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Cell-penetrating peptides: mechanism and kinetics of cargo delivery

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Abstract

Cell-penetrating peptides (CPPs) are short peptides of less than 30 amino acids that are able to penetrate cell membranes and translocate different cargoes into cells. The only common feature of these peptides appears to be that they are amphipathic and net positively charged. The mechanism of cell translocation is not known but it is apparently receptor and energy independent although, in certain cases, translocation can be partially mediated by endocytosis. Cargoes that are successfully internalized by CPPs range from small molecules to proteins and supramolecular particles. Most CPPs are inert or have very limited side effects. Their penetration into cells is rapid and initially first-order, with half-times from 5 to 20 min. The size of smaller cargoes does not affect the rate of internalization, but with larger cargoes, the rate is substantially decreased. CPPs are novel vehicles for the translocation of cargo into cells, whose properties make them potential drug delivery agents, of interest for future use.

Keywords: Drug delivery; Membrane translocation; Cellular uptake; Endocytosis; Toxicity

Contents

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1. Introduction

Over the last 10 years, it has been found that certain peptides and proteins can penetrate the cell membrane and enter the cell. A variety of cargo molecules can be attached to these peptides and proteins and translocated into the cell. Carrier peptides and proteins thus constitute a new class of potential drug delivery vectors. Currently, about hundred such peptides and a few proteins are known. In this review, we shall concentrate on the carrier peptides and aim to give an up to date survey of their structure, kinetics of cell penetration, cargo delivery potential, and undesirable side effects. Transduction proteins will be only briefly mentioned here, since they are covered in a separate review. However, in most cases only fractions of the transduction protein sequence are necessary for translocation and internalization of cargo into the cells, so these peptides will be included in the review.

Membranes of eucaryotic cells and organelles, as well as the cell wall and membrane of pathogenic microorganisms, constitute a serious barrier for the access of hydrophilic drugs to their target molecules inside the cell structures. To overcome problems of conventional and gene drug delivery, various techniques have been developed. A conventional procedure for delivering genetic material is to use viral vectors, but treating genetic disorders with this method has met with only limited success [1]. Alternative nonviral methods, such as electroporation, microinjection, and the use of liposomes, have been developed for conventional and gene drug delivery. These methods have been proved to be effective in vitro and for research purposes, but show limited potential for delivery in vivo due to toxicity, cell damage, and immunogenicity. They are also technically demanding in their application, lack tissue and cell specificity, and can deliver material to only a limited amount of cells. In view of these considerations, transduction peptides and proteins offer a promising new tool for noninvasive delivery of hydrophilic drugs and genetic material.

2. Cell-penetrating peptides—an overview

2.1. Terminology, classification, and structure

A unified terminology and classification of the carrier peptides has not yet been developed. The commonly used name is "cell-penetrating peptide" (CPP) which was more widely introduced in a review article [2] and in the first book [3] on this subject. Very generally, CPPs are up to 30 amino acid amphiphilic peptides, which can be internalized by cells by mechanisms that require no energy and are receptor mediated or not. According to a recently suggested classification, CPPs can be arranged in three classes: protein derived CPPs, model peptides, and designed CPPs [4]. Protein derived CPPs usually consist of the minimal effective partial sequence of the parent translocation protein, and are known also as protein transduction domains or membrane translocation sequences. Model CPPs comprise sequences that have been designed with the aim of producing well defined amphipathic α-helical structures or of mimicking the structures of known CPPs. Designed CPPs are usually chimeric peptides composed of a hydrophilic and a hydrophobic domain of different origin. The structures and origins of the most prominent representative CPPs are shown in Table 1.

Table 1
Examples of cell-

Name sequence
Penetratin
RQIKIWFQNRR
Tat
CGRKKRRQRR
pVEC
LLILRRRIRKQ
MAP
KLALKLALKA
$(Arg)_7$
RRRRRRR
MPG
GALFLGFLGA
Transportan
GWTLNSAGYL

[a] These pept

Table 1
Examples of cell-penetrating peptides (CPPs)

Name sequence	Class source	Ref.
Penetratin RQIKIWFQNRRMKWKK[a]	**protein derived CPP** *Drosophila* Antennapedia homeodomain (amino acids 43–58)	[5]
Tat CGRKKRRQRRRPPQC[a]	**protein derived CPP** protein from human immunodeficiency virus 1 (amino acids 48–60)	[6]
pVEC LLIILRRRIRKQAHAHSK-amide	**protein derived CPP** derived from murine vascular endothelial cadherin	[7]
MAP KLALKLALKALKAALKLA-amide	**model peptide**	[8]
$(Arg)_7$ RRRRRRR	**model peptide**	[9]
MPG GALFLGFLGAAGSTMGAWSQPKSKRKV	**designed CPP** peptide derived from fusion sequence of HIV-1 gp41 protein coupled to peptide derived from the nuclear localization sequence of SV40 T-antigen	[10]
Transportan GWTLNSAGYLLGKINLKALAALAKISIL-amide	**designed CPP** minimal active part of galanin (amino acids 1–12) coupled to mastoparan via Lys^{13}	[11]

[a] These peptides can be amidated at C-terminus or not.

As can be seen, CPPs from different classes do not share common amino acid sequence motifs. The only two common features of all CPPs appear to be a positive charge and amphipathicity. All known CPPs are net positively charged at physiological pH, and incorporate from approximately 17% (for instance hCT(9-32) [12]) to 100% (polyarginines [9,13]) of positively charged amino acids. Most CPPs have only one negative charge–at the C-terminus–or even none, when the C-terminus is amidated. This does not mean that negative charges would prevent internalization of CPP into the cells as shown by the example of VT5, a representative model peptide that carries four negative charges in addition to five positive ones, and is effectively internalized [14]. With the exception of polycationic homopolymers (polyarginines, polylysines and polyornithines [9,15,16]), all other CPPs are amphipathic. Some of them adopt amphipathic character when in an α-helical structure, as for instance the model peptide MAP [8], while others have distinct hydrophobic and hydrophilic parts, as, for instance, the chimeric CPP transportan [11].

2.2. Penetration of CPPs in vitro and in vivo

The transport of CPPs across membranes has been studied using cultured cells, artificial lipid vesicles, tissues and in vivo.

A number of different mammalian cells have been used. CPPs were successfully internalized in primary cells such as those from rat brain and rat spinal cord [5], calf aorta [17], porcine and human umbilical vein endothelium [8], and in osteoclast culture [18] but, most often, cell lines have been used. The main cell lines employed in the internalization experiments are listed in Table 2.

No special cell cultivating procedures are needed for internalization studies with cell lines. Cells are usually grown to 70–80% confluence in flasks, dishes or coverslips. The layers of cells can be incubated with a solution of CPP as such or detached from the surface and a suspension of cells prepared prior to incubation with CPP in order to assure a uniformly accessible cell surface. However, detachment of cells may have an impact on the kinetics and efficiency of internalization. Scraping cells from the dish surface will result in a number of broken cells, and trypsin treatment could affect cell surface proteins. When the internalization of CPP is monitored directly by confocal microscopy or other imaging techniques, the cells are usually fixed by formaldehyde or paraformaldehyde—milder fixation agents than acetone and methanol [41–43]. It has been argued recently that even mild fixation can affect the internalization of some CPPs [32]. However, internalization of penetratin, Tat and trans-

Table 2
Frequently used cell lines in which internalization of labelled or cargo conjugated cell-penetrating peptide (CPP) was confirmed

