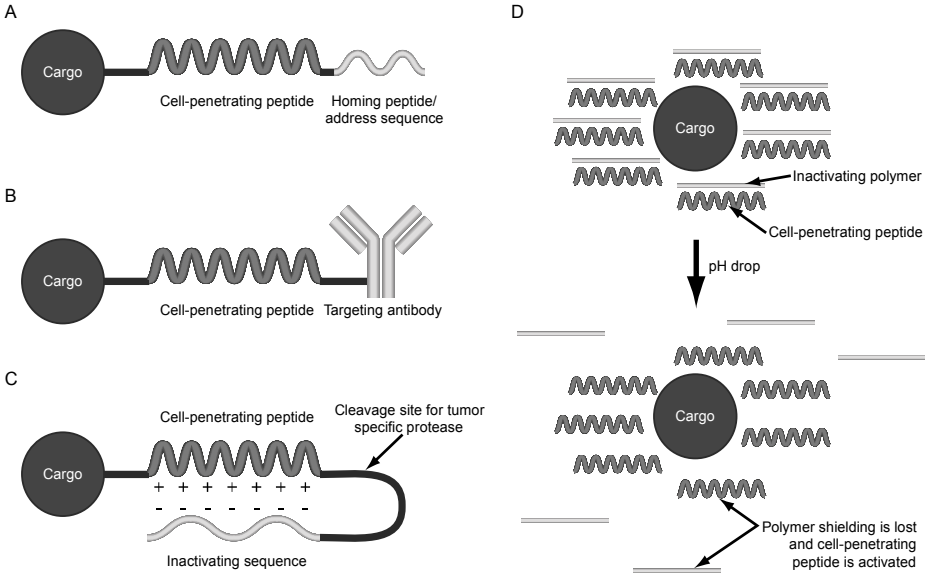

Figure 4. CPP targeting and activation strategies. CPPs can be extended with homing/address sequences (A) or linked to targeting antibodies (B). CPPs might be activated through enzymatic cleavage of inactivating sequences (C) or by low pH using pH sensitive polymers or linkers (D).

Targeted CPP mediated cargo delivery can often be achieved by using CPP-conjugated specific monoclonal antibodies (mAb) (Figure 4B). When a mAb has some therapeutic effects *per se*, then by adding a delivery vector to it allows to design interesting dual therapeutic effect strategies which can be enhanced by additional functional entities such as cytotoxic molecules. CPP-antibody conjugates and targeting moieties have also found successful use in polymer-based delivery systems and as components of liposomes [84–86]. In the latter systems the CPP can be deactivated until the nanoparticles reach their dedicated destination, e.g. a tumor. Similar activatable concept can be incorporated into other CPP technologies too, which are described in the following paragraph.

1.2.2. Activatable CPPs (ACPPs)

Matrix metalloproteinases (MMPs) are expressed in upregulated levels in many types of tumors and are mediators of tissue invasion and metastasis [87]. There are several reports showing these proteases could be exploited to activate the cell-penetrating property of certain peptides [88]. The idea behind ACPP strategy is based on the prodrug concept – a specific protease cleaves the CPP sequence from a negatively charged shielding domain which then allows CPP-conjugated bioactive molecules to enter cells [89–91] (Figure 4C). In addition to tumor specific MMPs, other enzymes can be exploited as well. However, choosing exactly which enzymes to use is often not a trivial task. Thus, in order to find specific cleavage sites from a wider range of possible targets, phage display selections have been carried out to identify unique sequences for efficient labeling of tumors and their metastases [92]. As an advantage of phage display method, ACCPs with cleavage sites for unknown proteases can be found [93].

Other activation strategies take advantage of changes in the microenvironment of dedicated target sites, thus avoiding the need for cell specific proteins. It is well known that rapidly dividing advanced solid tumors have higher need for oxygen. This results in an imbalance between oxygen supply and consumption caused by abnormal physiology of blood microvessels and their increased diffusion distance from tumor cells [94]. While tumor hypoxia and hypoxia-related low pH of tumor microenvironment can seriously hamper the effects of radiotherapy and oxygen dependent cytotoxic agents as well as the outcome of photodynamic therapy [94], it can be effectively exploited in CPP based delivery systems. In these systems drug particles are layered with CPPs which are inactivated by anionic polymers containing e.g. pH sensitive sulfonamide (PSD) group. Due to the aforementioned lower pH at tumor sites, the shielding polymers become protonated and disassociate from CPPs, allowing the liberation of their cargos [95, 96] (Figure 4D). Further use of pH dependence is to employ CPP-modified liposomes that are covered with long polyethylene glycol (PEG) chains. The PEG chains exceed the length of the CPPs and sterically shield them from interacting with cell membranes. However, when reaching tumor

sites, the PEG chains, attached to the liposome via a pH sensitive hydrazone linker, are cleaved and cell penetration properties are activated [97].

1.2.3. Inherently bioactive CPPs

Protein-protein interactions play a key role in cellular signaling pathways and it is known that peptide ligands can mimic these interactions. Thus it is not counterintuitive that intracellularly delivered CPPs by themselves could possess intrinsic biological activities, at least when designed accordingly. However, the border between inherently bioactive CPPs, CPPs fused with small peptide cargos, and chimeric CPPs can be ill-defined and a gray area in terminology exists in the literature.

Usually the bioactive CPPs have been derived from proteins of interest by choosing their cell-permeable domains which would still retain the parent protein related biological effect (Figure 5). For example, N-terminal part of p14ARF protein (ARF^{1-22}) is internalized efficiently into cells and once internalized, it increases p53 activity by inhibition of HMD2 protein and decreases proliferation of tumor cells [98]. Initiation of apoptotic machinery is also demonstrated in another, principally different but elegant study, where the apoptotic BH3 domain of a Bcl-2 family protein was used. This domain induces apoptosis similarly to the protein that it is derived from but it cannot cross cell membranes. However, by a certain technique called stapling its structure could be confined into α-helical conformation which converts the BH3 apoptotic domain into a bioactive CPP [99] (Figure 5).

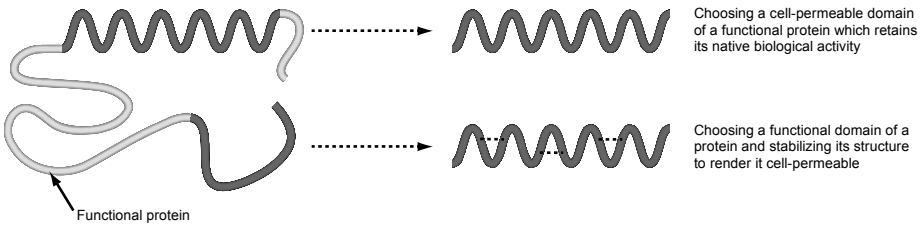

Figure 5. CPPs with intrinsic biological activity. Cell-permeable sequences from within a functional protein could be searched which retain the biological effect of the parent protein (upper), alternatively a functional domain of a protein can be chosen and converted into a cell-penetrating peptide by stabilizing its structure via stapling method (lower).

Human Cyt c, a key player in regulated cell death mechanisms, seems an interesting target in the search for tumor cell apoptosis inducing peptides. There are methods available that can be used for predicting cell-penetrating sequences from within proteins [100, 101] and by applying one of them to Cyt c an effi-

cient cell-penetrating region which retained the original apoptotic activity was discovered (Paper III) [102]. An interesting dual targeting strategy was introduced when the peptide was extended by a nuclear pore complex targeting ligand, which led to an even more potent apoptosis inducer. A problem with apoptosis induction is its specificity (an often occurring problem when working with CPPs). Hence, to render this system cancer specific, an additional tumor homing ligand could be conjugated to the construct, however, this strategy remains so far untested [102].

Dual properties of cellular entry and receptor activation have been shown for a number of other peptides, e.g. a scorpion venom derived peptide modulates intracellular Ca^{2+} levels [103], a peptide derived from GPRC intracellular G-protein binding domain internalizes and activates its related G-protein signaling pathway which results in blood vessel contraction [104], and toll-like receptor 4 (TLR-4) adaptor protein mimicking peptide efficiently reduces the cellular response to inflammatory stimuli [105]. Interestingly, CPPs derived from prions reduce pathogenic prion isoforms upon internalization [106].

It appears that certain CPPs can have effects on endocytic trafficking. For example, vasostatin 1 seems to stimulate caveolae-dependent endocytosis via the phosphatidylinositol-3 kinase (PI3K) dependent pathway [107] and CPPs have been shown to induce F-actin network remodeling/reorganization and activation of macropinocytosis [108–110]. This is suggested to be mediated at least partially by CPP interactions with cell membrane heparan sulfates (HS) linked with specific membrane core proteins (syndecans or glypicans). The latter molecules can activate numerous signaling pathways including ligand internalization events, and their expression can be tissue type dependent [111] which might explain why CPPs can behave differently in seemingly similar conditions. However, these effects are still relatively poorly studied in relation with CPPs.

1.2.4. Increasing the bioavailability of CPPs

As stated in the introductory section of this chapter, the requirement for cargo delivery vehicles arises from low bioavailability of certain drugs. However, while CPPs aim to increase it, they often do not reach their full potential due to roughly three factors: (i) CPPs are relatively vulnerable to the degrading capacity of serum due to their peptidic origin; (ii) CPPs seem to exploit endocytic routes to gain access to cells and thus their entrapment in endosomes remains an issue; and (iii) for *in vivo* use CPPs together with their respective cargos are cleared too fast from the blood circulation through renal clearance.

