[Frontiers in Bioscience, Elite, 5, 509-516, January 1, 2013]

Cell-penetrating peptides: from cell cultures to *in vivo* applications

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1. ABSTRACT

The field of gene therapy is starting to move towards clinical applications but is currently limited by the lack of efficient delivery systems. Cell-penetrating peptides provide a means of cellular delivery for gene therapy applications as well as delivery of traditional drugs. Using cell-penetrating peptides a range of different cargos have been successfully delivered into a number of cell types, *in vitro* as well as *in vivo*. In this review we discuss uptake mechanisms of different cell-penetrating peptides, with or without cargo. The transition from *in vitro* to *in vivo* applications and strategies to increase the bioavailability of cell-penetrating peptides are also discussed.

2. INTRODUCTION

Nucleic acid based therapeutics, or gene therapy, is growing and highly promising. Gene therapy was initially defined as replacement or complementation of dysfunctional or missing genes. This was later extended to include modulation of gene expression, both endogenous and exogenous genes such as viral genes. siRNA (1), antisense oligonucleotides (ASON) (2) and microRNA (miRNA) (3) are being investigated for gene therapy applications and have a huge potential to cure a number of diseases. In most cases the challenge is not the gene regulation itself but rather the delivery into the cell (4). The most commonly used methods for delivery of nucleic acid

Table 1. CPPs mentioned in this review

Peptides	Amino acid sequence	Net charge	Ref
Protein derived			
Penetratin	RQIKIWFQNRRMKWKK-NH_2	+8	(8)
Tat(48-60)	GRKKRRQRRRQC	+8	(10)
Tat-DRBD	144 amino acid recombinantly expressed fusion protein		(22)
Chimeric			
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-NH_2	+5	(11)
TP10	AGYLLGKINLKALAALAKKIL-NH_2	+5	(12)
PepFect 3	Stearyl-AGYLLGKINLKALAALAKKIL-NH_2	+4	(7)
PepFect 6	Stearyl-AGYLLGK(εNH[a])INLKALAALAKKIL-NH_2	+4[b]	(15)
PepFect 14	Stearyl-AGYLLGKLLOOLAAAALOOLL-NH_2	+5	(16)
RVG-9R	YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGrrrrrrrrr	+11	(23)
Designed			
Polyarginine	R_n	+n	(17)
R7W	Fl-RRRRRRRW-NH_2	+7	(24)
RXR	$(RXR)_4$	+8	(18)
Pep-1	Ac-KETWWETWWTEWSQPKKKRKV-Cya	+3	(25)
PEG-Pep-3	PEG-KWFETWFTEWPKKRK-Cya	+3	(26)
CADY	Ac-GLWRALWRLLRSLWRLLWRA-Cya	+5	(21)
ppTG20	GLFRALLRLLRSLWRLLLRA	+5	(20)

Abbreviations: O=Ornithine, a= lysine-tree with trifluoromethylquinoline derivative modifications, for structure see (15), b=net charge of peptide backbone, r=D-arginine, n=integer 6-12, Fl=fluorescein, X=6-aminohexanoic acid, Ac=Acetyl,Cya=Cysteamide, PEG=polyethylene glycol

based therapeutics have several drawbacks such as immunogenicity problems and insertional mutagenesis with viral vectors (5), and low efficacy of many of the non-viral methods, therefore there is a need for improved delivery systems.

A simple definition of a cell-penetrating peptide (CPP) is a peptide that alone or together with a cargo is capable of crossing the cell membrane and translocate into the cell. In most cases, CPPs consist of less than 30 amino acids, are positively charged and are often amphipathic. CPPs have been used to deliver a large range of different cargoes, ranging from small organic compounds and fluorescent markers to antisense oligonucleotides, large nucleic acids, plasmids and proteins (4, 6). Cargoes can either be linked to the CPP with covalent bonds, for example disulfide bridges (6), or by non-covalent interactions (7). The first CPPs to be discovered were penetratin, derived from the Drosophila Antennapedia homeodomain (8) and Tat, derived from amino acids of HIV Tat protein (9, 10). Today there are hundreds of known CPPs, they can be subdivided into protein-derived (such as penetratin and Tat) also known as protein transduction domains, chimeric (composed of two or more naturally occurring sequences) and designed peptides. Table 1 lists the peptides discussed in this review. The numbers of designed and chimeric peptides are rapidly increasing and many of these peptides have significantly higher cell penetrating capabilities than the early protein derived CPPs. Despite this, early CPPs such as Tat are still being widely used and there are numerous examples of modified variants of Tat with improved cell penetration.

