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Evaluation of peptide-mediated nucleic acid delivery

Department of Pharmaceutics, Utrecht Institute of Pharmaceutical Sciences (UIPS), Utrecht University

Anastasia Tomatsidou
Student number: 3711420
Drug Innovation Masters
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Supervisor: Markus de Raad
Examiner: Dr. Enrico Mastrobattista

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Introduction

Gene therapy can be defined as the treatment of a disease by the delivery and expression of genetic material in the cells of a patient (1). Gene therapy can theoretically cure a wide range of diseases. The completion of the Human Genome Project has led to the identification of several disease-causing genes, which expanded the knowledge on the molecular background of various diseases (2,3). Genetic diseases, such as cystic fibrosis (4) and muscular dystrophy (5), can be treated through the restoration of gene function of deficient cells. Other afflictions, such as cardiovascular (6,7), neurological (8), infectious (9), wound healing (10), tissue engineering (11,12) and cancer (13,14), can also be treated using gene therapy. Treatment can be achieved through the transfer of genes into the affected cells in order to modify the expression of existing genes. Alternatively, gene transfer can lead to increase/decrease of the level of naturally occurring proteins or production of cytotoxic proteins.

Although the first clinical trial initiated in 1990 (15), it was not before 2000 that different research groups published positive data on clinical trials using gene therapy (3). After the report of the first successful clinical trial for the treatment of γc-SCID in 2000 (16), more studies followed (17,18). Recently, the first gene therapy for the treatment of lipoprotein lipase deficiency (LPLD) was approved in Europe (19,20). In conclusion, gene therapy seems today more accessible and promising than ever.

Nucleic acids, such as plasmid DNA (pDNA), small interfering RNA (siRNA), microRNA (miRNA) and oligonucleotides (ONs), can be used in gene therapy applications (21). These molecules exhibit high specificity and low toxicity (22). However, the high charge distribution, the large molecular weight and the hydrophilicity make them impermeable to cellular membranes (21-23). Thus, nucleic acids need the assistance of a delivery system which can efficiently deliver them inside the target cell. However, the development of the most optimal delivery system still remains a main issue (24).

Gene delivery systems are generally divided into viral and non-viral (24). Viral delivery systems on the one hand, are being successfully used in gene therapy, although they exhibit immunogenicity, cytotoxicity and tumorigenicity (25,26). Non-viral delivery systems on the other hand, are not as efficient as viral vectors but they offer a safe alternative (3,27). A wide range of non-viral vectors has been developed, including systems based on cationic lipids, cationic polymers and peptides. Even though cationic lipids and cationic polymers have been extensively studied as potential gene delivery systems, they need to be improved. For instance, the uncontrolled synthesis, which can result in random polymer formation, and the cellular toxicity pose difficulties in their utilization (23).

Non-viral vectors need to overcome several biological barriers, including the cell membrane, the intracellular environment and the nuclear envelope (*fig.1*), in order to achieve successful delivery of their cargo into somatic cells. Firstly, non-viral vectors must be able to strongly condense and protect their nucleic acid cargo (28). Many gene delivery systems have a net positive charge so that they can compact the negatively charged nucleic acids into nano-sized particles. Secondly, they have to bind to the cellular membrane or cell-surface receptors in order to be internalized. Since the majority of the cellular membranes have a net negative charge, cellular uptake of these positively charged formulations is possible (29). Upon binding, the delivery system will be internalized mainly via endocytosis,

as the cellular membrane of mammalian cells does not allow the passage of large (>500Da), charged molecules (30-32). After endocytosis, the complexes are trapped in endocytic compartments, called endosomes. The endosomes normally follow the lysosomal pathway which leads to degradation of the complexes by the acidic pH and the enzymes in the lysosomes. As a result, the trapped particles have to escape the endosome to avoid degradation (29,31,33). After achieving endosomal escape, the particles are released in the cytoplasm. In the case of pDNA, it has to cross the cytoplasm, reach and enter the nucleus. If the carrier and the cargo are dissociated, pDNA is released in the cytoplasm, where it becomes static and has a half-life of 50-90 min because of nuclease attack (29,34,35). Carrier associated or dissociated pDNA, that achieves to enter the nucleus intact, can be expressed. In contrast, siRNA and miRNA do not have to enter the nucleus as the RNA interference (RNAi) machinery is located in the cytoplasm.

It becomes evident that non-viral vectors are required to have several characteristics in order to achieve successful gene delivery.