Several strategies have been used to successfully tackle the aforementioned drawbacks. The peptidic carriers are often N-terminally modified by fatty acids, such as stearic acid, or cholesterol [52, 60, 64] (Figure 6A). Also C-terminal cysteamidation appears to be essential for the stability and penetration mechanism of certain CPPs (Figure 6B), such as MPG, Pep and CADY peptides

[112]. As another example, peptides can be fused together with histidine containing domains. These amino acids are not protonated at physiological pH but become charged at low pH of endosomes. This in turn leads to the endosome destabilization and subsequent cargo release. For example this strategy is used in EB1 peptide for the delivery of siRNA [113]. Alternatively short fusogenic peptides can be exploited to promote endosomal release [113–115] (Figure 6C).

The stability of arginine-rich peptides can be enhanced considerably by using D-amino acids as their building blocks [34, 45, 56] or synthesizing their retro-inverso analogs [116–118]. The latter can however at least in some cases lead to considerable toxicity, contrary to their parent L-peptides [119]. As another strategy, non-natural amino acids could be introduced into peptide sequences (Figure 6D).

Arginine residues can be modified so that their side chains are moved form α-carbon atoms (native peptides) to nitrogen atoms (guanidinium containing peptoids), thereby improving both the stability and activity of the peptide [34]. Additionally, branched and dendrimeric peptides have also been shown to increase uptake [34].

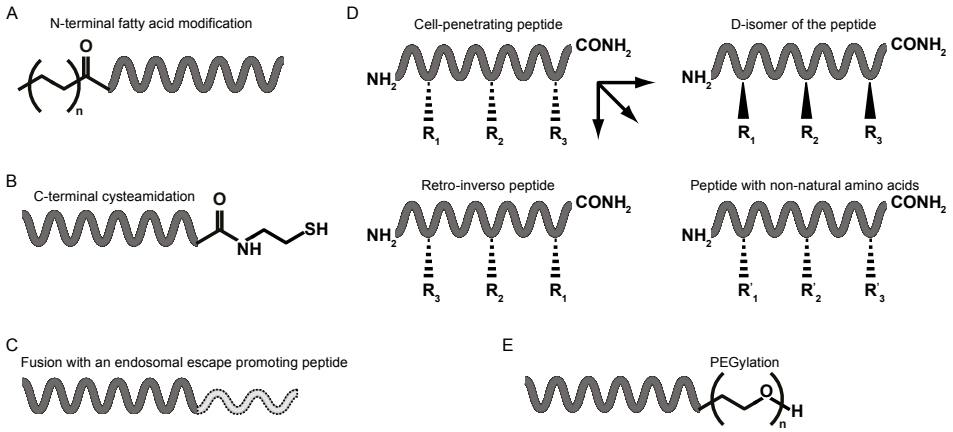

Figure 6. Means to increase bioavailability of CPPs. N-terminal modification with a fatty acid makes CPPs more stable against serum proteins and enhances their non-covalent cargo complexation (A), C-terminal cysteamidation is required for the activity of certain peptides (B), endosomal escape promoting peptides or other moieties might increase biological activity of CPPs (C), D-isomer of peptides as well as retro-inverso analogs and exploitment of non-natural amino acids has been shown to increase CPP stability both *in vitro* and *in vivo* (D), and peptide PEGylation has been shown to increase blood circulation half-life (E).

While incorporation of non-natural amino acids into CPPs increases their serum stability, the fast *in vivo* renal clearance remains still an issue that must be addressed. As already discussed above, this could be partially solved by using

non-covalently complexed large enough CPP nanoparticles. Another strategy to overcome this issue is functionalizing peptide nanoparticles with polyethylene glycol (PEG) chains [120, 121] (Figure 6E).

The abovementioned strategies to improve CPP based cargo delivery systems could make these peptides promising therapeutic vectors. A handful of CPP-based drug delivery vehicles that have successfully entered clinical trials indicate that the chosen path can indeed be fruitful [45, 122].

1.3. Internalization mechanisms of CPPs

The previous chapters contain numerous demonstrations how different types of cargos can be delivered to their required intracellular targets and mediate their dedicated biological effect. Further, these examples are crowned by a handful of ongoing clinical trials in which peptide-mediated drug delivery strategies are exploited. Although being extensively studied since their discovery, there is still no clear consensus in the literature regarding which uptake mechanisms CPPs use when gaining access to cells.

There are numerous methods to study CPP membrane interactions and involvement of endocytosis in the uptake. Internalization mechanisms studies mainly concentrate on registration of cellular entry after inhibiting certain endocytosis routes either by low temperature, by chemical inhibitors, or by knocking down certain pathway related genes. Alternatively co-incubation with specific endocytosis tracer molecules has been used as well.

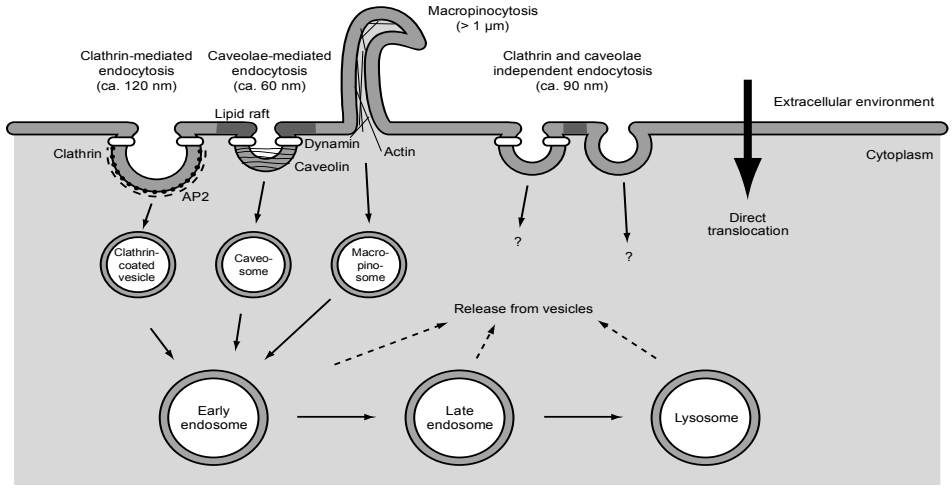

Figure 7. CPP uptake routes. Each fluid phase endocytosis pathway (CME, macropinocytosis, caveolae-mediated endocytosis, and clathrin/caveolae independent endocytosis) have been shown to be involved in CPP uptake. Some CPPs have been shown to directly translocate through biological membranes

Generally it is agreed though, that the main entry mechanism of larger CPP-cargo complexes/conjugates is endocytosis and that several endocytic pathways may be involved in parallel (Figure 7). Prior being endocytosed, CPPs interact commonly with cell membranes and these interaction can be multifaceted, depending largely on the type of a CPP (e.g. paCPP, saCPP or naCPP), the cell membrane composition (e.g. amount of cell surface GAGs, and the ratio of negatively and neutrally charged phospholipids) and the properties of the attached cargo molecule. Additionally, the interactions with receptors, cytoskeleton and specific kinases cannot be overruled either. Importantly, it can clearly be concluded when analyzing different CPP uptake mechanisms studies that the obtained results must be interpreted in the context of the CPP and cargo together. In the following paragraphs an overview is given regarding the aspects of CPP uptake mechanisms.

1.3.1. Different types of endocytosis

Endocytosis is a regulated process of internalizing molecules from extracellular environment to intracellular milieu and because it is heavily involved in CPP uptake then before talking about uptake routes involved in CPP internalization a short background of different types of endocytosis is provided. Coarsely, fluid-phase endocytosis can be subdivided into four different separate groups: clathrin-mediated endocytosis (CME), lipid raft/caveolae-dependent endocytosis, clathrin- and caveolin independent endocytosis, and macropinocytosis.

Clathrin-mediated endocytosis (CME)

CME is the most studied endocytosis pathway which is involved in many important cellular processes, such as controlling the number of signaling receptors on the cell membrane [123], recycling of synaptic vesicle membrane proteins in neurotransmission [124], and uptake of essential nutrients, for example cholesterol and iron [125, 126], to name a few. CME starts when a specific motif binds to its receptor after which the receptors are relocated on the plasma membrane into certain “hot-spots” where the clathrin coated pits start to form [127]. This is mediated by the assembly of cytosolic proteins, the most important of which is clathrin that forms a certain cage-like scaffold around the newly formed invagination. Clathrin is a three legged triskelion structure consisting of three clathrin heavy chains which are tightly bound to clathrin light chains [126]. Certain adaptor protein complexes such as AP2 and AP180 are required in the process, which together with clathrin are essential for vesicle formation in CME.

In order to internalize the receptor bound material, the clathrin coated pits/invaginations must be pinched off from the plasma membrane. This is thought to be mediated by a GTPase dynamin, which self-assembles around the neck of the invagination and pinches the vesicle off the cell membrane; however other GTPases may be involved in the maturation process as well [127].