Transportan is a typical example of a chimeric CPP, based on a short galanin sequence fused to a sequence from the wasp venom peptide mastoparan (11). Following the original transportan, several truncated versions have been synthesized (12), the most efficient being transportan 10 (TP10) (13). A second modification was the addition of a stearyl moiety to TP10 which enhanced the efficacy (7, 14). This new peptide was named PepFect 3. Since then, PepFect 3 has been further modified, the most successful modifications being addition of trifluoromethylquinoline derivatives (PepFect 6) (15) and replacement of the lysines with ornithines together with rearrangements of hydrophobic residues (PepFect 14) (16) (Table 1).

A common group of synthetic CPPs are arginine-rich CPPs. The simplest ones are polyarginines of different lengths, most commonly R8 and R9 (17). These have also been modified into more effective variants, including fatty acid modifications such as stearylation and $(RXR)_4$, a peptide consisting of alternating arginine and 6-aminohexanoic acid residues (18, 19). Generally the addition of non-natural amino acids or D-amino acids to CPPs have been shown to increase the efficacy and stability of CPPs. Tryptophan-containing peptides have also been widely studied, many of the protein derived CPPs contain tryptophan which is commonly present in transmembrane regions of proteins. The addition of tryptophan to CPPs has been shown to increase the uptake and lower the toxicity of R7 (20) and the tryptophan containing peptide CADY forms a secondary amphipathic helical structure with tryptophans on one side of the helix (21).

3. LOADING STRATEGIES

Early CPP cargoes were in most cases conjugated to the peptides by covalent bonding. This is still the preferred method for delivery of small molecular drugs and for coupling of fluorescent markers. In the case of oligonucleotide delivery, non-covalent complexation has many advantages over covalent coupling, including ease of preparation, low cost and the ability to use the same peptide structure for a number of different cargoes. Cargoes ranging from siRNA to plasmids and even proteins have been successfully delivered into cells using this technique (27). Non-covalent CPP-oligonucleotide complexes typically form nanoparticles with a size of around 100 nm in diameter (21, 28, 29). The complexes are generally

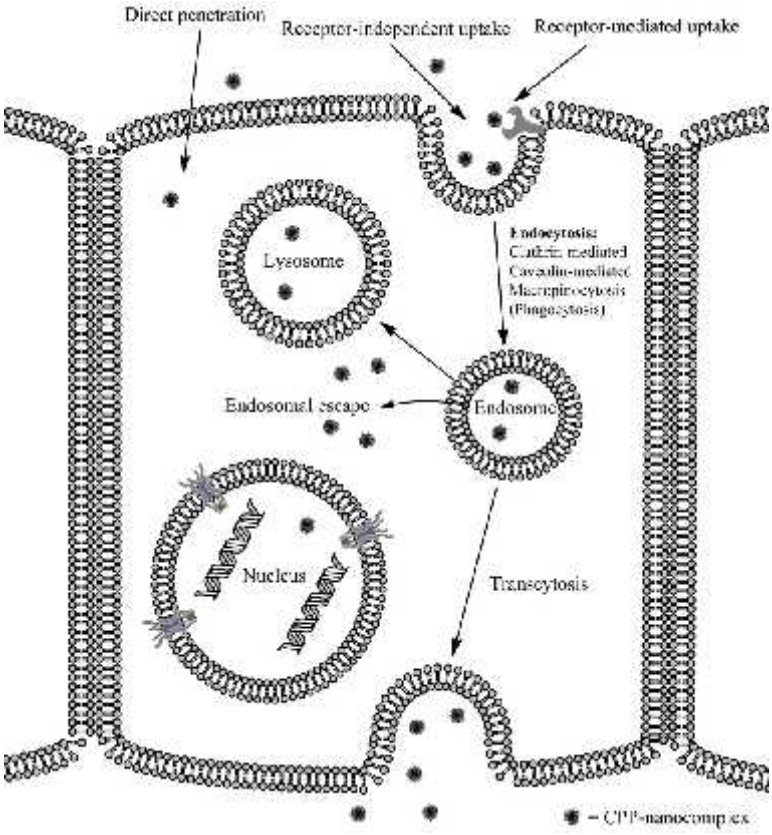

Figure 1. Different uptake pathways for CPPs. CPP uptake is divided between direct uptake and endocytosis, which is further divided in clathrin-mediated, caveolin-mediated, macropinocytosis and phagocytosis. Endocytosis can either be receptor-independent or receptor-mediated. In the case of endosomal uptake, endosomal escape is necessary to avoid degradation in the lysosome. Avoiding the lysosomal pathway as well as endosomal escape is a requirement for efficient transcytosis of CPPs across epithelial layers and deeper into tissues or into the brain.

assumed to have a positively charged surface due to the positively charged peptides encapsulating the oligonucleotide and a surplus of positive over negative charges. Recent measurements of zeta potential however indicated a net negative charge for PepFect-oligonucleotide complexes in biorelevant medium (16). This negative charge might be caused by oligonucleotide presence at the surface of the complexes; if this is the case the nucleic acids might also play a role in the recognition and uptake of complexes.