Non-viral vectors should strongly condense the nucleic acid cargo into nano-sized particles. In addition, they should protect it against nucleases and against the harsh conditions in the cellular environment. Also, they should be able to mediate cellular entry, endosomal escape and nuclear import, since the non-viral vector/nucleic acid complexes have to be efficiently delivered into the nucleus of the target cell (28).

Peptides constitute an advantageous alternative over other non-viral gene delivery systems as they possess the above mentioned characteristics (28). Furthermore, they are easy to produce, relatively stable, non-toxic and non-immunogenic (2,23). Peptides can be categorized, depending on their function, as: DNA-condensing peptides, which condense nucleic acids, cell-penetrating peptides (CPPs), which mediate membrane translocation and nuclear localization signal (NLS) peptides, which target DNA to the nucleus. To date, numerous peptides, which belong to these three different categories, have been studied for the ability to mediate nucleic acid delivery.

The aim of this thesis is to present an overview of functional DNA condensing, cell-penetrating and nuclear localization signal peptides, already described in literature. In addition, it aims to evaluate them on their functionality, in order to select the peptides which can be successfully used for nucleic acid delivery.

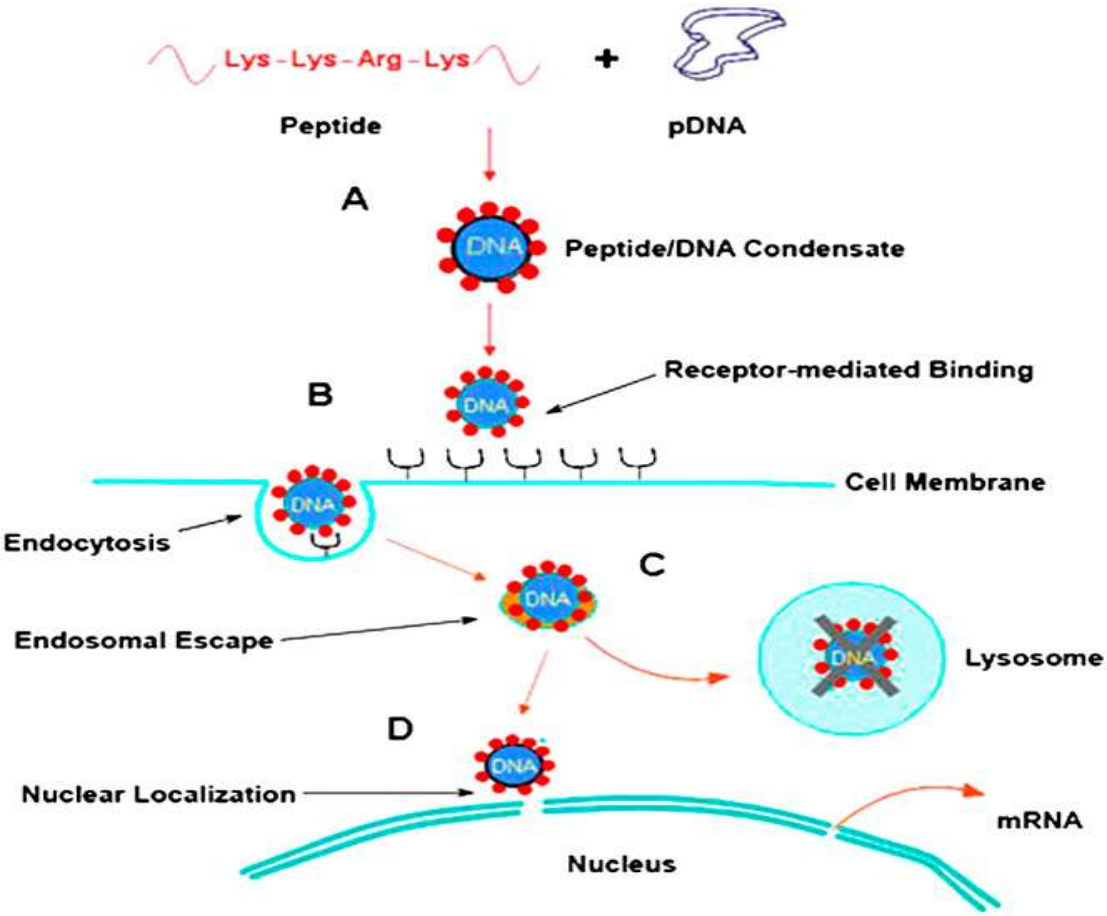

Figure 1. Barriers in peptide-guided gene delivery: (A) Strong association of the peptide with the DNA into nano-sized particles (B) Target and binding of the particles to cell-specific surface receptors (C) Disruption of the endosomal membrane (D) Nuclear targeting and expression of the DNA ({{100 Martin,M.E. 2007}})