The endocytosed clathrin coated vesicles are uncoated and delivered to early endosomes which undergo acidification by ATP-dependent proton pumps. During the acidification receptors disassociate from their ligands and some are transported back to the cell membrane. Early endosomes traffic from cell periphery to the perinuclear area and fuse with late endosomes or multivesicular bodies. Later on, these vesicles are matured to lysosomes in which the internalized constituents are enzymatically degraded into nutrients [128] (Figure 7).

To inhibit CME in the experiments included in this thesis, we used hypertonic sucrose solution which is shown to dismantle clathrin structures, and chlorpromazine which is used to deplete clathrin and AP2 adaptor protein complex from the plasma membrane to the endosomal membranes (see Methodological considerations chapter for more details).

Macropinocytosis

Macropinocytosis is regarded as a rather nonspecific internalization route because it does not generally involve specific receptors. Macropinocytosis accompanies membrane ruffling which is usually induced by growth-factors in all types of cells (only in macrophages and dendritic cells can macropinocytosis be constitutively active) and during membrane ruffling, lamellopodia are formed. These structures are cell membrane protrusions which collapse and subsequently form macropinosomes and engulf large amount of extracellular material (Figure 7). The process is dynamin-independent [127]. Interestingly, growth-factor induced macropinosomes seem not to go through degradation pathway but are instead involved in recycling pathways [129].

Membrane ruffling, lamellopodia formation and macropinosome processing require extensive actin remodeling at the cell membrane. Hence it is not surprising that many actin binding proteins, such as Rho-family of GTPases like Rac1 and Cdc42, are involved in this [130]. Additionally, PI3K is required for macropinosome closure in macrophages [131] and for insulin-induced membrane ruffling [132], suggesting that events involved in macropinocytosis can be cell type dependent.

Since PI3K is required for macropinocytosis, we used PI3K inhibitor wortmannin to interfere with this endocytosis pathway. We also used cytochalasin D to inhibit macropinocytosis by using its property to block actin polymerization. More details are provided in the Methodological considerations chapter.

Lipid raft/caveolae-mediated endocytosis

Flask-shaped caveolae, invaginations in the plasma membrane, are abundant in many types of cells. They colocalize with cholesterol and sphingolipid-rich membrane domains which contain many types of signaling molecules and membrane transporters [133]. Caveolae are defined by caveolin, a dimeric cholesterol binding protein, which inserts into the inner leaflet of cell membrane and coats the inner surface of the membrane invaginations [127] (Figure 7). The role of caveolin in lipid raft endocytosis is complex. In one hand, the lack of

caveolin leads to inability of cells to bind and take up serum albumin, but on the other hand caveolin-null mice have normal albumin levels in serum and interstitial space [127].

Also, it seems that caveolae are rather static structures on cell membranes and their internalization is initiated via signaling events. The signaling may involve crosslinking of caveolae-bound surface receptors which triggers uptake of their ligands, somewhat similarly to CME. It is even hypothesized that caveolin is not required for uptake, instead it inhibits it, and that internalization is triggered only after the inhibition is eliminated [134]. After activation, most cell types internalize caveolae slowly with a half-time of more than 20 min, and the overall volume of fluid-phase constituents remains small, except in endothelial cells where caveolae can accommodate 10–20% of the cell membrane [127] .

To inhibit this pathway, we used nystatin, an antifungal cholesterol depleting drug. Upon depleting cholesterol the membrane composition of lipid rafts is changed, which prevents their incorporation for endocytosis (see Methodological considerations chapter).

Clathrin- and caveolin independent endocytosis

This type of endocytosis is less studied, and compared to the other endocytic routes this pathway remains poorly defined. Clathrin- and caveolin independent endocytosis are further subdivided into dynamin-dependent and dynamin-independent routes (Figure 7). However, similarly to caveolae-dependent endocytosis, lipid rich structures are required for internalization in this route. Clathrin- and caveolin independent pathway is for example required for internalization of interleukin-2 receptor, for high and sustained synaptic activity, and for rapid endocytosis by neuroendocrine cells [127].

1.3.2. CPP interactions with cell membrane – the role of negatively charged proteoglycans

As described above, the first step in the internalization of polycationic CPPs is their binding to anionic cell membrane components. This happens via electrostatic interactions and hydrogen bonding with cell membrane carboxylates, phosphates and sulfates. These functional groups are present in membrane phospholipids, fatty acids and GAG-containing heparan sulfate proteoglycans (HSPGs) [34]. Hydrogen bond donation depends of course on the amino acid composition of a CPP. For example, arginine has a guanidinium group in its side chain which allows formation of bidentate bonds, while lysine and ornithine with their single ammonium group can donate only one hydrogen bond.

In polar solutions both guanidinium and ammonium groups have negligible hydration shells and hence are weakly associated with counter ions [135]. However, when being close to cell membrane, i.e. in a locally less polar environ-

ment, the association with phosphate or sulfate counter ions increases [136]. This process is partially driven by the entropy increase which originates from the release of weakly associated counter ions in water solution when the amino acids bind to cell membrane counter ions [137]. Theoretically the strength of this association is stronger when the amino acids insert deeper into the non-polar lipid bilayer, which should be easier for slightly more hydrophobic arginine than lysine [138].

The binding of positively charged amino acids to cell membrane anionic proteoglycans is not merely a biophysical phenomenon. Proteoglycans are involved in many cellular processes, such as growth factor signaling, proliferation, cell adhesion, endocytosis etc. [139], and their expression on the cell surface is precisely controlled. For example, expression of glypicans and syndecans depends strongly on tissue type, developmental stage and pathological state, and they take part in the activation of signal transduction pathways and ligand internalization events [140]. Proteoglycan containing cell membrane microdomains are directly involved in reorganization of cytoskeleton and F-actin polymerization by activation of protein kinase C and Rho/Rac GTPases [127, 141–144] which increases membrane fluidity and can lead to induction macropinocytosis or other endocytosis pathways [108]. Since different CPPs feature different charge densities, arginine content and other properties, it is thus not surprising that depending on the particular expression of GAGs on the cell surface the first binding event of CPPs can differ largely between even seemingly similar experimental setups.

Polycationic naCPPs do not interact with membrane lipids, unlike paCPPs and saCPPs, but require anionic counterparts such as GAGs for interaction [24]. Contrary to paCPPs which bind to both neutral and anionic lipids in a membrane [21–26], saCPPs bind predominantly to membranes which contain at least 20% of anionic lipids [24, 30]. Hence, paCPPs can induce membrane perturbation while saCPPs generally do not when the anionic lipid content in a membrane is low (with some exceptions, e.g. MAP peptide) [31]. In the presence of membrane potential these peptides might induce transient pore formation which can lead to endocytosis independent internalization when no or small cargos are used [145–148]. This occurs often without cytotoxicity; however membrane repair response might mask it [149].

Both for endocytosis induction and pore formation it is necessary that the peptide could induce negative Gaussian curvature within the cell membrane [137]. Lysine residues can induce curvature only in one dimension as opposed to two-dimensional induction by arginines. This probably explains partly why arginines are more important for cellular penetration than lysines in purely cationic CPPs. However, hydrophobic amino acids can support curvature formation as well, suggesting that in case of amphipathic CPPs the overall differences between arginine and lysine containing CPPs could be somewhat reduced [137].

1.3.3. Endocytosis as a key contributor to CPP internalization

All the evidence indicates that endocytosis is involved almost exclusively in the uptake of these peptides when conjugated with larger cargos or when CPP/cargo nano-sized complexes are used. In this process all endocytosis subtypes – macropinocytosis, clathrin-mediated, and caveolin/lipid raft dependent endocytosis – have been shown to be involved [150], however, the conclusions about which CPPs use which endocytic routes in which extent are not always converging [151–158].

However, the lack of consensus is not surprising when the importance of the initial binding event is considered, as discussed in the previous paragraph. Also, somewhat contradicting results can be explained by differences in experimental setups – in some cases only fluorescently labeled CPPs are studied whereas in other cases some biological effect is considered [45]. Even further, in different biological assays the readout could be produced in different cellular compartments, e.g. splice correction assay [159] reflects whether the CPP delivers its cargo effectively into the nucleolus while siRNA delivery studies reflect only access to cytoplasm [160]. Despite diverging results, generally the naCPPs seem more prone to be taken up by cells via macropinocytosis [108] while paCPPs and saCPPs rather tend to prefer CME [158].

The involvement of endocytic routes has been assessed using genetic and pharmacological tools [70]. Both of these methods have advantages and disadvantages. While pharmacological endocytosis inhibitors can in certain cases be unspecific and possess toxicity, their effects are transient and believably short enough so that a cell does not activate compensatory pathways during an experiment. Genetic tools on the other hand can be very specific in knocking down a certain endocytosis pathway, but the complete shut-down of a route raises a question about switching on certain compensatory mechanisms and a concern regarding long term effects on normal physiological state of cells.

1.3.4. Endosomal escape is required for increased bioactivity

CPPs tend more than often to remain entrapped into endocytic vesicles, regardless which endocytic routes are exploited, and hence their biological potential is not fully met [44]. Indeed several groups have observed that quantitative uptake of fluorescently labelled CPP-cargo constructs does not always correlate with the biological effect of the attached cargo molecule. Often co-treatment of cells with known endosomolytic agents such as chloroquine is needed for biologically significant effects [64–66, 114, 161, 162]. Therefore, it is important to develop methods to increase the rate of endosomal escape of CPPs and their cargos.