Combinations of CPPs and other delivery systems have also been attempted using liposomes, polymer nanoparticles, inorganic nanoparticles and exosomes for drug delivery or imaging/diagnostics. The addition of cell penetrating and targeting peptides to nanoparticles used for drug delivery or imaging could potentially increase the effect of the drug and/or aid in delivery across the blood-brain barrier (23, 30-32).

4. UPTAKE MECHANISMS

Initially CPPs were thought to penetrate cell membranes via a direct penetration mechanism; microscopy studies of fluorescently labeled CPP uptake and the apparent lack of temperature dependence for penetration lead to the conclusion that endocytosis was not the major means of uptake. Many of the observations of direct penetration were later found to be microscopy artifacts (33) and most did not take the contribution of the cargo on the mechanism of uptake into account. The predominating view was shifted toward endocytic uptake and evidence for different types of endocytosis was found in a number of studies (Figure 1) (34-36). Given that many CPPs seem to enter cells by endocytosis the further intracellular fate of the CPPs becomes more important to control. Following the normal trafficking the CPPs and cargoes intended to reach intracellular locations would be degraded in lysosomes. Thus, in order to prevent lysosomal degradation the ability to “escape” out of endosomes becomes an important feature. Several different modifications have been introduced to enhance the endosomal escape of cell penetrating peptides, ranging from fatty acids and specific amino acid sequences to endosomolytic groups covalently coupled to the peptide backbone. Endosomal escape is generally thought to be facilitated by increasing the peptides ability to interact with the endosomal membrane (fatty acid modifications, hydrophobic amino acids) or by acting as “proton sponges” causing proton influx into the endosome and thereby

destabilizing the endosomal membrane, releasing the entrapped peptides with its cargo (15, 37).

The endocytic uptake of CPPs is still generally considered to be receptor independent. However, the exact means of endocytosis has not been determined and might also vary depending on the peptide and cell type as well as the cargo. Some studies have indicated macropinocytosis as the most important means of endocytosis (38) but this has not been generalized to all CPPs. Clathrin-mediated and caveolae-mediated endocytosis has also been observed in several studies. Cell-surface bound heparan sulfate proteoglycan (HSPG) can be a binding target for cationic CPPs as these molecules are polyanionic. HSPG has been shown to induce clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis, when binding to a CPP-nanocomplex (39). Seemingly contradictory, the secondarily amphipathic CADY peptide was recently reported to follow a non-endocytic uptake pathway; this was supported by colocalization studies, endocytosis inhibition and electron microscopy (21). This highlights the difference in uptake between different CPPs and the importance of mechanistic studies of uptake for different classes of CPPs. A distinction between endocytic and non-endocytic CPPs might be needed, but the matter is complicated by the fact that some CPPs have displayed both types of uptake (40).

4.1. Scavenger receptors

Recently, the involvement of class A scavenger receptors in the uptake of PepFect 14-SCO nanocomplexes has been reported (28). Uptake by scavenger receptors is endocytosis-mediated. RNAi knockdown of class A scavenger receptors 3 and 5 lead to significant decreases in CPP uptake and specific inhibition of the scavenger receptors using inhibitory ligands lead to a complete loss of PepFect 14-mediated oligonucleotide uptake. This is the first example of scavenger receptor-mediated CPP-uptake, most likely scavenger receptors also play an important role in the uptake of other CPPs than PepFect 14.

4.2. Cargo contribution

Verdurmen *et al*. showed that arginine-rich CPPs induce acid sphingomyelinase (ASMase) translocation from internal compartments to the plasma membrane. ASMase hydrolyzes sphingomyelin producing ceramide-rich microdomains at the outer leaflet of the plasma membrane. The border between the ceramide-enriched domain and plasma membrane is thought to be the site of translocation for arginine-rich CPPs (41). The ASMase/ceramide-induced translocation was found to be endocytosis-independent. In another study; clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis were shown to be involved in the uptake of several arginine-rich peptides when administered without cargo molecules (40).