DNA condensing peptides

The formation of stable nucleic acid/peptide complexes is vital for successful and efficient gene delivery (24). DNA condensing peptides are able to tightly condense nucleic acid cargo into compact, nano-sized particles (36). These peptides are usually cationic and rich in basic amino acids, such as arginine and lysine. Therefore, they are able to non-specifically interact with the negatively charged phosphate backbone of nucleic acids through electrostatic interactions (28,37).

A wide range of non-natural DNA condensing peptides is currently available. The simplest peptides are linear and contain 6 to 20 residues (38,39), while more complex ones have longer chains or they contain substitutions or additions of amino acids or chemical groups (40). In addition, natural and synthetic DNA condensing peptides are frequently coupled to cationic lipids, cationic polymers and viral proteins or they are inserted into recombinant proteins (41).

It has been reported that the minimum number of lysine and arginine residues per peptide required to condense DNA is 8 and 9 respectively, while peptides with 13 or more positive charges have the ability to strongly condense DNA into stable nano-particles (42-44). The design of DNA condensing peptides with variations on the residue content and/or the structure of the peptide chain has also been reported. For example, branched cationic peptides and cross-linking peptides have been shown to efficiently condense and protect DNA (45,46).

Except from synthetic peptides, natural derived DNA binding peptides can also be used for DNA condensation. For instance, human histone H1 has been shown to have the ability to bind and condense transgene DNA through specific DNA binding domains (47). Peptides derived from protamine have also been found to efficiently function as DNA condensing (39,48,49).

DNA condensing peptide requirements

There are two requirements that a DNA condensing peptide has to fulfill in order to be able to efficiently bind DNA: (a) DNA condensation into compact, stable, nano-sized particles and (b) DNA protection against physical or enzymatic degradation.

Many different techniques are used in order to determine the ability of DNA condensation and protection by peptides. However, only certain are frequently used. The gel retardation assay and the dye displacement assay are techniques which are mainly used for the study of the binding affinity of a peptide/protein to DNA. In addition, the nuclease protection assay and the stability test are techniques which are mainly used for the assessment of the ability of DNA binding peptides to protect the DNA from enzymatic and physical degradation respectively.

Evaluation of DNA condensing peptides

DNA condensing peptides were evaluated on the ability to condense and protect DNA. The majority of the peptides included here, utilized one or a combination of the above mentioned techniques for the assessment of the DNA condensation and protection capacity.

Even though the evaluation of the peptides is feasible, head-to-head comparison is problematic, especially due to the utilization of variable experimental setups. For instance, the use of different DNA concentrations or peptide/DNA ratio units (e.g. charge ratio or weight/weight ratio) or different buffers during the preparation of the complexes, hinders direct comparison of DNA condensing peptides. The utilization of a variety of techniques for the determination of the DNA binding and protection ability also hampers direct comparison.

Evaluation and selection of functional DNA condensing peptides

The number of the available studies on DNA condensing peptides is extensive since many different peptides have been studied and used for nucleic acid binding either alone or as a part of a delivery system. DNA condensing peptides which were tested for the ability to bind DNA without being coupled to cationic lipids or polymers or combined with proteins were included. This inclusion criterion was set because the combination of a peptide with a cationic lipid or polymer or protein leads to the creation of a complex delivery system. The delivery system can influence the ability of a single peptide to bind DNA, resulting in a biased evaluation.

A total of about 80 different synthetic and natural DNA condensing peptides have been scanned and evaluated on the ability to condense and protect DNA. An overview of these peptides can be found on **Table 4** of the appendix. However, merely the peptides with the best DNA condensation and protection capacity were selected and reported. Twenty-three peptides were evaluated as best scoring. An overview of these peptides can be found on **Table 1**.