One possible way to do that is to fuse CPPs with fusogenic peptides, e.g. to HA2 [155, 163], or with histidines that become protonated at endosomal pH and thereby increase destabilization of endosome membranes [113, 164]. By

attaching a fatty acid molecule to a CPP, its endosomal escape can be enhanced even further [56]. For example attachment of stearyl groups to arginine-rich peptides and TP10 has resulted in increased biological activity of the delivered steric block ONs, siRNA and plasmids [32, 64–66, 154, 165, 166]. Other fatty acids have been tested as well but with varying efficacy [167–169]. In addition to the attached stearyl group, covalent attachment of chloroquine analogs to TP10 have increased its endosomal release even further, proved by the observed strong RNA interference (RNAi) effects upon non-covalent delivery of siRNAs [66].

Other methods have been tested as well, such as conjugating photosensitizers to CPPs [170, 171] or using excess Ca^{2+} ions in the extracellular medium [172], but these methods are less explored and are more difficult to implement in biologically relevant systems.

1.3.5. Direct translocation

Most CPPs do not seem to translocate directly over cell membrane and endocytic routes are required, as discussed above. Nevertheless, direct translocation has been shown to be involved in uptake according to some reports studying unconjugated CPPs. For example, translocation of free Tat peptide has been reported to be independent of endocytosis, since the peptide frequently internalizes at low temperature and into genetically modified cells lacking certain endocytic pathways [173]. MPG and Pep are two other peptides that also seem to translocate cells via direct penetration mechanisms, even when conjugated to cargos [17, 62], although the evidence of this is inconclusive according to some other investigations [174]. Additionally, based on microscopy observations and flow cytometry experiments, there seems to be a certain concentration threshold above which the direct translocation of cationic CPPs could be favored [108, 144].

However, according to studies on CPP structural polymorphisms, purely cationic CPPs do not seem to interact with lipid bilayers which in that case should exclude their direct translocation [33]. According to the latter study only amphipathic peptides possess membrane-active properties and, at least hypothetically, could be translocated in an endocytosis independent manner. This is supported for example by an observation that under certain circumstances TP10 forms transient pores in large unilamellar vesicles (LUVs) and promotes calcein leakage from these vesicles [175]. However, to induce that large membrane disturbance, high peptide concentrations are needed that are generally toxic to cells.

1.4. Internalization kinetics of CPPs

The rate of CPP uptake is an important parameter when evaluating CPPs for drug delivery purposes because it provides more information on different aspects of CPP uptake as compared to simple end-point studies. As discussed above, CPP uptake mechanisms depend heavily on concentration and cargo type and before drawing conclusions, origin of the readout must be carefully analyzed. End-point studies using labeled peptides, for example, often fail to distinguish the biologically available fraction of the internalized peptide from the endosome-bound material. In addition to benefits for CPP mechanistic studies, measuring kinetics can provide us with valuable comparative information about how different cargoes and chemical modifications affect CPP uptake.

Despite the above presented advantages, not many studies have concentrated on measuring uptake kinetics of CPPs. The few studies performed include ^{125}I-biotinyl-transportan kinetics in Bowes' melanoma cells [10], NBD penetratin kinetics in K562 cells [176, 177], [^{99m}Tc]Tat [178] and fluoresceinyl-Tat kinetics in Jurkat cells [179]. Internalization kinetics of rhodamine-labeled Tat, polyarginine [180, 181], transportan and penetratin [181] have been measured. More recently, the uptake kinetics of modified fluorescein-labeled polyarginine [182], fluorescein-labeled programmed cell death inducing cyclic hexapeptide conjugated to an arginine rich CPP [183], and fluorescein or TAMRA labeled L- or D-isomer of polyarginine [184] has been reported. We have measured, as presented in this thesis, the cytosolic delivery kinetics of CPPs using a quenched fluorescence assay (Paper I) and a bioluminescence based assay (Paper II and IV).

The kinetic studies have shown that CPP uptake via endocytic routes can be, in some cases, very fast (Paper II), with uptake half-times as low as 2–12 min [178, 180, 185]. In most cases, however, the uptake is slower, with half-times between 20–60 min [177, 185–187]. It should be noted that by comparing only uptake half-times it is difficult to draw conclusions on the uptake mechanisms because endocytosis, especially CME, can be very fast too [127]. To thoroughly analyze CPP internalization mechanisms, different endocytic routes should be systematically inhibited and uptake kinetics measured, preferably using assays with biological readouts.

2. AIMS OF THE STUDY

The general aim of this thesis was to characterize CPPs in terms of their cellular internalization kinetics, mechanisms, and bioactivity. The objective included the assessment of kinetic assays' advantages over conventional single end-point studies and categorizing CPPs based on their uptake kinetics profiles. The goal also included testing the applicability of predicted CPPs with intrinsic biological effects. The precise goals of each paper are described below.

Paper I To determine kinetic parameters of CPP cytosolic internalization using a quenched fluorescence assay and to study the effects of endocytosis inhibitors on these parameters.

Paper II To determine CPP cytosolic cargo delivery kinetics profiles in complete cell growth media using a bioluminescence assay and to categorize CPPs based on these data.

Paper III To test applicability of developing CPPs with intrinsic biological properties by predicting cell permeable sequences from apoptosis inducing human cytochrome c protein and to test and improve the predicted peptide's bioactivity to promote apoptosis.

Paper IV To determine the effects of endocytosis inhibitors on CPP cytosolic cargo delivery kinetics profiles in complete cell growth media using a bioluminescence assay in order to assess CPP uptake mechanisms.

3. METHODOLOGICAL CONSIDERATIONS

The methods and materials used in the current thesis are presented in detail in each publication. This chapter describes some theoretical and practical aspects of the used protocols together with brief explanation and background of the selected methods.

3.1. Selection of CPPs

In this thesis several well-characterized CPPs were used to compare their uptake kinetics, profiles and mechanisms (Paper I, II and IV); additionally novel CPPs with inherent biological activities predicted from proteins with known functions (Paper III) were used as well (Table 1).

Table 1. CPPs used in this thesis.

CPP	Assay	Paper	Ref.
pVec	Uptake kinetics (quenched fluorescence, bioluminescence)	I, II, IV	[29]
M918	Uptake kinetics (quenched fluorescence, bioluminescence)	I, II, IV	[188]
TP10	Uptake kinetics (quenched fluorescence, bioluminescence)	I, II, IV	[189]
Penetratin	Uptake kinetics (bioluminescence)	II, IV	[5]
EB1	Uptake kinetics (bioluminescence)	II, IV	[113]
MAP	Uptake kinetics (bioluminescence)	II, IV	[15]
Tat	Uptake kinetics (bioluminescence)	II, IV	[6]
TP10(Cys)	Uptake kinetics (bioluminescence)	II, IV	[189]
Nup153–Cyt c	Apoptosis induction	III	[102]

In Paper I, three CPPs were studied to assess their uptake kinetics and mechanisms in a quenched fluorescence assay – pVec, derived from murine vascular endothelial cadherin, M918, derived from p14ARF; and a designed chimeric peptide TP10, derived from galanin and mastoparan.

In Paper II and IV, more thorough comparison of uptake profiles and effects of endocytosis inhibitors of eight CPPs was carried out using a bioluminescence assay. In addition to previously mentioned pVec, M918 and TP10, also penetratin, derived from Antennapedia homeodomain; EB1, secondary amphipathic peptide developed from penetratin; MAP, model amphipathic peptide; Tat, HIV-1 TAT transactivator derived peptide; and TP10(Cys), a TP10 version where the Cys residue was attached to Lys^7, were used.

In Paper III, the applicability of a predicted CPP for protein mimicry was studied by using a cell-permeable apoptotic cytochrome c analogue.

3.2. Peptide synthesis

All the used peptides were prepared by solid phase peptide synthesis (SPPS), a robust and effective method for production of peptides, introduced by Bruce Merrifield in 1963 [190]. According to this method, amino acids are subsequently anchored to small porous insoluble beads, resin. The resin is of polymeric origin and can be functionalized by different reactive groups, linkers, in order to facilitate coupling of amino acids and growth of peptide chain. The linkers are designed to be stable in chemical conditions required for SPPS, so that the growing peptide chain could remain covalently attached to the resin during the synthesis, but sensitive to specific conditions such as strong acids (e.g. trifluoroacetic acid (TFA) or anhydrous hydrofluoric acid (HF), depending on the SPPS chemistry) so that the final peptide chain could be cleaved from the resin after being driven the reaction to completion. The use of a solid support allows having excess of reagents in each synthesis step which can be easily washed away when using appropriate filter-equipped reaction vessels. We used 4-methylbenzhydrylamine (MBHA) and Rink-amide MBHA resins which creates C-terminally amidated peptides, meaning that the final peptides contain one less negative charge and are biologically more stable than their carboxyl group containing analogues.

Chemically reactive functional groups of the coupled amino acids must be protected with certain protecting groups to suppress unwanted side reactions. N^{α}-atoms of amino acids are reactive as well and must be temporarily protected during synthesis. The used temporary protecting group can be either a 9-fluorenylmethyloxycarbonyl (Fmoc) or a *tert*-butyloxycarbonyl (*t*-Boc) group, defining the chemistry of SPPS.