Cell line	CPP	Label/cargo	Reference
3T3	penetratin	oligonucleotide (20 mer)	[19]
	Tat	oligonucleotide (20 mer), phage	[19,20]
	MPG	plasmid	[21]
A9	pVEC	peptide nucleic acid (6 mer), streptavidin-FITC	[7]
A431	penetratin	liposome	[22]
	Tat	liposome, phage	[20,22]
ADR	penetratin	liposome	[22]
	Tat	liposome	[22]
AEC	penetratin	peptide nucleic acid (6 mer)	[7]
	MAP	fluorescein	[8]
	pVEC	peptide nucleic acid (6 mer), streptavidin-FITC	[7]
bEnd	penetratin	peptide nucleic acid (6 mer)	[7]
	pVEC	peptide nucleic acid (6 mer)	[7]
BM	MAP	peptide (5 mer)	[23]
Bowes	penetratin	peptide nucleic acid (6 mer), streptavidin-FITC, biotin, avidin, ^{125}I, peptide (5 mer), peptide nucleic acid (21 mer)	[7,11,23–25]
	Tat	peptide (5 mer)	[23]
	transportan	^{125}I, biotin, peptide (5 mer)	[11,23]
	transportan-10	biotin	[26]
	pVEC	peptide nucleic acid (6 mer), streptavidin-FITC	[7]
BRL	transportan	green fluorescent protein, antibodies	[27]
C17.2	Tat	ferromagnetic particles	[28]
C26	penetratin	liposome	[22]
	Tat	liposome	[22]
Caco-2	penetratin	fluorescein	[29]
	Tat	Tc-99m	[30]
	transportan	fluorescein	[29]
	transportan-10	fluorescein	[29]
CCL39	Tat	fluorescein	[6]
CEM	transportan	peptide nucleic acid	[31]
CHO K-1	Tat	fluorescein	[32]
	(Arg)9	fluorescein	[32]
Cos-1	Tat	phage	[20]
Cos-7	transportan	green fluorescent protein, antibodies	[27]
DU145	penetratin	peptide nucleic acid (11 mer) (82)	[33]
ECV 304	MAP	fluorescein	[8]
GH	Tat	fluorescein	[6]
H9C2	Tat	liposome	[34]
	MTS-NLS	phosphorothioate (26 mer)	[35]
HBCEC	penetratin	peptide nucleic acid (6 mer), streptavidin-FITC	[7]
	pVEC	peptide nucleic acid (6 mer), streptavidin-FITC	[7]
HeLa	Tat	fluorescein, phage	[6,20,30]
	(Arg)9	fluorescein	[32]
HEP G2	MAP	fluorescein	[8]
HS-68	MPG	plasmid	[21]
HTB 9	penetratin	liposome	[22]
	Tat	liposome	[22]
JR8	penetratin	peptide nucleic acid (13 mer)	[36]
Jurkat	Tat	fluorescein, Tc-99m	[32,34]
	transportan	peptide nucleic acid (12–16 mer)	[31]
	(Arg)9	fluorescein	[13,32]
K 562	penetratin	7-nitrobenz-2-oxo-1, 3-diazol-4-yl-group	[37]
KB3-1	Tat	fluorescein	[30]
MBT-2	Tat	liposome	[22]
MCF-7	penetratin	liposome	[22]
	Tat	liposome	[22]
NG108-15	Tat	antitetanus F(ab′)2 fragment	[38]
PC-12	penetratin	oligonucleotide (21 mer)	[39]
Rev-12-T6	Tat	antitetanus F(ab′)2 fragment	[38]
Rin m5F	transportan	biotin	[26]
SEZ	MAP	fluorescein	[8]
SK-BR-3	penetratin	liposome	[22]
	Tat	fluorescein	[30]
T84	Tat	phosphopeptide (10 mer)	[40]

	Reference
	[20]
ent	[27]
ıdies	
c	[33]
(82)	
	[8]
	[6]
	[34]
ate	[35]
c	[7]
c	[7]
	[6,20,30]
	[32]
	[8]
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	[22]
	[22]
c	[36]
	[32,34]
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oxo-1,	[37]
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	[30]
	[22]
	[22]
	[22]
	[38]
e	[39]
b′)2	[38]
	[26]
	[8]
	[22]
	[30]
e	[40]

portan was detected in both fixed and live cells from several cell lines (Bowes, Jurkat, HeLa, Caco-2) [11,23,24,29,30,32,44].

Only a few internalization experiments have been performed in vivo in whole organisms and even fewer ex vivo in isolated tissue. Ex vivo tissue experiments were done mainly in isolated blood vessels. Blood vessel endothelial tissue from isolated mouse aorta rings successfully took up the caveolin-1 scaffolding domain peptide attached to penetratin, resulting in inhibition of acetylcholine-induced vasodilatation and nitric oxide production [45]. The same peptide, used systemically in vivo in mice, suppressed acute inflammation and vascular leak as efficiently as glucocorticoid or endothelial nitric oxide synthetase inhibitors [45]. Most in vivo experiments were carried out with penetratin and Tat. Besides the blood vessel cells (see above), penetratin was successfully employed in vivo to enter peritoneal exudate cells [46] and to reach brain and spinal cord cells, in some cases also by passing the blood–brain barrier [25,47,48]. Tat was used in vivo to deliver active P-galactosidase in cells from all tissues of mouse [49]. Although ex vivo and, in particular, in vivo experiments that resulted in clear physiological responses are of exceptional importance for understanding the mechanism of internalization, and particularly for demonstrating the drug delivery potential of CPPs, these experiments are not suitable for studies of kinetics of internalization because of difficulties in obtaining sufficient kinetic data under well defined and reproducible experimental conditions. Proper kinetic experiments are almost exclusively obtained with cells from cell lines.

2.3. Mechanism of penetration

Mechanism of internalization of CPP has not been resolved yet. In spite of some common features of these peptides, particularly their highly cationic nature, their structural diversity (see Table 1) has fuelled the idea that the transduction mechanism is not the same for CPPs of different types.

A number of investigations of CPP internalization in cell lines have been carried out under conditions that should prevent active transport of CPPs and their translocation by endocytotic pathways. Efficient translocation was observed at low temperatures (0 to +4 °C) and in the presence of many different inhibitors of endocytosis [5–8,11,24,26,27,29,30,32,37,44,50,51]. Recent results showed, however, that the role of endocytosis in internalization of CPP is not negligible [52,53]. At least for some CPPs endocytosis could be an exclusive or alternative mechanism of internalization. It was shown that the internalization of penetratin and protegrin-1 derived SynB peptides into the live cells is related to endocytotic processes [54] and that Tat derived CPPs do not enter live cells at low temperature [32] and are not internalized into liposomes [55]. Recently it was suggested that Tat derived CPPs enter cells primarily by lipid raft-mediated macropinocytosis that is stimulated by cell-surface binding of Tat derived CPPs [56].

For penetratin and Tat an inverted micelle mechanism was suggested, in which positively charged peptides interact with negatively charged phospholipids to convert part of the membrane into an inverted micelle structure that can open on either the intracellular or the extracellular side of the membrane [5,52]. On the other hand, internalization of some CPPs appears to be very much affected by the membrane composition. Examples are pVEC and the transportan analogue, transportan-10, that may employ a carpet model for translocation [40]. Both peptides are toxic to microbes but non-toxic to mammalian HeLa cells, which differ in membrane composition [57]. The translocation of peptide that adopts an α-helical structure when in contact with membrane, as for instance MAP and, partly, transportan [58], could be associated with pore formation [59,60]. Knowing the internalization mechanism would be of help in designing a proper kinetic scheme as the basis for analyzing the kinetic data. Nevertheless, we have constructed a hypothetical translocation scheme for CPPs, based on the known and postulated processes that occur during internalization (Fig. 1).

It is obvious that the internalization of CPP is a multistep process, represented in Fig. 1 as a sequence of equilibrium states with a non-equilibrium step in the middle, namely the degradation of CPP in the cell. All steps of the scheme can be considered as realistic. The first step of internalization is the interaction of CPP with the cell surface. It has been shown that cationic CPP molecules bind electrostatically to the exposed charged parts of the phospholipids and that loosely bound CPP can be removed from the cell

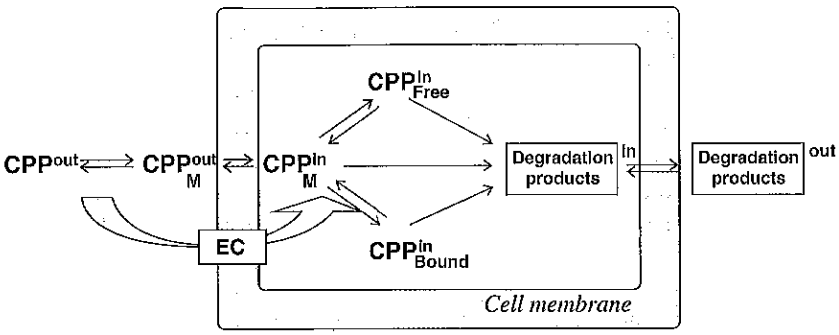

Fig. 1. Simplified kinetic scheme for cell-penetrating peptide internalization. CPP is cell-penetrating peptide; in and out represent the proportion of CPP or its degradation products inside and outside the cell, respectively; label M denotes membrane bound CPP; Free means internalized but non-bound CPP (for instance in cytosol); Bound means the fraction of CPP that is interacting with inner cell structures (intracellular membranes, proteins, etc); Degradation products result from proteolytic cleavage of the CPP in the cell; EC denotes endocytosis.

surface by ice-cold acidic solution [11]. Membrane binding of Tat was quantified by isothermal titration calorimetry [61]. The reaction enthalpy ΔH° was around −1.5 kcal/mol peptide, yielding a very small corrected intrinsic binding constant of 1–10 M^{-1}. It was also shown that heparan sulphate is a much better candidate for the first binding site of Tat, since ΔH° of around −4.6 kcal per mol peptide was obtained for its interaction with Tat, resulting in an equilibrium binding constant of around 6×10^{5} M^{-1} [62].

The interaction of CPP with lipid bilayer was also studied by molecular modelling [26,63]. The results with penetratin and transportan and their analogues point to the importance of the charge gradient in the core of the lipid bilayer for the translocation of CPPs. Accumulation of labelled CPPs in or at the cell membrane was readily observed by confocal microscopy.