Certain DNA condensing peptides were evaluated as best scoring depending on the units of the peptide/DNA ratio. More specifically, the selected peptides could tightly condense and fully protect DNA at lower DNA/peptide ratio units compared to other peptides of the same study. Even though the DNA concentrations and the peptide/DNA ratio units differ from study to study, the peptide/DNA ratios were expressed as charge ratios for the majority of the selected peptides. The best scoring peptides exhibited strong DNA condensation at a charge ratio (+/-) ranging from 0.5 to 4:1. For instance, the peptides K18 and pK17 could strongly condense DNA at a charge ratio (+/-) equal of higher than 0.4:1. In contrast, peptide P2, synthesized and studied together with K18 and pK17, was unable to tightly condense DNA even at a charge ratio (+/-) of 4:1 (48). As a result, K18 and pK17 were selected and evaluated as excellent DNA binders. In addition, the selected CPPs could fully protect DNA at charge ratio units similar or higher than the units essential for tight DNA condensation.

The peptide collection included both synthetic and natural peptides. However, only 5 out of 23 peptides were natural: the dimeric H9 peptide and the $(SPKR)_4$ peptide were derived from human histone H1, P1 and P2 peptides were derived from salmon protamine and the μ (mu) peptide was derived from the condensed core of the adenovirus (39,49-52). In general, the number of the studies on natural peptides was smaller compared to the number of the studies on synthetic peptides. Moreover, there were natural peptides available, which have not been tested for their DNA condensing and protection ability and as a result they could not be included in the evaluation. The selected natural peptides showed

excellent DNA condensing ability. Nevertheless, the μ and the $(SPKR)_4$ peptides have not been tested for DNA protection.

The 18 best scoring synthetic peptides included linear polyarginines and polylysines, linear and branched arginine and lysine-containing peptides and cross-linking peptides. The number of polylysine or lysine-containing peptides was bigger compared to the number of polyarginine or arginine-containing peptides. In addition, there were 6 cross-linking peptides, rich in lysine residues. Cross-linking peptides possess high DNA binding capacity due to the introduction of the cysteine residues in the peptide chain. Cysteines are able to form disulfide bonds inside the peptide, upon spontaneous oxidization, leading to the formation of nano-sized, stabilized DNA condensates (46,53,54). The majority of the selected synthetic DNA condensing peptides had an excellent ability to condense and protect DNA, except from R15 and R3V6 (44,55). Moreover, 5 out of 18 synthetic peptides were not tested for the ability to protect DNA. However, they were evaluated as excellent DNA binders. These peptides included the polylysines K18, K20, AlkCWK18, DiAlkCWK18 and AlkCYK18 (39,42,43,56).

Table 1. Overview of the best scoring DNA condensing peptides

Name	Sequence	DNA condensation	DNA protection	Reference
Arginine-15	R15	+	++(E)	(44)
RV	R3V6	+	+(E)	(55)
Branched polyarginine	$(RRRR)_2KGGC$	++	++(P)	(45)
R6p	CHR6HC	++	++(E)	(53)
pK17	K17	++	++(E)	(48)
Lysine-18	K18	++	ND	(39)
Lysine-20	K20	++	ND	(56)
K8	YKAK8WK	++	++(E)	(48)
Branched polylysine	$(KKKK)_2KGGC$	++	++(P)	(45)
K6p	CHK6HC	++	++(E)	(53)
K12p	CHK6H2K6HC	++	++(E)	(53)
AlkCWKn	AlkCWK18	++	ND	(43,56)
AlkCWKn dimer	DiAlkCWK18	++	ND	(43)
AlkCYKn	AlkCYK18	++	ND	(56)
McKenzie III	CWK8CK8C	++	++(P)	(54)
McKenzie IV	CWK5CK5CK5C	++	++(P)	(54)
McKenzie 11	CK4CK4C	++	++(P)	(46)
McKenzie 12	CK4HK3C	++	+(P)	(46)
H9 dimer (H9-2)	$(KTPKKAKKP)_2$	++	++(E)	(39)
P1	PRRRRSSSRPIRRRRPRRASRR	++	++(P)	(49)
P2	RRRRPRRVSRRRRRRGGRRRR	++	++(P)	(49)
$(SPKR)_4$	SPKRSPKRSPKRSPKR	++	ND	(50)
μ peptide	MRRAHHRRRRASHRRMRGG	++	ND	(51,52)

(++)= excellent, (+)= good, ND= non-defined, (E)= enzymatic degradation, (P)= physical degradation

Since the peptides selected and reported here, could efficiently condense and protect DNA, they were also studied for their transfection efficiency. The studies have shown that these peptides could mediate *in vitro* DNA delivery either alone or with the assistance of a delivery system or a commercially available lysosomotropic agent. The majority of them could deliver DNA in the presence of chloroquine. However, the synthetic peptides R15 (44), R6p, K6p and K12p (53) could mediate DNA delivery into the target cell without any assistance.