In each synthesis cycle of SPPS, the alpha-carboxyl group of a N^{α}-protected amino acid is activated, then the activated amino acid is coupled to the growing peptide chain on the solid support via formation of a peptide bond, and the temporary protecting group Fmoc or *t*-Boc is subsequently removed by base or acid, respectively. Every step is followed by appropriate washing steps to remove excess reagents.

The peptide is finally cleaved from the resin and permanent protection groups are removed from chemically reactive amino acid side chains by TFA (Fmoc chemistry) or HF (*t*-Boc chemistry) in the presence of scavenger molecules. These molecules are used to neutralize the removed chemically reactive species from the protection groups which may otherways give rise to side reactions and change the structure of the final peptide. Then the peptide is purified by reversed phase HPLC and molecular mass is determined by mass spectrometry.

3.3. Choice of cargo molecules

The cargo molecules that were conjugated to the synthesized CPPs were chosen based on characteristics of the used assays. CPPs were conjugated to a fluorescently labeled cargo peptide in the quenched fluorescence assay (Figure 8A) for the estimation of cytoplasmic cargo delivery kinetics using a fluorometer (Paper I) and for visualizing the cellular distribution of internalized peptides by confocal microscopy (Paper III).

To improve the cellular internalization kinetics studies, in Paper II and IV luciferin-linker was used as a cargo molecule attached to CPPs via a disulfide bridge. In the cytoplasm the luciferin-linker is cleaved from the transporter and the linker goes through a conformational change and is spontaneously released from the luciferin molecule (Figure 8B). Therefore, this semi-biological readout system is suitable for measuring CPP internalization kinetics, making the assay more biologically relevant.

In Paper III, an intrinsically bioactive CPP was predicted and chosen, meaning that the cargo and transporter could not be formally separated (i.e. the CPP *is* its own cargo). This approach was used to demonstrate the applicability of protein mimicry with intracellular targets without the need for an additional transporter system.

3.4. CPP and cargo conjugation through a disulfide bond

In each paper, CPP-cargo constructs conjugated via a disulfide bridge have been utilized. We chose this strategy due to a common observation that disulfide bonds are readily cleaved in cytoplasmic environment due to its high glutathione concentration. This strategy has been extensively used in the literature [158, 191–193], because it allows registration of cytoplasmic portion of the internalized conjugates, while not taking into account endosomally entrapped material. This eliminates an often occurring drawback of many other CPP internalization studies where fluorescently labeled CPPs that are trapped in endosomes lead to overestimation of biologically available constructs [45].

There are, however, some concerns found in the literature regarding this strategy. Namely, it has been reported that disulfide bonds can be partially reduced on cell membranes of some cells and in vesicles by protein disulfide isomerase or gamma-interferon-inducible lysosomal thiol reductase [194]. The reduction might depend even on the peptide sequence itself [195]. These results indicate that it might be necessary to determine the reduction capability separately for each peptide and cell type combination. Nevertheless, it has been suggested that despite these concerns the vast majority of reduction takes place in cytoplasm [196] and in accordance with the latter we have not seen any change in extracellular sulfhydryl content in our assays.

3.5. Cell cultures

Two different cell types were used in this thesis. In Paper I HeLa cells were used. HeLa cells are immortalized cervical cancer cells taken from Henrietta Lacks in 1951. These cells grow rapidly and are relatively robust making their handling easy which is the main reason why HeLa cells have been the cell line of choice in many research areas including in CPP field. In Paper II and IV a modified HeLa cell line was used, HeLa pLuc 705 cells. The latter cell line is stably transfected with a luciferase gene which is interrupted by intron 2 of β-globin pre-mRNA carrying a cryptic splice site. These cells have been extensively used by our and other groups for estimating delivery efficacy of splice correcting oligonucleotides and for assessing uptake routes of different CPPs. Therefore, we chose this cell line for our follow-up CPP uptake kinetic studies in order to be able to compare the data presented in this thesis (Paper II and IV) with earlier results obtained with the splice correction assay by our group.

U373MG human astrocytoma cells were used in paper III to study CPPs with native apoptotic characteristics. This cell line was chosen because it is well characterized and has previously been used by the group of Prof. J. Howl with whom we collaborate for studying apoptotic events.

3.6. Measuring uptake kinetics of CPPs

The efficacy of CPPs and their uptake mechanisms are usually assessed using static end-point assays. However, these experiments do not necessarily reflect all aspects of CPP internalization and the kinetic uptake studies can shed additional light on the matter.

We therefore chose to assess the uptake kinetics and internalization mechanisms of selected CPPs using a quenched fluorescence assay (Paper I). In this assay we labeled a short peptide cargo with a fluorophore while the used CPPs carried a corresponding fluorescence quencher. To conjugate the cargo with CPPs we decided to use a disulfide bridge as stated above because this will be readily cleaved in cell's cytoplasm leading to spatial separation of the fluorophore and the quencher, which leads to increased fluorescence (Figure 8A). Using this assay it is possible to quantitatively measure the total CPP uptake directly in cell suspension using a fluorometer.

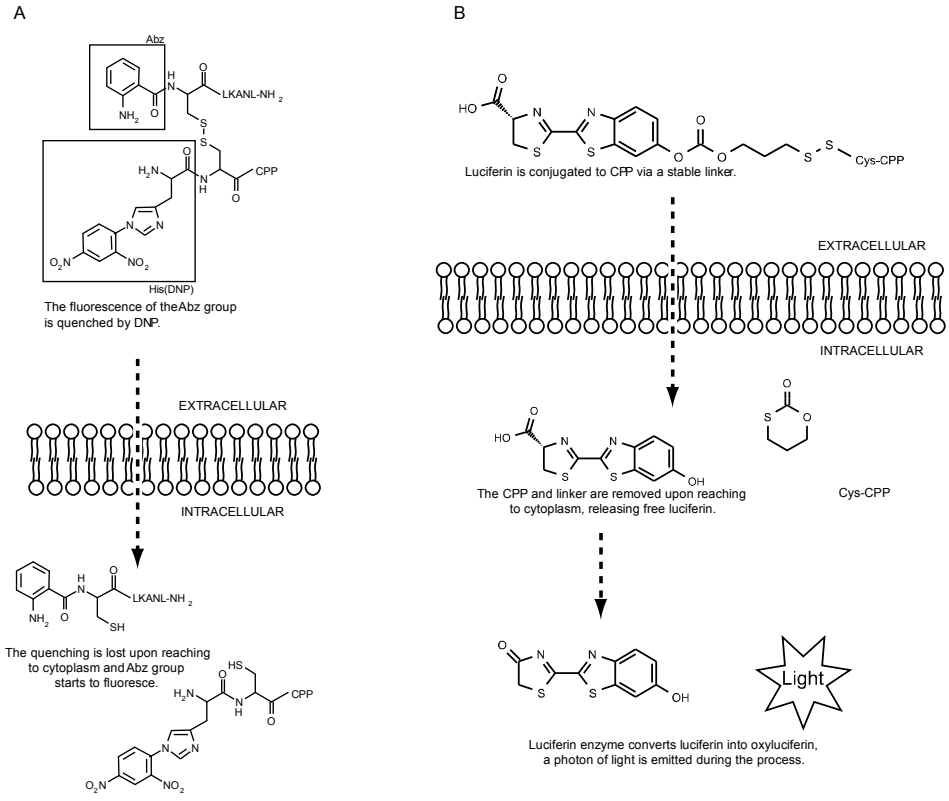

Figure 8. Assays to measure uptake kinetics of CPPs used in this thesis. (A) In the quenched fluorescence assay the fluorophore and its quencher are physically separated from each other and fluorescence is produced when the conjugate is reduced in the cytoplasm. (B) In the bioluminescence based assay luciferin is conjugated to CPPs. When the conjugate reaches cytoplasm, the luciferin is released and luciferase enzyme converts luciferin into oxyluciferin and a photon of light is emitted.

To further improve the performance of uptake kinetic assays in the follow up studies (Paper II and IV), we changed the setup and used luciferin as a cargo. Again, we conjugated it to CPPs over a disulfide bridge. When the conjugate reaches cytoplasm, the linker is reduced and free luciferin is released which is oxidized by luciferase enzyme, emitting a photon of light (Figure 8B). The luminescence can be read by luminometer and total uptake estimated at any time point. This assay has two important advantages over the previously used quenched fluorescence assay – firstly the assay is semi-biological, and secondly adherent cells can be measured. Furthermore, any cell line that is stably or transiently transfected with luciferase gene can be used to characterize the delivery using this assay.

3.7. Interfering with uptake pathways by using endocytosis inhibitors

Because various endocytic routes are involved in CPP uptake, as discussed above, we aimed to interfere with these pathways using different endocytosis inhibitors to elucidate the uptake mechanisms of CPPs. In Paper I, four different inhibitors were used – chlorpromazine (10 μM), wortmannin (50 nM), sucrose (0.4 M) and cytochalasin D (4 μM) whereas in Paper IV chlorpromazine (10 μM), cytochalasin D (4 μM), nystatin (50 μM) and chloroquine (100 μM) was used (Table 2).

Table 2. Endocytosis inhibitors used in this thesis.