Both endosomal and non-endosomal pathways have also been observed for Tat. Fluorescently labeled Tat was taken up by cells at 4°C, at this temperature all types of endocytic uptake should be inhibited. Despite this a Tat fusion protein, Tat-bt-SAv, was shown to be completely restricted to endocytosis-dependent uptake mechanisms (42). These findings show that uptake mechanisms of CPPs coupled to cargo and CPPs alone could differ and highlight the importance of separating the mechanism of translocation between these two systems.

4.3. Methods to evaluate CPP uptake

Labeling peptides with fluorescent molecules is the most common method of studying the uptake and cellular transport of peptides with or without cargo (43). This is usually done by covalent linkage of CPPs to fluorescein or other fluorescent dyes. The labeled peptides can then be used in a range of different microscopy based uptake assays or for *in vivo* applications. However, one disadvantage with the labeling is that it might affect the chemical properties and thus might affect the uptake mechanisms of the peptides or peptide-cargo complexes. Another approach is to use a fluorescent cargo, for example a labeled siRNA. The drawback in this case is that microscopy only reveals cellular trafficking of the cargo itself and does not give any information about the intracellular fate of the peptide. Non-covalent complexation with different oligonucleotide-cargos has extensively been used to evaluate the efficiency of novel CPPs; one common method is luciferase based assays using splice correcting oligonucleotides or luciferase expressing plasmids. These methods generally provide a fast, easy and cheap way to assess the efficiency of CPPs.

The criteria for being a “good CPP” are usually based on *in vitro* efficiency of a CPP, not the efficiency or applicability of a CPP *in vivo*. In order to cope with this, CPP assays must evolve from simple cell culture delivery tests to systems more closely resembling an *in vivo* system. Transcytosis across vascular endothelial cells could be modeled *in vitro* by three-dimensional setups, ex. Transwell (Corning) complemented with an appropriate cell line for the application, i.e. vascular endothelial) cells for intravenous delivery, blood-brain barrier-derived endothelial cells for intravenous delivery of brain-targeted nanoparticles, epidermal cells for delivery across the skin or epithelial colorectal cells (Caco-2) for oral delivery (44).

5. *IN VIVO* APPLICATIONS

The first proof of concept for applications of CPPs *in vivo* was published by Dowdy *et al* in 1999, in a study on intraperitoneal delivery of Tat-β-galactosidase fusion protein (45). Following this, CPPs have been used in several *in vivo* studies, including systemic administration of PF6-siRNA complexes (15), gene delivery by intravenous injection of ppTG20-plasmid complexes (20), intratracheal delivery of human recombinant caspase-3 mediated by non-covalent Pep-1 (46), and biodistribution and tumor accumulation of fluorescently labeled arginine rich CPPs in tumor xenografted mice (47). Delivery routes have ranged from intramuscular and systemic delivery to nasal and dermal delivery (48, 49). There are several examples of CPPs combined with targeting peptides for selective delivery to specific targets, in many cases cancer targeting peptides obtained by combination of peptide-based receptor ligands for selectivity and CPPs for increased uptake.

These peptides could either carry a classical chemotherapeutic as a cargo, nucleic acid based therapeutics or have an effect on their own. Several different p53 derived peptides linked to CPPs have been shown to restore the p53 functions in cancer cells (50, 51). Recently, CADY-doxorubicin complexes were shown to be more efficient for delivery than the doxorubicin alone or in liposomal formulations. Furthermore the CADY-doxorubicin complexes had longer blood circulation time than a polyethylene glycol(PEG)-modified liposomal doxorubicin formulation approved for clinical use (52).

CPPs have also been suggested as delivery vehicles for drug delivery across the blood-brain barrier Successful brain delivery in animals has been reported but the percentage of peptide taken up in the brain is still low. One reason for this could be that most CPPs are designed for delivery into the cytosol rather than to promote transcytosis across an endothelium. A chimeric peptide derived from rabies virus glycoprotein coupled to nonaarginine (RVG-9R) showed significantly increased delivery of siRNA to the brain through intravenous injection (23). Nasal delivery routes have also been investigated such as the coupling of Tat to copolymer micelles for delivery to the brain through the nasal cavity (48).

5.1. Clearance of nanoparticles

Organ tissue fenestration, elimination by macrophages and short half-life of nanoparticles in blood are some of the problems associated with drug delivery. In a study by Sarko *et al*. the half-life of some CPPs in human serum was found to be highly dependent on the peptide structure. Peptides containing at least one RR bond were found to have significantly shorter half-lives than peptides without RR bonds such as TP10 and MAP. TP10 and MAP were both found to have half-lives of over 72 hours in human serum (53).