Endocytosis is included into the scheme since recent data strongly suggest its role in translocation mechanism for some CPPs, as discussed above.

Peptidolytic degradation of CPP in the cell is a proven fact which has been confirmed, for example for transportan in vitro [11] and for pVEC also quasi in vivo, in the lysate of non-digested cells [7]. Finally, the release of the degradation products from the cells cannot be excluded. Thus, it seems that the general features of the scheme shown in Fig. 1 are justified.

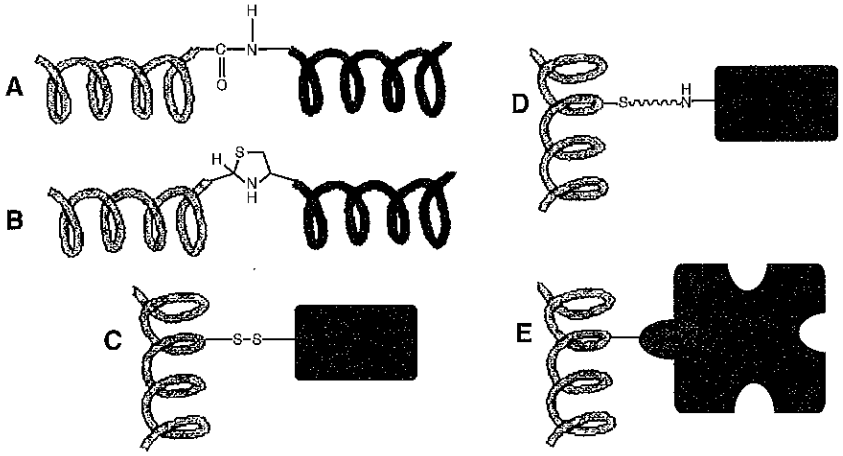

Fig. 2. Attachment of cargo to the cell-penetrating peptide. CPP is blue, cargo is shown in green. A, B, C, and D represent cargo covalently bound to the CPP via a peptide bond, thiazolidine ring, disulphide bridge, and bifunctional linker molecule, respectively. (E) A large cargo molecule (for instance streptavidin, shown in dark green) is non-covalently bound to a smaller cargo (for instance biotin, shown in light green) that is covalently attached to the CPP.

The scheme, however, is too complex to allow complete kinetic evaluation but it has been very useful in planning kinetic experiments and for interpreting the results.

2.4. Cargo

The main applicative potential of CPPs is the possibility of attaching biologically active cargo and translocating it into cells. Cargo can be attached in different ways. The link between the CPP and cargo is usually a covalent bond. When the cargo is a peptide or protein, CPP and cargo are most often synthesized or expressed in tandem as fusion protein [64–66]. Alternatively, a suitable amino acid side-chain or bifunctional spacer molecule can be used. To couple cargo to transportan, the thiol group of cysteine, amino group of lysine, or bifunctional cross-linker SMCC was used [11,25]. The thiol group of cysteine has the reductive environment in the cell will readily cleave the disulphide bridge between CPP and cargo, resulting in the release of cargo. Attachment of cargo to CPP can be achieved also by non-covalent bonds, employing for instance the interaction of avidin to a biotin-CPP construct [27]; this method has been used very seldom. An overview of the most important modes of cargo attachment is given in Fig. 2.

A large number of cargo molecules have been effectively delivered into cells via CPPs, including small molecules, peptides, fragments of DNA and PNA, proteins, phages, liposomes and magnetic nanoparticles (Table 2). Some of the proteins delivered by CPPs are shown in Table 3.

2.5. Side effects

The toxic effects of CPPs must be kept at a minimum if they are to be used as drug delivery vehicles. The in vitro toxicity of CPPs has been frequently characterized, but in vivo studies are limited. In general, two types of toxic effect have been observed: toxic effects on membranes of cells and organelles, and toxic effects resulting from the specific interaction of CPPs with cell components.

CPPs from the class of model peptides, such as MAP, and some designed CPPs, such as transportan, resemble in structure the antimicrobial lytic peptides that kill microbial cells by disrupting their cell membranes [70,71]. MAP in concentrations over 1 μM has been observed to exert a rather strong toxic effect on various cell lines [8,17,23] by the trypan blue exclusion [72], MTT [73], fluorescein leakage [17], and 2-deoxyglucose-6-phosphate leakage [74] tests. Transportan showed somewhat lower toxicity than MAP, starting at 5 μM concentration, as observed by the glucose leakage test using BMC cell line [23]. Penetratin causes very little disturbance to membranes [23], while Tat appears to cause practically no harm to cell membranes [6,23].

Due to the net positive charge of most CPPs, strong binding to polyanions is expected. Indeed, penetratin interacts with heparin, polysialic acid and nucleic acids [75,76]. The interaction appears to be important for internalization but whether it is the cause of any side effects in vivo (see below) is not known.

Diverse specific effects of CPPs have been observed. Transportan is derived from galanin and mastoparan and retains some properties of both peptides. It inhibits galanin binding to galanin type-1 receptor (K_D=17.4 nM) and modulates the activities of heterotrimeric G-proteins [11]. Inhibition of GTPase activity by transportan was observed with G_s—but not with other types of G-proteins. Although this could be a serious obstacle to the use of transportan as a drug delivery vehicle, it must be

Table 3
Proteins translocated into cells and tissue by different cell-penetrating peptides (CPP)

CPP	Protein translocated	Target cells/tissue	Ref.
Penetratin	p16 (26 kDa)[a]	human fibroblasts	[67]
Tat	β-galactosidase (120 kDa)[a]	mouse in vivo, many tissue	[49]
	green fluorescence protein (28 kDa)[a]	mouse muscle cells and tissue	[68]
	small GTPase Rho (21 kDa)[a]	avian osteoclasts	[18]
	caspase-3 (32 kDa)[a]	HIV-infected cells	[69]
Transportan	green fluorescence protein (28 kDa)[b] avidin (66 kDa)[b] antibodies (150 kDa)[b]	various cell lines (Bowes, BRL, COS-7)	[27]

The approximate molecular mass of cargo is indicated in parenthesis.
[a] CPP and cargo expressed as fused protein.
[b] Cargo chemically coupled to CPP.

stressed that the inhibition of GTPase occurs at 10 times higher concentrations than those used in delivery experiments. In any case, the problem was overcome by truncating transportan, resulting in transportan-10 which has no effect on G-proteins [26]. The parent protein of Tat-peptides, HIV transcription factor Tat, in addition to activating gene expression and replication, is also involved in a number of processes including angiogenesis [77] and apoptosis [78,79]. Some of these effects can be produced also with Tat derived CPPs [80].

Toxicity and undesirable side effects have not been detected in most in vivo applications of CPPs (cf. [9,25,49]). However, when 10 μg or more of penetratin was applied in rat by intrastriatal injection, neurotoxic cell death and recruitment of inflammatory cells in brain was detected; an effect which was much decreased at a dose of 1 μg [47].

3. Kinetics of penetration and cargo delivery

3.1. Methodology

3.1.1. Monitoring internalization of CPPs and cargo

CPPs cannot usually be observed directly and must be appropriately labelled to allow reliable detection of the small amounts that are usually internalized. Labelled CPP or cargo can be detected by radioactivity, fluorescence emission, fluorescence quenching, specific labelling with dyes or by enzymatic activity of the cargo. Some of the detection methods are very convenient for visualizing CPP internalized in cells but do not have a sufficiently rapid response time for kinetic studies. An example is the visualization of internalized biotinylated transportan by labelled streptavidin, using confocal microscopy (see Ref. [11]). In most internalization kinetic studies, however, the CPP itself or the cargo molecules in CPP-cargo constructs have been labelled by radioactive isotopes or fluorophores.

In principle, the CPP or cargo molecules could be labelled with a variety of different radionuclides. Most frequently, CPPs are radiolabelled by ^{125}I-iodination using the chloramine-T method [81]; see for instance [11]. This method can be exploited only when Tyr is present in the sequence. An alternative method of labelling CPPs was the use of radioactive technetium (^{99m}Tc) chelated to Tat [34]. To our knowledge, labelling of CPPs with other radionuclides has not been performed.

Alternatively, radioactively labelled cargo molecules can be used. This has the advantage that the delivery of a cargo molecule into cells can be traced directly and, furthermore, that there may be a large variety of commercially available labelled compounds for use as cargo. One example is the use of a commercially available radiolabelled antineoplastic agent, ^{14}C-doxorubicin, as cargo coupled to penetratin [48]. The high sensitivity obtainable by using radioactively labelled CPP or CPP-cargo construct is the main advantage of this method over others. The disadvantage is the necessity of separating the cell-internalized fraction of the labelled compound from the bulk that remains in the incubation solution. This prevents the design of kinetic experiments that require continuous monitoring of the amount of cell-internalized CPP or CPP-cargo construct. Additionally, the fraction of CPP or CPP-cargo construct that may be firmly inserted into the cell membranes cannot be easily differentiated from the fraction residing in cytoplasm and in organelles.