Inhibitor	Inhibition mechanism	Inhibition effect		
		CME*	MP*	C/LR*
Chlorpromazine	Clathrin/AP2 depletion from plasma membrane to endosomal membranes	+++	+	–
Sucrose	Dispersion of clathrin from plasma membrane	+++	+	+
Wortmannin	Phosphatidylinositol 3-kinase (PI3K) inhibitor	+	+++	+
Cytochalasin D	Blocking of actin polymerization, disassembly of actin cytoskeleton	+	+++	+
Nystatin	Cholesterol depletion from plasma membrane	–	–	+++
Chloroquine	Slows down acidification of endosomal vesicles, promotes endosomal escape	+	+	+

* CME – clathrin mediated endocytosis; MP – macropinocytosis; C/LR – caveolae/lipid raft mediated endocytosis

Both chlorpromazine and sucrose were used to inhibit clathrin mediated endocytosis (CME), also referred to as receptor mediated endocytosis. In CME a specific coat protein clathrin, containing light and heavy chains, is required to self-assemble on the intracellular face of cell membrane after which clathrin coated pits could be formed. Using hypertonic sucrose solution has been a popular method to disperse clathrin structures on the plasma membrane and thus to inhibit CME [197]. However, because it has been shown to affect all major endocytic routes to some extent [198] its lack of specificity should be considered.

Therefore we used hypertonic sucrose solution only in Paper I and in conjunction with a more specific CME inhibitor chlorpromazine. Chlorpromazine, a cationic amphipathic drug, triggers the depletion of clathrin and AP2 adaptor protein complex from the plasma membrane and leads to their artificial assembly on endosomal membranes [198]. However, it should be considered

that because chlorpromazine is amphipathic, it can insert into plasma membrane and change its fluidity. It can also inhibit phospholipase C which is important for actin dynamics and macropinocytosis, both factors which could give rise to some misinterpretations [198].

Wortmannin is an inhibitor of PI3K. PI3K-generated lipid mediators are highly needed in the reorganization of the actin cytoskeleton because these molecules regulate nucleation, elongation and bundling of actin filaments [199, 200]. These processes are important for macropinocytosis, which is why wortmannin has been used to inhibit this endocytosis route [198]. However, side effects can occur when using wortmannin because phosphatidylinositides link scaffold and signaling proteins to cell membrane, including certain clathrin adaptors and dynamin GTPase [200, 201]. As a side effect, CME and caveolae-mediated endocytosis might therefore be inhibited to some extent [198].

As mentioned above, rearrangement of actin cytoskeleton is required for macropinocytosis and macropinosomes are coated with F-actin [202]. Cytochalasin D blocks actin polymerization and disassembles the existing actin cytoskeleton thereby inhibiting membrane ruffling and macropinocytosis [198]. However, in addition to being extensively involved in the latter pathway, actin cytoskeleton is required partly in CME and caveolae-mediated endocytosis as well and, therefore, unspecific events can take place when using cytochalasin D [198].

Nystatin, in essence an antifungal drug, has been used to inhibit lipid raft/caveolae-mediated endocytosis due to its property to form large aggregates upon binding to cholesterol [198]. This leads to changes in caveolar shape and inhibition of lipid-raft ligands' binding and subsequent internalization, which makes nystatin quite specific endocytosis inhibitor that does not affect CME or macropinocytosis.

Chloroquine, a weak base, is cell membrane permeable if deprotonated (10% at physiological pH), but when protonated, it cannot cross the membrane. Therefore, chloroquine accumulates in acidic vesicles such as early endosomes, slows down their acidification and induces their swelling and promotes rupture via a proton sponge effect [203]. Hence chloroquine treatment is traditionally aimed to enhance endosomal escape of the internalized material to increase its bioavailability. However, chloroquine might also possess other effects – it inhibits ligand uptake, receptor recycling, and vesicle recycling which can slow down the overall endocytosis pathway [204]. We measured uptake kinetics of luciferin-conjugated CPPs in presence of chloroquine to observe which of these effects prevail in early incubation times (Paper IV).

3.8. Toxicity studies

Several assays were used in this thesis to assess possible cytotoxic effects of CPP conjugates. To monitor whether there is an increase in extracellular thiol concentration, e.g. due to disulfide bridge cleavage of CPP-cargo conjugates prior cellular entry or because of membranolytic effects and outflow of a cytosolic small molecule glutathione into extracellular media, we incubated cells with Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid) or DTNB) at various time points (Paper I, II). DTNB reacts with free thiols, upon which 2-nitro-5-thiobenzoate (TNB^-) is released which in turn ionizes to quantifiable yellow TNB^{2-}.

The integrity of plasma membrane was also analyzed by an assay which measures leakage of lactate dehydrogenase (LDH) from damaged cells (Paper I, II and IV). LDH is an enzyme required to convert lactate to pyruvate, with accompanying conversion of NAD^+ to NADH. In the commercially available CytoTox-ONE™ assay used in this thesis, NADH is used in turn to convert resazurin to resofurin, the fluorescent end product of the assay, which is proportional to the released LDH and therefore proportional to membrane leakage.

In Paper III the cell viability was measured using the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion assay. MTT is reduced to purple formazan by mitochondrial dehydrogenases of living metabolically active cells. Formazan, and hence mitochondrial dehydrogenase activity and activity of living cells, is quantifiable by spectrophotometry.

For assessing apoptosis in Paper III, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed. In this method fragmented DNA, resulting from apoptotic signaling events, is labeled and detected. The enzyme terminal deoxynucleotidyl transferase is used to add labeled dUTPs to the DNA nicks, and the label is visualized by confocal microscopy.

The apoptotic events in Paper III were also described by measuring caspase-3 activity in intact cells. Caspase-3 is a cysteine-aspartic acid protease which is activated in apoptotic cells. Caspase-3 in turn activates caspases 6, 7 and 9, which are essential for apoptosis progression. Caspase-3 activity was measured by cell permeable NucView™ 488 Caspase-3 substrate. The substrate is a fluorogenic DNA dye coupled to a caspase-3 specific DEVD substrate moiety. When activated caspase-3 has cleaved the DEVD moiety, the DNA dye migrates to the cell nucleus, binds to DNA and becomes fluorescent, which can be visualized by confocal microscopy.

4. RESULTS AND DISCUSSION

In the four papers included in this thesis (three published articles and one manuscript) selected CPPs are characterized in terms of their cellular internalization kinetics, uptake mechanisms and bioactivity. The kinetic assays used in Paper I, II and IV were designed to provide data on how efficiently, fast, and according to which kinetic profile cargos are transported into cytoplasm by different CPPs. Because the readout of the kinetic assays depends on whether the conjugates have reached the cytosol or not, conclusions regarding biological availability of these cargo-CPP constructs can also be made. In Paper III, cellular translocation capability and bioactivity of apoptosis inducing peptides, which mimic the cytochrome c protein, was studied and a novel cell-penetrating peptide with intrinsic biological activity was discovered. In this chapter, the results of each paper will be summarized and discussed.

4.1. Endocytosis inhibitors affect kinetic constants of CPP uptake, as assessed by a quenched fluorescence assay (Paper I)

So far only few studies have concentrated on measuring real-time CPP uptake kinetics to compare the efficacy or uptake mechanisms of CPPs. Studies have often relied on end-point measurements, but in some cases this strategy may provide biased results. This is because in end-point investigations efficient CPPs with high turnover and less efficient CPPs with low turnover might not be distinguished. Furthermore, the endosomally entrapped portion of CPPs is often not accounted for, leading to overestimation of CPP bioavailability to required cellular compartments [205]. This led us to measure CPP uptake kinetics in conditions where the registered signal would arise only, or as much as possible, from the cytoplasm.

We labeled three CPPs (M918, TP10 and pVec) with a fluorescence quencher dinitrophenol (DNP) and conjugated the peptides over a disulfide bridge to a pentapeptide cargo molecule, labeled with a fluorophore 2-amino benzoic acid (Abz). We used the conjugates to compare these three CPPs in terms of their cargo delivery kinetics and efficacy into HeLa cells. The cells were suspended in HKRg buffer and the uptake was measured in the presence and absence of endocytosis inhibitors chlorpromazine, wortmannin, sucrose and cytochalasin D (see Methodological considerations chapter). First order uptake kinetics equation described the uptake kinetics profile well, according to the formula

$$Y = Y_{max} \cdot [1 - \exp(-K \cdot x)] \qquad \text{(Eq. 1)},$$

where Y is the uptake level (picomoles of internalized peptide), Y_{max} is the maximal uptake level, K is the first-order rate constant in s^{-1}, and x is time in s.

In general terms of total uptake, M918 peptide outperforms pVec which is in turn superior to TP10 in this assay. Interestingly, while the uptake rate of M918 peptide increases with concentration, the rate constant of pVec and TP10 is higher at lower concentration. When CPP concentration was increased 5 times (from 1 μM to 5 μM) the total uptake of M918 increased by a factor of 3.7, while uptake of pVec and TP10 increased by 6.3 and 10 times, respectively.

Endocytosis inhibitors affect both the first-order rate constant and the total uptake level of CPP conjugates. Interestingly, the inhibitors affect the M918 peptide most while pVec is least influenced by them. While all the used inhibitors lowered the total uptake of M918, sucrose and wortmannin increased the rate constant. For TP10, the uptake is lowered and rate constant increased by chlorpromazine, wortmannin and sucrose, while cytochalasin D slightly increases the overall uptake but has no effect on the uptake rate. For pVec, only sucrose is capable of lowering its total uptake and increasing its rate constant. These data suggest that different competing uptake mechanisms with different efficacies may be involved simultaneously in uptake of different CPPs. While uptake of pVec and M918 seem to be dependent on both macropinocytosis and CME, the predominant cellular entry route for TP10 is consistent with CME. These findings are in line with the previously published results using different assays [158, 206, 207].