Morris *et al*. showed an improvement of ASON-delivery *in vivo* through PEGylation of Pep-3. PEG-Pep-3 had an increased stability *in vivo* compared to the non-PEGylated Pep-3, thus enhancing the delivery of ASONs. The stabilizing effect of CPP-nanocomplex PEGylation might be similar to the decrease of serum protein opsonization on PEGylated liposomes compared to non-PEGylated liposomes (54). CPP complex half-life in blood was also found to be correlated to the amount of serum proteins associated to liposomes. PEGylation is a commonly used method to increase half-life in blood and water solubility while decreasing toxicity and immunogenicity of nanoparticles and has also been used for proteins, dendrimers, small molecules (55) and even viruses (56).

Nanoparticles delivered *in vivo* are generally sequestered by the mononuclear phagocyte system (MPS) causing depletion of bioavailable nanoparticles. This is also the case for CPP nanocomplexes (15, 23, 57), displaying sequestration by liver, spleen and kidneys. The MPS consist of primarily monocytes and macrophages; the latter involves Kupffer cells which are liver macrophages. Kupffer cells have been shown to play a major role in removal of nanoparticles, from the blood stream. Gadolinium chloride (58) and liposomes with clodronate (59) have been used to induce transient apoptosis of macrophages in the liver and spleen for subsequent administration of nanoparticles, leading to increased bioavailability due to decreased sequestration by macrophages. Macrophage apoptosis is however not warranted in *in vivo* applications as the overall immune system is suppressed. A similar train of thought was used by Haisma *et al*. in a study where they prevented liver macrophage sequestration of adenoviral vectors by pretreating mice with polyinosinic acid, a ligand for class A scavenger receptors. An increase in transgene expression was seen in all tissues following adenoviral delivery (60).

On the relevance of size, long-circulating non-deformable particles should not exceed 200 nm in order to bypass splenic filtration (61). Nanoparticles including CPP complexes in the 100 nm size range are in theory too large for kidney filtration (62, 63). However, a recent study has suggested that positively charged, self-assembled polycation-siRNA nanoparticles accumulate in the kidney glomerular basement membrane followed by disassembly by negatively charged proteoglycans. This mechanism might also be involved in the clearance of non-covalent CPP-siRNA nanoparticles with a positive zeta-potential. Negatively charged complexes are however less likely to disassemble due to charge repulsion (62).

6. FUTURE ASPECTS

For systemic delivery of nanocomplexes, renal clearance and hepatic uptake need to be avoided. This might be done by protection of nanocomplexes against opsonization by serum proteins, , by inhibiting non-specific receptor interaction to nanocomplexes and improving targeting of nanocomplexes to specific receptors, thereby improving the overall bioavailability of peptide nanocomplexes. An alternative strategy might be to design peptides for rapid endothelial uptake, i.e. through transcytosis past the vascular endothelium or into target tissues. The role of scavenger receptors in CPP uptake needs to be further studied. Possibly, inhibition of class A scavenger receptor-mediated uptake by macrophages, could be a strategy to avoid sequestration of CPP-nanocomplexes in the blood stream.

As the field of CPPs matures, the focus will most likely shift towards *in vivo* studies and clinical applications. New peptide modifications will be required to improve the blood circulation time and stability of peptide based delivery systems and further studies of side effects and immune responses will be needed to determine *in vivo* toxicity and immunogenicity of CPPs. Similarly, improved *in vitro* systems for assessing the function and bioavailability of CPP-nanocomplexes are needed to more precisely determine the potential for use *in vivo*.

Provided that the difficulties can be overcome, CPPs will certainly play a key role in the field of drug delivery; efficient delivery combined with the therapeutic

potential of siRNA could replace a number of existing drugs and yield new treatment possibilities. After almost 30 years of research within the field of CPPs, clinical trials have been scarce. However, the authors of this review are optimistic about the future as the number of clinical trials is rising. Currently there are about 25 ongoing clinical phase I/II trials where CPPs are used as delivery systems and the first phase III trials are expected to be started in the near future.

7. ACKNOWLEDGEMENTS

Jakob Regberg and NK Jonas Eriksson equally contributed to this article.