The most frequently used fluorophores are fluorescein and fluorescein derivatives [17,34,48], DNB (7-nitrobenz-2-oxo-1,3-diazol-4-yl) [37] and Abz (2-amino benzoic acid) [23,82]. After incubating cells with labelled CPP, the solution is removed and the concentration of labelled CPP inside the cells determined directly (cf. [82]) or by fluorescence flow cytometry [37]. Protocols have been developed to determine the amount of internalized CPP from cell lysate using HPLC [8]. The fluorescence of labelled CPP in the incubation solution may be quenched after a selected incubation time by adding a suitable quencher, allowing the fluorescence of the labelled CPP inside the cells to be quantitated, since the quencher is unable to penetrate into the cells. For example, the fluorescence of NBD-penetratin in the incubation solution was irreversibly quenched by the addition of sodium dithionite, and the concentration of internalized NBD-penetratin determined without any additional separation procedure of cells and incubation solution [37].

Confocal laser scanning microscopy has been used to monitor CPP uptake in real time [83]. Attached cells are incubated with fluorescently labelled peptide

and the time course of the fluorescence inside and outside the cells is monitored. The signal is corrected for the superimposition of the background fluorescence resulting from non-ideal confocal properties of the microscope.

Recently, a new strategy has been developed. CPP-cargo constructs were synthesized in which small pentapeptide cargo molecules labelled with Abz fluorophore were coupled to different CPPs (transportan, penetratin, Tat and MAP) labelled with the fluorescence quencher 2-nitrotyrosine via a disulphide bond that is reductively cleaved inside the cell [23]. The intensity of the resulting fluorescence inside the cells is proportional to the amount of the internalized and liberated cargo, while the fluorescence of uncleaved CPP-cargo construct in the incubation solution remains quenched. In this way, cargo internalization into cells by a CPP can be monitored continuously and quantitatively in real time. The method has a number of advantages. Firstly, elimination of the non-internalized portion of cargo is not needed. Secondly, more quality kinetic data can be obtained from a single experiment than by any other known method. It is important that all experimental points obtained from each complete time course of internalization are measured in virtually identical conditions (same set of cells, same incubation solution, etc.), decreasing the scattering of data. Because the disulphide bond is reduced by glutathione in the cytoplasm, as confirmed by in vitro experiments, any CPP-cargo construct associated with cell membranes has less chance of being reduced and hence observed. Consequently, it is mainly the concentration of free cargo in the inner cellular solution that is determined. This approach would definitely be the method of choice for studying kinetics of internalization of CPP-cargo constructs in the micromolar and submicromolar concentration range.

The lower sensitivity of methods based on fluorescence is the main drawback compared to methods based on radioactivity. The detection limit of fluorescence methods is close to the concentrations where some CPPs (MAP and transportan, see [23]) could disturb membranes. For these CPPs, following internalization at lower concentrations (below 0.1 μM) would be much safer. This can be reliably achieved by use of radioactively labelled CPPs.

3.1.2. Approaches to determining the kinetics of transduction

Kinetic studies of internalization of CPP into cells involve incubation of cells with CPP at suitable concentrations, detection of the amount of CPP internalized into the cells as a function of time, and subsequent analysis of the collected data.

Kinetics can be followed in layers of cultured cells, but it is better to work with detached cells, which expose their entire surface to the CPP in the incubation medium, enabling more accurate determination of the amount of internalized CPP. With detached cells, it is also easier to remove CPP that is adsorbed on the cell surface. This is usually done by short treatment of cells with acidic solution or trypsin [11,82]. The procedure is important in order to avoid artifacts.

Normally, the total amount of internalized CPP is determined. Sometimes, however, it is of interest to determine separately the fraction of CPP that is free in the cytosol and the fraction that is firmly associated with membranes. Attempts have been made to approximate these fractions by determining the partition coefficients of CPPs between water and lipid environment, using octanol as a model of the lipid part [11]. The results of this approach are unreliable, mainly because octanol is not an adequate model of the membrane [84].

Until the mechanism of internalization is not understood, any approach to internalization kinetics can only be phenomenological. According to the current state of the art, the putative scheme shown in Fig. 1 is too complex for accurate kinetic treatment, therefore simplifications and approximations are necessary. Very often the initial rate of the internalization process is determined, assuming that, at least in the early phase, the internalization of CPP is a first-order process approximated by the equation

$$\mathrm{CPP}^{\mathrm{out}} \xrightarrow{k^{\mathrm{I}}} \mathrm{CPP}^{\mathrm{in}} ,$$

where k^{I} is an apparent first-order rate constant. This simplification has yielded valuable information in uptake studies of CPP.

The simplest way to measure kinetics of CPP internalization is a so-called one point experiment in which the amount of internalized CPP is determined at only one time point. The benefits of simplicity, speed

and small amount of material used are lost in the fact that a one point experiment cannot yield kinetic constants and, consequently, the information obtained can only be useful for rough evaluation and comparison. The approach is similar to some enzyme kinetics studies, where the time interval within which the dependence of the amount of transformed substrate with time is quasi-linear is first determined and then only one point inside this interval is used for the main experiment.

It is more reliable and informative to monitor the concentration of internalized CPP as a function of time. Kinetic data should be collected over a time interval that is long enough to approach concentration equilibrium between external and internalized CPP. This can be done at one fixed concentration of CPP or, better, at several concentrations of CPP. Data obtained at a fixed concentration of CPP allow the rate constant of internalization to be calculated, together with the yield of internalization at that concentration. The quality of the calculated kinetic parameters depends greatly on the number and reliability of the experimental points.

The equation for first-order kinetics is

$$[A] = [A]_\infty \left(1 - e^{-kt}\right)$$

where $[A]$ is the concentration of internalized CPP at time point t, $[A]_\infty$ the final concentration of CPP inside the cells, and k the first-order rate constant. Parameters k and $[A]_\infty$ are obtained by fitting. Comparison of the initial concentration of CPP outside the cells ($[A]_0$) with the estimated value of $[A]_\infty$ enables the internalization yield to be calculated. The first-order rate constant k can be converted into the half-time of internalization ($t_{0.5}$) using the equation $t_{0.5}=\ln 2/k$.

If goodness of fit is not satisfactory, the proposed first-order rate mechanism is probably not obeyed. In this case, other equations should be tested, describing more complex internalization mechanisms or models. Even if the fitting is good, one must be always aware that the results apply to the chosen concentration of CPP only and that at other concentrations CPP could internalize according to a different model.

The best approach is to follow the progress curves of internalization at several initial concentrations of CPP. This is demanding since a great number of experimental points are required, but the method yields important advantages. The kinetic parameters obtained are valid for the whole range of CPP concentrations examined and are therefore much more reliable. Even more importantly, the procedure allows the order of the reaction to be checked and the more complex kinetic models to be verified. For a first-order kinetics model, the rate constant should not be dependent on the initial concentration of CPP, while second-order kinetics demand linear dependence of the first order rate constant on the initial concentration of CPP. This can be verified by treating each progress curve separately using a suitable computer program as described above for a fixed concentration experiment and checking the concentration dependence of the rate constants derived. Some computer programs (cf. Dynafit, or a program of Stojan [85]) can fit all the progress curves simultaneously, using either the explicitly derived equation for the predicted mechanism or a numerical treatment of the system when the explicit equations cannot be obtained due to the complexity of the kinetic scheme.

3.2. Results

In spite of the importance of kinetic data for insight into the mechanism of internalization and for the potential application of CPPs in drug delivery, it is surprising how few proper kinetic analyses have been carried out so far. One general result of these experiments is that the internalization of CPP is a rather fast process. At 37 °C, the maximal concentration of internalized peptide is achieved after less than 1 h of incubation of the cells, or even faster. Characteristically, small cargoes (small peptide or fluorescent labels) usually do not substantially affect the rate of internalization, while larger hydrophilic cargoes (proteins) are internalized more slowly. An overview of the results obtained with the main CPPs is given below.

Transportan is the CPP for which the kinetics has been studied most thoroughly, using mainly Bowes cells. Internalization of biotinyl-transportan was shown to be rapid from visualization studies by indirect immunofluorescence using confocal microscopy [11]. The cells were intensely stained after 1 min incubation at 37 °C with 10 μM biotinyl-transportan. After the first 5 min, the peptide was

detected mostly in the plasma membrane and cytosolic membrane structures (endosomes, endoplasmic reticulum, Golgi). After approximately 20 min, biotinyl-transportan was concentrated in the nuclear membrane and the nuclei.