Cell-penetrating peptides (CPPs)

The cell-penetrating peptide (CPP) category is comprised of many different peptides which have differences in origin (natural, chimeric or synthetic peptides) (57), structure (low amphipathic or high amphipathic peptides) (58) and function (fusogenic or endosomolytic peptides) (36). Functional groups (59) and cationic lipids or polymers (60) are often introduced in the CPP chain, in order to make them more efficient. The CPP chains have variable lengths, from 5 to 30 amino acids. In addition, they usually have a net positive charge (21).

CPPs can bind nucleic acids either covalently, through chemical linkage, or non-covalently, through electrostatic and hydrophobic interactions (22). Moreover, CPPs and CPP/nucleic acid particles can use several uptake mechanisms in order to enter cells. The way of cellular uptake depends on the peptide characteristics (for example sequence, size and structure), the type/structure of the cargo and the experimental conditions, such as the cell type (61). Cellular uptake can be divided into two routes: (a) direct translocation through the lipid bilayer and (b) energy-dependent route or also known as endocytosis (58,62).

The first studies on the cellular entry of CPPs led to the discovery of direct translocation which describes the direct penetration of CPP through cellular membranes in an energy-independent manner (21). The electrostatic interactions, the hydrogen bonding and the structure of the CPP (α-helical or β-barrel) can induce direct translocation through the lipid bilayer (58). Diverse models have been suggested to describe this interaction, such as the pore formation model, the carpet model and the inverted micelle-mediated model (21,62). Nevertheless, recent studies questioned the idea of direct translocation, leading to the endocytosis route, as the main uptake mechanism (21).

CPPs and CPP/nucleic acid particles can enter cells via several endocytotic routes, ending up at different cytoplasmic compartments. The energy-dependent uptake mechanism can be divided into three main pathways which are clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis (21,58,62). The existence of this variety of endocytotic pathways is associated with the diversity of the physical and chemical characteristics of each CPP, the biophysical properties of the used nucleic acids and the composition of the cellular membrane of the target cell (58). In the case of endocytosis, nucleic acids must escape the endosomes and be released in the cytoplasm and/or transferred into the nucleus (58).

In conclusion, CPPs are able to induce membrane translocation and/or endosomal escape for the delivery of nucleic acids into the cytosol or nucleus (63).

Here CPPs are divided depending on their function to fusogenic and endosomolytic.

Fusogenic cell-penetrating peptides

Many fusogenic cell-penetrating peptides, such as Tat, melittin and penetratin, derive from domains of proteins that interact with cellular membranes. These domains are called protein transduction domains (PTDs). Fusogenic peptides can also be synthetic amphipathic, such as GALA, KALA and ppTG20. This class of cell-penetrating peptides adopts α-helical structures at endosomal pH. The α-helical structures result in hydrophobic and hydrophilic faces, which induce disruption and pore formation, upon interaction with the endosomal membrane (28,36).

Endosomolytic cell-penetrating peptides

There are several synthetic cell-penetrating peptides that become endosomolytic at lower pH, thus escaping the endosome. When trapped inside the acidic environment of the early or late endosome, the peptides are able to buffer against the proton pump to induce lysis or interact with the endosomal membrane to cause pore formation (36). Histidine-rich peptides, like H5WYG, and amphipathic fusogenic peptides, like GALA, are the main representatives of this class of cell-penetrating peptides. On the one hand, histidine-rich peptides, mediate endosomal escape taking advantage of the "proton sponge effect". More specifically, additional protons are pumped into the endosome, due to proton absorption from the histidine imidazole groups. This phenomenon causes chloride ion and water influx, which leads to osmotic swelling and subsequent disruption of the endosomal membrane (64). As a result, the peptide/DNA complex is released. On the other hand, amphipathic fusogenic peptides are able to form pores to the endosomal membrane, since they adopt α-helical structures, when trapped in the acidic endosomal environment (65).

Cell-penetrating peptide requirements

The only requirement that a cell-penetrating peptide has to meet, in order to efficiently mediate nucleic acid delivery, is cellular entry. However, cellular entry has to be qualitatively and quantitatively examined. In other words, it is necessary to use methods which can visualize the cell-penetrating ability and also quantify the number of the intact peptide and cargo molecules inside the cell (66).

Fluorescence microscopy, on fixed or live cells, and flow cytometry are the most frequently used techniques for qualitative and quantitative analysis.