8. REFERENCES

1. D Castanotto, JJ Rossi: The promises and pitfalls of RNA-interference-based therapeutics. *Nature* 457, 426-433 (2009)

2. CF Bennett, EE Swayze: RNA Targeting Therapeutics: Molecular Mechanisms of Antisense Oligonucleotides as a Therapeutic Platform. *Annu Rev Pharmacol Toxicol* 50, 259-293 (2010)

3. T Bratkovič, G Glavan, B Štrukelj, M Živin, B Rogelj: Exploiting microRNAs for cell engineering and therapy. *Biotechnol Adv* 30, 753-765 (2012)

4. F Heitz, MC Morris, G Divita: Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br J Pharmacol* 157, 195-206 (2009)

5. R Heilbronn, S Weger: Viral vectors for gene transfer: current status of gene therapeutics. *Handb Exp Pharmacol* 197, 143-170 (2010)

6. M Zorko, Ü Langel: Cell-penetrating peptides: mechanism and kinetics of cargo delivery. *Adv Drug Deliv Rev* 57, 529-545 (2005)

7. M Mäe, S EL Andaloussi, P Lundin, N Oskolkov, HJ Johansson, P Guterstam, Ü Langel: A stearylated CPP for delivery of splice correcting oligonucleotides using a non-covalent co-incubation strategy. *J Controlled Release* 134, 221-227 (2009)

8. D Derossi, AH Joliot, G Chassaing, A Prochiantz: The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* 269, 10444-10450 (1994)

9. JP Richard, K Melikov, H Brooks, P Prevot, B Lebleu, LV Chernomordik: Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. *J Biol Chem* 280, 15300-15306 (2005)

10. E Vivès, 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, 16010-16017 (1997)

11. M Pooga, M Hällbrink, M Zorko, Ü Langel: Cell penetration by transportan. *FASEB J* 12, 67-77 (1998)

12. U Soomets, M Lindgren, X Gallet, M Hällbrink, A Elmquist, L Balaspiri, M Zorko, M Pooga, R Brasseur, Ü Langel: Deletion analogues of transportan. *Biochim Biophys Acta* 1467, 165-176 (2000)

13. K Kilk, S EL-Andaloussi, P Järver, A Meikas, A Valkna, T Bartfai, P Kogerman, M Metsis, Ü Langel: Evaluation of transportan 10 in PEI mediated plasmid delivery assay. *J Controlled Release* 103, 511-523 (2005)

14. S Futaki, W Ohashi, T Suzuki, M Niwa, S Tanaka, K Ueda, H Harashima, Y Sugiura: Stearylated arginine-rich peptides: a new class of transfection systems. *Bioconjug Chem* 12, 1005-1011 (2001)

15. S EL-Andaloussi, T Lehto, I Mager, K Rosenthal-Aizman, II Oprea, OE Simonson, H Sork, K Ezzat, DM Copolovici, K Kurrikoff, JR Viola, EM Zaghloul, R Sillard, HJ Johansson, F Said Hassane, P Guterstam, J Suhorutsenko, PM Moreno, N Oskolkov, J Hälldin, U Tedebark, A Metspalu, B Lebleu, J Lehtio, CIE Smith, Ü Langel: Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo. *Nucleic Acids Res* 39, 3972-3987 (2011)

16. K Ezzat, S EL-Andaloussi, EM Zaghloul, T Lehto, S Lindberg, PM Moreno, JR Viola, T Magdy, R Abdo, P Guterstam, R Sillard, SM Hammond, MJ Wood, AA Arzumanov, MJ Gait, CIE Smith, M Hällbrink, Ü Langel: PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. *Nucleic Acids Res* 39, 5284-5298 (2011)

17. N Schmidt, A Mishra, GH Lai, GCL Wong: Arginine-rich cell-penetrating peptides. *FEBS Lett* 584, 1806-1813 (2010)

18. R Abes, HM Moulton, P Clair, ST Yang, S Abes, K Melikov, P Prevot, DS Youngblood, PL Iversen, LV Chernomordik, B Lebleu: Delivery of steric block morpholino oligomers by (R-X-R)4 peptides: structure-activity studies. *Nucleic Acids Res* 36, 6343-6354 (2008)

19. JB Rothbard, E Kreider, CL VanDeusen, L Wright, BL Wylie, PA Wender: Arginine-rich molecular transporters for drug delivery: role of backbone spacing in cellular uptake. *J Med Chem* 45, 3612-3618 (2002)

20. K Rittner, A Benavente, A Bompard-Sorlet, F Heitz, G Divita, R Brasseur, E Jacobs: New basic membrane-destabilizing peptides for plasmid-based gene delivery *in vitro* and *in vivo*. *Molecular Therapy* 5, 104-114 (2002)

21. L Crombez, G Aldrian-Herrada, K Konate, QN Nguyen, GK McMaster, R Brasseur, F Heitz, G Divita: A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells. *Mol Ther* 17, 95-103 (2009)