With [^{125}I]-transportan it was shown that more than 50% of the maximal internalization of transportan in Bowes cells is achieved in approximately 3 min after starting the incubation of cells. The time course of uptake was similar for all concentrations of transportan (5–500 nM), showing a very fast initial increase of transportan concentration in the cells. The maximal intracellular concentration was achieved after approximately 20 min, after which the radioactivity in the cells slowly decreased, while the fraction of radioactivity outside the cells started to increase. The decrease was interpreted as outflow of radioactively labelled fragments of degraded transportan from the cells. It was calculated that the maximal concentration of [^{125}I]-transportan inside the cells was around 2-fold higher than the concentration of free ligand outside the cells.

The observed time course of radioactivity in the cells was analyzed according to the following kinetic model:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_3} C \underset{k_5}{\overset{k_4}{\rightleftharpoons}} D$$

where A and B represent the concentrations of [^{125}I]-biotinyl-transportan outside and inside the cells, C and D the concentrations of radioactive peptide fragment (or fragments) inside and outside the cells, and k's are the first-order rate constants for the corresponding processes. This scheme closely resembles that shown in Fig. 1. The amount of the radioactive peptides in the cells (B+C) and the corresponding rate constants were calculated by a numerical treatment of the experimental data using a modified regression computer program of Stojan [85]. The advantage of this approach was the simultaneous fitting of the curves for all five initial concentrations (A_0) of [^{125}I]-biotinyl-transportan. The following values for the rate constants were obtained: k_1=0.019 min^{-1}, k_2=0.15 min^{-1}, k_3=0.058 min^{-1}, k_4=0.27 min^{-1} and k_5=0.0039 min^{-1}. The ratio between the constants for uptake of transportan (k_1) and its release (k_2) and the ratio of volumes outside and inside the cells are in accordance with the maximal accumulation of [^{125}I]-biotinyl-transportan detected in the cells (see above). On the other hand, the ratio of the constants for uptake (k_4) and release (k_5) of the degradation products corresponds to the ratio of intracellular (2–5 μl) and extracellular (100 μl) volumes.

This suggests that the degradation products do not accumulate in the membranes and that transportan could be degraded in the cells.

Fundamental kinetic studies were carried out also with a number of transportan analogues [26,82]. Most of the analogues have retained the ability to penetrate Bowes cells. The results showed the great importance of the mastoparan part and its amphipathic properties for the ability of this type of peptide to cross the cellular membranes while modifications in the galanin part have a much less dramatic effect. The most important result, however, is the discovery that transportan-10 is an effective cell-penetrating peptide that does not interfere with the activation process of G-proteins [26].

Although penetratin was the first CPP described [5], very few kinetic data on its internalization into cells is available. Drin et al. studied the time course of internalization of NBD-labelled penetratin into K562 cells [37]. They showed a rapid uptake of penetratin (1.6 μM initial concentration at 37 °C), reaching a plateau of the concentration of the internalized peptide after around 1 h of incubation. From their data ([37], Fig. 3) the value of to 5 of 17 min was recalculated assuming first-order kinetics. Hällbrink et al. have followed the uptake of penetratin associated with a small pentapeptide cargo into Bowes cells [23]. The time course of the penetratin uptake was roughly consistent with first-order kinetics (1 μM initial concentration at 37 °C) and with $t_{0.5}$ of around 58 min. It is difficult to say whether the discrepancy between the results in [37] and those obtained in [23] are due to different cells or reflect the impact of cargoes. Although a number of different penetratin analogues have been synthesized [5,82,86], none has been used in kinetic experiments.

More than 10 Tat-derived short peptides have been shown to translocate into different cells [87]. Kinetic experiments were carried out with fluorescein-labelled Tat (49-57) [88] and Tat (48-60) coupled to small

peptide cargo labelled with Abz [23]. The cell uptake of Tat (49-57) was analyzed according to Michaelis kinetics in parallel with penetratin and two model poly-Arg peptides, allowing only approximate comparison of the rate of internalization of these peptides and not giving proper kinetic parameters. It was shown that Tat (49-57) was internalized almost two times slower than penetratin [88]. This is not in accordance with the results of Hällbrink et al. who found that the first-order rate constant for Tat was 1.7-fold greater than that for penetratin [23]. In these studies, however, the peptides carried a small load that could affect the penetration rate. A number of Tat analogues were synthesized and tested on T-Jurkat cells [88]. All analogues exhibited diminished cellular uptake. These results suggest that the cationic residues of Tat play a principal role in its uptake, although detailed kinetic parameters for these analogues were not obtained.

The internalization of MAP was roughly linear for 60 min [8]. With longer incubation periods, the amount of internalized peptides started to decrease, due to enzymatic cleavage of MAP in the incubation solution. The rate of internalization is approximately in accordance with first-order kinetics. A similar result was obtained by Hällbrink et al. [23], who observed approximately first order internalization kinetics of a small peptide cargo attached to MAP. The concentration dependence of internalization was also almost linear in the range from 0.1 to 10 μM [23]. Studies of the internalization behaviour of 15 MAP analogues [17] using the "one point experiment" (the amount of internalized peptide after 30 min incubation at 4.5 μM initial concentration) showed that helical amphipathicity was the only essential structural requirement for the translocation of peptides into the cells.

3.3. Comparison of kinetics of uptake of different CPPs and the effect of cargo

Only one complete comparative study on the rate and yield of internalization of small cargo with transportan, penetratin, Tat and MAP has been undertaken so far [23]. The approximately first-order kinetics of the initial internalization of cargo was observed with all the CPPs. The fastest uptake was with MAP ($t_{0.5}$=7 min), followed by transportan ($t_{0.5}$=12 min), Tat ($t_{0.5}$=34 min), and penetratin ($t_{0.5}$=56 min). The efficiency of cargo delivery into the cells, expressed by the apparent cell transport equilibrium constant (K_{io}), matches the rate of the cellular uptake. The fastest penetrator, MAP, was also the most effective delivery vector (K_{io}=417), followed by transportan (K_{io}=353), Tat (K_{io}=107), and penetratin (K_{io}=71). The efficiency and rate of cargo delivery into the cells is compensated by the degree of membrane leakage. The most efficient CPP, MAP, caused extensive leakage of radioactive 2-[^{3}H]-deoxyglucose-6-phosphate from the cells at only 1 μM concentration. Transportan was not effective at this concentration but at higher concentrations membrane leakage increased. Tat did not induce any leakage at all, even at the highest concentrations used (20 μM), and the effect of penetratin was negligible.

The effect of cargo on translocation velocity is also of great interest. Those used so far were very different, both structurally and in size, ranging from small organic molecules (cf. biotin, fluorescein), through peptides, DNA and PNA fragments, to proteins of around 150 kDa. It is reasonable to expect that cargo should have an important influence on the rate of internalization of CPP-cargo constructs. It was shown that small cargoes do not usually influence the rate of translocation substantially (cf. [11]). Kinetic data on the translocation of a CPP coupled to medium and large size cargoes are not available. From immunofluorescence studies utilizing confocal microscopy it was concluded that transportan attached to protein cargoes of up to 150 kDa internalize more slowly than transportan itself—as estimated visually from the dynamics of the appearance of fluorescence in the cells [11,27]. Biotinyl-transportan could be detected in cells after a few minutes and reached maximal intracellular concentration after 20 min of incubation [11]. Transportan attached to green fluorescence protein, avidin-TRITC, and polyclonal antibody, respectively, was detected in the cytoplasm much later, on average after 30 min of incubation. However, in some cases, for instance with monoclonal antibody against biotin non-covalently attached to biotinyl-transportan, the construct was detected more than 2 h later [27]. Proteins were translocated also by Tat carrier [49]. Successful translocation of partially denatured β-

Table 4
The half-time ($t_{0.5}$) of
CPPs into different c

CPP	Lab
Penetratin	per
	NB
	NB
Tat	per
	flu
	pep
	rho
MAP	per
Transportan	per
	Ab
	125
Transportan-10	Ab

Values of $t_{0.5}$ were
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4. Conclusions

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Table 4
The half-time ($t_{0.5}$) of the internalization of main representatives of CPPs into different cell lines; T=37 °C, pH=7.4

CPP	Label/cargo	Cell line	$t_{0.5}$ (min)	Reference
Penetratin	pentapeptide	Bowes	60	[23]
	NBD	K562	45[a]	[54]
	NBD	K562	20[a]	[37]
Tat	pentapeptide	Bowes	30	[23]
	fluorescein	Jurkat	20[a]	[32]
	peptide chelator	Jurkat	1	[34]
	rhodamine	HeLa	1[a]	[44]
MAP	pentapeptide	Bowes	10	[23]
Transportan	pentapeptide	Bowes	10	[23]
	Abz	Caco-2	15[a]	[29]
	^{125}I	Bowes	35	[11]
Transportan-10	Abz	Caco-2	25[a]	[29]

Values of $t_{0.5}$ were calculated from first-order rate constants or assessed from the figures, as indicated.

[a] The value was recalculated from the kinetic data presented in the figure from the indicated reference.

galactosidase into different mouse cells was achieved but the experiments do not allow a comparison of the rate of translocation with the Tat-β-galactosidase construct and Tat itself.