These data reveal that when CPPs are compared in terms of their uptake efficiency, kinetic data provide a more thorough picture of it than end-point assays, because CPP concentration affects both the kinetics and the total uptake of CPPs and importantly this relationship is not necessarily linear or similar for different CPPs. These points should be taken into serious consideration when designing end-point studies.

4.2. Based on CPP uptake kinetic profiles in complete media, there are two distinct groups of CPPs, as assessed by a bioluminescence assay (Paper II)

The previously used quenched fluorescence assay has certain drawbacks (such as the presence of background signal and limitation to measure pre-suspended cells) that we aimed to overcome in next studies. Therefore, a bioluminescence based assay was used to assess uptake kinetics of CPPs in adherent cells and in complete cell growth media. This assay has two important advantages. Firstly, the readout is biological which lowers the probability of measuring artifacts, and secondly, the assay has near-zero background signal which aids in the data interpretation process.

We measured uptake kinetics of eight different CPPs (Tat, TP10, TP10(Cys), MAP, pVec, penetratin, EB1 and M918) and our results show that in complete media the CPPs display either a fast exponential profile or slower sigmoidal profile. The profile depends on the specific CPP and to some extent also on its

concentration. At all concentrations, Tat peptide displayed a fast cytosolic entry with a decaying exponential profile. The uptake is similarly to membrane permeable free luciferin used as a positive control. On the other hand, EB1, TP10(Cys), M918, penetratin and pVec displayed a slower sigmoidal profile. Interestingly, TP10 and MAP fall into the both categories, depending on their concentration. 10 μM TP10 and 5–10 μM MAP peptide fall into the fast internalizing group, whereas at lower concentrations they behave according to the sigmoidal slow uptake profile.

EB1 peptide was the only peptide from the sigmoidal group which displayed a certain phase change in its behavior, more precisely, at 10 μM concentration its maximal uptake rate occurred 30 min earlier than at its lower concentration and earlier than for any other peptide in the sigmoidal group. This is in accordance with the endosomolytic design of this peptide.

TP10 and TP10(Cys) differ only in the position where luciferin was coupled. In the case of TP10, luciferin was conjugated to the peptide's N-terminus, in case of TP10(Cys) cysteine was coupled to the side chain Lys^7 and the cargo was attached to that position. This rather small modification led interestingly to two completely different uptake profiles. While TP10 fell into the fast internalization group at higher concentrations, TP10(Cys) always followed the sigmoidal profile.

For the sigmoidal uptake profile peptides, we also estimated the time at which the maximal uptake rate occurred. In most cases the maximal uptake rate occurred approximately after 30–60 min incubation except for 2.5 μM MAP, 5 μM TP10, and 10 μM EB1. At the mentioned concentrations, these peptides displayed the fastest cytosolic internalization well before 10 min. It should be noted that when the concentration was raised above this threshold, TP10 and MAP peptide moved from the sigmoidal uptake group to the fast, free luciferin-like group. EB1 was not tested at higher concentrations.

These data collectively support the idea that kinetic studies indeed provide more data on the differences between CPPs compared to simple end-point studies.

4.3. Apoptosis can be induced by inherently bioactive cell-permeable cytochrome c analogues (Paper III)

In addition to CPP uptake mechanism studies where a reporter/cargo molecule is covalently attached to these carrier molecules, it was also examined how inherently bioactive cell-penetrating peptides could be exploited to mediate their dedicated biological effects. Induction of apoptosis by cytochrome c (Cyt c) analogues was selected as a model system and cell-permeable sequences from within human Cyt c were predicted by QSAR analysis.

Cyt $c^{77–101}$ was found to have cellular internalization properties. It localized to endoplasmic reticulum (ER) and was capable of inducing apoptosis with

LD_{50} at 81 μM. However, when the Cyt $c^{77–101}$ was N-terminally extended with a sequence derived from the nucleoporin 153 (Nup153) protein, $Nup153^{980–987}$, in order to target nuclear pore complex (NPC), the resulting chimeric peptide Nup153-Cyt c changed its localization from ER to perinuclear region. At longer incubation times Nup153-Cyt c induced considerable redistribution of the NPC protein Nup153 from the perinuclear area to the cytosol and nucleus. Most importantly, apoptogenicity of the resulting chimera was at the same time increased remarkably (LD_{50} 0.7 μM).

Taken together, this demonstrates that CPPs with intrinsic biological activities could be developed via rather simple steps, which eases biotechnological development and could sometimes eliminate problematic cargo conjugation problems. Furthermore, in protein mimicry by chimeric cell-permeable Cyt c analogues, the nuclear pore complex poses as a novel target for apoptosis induction for therapeutic purposes. In future studies, cell type specificity might be added to the system by incorporating specific peptidic address motifs into the molecule thereby increasing the potential applicability of this strategy.

4.4. Endocytosis inhibitors affect the CPP uptake kinetic profiles, as assessed by a bioluminescence assay (Paper IV)

The compelling results of Paper II regarding the clearly distinct uptake kinetic profiles of the fast and slow internalizing group peptides lead to the question whether the fast profile might reflect the endocytosis independent internalization route. To assess this we used the same bioluminescence based assay as in Paper II to measure the cytosolic entry kinetics of eight different CPPs (Tat, TP10, TP10(Cys), MAP, pVec, penetratin, EB1 and M918) in the presence of certain endocytosis inhibitors.

In order to interfere with different types of endocytosis we used chlorpromazine (Cpz) to inhibit clathrin mediated endocytosis (CME), cytochalasin D (CyD) to inhibit macropinocytosis (MP), and nystatin (Nys) to inhibit caveolae/lipid raft dependent endocytosis (C/LR) (Table 2). It is known that in some cases endocytosis inhibitors might not be exclusively specific but nevertheless useful because by using chemical inhibitors long term activation of compensatory transport pathways could be minimized [198, 208]. We chose the mentioned inhibitors to have minimized cross-inhibition effects [198]. In addition, chloroquine (CQ) was used (Table 2), which is conventionally exploited to inhibit acidification rate of early endosomes and to promote the long term release of the endosomally entrapped material; however the decelerated acidification is accompanied by slowing down the vesicle recycling which could in turn slow down the overall endocytosis rate [203, 204]. By using CQ we aimed to test which of these mechanisms prevail in early uptake kinetics.

First we studied effects of the used endocytosis inhibitors on the overall cytosolic entry of luciferin-CPP conjugates in various time points (15, 30, 60 and 120 min). This analysis revealed that mostly macropinocytosis seems to be involved in the uptake of these conjugates, except for the Tat peptide conjugate. However, the overall uptake inhibition level depends both on the incubation time and luciferin-CPP concentration. This is in line with some previously published studies [46, 69]; even the nuclear delivery of M918, penetratin and Tat has been found to be strongly dependent on macropinocytosis [158]. However, CME seems to be involved much less in the cytoplasmic entry of luciferin-TP10 conjugate as opposed to its nuclear delivery [158], reflecting the differences between the results obtained by different readouts and cargos.

In addition to the previously mentioned analysis of the overall uptake in selected time points we also studied and characterized the uptake rate kinetics curves. This showed that co-incubation with endocytosis inhibitors can significantly change the kinetic behavior of CPPs, suggesting thereby more clearly the involvment of CME and caveolae/lipid raft mediated endocytosis in the cytosolic entry of luciferin-CPP conjugates. Interestingly we found that caveolae/lipid raft mediated endocytosis is more involved at higher conjugate concentration of all the slow uptake group peptides whereas CME is involved at lower concentration of penetratin and EB1 contrary to M918 and pVec.

In conclusion, in Paper IV we demonstrated that in the cytosolic delivery of luciferin-CPP conjugates the prevailing uptake route is macropinocytosis, even for the fast internalizing group peptides. To a smaller extent CME and caveolae/lipid-raft dependent endocytosis are used too but their involvement depends on the peptide concentration in case of certain CPPs. The employment of these pathways is more clearly revealed when analyzing the kinetic parameters or shapes of the uptake rate kinetics curves than when comparing the overall uptake in selected time points. This reflects the importance of kinetic studies when analyzing CPP uptake mechanisms and this information might be essential for designing peptide-based delivery vectors. The used endocytosis sub-type might be important for defining the intracellular fate and targets of the internalized material, similarly to some internalized receptors and their ligands [198, 208].

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SUMMARY

Cell-penetrating peptides appear attractive candidates for delivering many types of cargoes to the intracellular milieu of cells *in vitro* and *in vivo*. Which pathways exactly CPPs use to gain access to the required cellular compartments, e.g. the cytoplasm or nucleus, has nevertheless remained a question to be answered so far, despite the numerous published studies to address this issue. It seems to be agreed, however, that mostly a combination of different endocytosis subtypes is exploited while in some settings direct membrane translocation cannot be excluded.