22. A Eguchi, BR Meade, YC Chang, CT Fredrickson, K Willert, N Puri, SF Dowdy: Efficient siRNA delivery into

primary cells by a peptide transduction domain-dsRNA binding domain fusion protein. *Nat Biotechnol* 27, 567-571 (2009)

23. P Kumar, H Wu, JL McBride, KE Jung, MH Kim, BL Davidson, SK Lee, P Shankar and N Manjunath: Transvascular delivery of small interfering RNA to the central nervous system. *Nature* 448, 39-43 (2007)
24. JR Maiolo, M Ferrer, EA Ottinger: Effects of cargo molecules on the cellular uptake of arginine-rich cell-penetrating peptides. *Biochim Biophys Acta* 1712, 161-172 (2005)

25. MC Morris, J Depollier, J Mery, F Heitz, G Divita: A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat Biotechnol* 19, 1173-1176 (2001)

26. MC Morris, E Gros, G Aldrian-Herrada, M Choob, J Archdeacon, F Heitz, G Divita: A non-covalent peptide-based carrier for in vivo delivery of DNA mimics. *Nucleic Acids Res* 35, e49-e49 (2007)

27. T Lehto, K Kurrikoff, Ü Langel: Cell-penetrating peptides for the delivery of nucleic acids. *Expert Opin Drug Deliv* 9, 823-836 (2012)

28. K Ezzat, H Helmfors, O Tudoran, C Juks, S Lindberg, K Padari, S EL-Andaloussi, M Pooga, Ü Langel: Scavenger receptor-mediated uptake of cell-penetrating peptide nanocomplexes with oligonucleotides. *FASEB J* 26, 1172-1180 (2012)

29. Y Arthanari, A Pluen, R Rajendran, H Aojula, C Demonacos: Delivery of therapeutic shRNA and siRNA by Tat fusion peptide targeting bcr--abl fusion gene in Chronic Myeloid Leukemia cells. *J Controlled Release* 145, 272-280 (2010)

30. E Ruoslahti: Peptides as targeting elements and tissue penetration devices for nanoparticles. *Adv Mater* (2012) in press

31. L Alvarez-Erviti, Y Seow, H Yin, C Betts, S Lakhal, MJ Wood: Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 29, 341-345 (2011)

32. Y Sakurai, H Hatakeyama, Y Sato, H Akita, K Takayama, S Kobayashi, S Futaki, H Harashima: Endosomal escape and the knockdown efficiency of liposomal-siRNA by the fusogenic peptide shGALA. *Biomaterials* 32, 5733-5742 (2011)

33. JP Richard, K Melikov, E Vives, C Ramos, B Verbeure, MJ Gait, LV Chernomordik, B Lebleu: Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J Biol Chem* 278, 585-590 (2003)

34. G Drin, S Cottin, E Blanc, AR Rees, J Temsamani: Studies on the internalization mechanism of cationic cell-penetrating peptides. *J Biol Chem* 278, 31192-31201 (2003)

35. P Lundin, H Johansson, P Guterstam, T Holm, M Hansen, Ü Langel, S EL-Andaloussi: Distinct uptake routes of cell-penetrating peptide conjugates. *Bioconjug Chem* 19, 2535-2542 (2008)

36. S Futaki, I Nakase, A Tadokoro, T Takeuchi, AT Jones: Arginine-rich peptides and their internalization mechanisms. *Biochem Soc Trans* 35, 784 (2007)

37. M Yamagata, T Kawano, K Shiba, T Mori, Y Katayama, T Niidome: Structural advantage of dendritic poly(l-lysine) for gene delivery into cells. *Bioorganic & Medicinal Chemistry* 15, 526-532 (2007)

38. JS Wadia, RV Stan, SF Dowdy: Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* 10, 310-315 (2004)

39. GMK Poon, J Gariépy: Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells. *Biochem Soc Trans* 35, 788 (2007)

40. F Duchardt, M Fotin-Mleczek, H Schwarz, R Fischer, R Brock: A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. *Traffic* 8, 848-866 (2007)

41. WPR Verdurmen, M Thanos, IR Ruttekolk, E Gulbins, R Brock: Cationic cell-penetrating peptides induce ceramide formation via acid sphingomyelinase: Implications for uptake. *J Controlled Release* 147, 171-179 (2010)

42. G Ter-Avetisyan, G Tünnemann, D Nowak, M Nitschke, A Herrmann, M Drab, MC Cardoso: Cell entry of arginine-rich peptides is independent of endocytosis. *J Biol Chem* 284, 3370 (2009)