In Table 4 kinetic data for the internalization of main representatives of CPPs into different cell lines are summarized. Only data of CPP internalization that were obtained in approximately comparable conditions (physiological temperature and pH) were selected and were presented in terms of $t_{0.5}$.

4. Conclusions and perspectives

The large number of different CPPs synthesized so far have been demonstrated to enter different cells, tissues and organs. They are able to promote translocation of various types of useful cargo, ranging from small molecules to proteins and large supramolecular particles, with great efficiency and reasonable velocity. Delivery of cargo has been achieved also into some systems that are usually difficult to cross, for instance the blood–brain barrier. As described above, CPPs have already been used successfully as cargo carriers for scientific purposes, opening up the potential for clinical delivery of drugs in the future. This was additionally supported by successful cargo delivery in vivo.

To use CPPs as drug delivery vehicles in practice, CPPs should be:

- small and simple to synthesize
- able to be coupled to different cargoes without losing their translocation properties
- tissue, cell and cell-compartment selective
- stable cargo carriers but without side effects.

Some of these properties have already been successfully realized. Most CPPs are relatively small and can be synthesized using traditional methods that can be automated. The coupling of cargo is not a problem in most cases but the decrease of rate and efficiency of translocation in the case of large cargo has not yet been overcome. Until the mechanism of penetration is known, this will be difficult to achieve. For this reason, more efforts should be invested in elucidating the penetration process. The same is true with the selectivity of CPPs. Incorporation of signalling peptide sequences into CPPs was suggested and undertaken in order to provide selective targeting of the cell nucleus in the case of gene delivery [89–91]. Selectivity toward cancer cells would be of great interest. For this purpose, the proteins specifically overexpressed in these cells could be used [92,93]. A similar approach could be exploited for antiviral drugs carried by CPPs designed to target specific surface proteins of virus-infected cells [94,95].

The stability of CPPs is a two edged problem. On the one hand, good extracellular stability would be profitable for efficient cargo delivery; on the other hand, high intracellular stability could result in accumulation of CPP inside the cells with all the potential, undesirable side effects. In general, the stability of peptides, both extracellularly and intracellularly, is poor due to the presence of peptidases and proteinases. Stability was enhanced by protecting the C-terminus by amidation and by synthesizing all-D CPPs that were resistant against degradation but retained full translocation potential (see for instance [50]). Side effects of CPPs have not been explored in detail yet, and for the moment, we are still far away from systematic pre-clinical and clinical trials of CPPs. However, as described in Section 2.5, most CPPs show only moderate or very limited side effects when applied in vivo and in vitro.

In conclusion, it is clear that CPPs are novel vehicles for the rapid translocation of cargo into cells, and exhibit the properties that make them potential drug delivery agents, making them interesting for practical use in the future.

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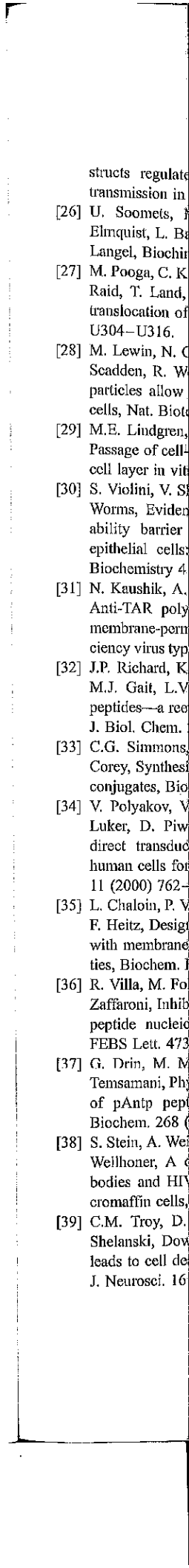
References

[1] P.D. Robins, H. Tahara, S.C. Ghivizzani, Viral vectors for gene therapy, Trends Biotechnol. 16 (1998) 35–40.
[2] M. Lindgren, M. Hällbrink, A. Prochiantz, Ü. Langel, Cell-penetrating peptides, Trends Pharmacol. Sci. 21 (2000) 99–103.
[3] Ü. Langel (Ed.), Cell-Penetrating Peptides, Processes and Applications, CRC Press, Boca Raton, USA, 2002.
[4] E. Eiríksdóttir, H. Myrberg, M. Hansen, Ü. Langel, Cellular uptake of cell-penetrating peptides, Drug Design Rev. Online 1 (2004) 161–173.
[5] D. Derossi, A.H. Joliot, G. Chassaing, A. Prochiantz, The 3rd helix of the Antennapedia homeodomain translocates through biological-membranes, J. Biol. Chem. 269 (1994) 10444–10450.
[6] E. Vivés, P. Brodin, B. Lebleu, A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus, J. Biol. Chem. 272 (1997) 16010–16017.
[7] A. Elmquist, M. Lindgren, T. Bartfai, Ü. Langel, VE-cadherin-derived cell-penetrating peptide, pVEC, with carrier functions, Exp. Cell Res. 269 (2001) 237–244.
[8] J. Oehlke, A. Scheller, B. Wiesner, E. Krause, M. Beyermann, E. Klauschenz, M. Melzig, M. Bienert, Cellular uptake of an α-helical amphipathic model peptide with the potential to deliver polar compounds into cell interior non-endocytotically, Biochim. Biophys. Acta 1414 (1998) 127–139.
[9] J.B. Rothbard, S. Garlington, Q. Lin, T. Kirschberg, E. Kreider, P.L. McGrane, P.A. Wender, P.A Khavari, Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation, Nat. Med. 6 (2000) 1253–1257.
[10] M.C. Morris, P. Vidal, L. Chaloin, F. Heitz, G. Divita, A new peptide vector for efficient delivery of oligonucleotides into mammalian cells, Nucleic Acids Res. 25 (1997) 2730–2736.
[11] M. Pooga, M. Hällbrink, M. Zorko, Ü. Langel, Cell penetration by transportan, FASEB J. 12 (1998) 67–77.
[12] M.C Schmidt, B. Rothen-Rutishauser, B. Rist, A. Beck-Sickinger, H. Wunderli-Allenspach, W. Rubas, W. Sadée, H.P. Merkle, Translocation of human calcitonin in respiratory nasal epithelium is associated with self-assembly in lipid membrane, Biochemistry 37 (1998) 16582–16590.
[13] D.J. Mitchell, D.T Kim, L. Steinman, C.G. Fathman, J.B. Rothbard, Polyarginine enters cells more efficiently than other polycationic homopolymers, J. Pept. Res. 56 (2000) 318–325.
[14] J. Oehlke, M. Beyermann, B. Wiesner, M. Melzig, H. Berger, E. Krause, M. Bienert, Evidence for extensive and non-specific translocation of oligopeptides across plasma membranes of mammalian cells, Biochim. Biophys. Acta 1330 (1997) 50–60.
[15] X.H. Zhou, A.L. Klibanov, L. Huang, Lipophilic polylysines mediate efficient DNA transfection in mammalian cells, Biochim. Biophys. Acta 1065 (1991) 8–14.
[16] E. Ramsay, Examination of the biophysical interaction between plasmid DNA and the polycations, polylysine and polyornithine, as a basis for their differential gene transfection in vitro, Int. J. Pharm. 210 (2000) 97–107.
[17] A. Scheller, J. Oehlke, B. Wiesner, M. Dathe, E. Krause, M. Beyermann, M. Melzig, M.J. Bienert, Structural requirements for cellular uptake of α-helical amphipathic peptide, J. Pept. Sci. 5 (1999) 185–194.
[18] M.A. Chellaiah, N. Soga, S. Swanson, S. McAllister, U. Alvarez, D.M. Wang, S.F. Dowdy, K.A. Hruska, Rho-A is critical for osteoclast podosome organization, motility and bone resorption, J. Biol. Chem. 275 (2000) 11993–12002.
[19] A. Astriab-Fisher, D.S. Sergueev, M. Fisher, B.R. Shaw, R.L. Juliano, Antisense inhibition of P-glycoprotein expression using peptide-oligonucleotide conjugates, Biochem. Pharmacol. 60 (2000) 83–90.
[20] A. Eguchi, T. Akuta, H. Okuyama, T. Senda, H. Yokoi, H. Inokuchi, S. Fujita, T. Hayakawa, K. Takeda, M. Hasegawa, M. Nakanishi, Protein transduction domain of HIV-1 Tat protein promotes efficient delivery of DNA into mammalian cells, J. Biol. Chem. 276 (2001) 26204–26210.
[21] M.C. Morris, L. Chaloin, J. Mery, F. Heitz, G. Divita, A novel potent strategy for gene delivery using a single peptide vector as a carrier, Nucleic Acids Res. 27 (1999) 3510–3517.
[22] Y.-L. Tseng, J.-J. Liu, R.-L. Hong, Translocation of liposomes into cancer cells by cell-penetrating peptides penetratin and TAT: a kinetic and efficacy study, Mol. Pharmacol. 62 (2002) 864–872.
[23] M. Hällbrink, A. Florén, A. Elmquist, M. Pooga, T. Bartfai, Ü. Langel, Quantification of cellular uptake and comparison of some cell penetrating peptides, Biochim. Biophys. Acta 1515 (2001) 101–109.
[24] K. Kilk, M. Magzoub, M. Pooga, L.E. Eriksson, Ü. Langel, A. Gräslund, Cellular internalization of a cargo complex with a novel peptide derived from the third helix of the islet-1 homeodomain. Comparison with the penetratin peptide, Bioconjug. Chem. 12 (2001) 911–916.
[25] M. Pooga, U. Soomets, M. Hällbrink, A. Valkna, K. Saar, K. Rezaei, U. Kahl, J.X. Hao, X.J. Xu, Z. Wiesenfeld-Hallin, T. Hökfelt, T. Bartfai, Ü. Langel, Cell penetrating PNA con-