Most of the studies of the CPP uptake mechanisms rely on measurement of overall peptide uptake in a single time point. This is often done using fluorescently labeled peptides, which generally cannot fully reflect the delivery of biologically available material. To overcome this issue biological readout systems have been developed but they also have certain drawbacks. For example, these assays are mainly end-point studies and are often carried out as side-investigations, thus the potential concentration dependent phenomena are often left unregistered.

We measured CPP uptake kinetics with an aim to avoid the aforementioned drawbacks. We implemented a quenched fluorescence and a bioluminescence based assay to assess the CPP uptake mechanisms which are involved in the cytoplasmic cargo delivery.

In addition to CPP uptake mechanism studies where a reporter/cargo molecule is covalently attached to these carrier molecules, it was also examined how inherently bioactive cell-penetrating peptides could be exploited to mediate their dedicated biological effects.

The results of Paper I, II, III and IV collectively suggest that:

1. when CPPs are compared in terms of their uptake efficiency kinetic data provides a more thorough picture of it than end-point assays;
2. in some cases the endocytosis inhibitors affect only the CPP cytoplasmic entry rate but not the overall uptake if incubation time is long enough while in other cases the trend is reversed;
3. CPPs with comparable overall uptake can have very different uptake rate kinetic profiles. The peptides cluster into two groups: the fast and slow cytosol-entering peptides while the fast group resembles closely the behavior of membrane permeable positive control;
4. endocytosis, mostly macropinocytosis, is highly involved in the cytosolic entry of CPPs, including in the case of the fast uptake group peptides;
5. the involvement of other endocytosis sub-types, CME and caveolae/lipid raft mediated endocytosis, depends on the particular CPP and its concentration;
6. CPPs with intrinsic biological effects, such as apoptosis inducing Cyt c peptide, could be developed using CPP prediction tools;
7. the parent protein apoptosis inducing properties can be retained in the cell-permeable sub domain and these properties could be enhanced and directed towards novel intracellular targets by specific targeting ligands.

SUMMARY IN ESTONIAN

Rakkusisenevate peptiidide sisenemiskineetika, mehhanismid ja vahetu bioloogiline aktiivsus

Üha enam uusi farmakoloogiliselt huvipakkuvaid molekule või ravimikandidaate, näiteks oligonukleotiidid ja terapeutilised valgud, omavad toimet rakusiseses keskkonnas. Paraku enamik neid molekule ei suuda ilma vastavate transportvektorite abita rakumembraani ületada ega jõua seega oma sihtmärkideni. Erinevaid võimalikke transportsüsteeme, millega nende molekulide rakkudesse jõudmist parandada, on mitmeid. Nendest üheks on rakkusisenevad peptiidid (RSP-d). Alates RSP-de avastatamisest 1990-ndatel aastate keskel, on neid peptiide ulatuslikult kasutatud erinevate lastmolekulide rakkudesse viimiseks ja paljude bioloogiliste protesside uurimiseks. Veelgi enam, välja on töötatud ka sellised RSP-d, mis omavad ise vahetut bioloogilist efekti. Seega RSP-de võimalikud rakendusalad on laiad.

RSP-d erinevad teineteisest oma füsikokeemiliste omaduste poolest ja seetõttu ei pruugi nad jagada täpselt samu rakkudesse sisenemise mehhanisme. Samas mõnesse kindlasse rakupiirkonda kohalejõudmine võib sõltuda just RSP-de sisenemisteest, mistõttu on oluline uurida RSP-de internalisatsiooni mehhanisme, kineetikat ja vahetuid bioloogilisi efekte.

Sisenemismehhanismide uurimisel tuleks silmas pidada mitmeid asjaolusid. Näiteks mõõtes RSP-de rakkudesse jõudmise kineetikat, saab nende transportmolekulide kohta detailsemat informatsiooni kui konventsionaalsetest lõpppunktmõõtmistest, kuna näiliselt sarnase efektiivsusega peptiidid võivad omada täiesti erinevaid kineetilisi profiile. Lisaks sellele võivad RSP-de lastmolekulid, kontsentratsioon ja eksperimendis kasutatav rakuliin mõjutada kogu transportsüsteemi omadusi ning muuta katsete tulemusi. Samuti võib eksperimenditulemuste interpreteerimist mõjutada see, milline oli mõõtmiseks valitud ajapunkt. Seetõttu me kitsendasime uuritavat probleemi ja analüüsisime neid mehhanisme, mis on seotud RSP-de jõudmisega HeLa rakkude tsütoplasmasse. Me mõõtsime kaheksa tuntud RSP (TP10, TP10(Cys), Tat, penetratin, pVec, MAP, M918 and EB1) rakkudesse sisenemise kineetikat kahel erineval meetodil – fluorestsentsi kustutamisel ja bioluminestsentsil põhineval meetodil.

Kineetikakatsete põhjal järeldasime, et teatud juhtudel mõjutavad endotsütoosi inhibiitorid ainult RSP-de rakkude tsütoplasmasse sisenemise kineetikakonstanti, kuid mitte absoluutset rakkudesse toimetatava materjali hulka (või vastupidi). Samuti leidsime, et sarnase efektiivsusega RSP-d võivad rakkudesse sisenemisel omada oluliselt erinevat kineetilist profiili. Mõnede RSP-de (näiteks Tat, MAP ja TP10) rakkudesse sisenemise kineetiline profiil võib olulisel määral sarnaneda teatud juhtudel rakumembraani vabalt ületava positiivse kontrolli profiiliga ning kogu protsess võib toimuda mõne minuti jooksul. Enamiku testitud RSP-de sisenemiskineetika oli aga märgatavalt aeglasem ja toimus ajalise viivitusega. Sellest hoolimata sisenesid kõik testitud RSP-d, k.a. kiire kineetilise profiili kohaselt sisenevad peptiidid, kasutades endotsütootilisi mehhanisme,

eelkõige makropinotsütoosi. Leidsime, et teiste vesikulaarsete transportteede (klatriini-vahendatud endotsütoos ja kaveoolide / lipiidparvede poolt vahendatud endotsütoos) hõlmamine sõltub konkreetsest RSP-st ja selle kontsentratsioonist. Näiteks penetratin'i ja EB1 sisenemine tsütoplasmasse sõltus kaveoolide vahendatud endotsütoosist eelkõige kõrgematel peptiidikontsentratsioonidel, kuid pVec ja M918 kasutasid nimetatud endotsütoosi mehhanismi eelkõige madalamatel kontsentratsioonidel.

Kuna paljud RSP-d on tuletatud looduslikult eksisteerivatest valkudest, on mõistlik eeldada, et lisaks lastmolekulide rakkudesse toimetamisele võib nii mõnigi RSP omada ise teatud vahetuid bioloogilisi efekte. Selle uurimiseks me valisime inimese tsütokroom c (Cyt c) valgu, mis normaaloludes paikneb mitokondrites ja osaleb muuhulgas raku apoptoosi esilekutsumisprotsessis. Pro-apoptootiliste signaalide ilmnemisel vabaneb mitokonditest tsütoplasmasse väike kogus Cyt c proteiini, mis tingib kaltsiumi vabanemise endoplasmaatilisest retiikulumist, mis omakorda suurendab postiivse tagasiside mehhanismi teel mitokondrites sisalduva Cyt c täiendavat vabanemist. Suur Cyt c hulk aktiveerib pro-apoptootilised kaspaasid, mis juhivad programmeeritud rakusurma protsessi.

Me hindasime vastavate ennustusalgoritmide abil, millised Cyt c lõigud võiksid suure tõenäosusega rakkudesse siseneda, ja testisime ennustatud järjestuste rakkudesse sisenemise määra ning apoptoosi esilekutsumise võimet. Leidsime, et ennustatud Cyt c analoog (Cyt $c^{77–101}$) siseneb rakkudesse efektiivselt, kolokaliseerub endoplasmaatilise retiikulumiga ja osaleb apoptoosi indutseerimises, nagu oodatud. Kui täiendasime seda Cyt c analoogi teatud nukleoporiini (Nup153) ligandiga, mis seondub tuuma poori kompleksiga, lokaliseerus Cyt c analoog perinukleaarsesse piirkonda. Lisaks sellele suurenes selle kimäärse peptiidi apoptoosi esile kutsumise võime märgatavalt. Rakkude peptiidiga töötlemise tulemusel lokaliseerus tuuma poori kompleksi Nup153 valk perinukleaarsest alast tsütoplasmasse ning rakutuuma. Seega kinnitasime uudse apoptoosis osaleva sihtmärgi olulisust nimetatud bioloogilise efekti esilekutsumises.

Võttes kokku käesolevas doktoritöös esitatud tulemused, võib väita, et kineetikal põhinevad mõõtmised on RSP-de rakkudesse sisenemise mehhanismide hindamisel võrreldes lõpp-punktmõõtmistega oluliselt informatiivsemad. Samuti me näitasime, et duaalsete omadustega RSP-de ennustuspõhine arendamine võib olla efektiivne viis uudsete ravimmolekulide transportsüsteemide väljatöötamiseks. Leitud tulemused võivad olla olulised biotehnoloogia- ja meditsiinialaste rakenduste juurutamisel.

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1. I. Mäger, E. Eiríksdóttir, K. Langel, S. El Andaloussi, Ü. Langel (2010) Assessing the uptake kinetics and internalization mechanisms of cell-penetrating peptides using a quenched fluorescence assay. *Biochim Biophys Acta*. **1798**(3):338–43.
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