43. F Madani, S Lindberg, Ü Langel, S Futaki, A Gräslund: Mechanisms of cellular uptake of cell-penetrating peptides. *Journal of Biophysics* 2011, 1-10 (2011)

44. ME Lindgren, MM Hällbrink, AM Elmquist, Ü Langel: Passage of cell-penetrating peptides across a human epithelial cell layer *in vitro*. *Biochem J* 377, 69-76 (2004)

45. SR Schwarze, A Ho, A Vocero-Akbani, SF Dowdy: In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 285, 1569-1572 (1999)

46. K Aoshiba, N Yokohori, N Atsushi: Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol* 28, 555-562 (2003)

47. I Nakase, Y Konishi, M Ueda, H Saji, S Futaki: Accumulation of arginine-rich cell-penetrating peptides in tumors and the potential for anticancer drug delivery in vivo. *J Controlled Release* 159, 181-188 (2012)

48. T Kanazawa, H Taki, K Tanaka, Y Takashima, H Okada: Cell-penetrating peptide-modified block copolymer micelles promote direct brain delivery via intranasal administration. *Pharm Res* 28, 2130-2139 (2011)

49. P Shah, P Desai, D Channer, M Singh: Enhanced skin permeation using polyarginine modified nanostructured lipid carriers. *J Control Release* (2012) in press

50. EL Snyder, BR Meade, CC Saenz, SF Dowdy: Treatment of terminal peritoneal carcinomatosis by a transducible p53-activating peptide. *PLoS Biol* 2, E36 (2004)

51. J Michl, B Scharf, A Schmidt, C Huynh, R Hannan, H von Gizycki, FK Friedman, P Brandt-Rauf, RL Fine, MR Pincus: PNC-28, a p53-derived peptide that is cytotoxic to cancer cells, blocks pancreatic cancer cell growth in vivo. *Int J Cancer* 119, 1577-1585 (2006)

52. Y Li, X Zheng, Z Cao, W Xu, J Zhang, M Gong: Self-assembled peptide (CADY-1) improved the clinical application of doxorubicin. *Int J Pharm* 434, 209-214 (2012)

53. D Sarko, B Beijer, R Garcia Boy, EM Nothelfer, K Leotta, M Eisenhut, A Altmann, U Haberkorn, W Mier: The pharmacokinetics of cell-penetrating peptides. *Mol Pharm* 7, 2224-2231 (2010)

54. A Chonn, SC Semple, PR Cullis: Association of blood proteins with large unilamellar liposomes *in vivo*. Relation to circulation lifetimes. *J Biol Chem* 267, 18759-18765 (1992)

55. MJ Joralemon, S McRae, T Emrick: PEGylated polymers for medicine: from conjugation to self-assembled systems. *Chem Commun* 46, 1377-1393 (2010)

56. P Wonganan, MA Croyle: PEGylated adenoviruses: from mice to monkeys. *Viruses* 2, 468-502 (2010)

57. S Pujals, E Sabido, T Tarrago, E Giralt: all-D proline-rich cell-penetrating peptides: a preliminary in vivo internalization study. *Biochem Soc Trans* 35, 794 (2007)

58. MJ Hardonk, FW Dijkhuis, CE Hulstaert, J Koudstaal: Heterogeneity of rat liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. *J Leukoc Biol* 52, 296-302 (1992)

59. CB Schmidt-Weber, M Rittig, E Buchner, I Hauser, I Schmidt, E Palombo-Kinne, F Emmrich, RW Kinne: Apoptotic cell death in activated monocytes following incorporation of clodronate-liposomes. *J Leukoc Biol* 60, 230-244 (1996)

60. HJ Haisma, JAAM Kamps, GK Kamps, JA Plantinga, MG Rots, AR Bellu: Polyinosinic acid enhances delivery of adenovirus vectors in vivo by preventing sequestration in liver macrophages. *J Gen Virol* 89, 1097-1105 (2008)

61. SM Moghimi, AC Hunter, JC Murray: Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacol Rev* 53, 283-318 (2001)

62. JE Zuckerman, CH Choi, H Han, ME Davis: Polycation-siRNA nanoparticles can disassemble at the kidney glomerular basement membrane. *Proc Natl Acad Sci U.S.A.* 109, 3137-3142 (2012)

63. J Wang, Z Lu, MG Wientjes, JL Au: Delivery of siRNA therapeutics: barriers and carriers. *AAPS J* 12, 492-503 (2010)

Key Words: Cell-penetrating peptides, Drug delivery, Gene therapy, *In vivo*, Clinical trial, Review

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