structs regulate galanin receptor levels and modify pain transmission in vivo, Nat. Biotechnol. 16 (1998) 857–861.
[26] U. Soomets, M. Lindgren, X. Gallet, M. Hällbrink, A. Elmquist, L. Balaspiri, M. Zorko, M. Pooga, R. Brasseur, Ü. Langel, Biochim. Biophys. Acta 1467 (2000) 165–176.
[27] M. Pooga, C. Kut, M. Kihlmark, M. Hällbrink, S. Fernaeus, R. Raid, T. Land, E. Hallberg, T. Bartfai, Ü. Langel, Cellular translocation of proteins by transportan, FASEB J. 15 (2001) U304–U316.
[28] M. Lewin, N. Carlesso, C.H Tung, X.W Tang, D. Gory, D.T. Scadden, R. Weissleder, Tat-peptide derived magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells, Nat. Biotechnol. 18 (2000) 410–414.
[29] M.E. Lindgren, M.M. Hällbrink, A.M. Elmquist, Ü. Langel, Passage of cell-penetrating peptides across a human epithelial cell layer in vitro, Biochem. J. 377 (2004) 69–76.
[30] S. Violini, V. Sharma, J.L. Prior, M. Dyszlewski, D. Piwnica-Worms, Evidence for a plasma membrane-mediated permeability barrier to tat basic domain in well-differentiated epithelial cells: lack of correlation with heparan sulfates, Biochemistry 41 (2002) 12652–12661.
[31] N. Kaushik, A. Basu, P. Palumbo, R.L. Myers, V.N. Pandey, Anti-TAR polyamide nucleotide analog conjugated with a membrane-permeating peptide inhibits human immunodeficiency virus type 1 production, J. Virol. 76 (2002) 3881–3891.
[32] J.P. Richard, K. Melikov, E. Vivés, C. Ramos, B. Verbeure, M.J. Gait, L.V. Chernomordik, B. Lebleu, Cell-penetrating peptides—a reevaluation of the mechanism of cellular uptake, J. Biol. Chem. 278 (2003) 585–590.
[33] C.G. Simmons, A.E. Pitts, L.D. Mayfield, J.W Shay, D.R. Corey, Synthesis and membrane permeability of PNA-peptide conjugates, Bioorg. Med. Chem. Lett. 7 (1997) 3001–3006.
[34] V. Polyakov, V. Sharma, J.L. Dahlheimer, C.M. Pica, G.D. Luker, D. Piwnica-Worms, Novel Tat-peptide chelates for direct transduction of technetium-99m and rhenium into human cells for imaging and radiotherapy, Bioconjug. Chem. 11 (2000) 762–771.
[35] L. Chaloin, P. Vidal, P. Lory, J. Mery, N. Lautredou, G. Divita, F. Heitz, Design of carrier peptide-oligonucleotide conjugates with membrane translocation and nuclear localization properties, Biochem. Biophys. Res. Commun. 243 (1998) 601–608.
[36] R. Villa, M. Folini, S. Lualdi, S. Veronese, M.G. Daidone, N. Zaffaroni, Inhibition of telomerase activity by cell-penetrating peptide nucleic acid construct in human melanoma cells, FEBS Lett. 473 (2000) 241–248.
[37] G. Drin, M. Mazel, P. Clair, D. Mathieu, M. Kaczorek, J. Temsamani, Physico-chemical requirements for cellular uptake of pAntp peptide—role of lipid-binding affinity, Eur. J. Biochem. 268 (2001) 1304–1314.
[38] S. Stein, A. Weiss, K. Adermann, P. Lazarovici, J. Hocman, H. Wellhoner, A disulfide conjugate between anti-tetanus antibodies and HIV (37-72) Tat neutralizes tetanus toxin inside cromaffin cells, FEBS Lett. 458 (1999) 383–386.
[39] C.M. Troy, D. Derossi, A. Prochiantz, L.A. Greene, M.L. Shelanski, Down regulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide–peroxynitrite pathway, J. Neurosci. 16 (1996) 253–261.
[40] C.T. Taylor, G.T. Furuta, K. Synnestvedt, S.P. Colgan, Phosphorylation-dependent targeting of cAMP response element binding protein to the ubiquitin/proteosome pathway in hypoxia, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 12091–12096.
[41] G. Griffiths (Ed.), Fine Structure Immunocytochemistry, Springer-Verlag, New York, USA, 1993.
[42] J.L. Perez, J.M. De Ona, J. Niubo, H. Villar, S. Melon, A. Garcia, R. Martin, Comparison of several fixation methods for cytomegalovirus antigenemia assay, J. Clin. Microbiol. 33 (1995) 1646–1649.
[43] D. DiDonato, D.L. Brasaemle, Fixation methods for the study of lipid droplets by immunofluorescence microscopy, J. Histochem. Cytochem. 51 (2003) 773–780.
[44] T. Suzuki, S. Futaki, M. Niwa, S. Tanaka, K. Ueda, Y. Sugiura, Possible existence of common internalization mechanisms among arginine-rich peptides, J. Biol. Chem. 277 (2002) 2437–2443.
[45] M. Bucci, J.P. Gratton, R.D. Rudic, L. Acevedo, F. Roviezzo, G. Cirino, W.C. Sessa, In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduced inflammation, Nat. Med. 6 (2000) 1362–1367.
[46] G.A. Pietersz, W.J. Li, V. Apostolopoulos, A 16-mer peptide (RQIKIWFQNRRMKWKK) from Antennapedia preferentially targets the class I pathway, Vaccine 19 (2001) 1397–1405.
[47] S.J. Bolton, D.N.C. Jones, J.G. Darker, D.S. Eggleston, A.J. Hunter, F.S. Walsh, Cellular uptake and spread of the cell-permeable peptide penetratin in adult rat brain, Eur. J. Neurosci. 12 (2000) 2847–2855.
[48] C. Rousselle, P. Clair, J.M. Lefauconnier, M. Kaczorek, J.M. Scherrmann, J. Temsamani, New advances in the transport of doxorubicin through the blood–brain barrier by a peptide vector mediated strategy, Mol. Pharmacol. 57 (2000) 679–686.
[49] S.R. Schwarze, A. Ho, A. Vocero-Akbani, S.F. Dowdy, In vivo protein transduction: delivery of a biologically active protein into the mouse, Science 285 (1999) 1569–1572.
[50] A. Elmquist, Ü. Langel, In vitro uptake and stability study of pVEC and its all-D analog, Biol. Chem. 384 (2003) 387–393.
[51] P.E.G. Thorén, D. Persson, P. Isakson, M. Goksör, A. Önfelt, B. Nordén, Uptake of analogs of penetratin, Tat(48-60) and oligoarginine in live cells, Biochem. Biophys. Res. Commun. 307 (2003) 100–107.
[52] E. Vivés, J.P. Richard, C. Rispal, B. Lebleu, TAT peptide internalization: seeking the mechanism of entry, Curr. Protein Pept. Sci. 4 (2003) 125–132.
[53] J.A. Leifert, J. Lindsay Whitton, 'Translocatory proteins' and 'Protein transduction domains': a critical analysis of their biological effects and the underlying mechanisms, Molec. Ther. 8 (2003) 13–20.
[54] G. Drin, S. Cottin, E. Blanc, A.R. Rees, J. Temsamani, Studies of the internalization mechanism of cationic cell-penetrating peptides, J. Biol. Chem. 278 (2003) 31192–31201.
[55] S.D. Krämer, H. Wunderli-Allenspach, No entry for TAT(44-57) into liposomes and intact MDCK cells: novel approach to