Evaluation of cell-penetrating peptides

CPPs were evaluated on quantitative cellular entry and transfection efficiency. The data resulting from the quantitative cellular entry analyses and the transfection studies were examined so that the peptides could be evaluated.

However, as mentioned above for DNA binding peptides, the evaluation of CPPs might be feasible but the head-to-head comparison is problematic. Especially the absence of a united protocol for the transfection experiments in mammalian cells hinders direct comparison. In more detail, the experimental conditions used in different studies have deviations concerning the DNA concentrations or the peptide/DNA ratio units, but also the cell lines, the presence or absence of serum or the utilization of controls during transfection. For example, the experimental setups used in the study of the cationic amphipathic peptide KALA and its derivative RAWA differ. KALA/pCMVLuc complexes were transfected into CV-1 cells at varying charge ratios and DNA amounts. The luciferase activity was maximal at a charge ratio (+/-) of 10:1, using 1.2 μg of DNA. The complexes were subsequently transfected into different mammalian cell lines, such as CaCo2 and HepG2, while dendrimer/DNA complexes and naked DNA were used as controls. KALA was shown to have similar or lower transfection efficiency, depending on the cell line, compared to the dendrimer (67). RAWA/pS2LUC condensates, on the other hand, were transfected into Cos-7 cells, at varying charge ratios and at DNA amounts ranging from 0.2 to 4 μg, in the presence

of chloroquine. The maximum luciferase activity was achieved at 4-8:1 charge ratio (+/-) and 0.5-1 μg of DNA. Variable cell lines, like HeLa and HUVECs, were treated with RAWA/pSV2LUC complexes at a charge ratio of 4:1 (+/-), using 2 μg of plasmid DNA. RAWA had lower, similar or higher transfection efficiency, depending on the cell line, compared to Lipofectamine plus, which was used as the internal control (68).

Evaluation of cell-penetrating peptides on cellular uptake and transfection efficiency

Although the number of the studies on CPPs is relatively big, only several CPPs were selected and reported. CPPs coupled to cationic lipids or polymers or conjugated with chemical groups or proteins were excluded. The combination of a peptide with a delivery system or the addition of a chemical group in the peptide chain would affect the cellular uptake and the transfection efficiency of the peptide. Therefore, the evaluation on the function of a single CPP would not be objective.

The selected CPPs were evaluated on quantitative cellular uptake and on transfection efficiency. However, the peptides which were only tested for cellular uptake and not on nucleic acid delivery can be found on **Table 5** of the appendix. Every selected peptide was tested for nucleic acid delivery and transfection, while only a small minority was also tested for cellular uptake. More specifically, the ability of each peptide to be internalized and to mediate cellular uptake and transfection of its nucleic acid cargo was compared to the respective control, utilized in the *in vitro* studies. Subsequently, these peptide abilities were evaluated as higher (+), similar (+/-) or lower (-) compared to the controls.

The utilization of controls in the assessment of the internalization and delivery of a complex into the target cell is necessary. The comparison of each CPP with the respective control provides important information on the efficiency of the peptide to transfer its nucleic acid cargo *in vitro*. Moreover, the potential *in vivo* application of the peptide can be defined.

A total of 27 different cell-penetrating peptides were evaluated and an overview can be found on **Table 2**. Only 2 out of the 27 peptides were studied for their ability to enter mammalian cells before binding their nucleic acid cargo. Peptide $bLFcin_6$, derived from bovine lactoferricin, showed an internalization ratio higher than that of the cell-penetrating peptides CPP5 and CPP6, but lower compared to that of TAT. $bLFcin_6$ could condense siRNA and mediate its delivery into Hela cells more efficiently than CPP5, CPP6 and TAT. However, the knockdown activity of the siRNA/$bLFcin_6$ condensate was similar to that of CPP5 and TAT (69). The M918 peptide was shown to translocate into various cell lines, such as CHO and Hifko, more efficiently than Penetratin and TP10. When M918 was conjugated to PNA, it was still able to be internalized, thus inducing splice correction in Hela pLuc 705 cells (70). In addition, the selected CPPs could bind, either covalently or non-covalently, different types of nucleic acids. The nucleic acids included here were pDNA, siRNA and PNA. The majority of the peptides mediated pDNA and siRNA delivery. However, Tat_{47-57} peptide and its derivatives could bind and deliver all three types of nucleic acids (71-73).

A wide range of controls have been utilized in cellular uptake and delivery studies of pDNA, siRNA and PNA complexed with the selected CPPs. The most frequently used controls

were the commercially available transfection reagent Lipofectamine and the cationic polymer PEI. In some cases, CPPs were compared to a combination of controls. For example, the efficiency of the *in vitro* pDNA transfer by the basic ampliphilic peptides ppTG1 and ppTG20 was compared to PEI, Lipofectamine and Superfect (74). Moreover, the cellular uptake and the transfection efficiency of the RV peptide R3V6 were compared to both Lipofectamine and PLL (55).

The ability of the selected CPPs to enter and transfect cells was usually compared to the same control. The only exception was Penetratin 1. The cellular uptake of Penetratin 1 conjugated to siRNA was compared to Lipofectamine, while the transfection efficiency was compared to a non-defined control (75). However, there were several CPPs whose cellular uptake or tranfection efficiency or both were not compared to controls. The amphipathic peptides CADY and MPG-8 were able to efficiently interact with siRNA. CADY could promote uptake of siRNA into mammalian cells, while the cellular uptake of MPG-8/siRNA complexes was not determined. Both peptides could efficiently mediate siRNA delivery thus inducing target knockdown. However, in both studies no controls were used in the assessment of the transfection efficiency (76,77). The *in vitro* transfection efficiencies of the histidine-rich peptide LAH4-L1 and the peptide for ocular delivery (POD) were also not compared to controls (76-80). As a result, the ability of these peptides to mediate nucleic acid entry and delivery into mammalian cells could not be evaluated.

After reviewing the data on Table 2, it becomes evident that the majority of the *in vitro* delivery studies utilized proper controls. The transfection efficiency of the majority of the peptides was similar to the respective controls. However, there were peptides which exhibited diverse transfection efficiencies depending on the experimental conditions. For instance, the RAWA peptide, a derivative of KALA, could efficiently transfer pDNA into Cos-7 cells. Its transfection efficiency was higher compared to different DNA transfer systems such as PEI, Superfect, Lipofectamine Plus and Geneporter. RAWA was also able to mediate pDNA transfer at different mammalian cell lines such as D17, Hela, HUVECs and NIH 3T3. However, the transfection efficiency of RAWA was lower, similar or higher, depending on the cell line, compared to Lipofectamine Plus (68).

Several peptides displayed higher transfection efficiency than the respective controls. The majority of them were implicated in pDNA delivery. For example, R15 could transfect 293T cells more efficiently that Lipofectin and Geneporter2 and other polyarginine peptides, such as R9 or R12 (44). ppTG1 and ppTG20/pDNA condensates were used to transfect Hela cells. The transfection efficiency of ppTG1 and ppTG20 was higher that PEI, Lipofectamine and Superfect but only at low DNA dose (74). MPG, LAH4 and Vpr(55-91) peptides could also transfect mammalian cells more efficiently compared to the respective controls (81-83). Even though the transfection efficiency of these peptides was evaluated as high, only that of R15 was significant. In addition, their ability to enter cells was not defined.

The transfection efficiency of every peptide implicated in PNA delivery was higher than the respective control. However, only Pep-3 was compared to Lipofectamine. Pep-3 could mediate delivery of antisense HypNA-pNA targeting cyclin B1 into variable mammalian cell lines, such as Jurkat T and HUVECs. The Pep-3 carrier was compared to other delivery methods including Lipofectamine and the cell-penetrating peptides Pep-2 and MPG. The antisense response provided by Pep-3 was higher that the response provided by the controls. The expression of cyclin B1 was significantly down-regulated on Jurkat T cells (84).