study membrane permeation of cell-penetrating peptides, Biochim. Biophys. Acta 1609 (2003) 161–169.
[56] J.S. Wadia, R.V. Stan, S.F. Dowdy, Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis, Nat. Med. 10 (3) (2004) 310–315.
[57] N. Nekhotiaeva, A. Elmquist, G.K. Rajarao, M. Hällbrink, Ü. Langel, L. Good, Cell entry and antimicrobial properties of eukaryotic cell-penetrating peptides, FASEB J. 17 (2003) U549–U563.
[58] M. Lindberg, J. Jarvet, Ü. Langel, A. Gräslund, Secondary structure and position of the cell-penetrating peptide transportan in SDS micelles as determined by NMR, Biochemistry 40 (2001) 3141–3149.
[59] E. Gazit, W.J Lee, P.T. Brey, Y.C. Shai, Mode of action of the antibacterial cecropin b2—a spectrofluorometric study, Biochemistry 33 (1994) 10681–10692.
[60] A. Zemel, D.R. Fattal, A. Ben-Shaul, Energetics and self-assembly of amphipathic peptide pores in lipid membranes, Biophys. J. 84 (2003) 2242–2255.
[61] A. Ziegler, X.L. Blatter, A. Seelig, J. Seelig, Protein transduction domains of HIV-1 and SIV TAT interact with charged lipid vesicles. Binding mechanism and thermodynamic analysis, Biochemistry 42 (2003) 9185–9194.
[62] A. Ziegler, J. Seelig, Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters, Biophys. J. 86 (2004) 254–263.
[63] O. Bouffioux, F. Basyn, R. Rezsohazy, R. Brasseur, Structure prediction of CPPs and iterative development of novel CPPs, in: Ü Langel (Ed.), Cell-Penetrating Peptides, Processes and Applications, CRC Press, Boca Raton, USA, 2002, pp. 187–222.
[64] Y.Z. Lin, S.Y. Yao, R.A. Veach, T.R. Torgerson, J. Hawiger, Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence, J. Biol. Chem. 270 (1995) 14255–14258.
[65] Y.Z. Lin, S.Y. Yao, J. Hawiger, Role of nuclear localization sequence in fibroblast growth factor-1-stimulated mitogenic pathway, J. Biol. Chem. 271 (1996) 5305–5308.
[66] M. Rojas, J.P. Donahue, Z.J. Tan, Y.Z. Lin, Genetic engineering of proteins with cell membrane permeability, Nat. Biotechnol. 16 (1998) 370–375.
[67] D. Kato, K. Miyazawa, M. Ruas, M. Starborg, I. Wada, T. Oka, T. Sakai, G. Peters, E. Hara, Features of replicative senescence induced by direct addition of antennapedia-p16(INK4A) fusion protein to human diploid fibroblasts, FEBS Lett. 427 (1998) 203–208.
[68] N.J. Caron, Y. Torrente, G. Camirand, M. Bujold, P. Chapdelaine, K. Leriche, N. Bresolin, J.P. Tremblay, Intracellular delivery of a Tat-eGFP fusion protein into muscle cells, Molec. Ther. 3 (2001) 310–318.
[69] A.M. Vocero-Akbani, N. Vander Heyden, N.A. Lissy, L. Ratner, S.F. Dowdy, Killing HIV-infected cells by transduction with an HIV protease-activated caspase-3 protein, Nat. Med. 5 (1999) 29–33.
[70] T. Ganz, R.I. Lehrer, Antimicrobial peptides of vertebrates, Curr. Opin. Immunol. 10 (1998) 41–44.
[71] M. Simmaco, G. Mignogna, D. Barra, Antimicrobial peptides from amphibian skin: what do they tell us? Biopolymers 47 (1998) 435–450.
[72] E. Walum, A. Peterson, L.J. Erkell, Photometric recording of cell viability using trypan blue in perfused cell cultures, Xenobiotica 15 (1985) 701–704.
[73] T. Mosmann, Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[74] E. Walum, A. Peterson, Tritiated 2-deoxy-D-glucose as probe for cell membrane permeability studies, Anal. Biochem. 120 (1982) 8–11.
[75] A.H. Joliot, A. Triller, M. Volovitch, C. Pernelle, A. Prochiantz, Alpha-2,8-polysialic acid is the neuronal surface receptor of antennapedia homeobox peptide, New Biol. 3 (1991) 1121–1134.
[76] A. Prochiantz, Messenger proteins: homeoproteins, TAT and others, Curr. Opin. Cell Biol. 12 (2000) 400–406.
[77] B. Ensoli, G. Barillari, S.Z. Salahuddin, R.C. Gallo, F. Wongstaal, Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients, Nature 345 (1990) 84–86.
[78] I.W. Park, C.K. Ullrich, E. Schoenberger, R.K. Ganju, J.E. Groopman, HIV-1 tat induces microvascular endothelial apoptosis through caspase activation, J. Immunol. 167 (2001) 2766–2771.
[79] S.H. Ramirez, J.F. Sanchez, C.A. Dimitri, H.A. Gelbard, S. Dewhurst, S.B. Maggirwar, Neurotrophins prevent HIV Tat-induced neuronal apoptosis via a nuclear factor-kappaB (NF-kappaB)-dependent mechanism, J. Neurochem. 78 (2001) 874–889.
[80] H. Jia, M. Lohr, S. Jezequel, D. Davis, S. Shaikh, D. Selwood, I. Zachary, Cystein-rich and basic domain HIV-1 Tat peptides inhibit angiogenesis and induce endothelial cell apoptosis, Biochem. Biophys. Res. Commun. 283 (2001) 469–479.
[81] J.R. Gavin, J. Roth, P. Jen, P. Freychet, Insulin receptors in human circulating cells and fibroblasts, Proc. Natl. Acad. Sci. U. S. A. 69 (1972) 747–751.
[82] M. Lindgren, X. Gallet, U. Soomets, M. Hällbrink, E. Bråkenhielm, M. Pooga, R. Brasseur, Ü. Langel, Translocation properties of novel cell penetrating transportan and penetratin analogues, Bioconjug. Chem. 11 (2000) 619–626.
[83] D. Lorenz, B. Wiesner, J. Zipper, A. Winkler, E. Krause, M. Beyermann, M. Lindau, M. Bienert, Mechanism of peptide-induced mast cell degranulation. Translocation and patch-clamp studies, J. Gen. Physiol. 112 (1998) 577–591.
[84] S.H. White, W.C. Wimley, Membrane protein folding and stability: physical principles, Annu. Rev. Biophys. Biomol. Struct. 28 (1999) 319–365.
[85] J. Stojan, Analysis of progress curves in an acetylcholinesterase reaction: a numerical integration treatment, J. Chem. Inf. Comput. Sci. 37 (1997) 1025–1027.
[86] D. Derossi, S. Calvet, A. Trembleau, A. Brunissen, G. Chassaing, A. Prochiantz, Cell internalization of the third

helix of the A
dent, J. Biol.
[87] A. Vivés, B. J
in: Ü. Langel
Applications,
[88] P.A. Wender,
Steinman, J.I
tion of mole
peptoid mole
97 (2000) 13
[89] K.H. Bremn
nuclear local
Opin. Mol. T
[90] R.I. Mahato,
delivery, Cur
[91] J. Hawiger,
intracellular
189–194.

helix of the Antennapedia homeodomain is receptor-independent, J. Biol. Chem. 271 (1996) 18188–18193.
[87] A. Vivés, B. Lebleu, The Tat-derived cell-penetrating peptides, in: Ü. Langel (Ed.), Cell-Penetrating Peptides, Processes and Applications, CRC Press, Boca Raton, USA, 2002, pp. 3–21.
[88] P.A. Wender, D.J. Mitchell, K. Pattabiraman, E.T. Pelkey, L. Steinman, J.B. Rothbard, The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 13003–13008.
[89] K.H. Bremner, L.W. Seymour, C.W. Pouton, Harnessing nuclear localization pathways for transgene delivery, Curr. Opin. Mol. Ther. 3 (2001) 170–177.
[90] R.I. Mahato, O.D. Monera, L.C. Smith, Peptide-based gene delivery, Curr. Opin. Mol. Ther. 1 (1999) 226–243.
[91] J. Hawiger, Cellular import of functional peptides to block intracellular signaling, Curr. Opin. Immunol. 9 (1997) 189–194.
[92] B.L. Lum, M.P. Gosland, S. Kaubisch, B.I. Sikic, Molecular targets in oncology: implications of the multidrug resistance gene, Pharmacotherapy 13 (1993) 88–109.
[93] M.N. Lango, D.M. Shin, J.R. Grandis, Targeting growth factor receptors: integration of novel therapeutics in the management of head and neck cancer, Curr. Opin. Oncol. 13 (2001) 168–175.
[94] A. Fraile-Ramos, T.N. Kledal, A. Pelchen-Matthews, K. Bowers, T.W. Schwartz, M. Marsh, The human cytomegalovirus US28 protein is located in endocytic vesicles and undergoes constitutive endocytosis and recycling, Mol. Cell. Biol. 12 (2001) 1737–1749.
[95] R.W. Doms, D. Trono, The plasma membrane as a combat zone in the HIV battlefield, Genes Dev. 14 (2000) 2677–2688.