Table 2. Overview of cell-penetrating peptides for nucleic acid delivery

Name	Sequence	Cellular uptake (peptide)	Cellular uptake (particle)	*In vitro* transfection	Control	Ref
pDNA delivery						
Arginine-15	R15	ND	ND	+	GP	(85)
RV	R3V6	ND	-	+/-	LF, PLL	(55)
KALA	WEAKLAKALAKALAKHLAK ALAKALKACEA	ND	Yes	+/-	DM	(67)
RAWA	RAWARALARALRALARALR ALAR	ND	ND	+	PEI, SF,LF+, GP	(68)
ppTG1, ppTG20	GLFKALLKLLKSLWKLLLKA, GLFRALLRLLRSLWRLLLRA	ND	ND	+	PEI, LF, SF	(74)
MPG	GALFLGFLGAAGSTMGAW SQPKSKRKV	ND	ND	+	LF	(81)
Tat_{47-57} oligomers	YGRKKRRQRRR	ND	ND	+	pLa	(71)
PolyTAT	CTATC polymerized	ND	ND	+/-	PEI	(86)
Tat derivative	C-5H-Tat-5H-C	ND	ND	+/-	PEI	(87)
PolyTat P2	YGRKKRRQRRR	ND	ND	+/-	LF	(88)
LAH4	KKALLALALHHLAHLALHLA LALKKA	ND	ND	+	PLL, PEI, DOTAP	(82)
LAH4-L1	KKALLAHALHLLALLALHLA HALKKA	ND	ND	Yes	No control	(78)
Vpr(55-91)	TGVEALIRILQQLLFIHFRIGC RHSRIGIIQQRRTRN	ND	ND	+	PEI	(83)
siRNA delivery						
EB1	LIRLWSHLIHIWFQNRRLK WKKK-amide	ND	ND	+/-	LF	(89)
MPG and MPGΔNLS	GALFLGFLGAAGSTMGAW SQPKKKRKV and GALFLGFLGAAGSTMGAW SQPKSKRKV	ND	ND	+/-	OF	(90)
MPG-8	bAFLGWLGAWGTMGWSP KK KRK-Cya	ND	ND	Yes	No control	(77)
MPGα	Ac-GALFLAFLAAALSLMGLWS QPKKKRKV-Cya	ND	-	+/-	LF	(91)
CADY	Ac-GLWRALWRLLRSLWRLLW RA-cysteamide	ND	Yes	Yes	No control	(76)
Tat_{47-57}	YGRKKRRQRRR	ND	+/-	-	LF	(72)
POD	GGG(ARKKAAKA)4	ND	ND	Yes	No control	(79)
Penetratin 1	CRQIKIWFQNRRMKWKK-NH_2	ND	+ (LF)	+/-	ND	(92)
Reducible poly(oligo-D-arginine)	rPOA	ND	ND	+/-	LF	(93)
$bLFcin_6$	RRWQWR	+	+	+/-	CPP5, CPP6	(69)
PNA delivery						
M918	MVTVLFRRLRIRRACGPPR VRV-NH2	+	ND	+	Pen, TP10	(70)

MAP	KLALKLALKALKAALKLA	ND	ND	+	PNA alone	(94)
Pep-3	Ac-KWFETWFTEWPKKRK-Cya	ND	Yes	+	LF, Pep-2, MPG	(84)
Tat, Pen, TP	CYGRKKRRQRRR-NH_2, CRQIKIWFQNRRMKWKK-NH_2, GWTLNSAGYLLGK*INLKALAALAKKIL-NH_2	ND	Yes	+	invPNA	(73)

(+)= higher than the control, (+/-)= similar to the control, (-)= lower than the control, ND= non-defined LF: lipofectamine, OF: oligofectamine, SF: superfect, GP: geneporter, FG: fugene, DM: dendrimer, PEI: polyethylamine, PLL: poly-L-lysine, pLa: poly-arginine

Nuclear localization signal (NLS) peptides

After cellular uptake and/or endosomal escape, condensed nucleic acids are released in the cytoplasm. Plasmid DNA in particular, has to find its way to the nucleus in order to be transcribed. Nuclear entry is relatively complicated because of the presence of the nuclear envelope which separates the nucleus from the cytoplasm. The nuclear envelope is covered by nuclear pore complexes (NPC) which are composed of many different proteins, called nucleoporins, and allow the transport of variant molecules. Small molecules, with a size up to 9nm in diameter, can diffuse passively, while larger molecules, with a size up to 25nm in diameter, can be actively transported through the NPC (95,96).

In order to ensure DNA targeting to the nucleus, nuclear localization signal (NLS) peptides have been utilized. NLS peptides are able to bind DNA either covalently, through chemical groups or peptide nucleic acids (PNAs) or non-covalently, through electrostatic interactions (31). In addition, they mediate nuclear DNA targeting through binding to importins which is followed by translocation through the NPC.

NLS peptides can be divided into classical and non-classical sequences (28). Classical sequences are comprised of short clusters of basic amino acids and can be divided into monopartite and bipartite peptides. Monopartite peptides, on the one hand, contain one cluster of basic amino acids, like the large tumor antigen of the simian virus (SV40), which is one of the most frequently studied NLS (28,31,97). Bipartite peptides, on the other hand, have two clusters of basic amino acids divided by 10-12 neutral residues, such as the peptide **KR**PAATKKAGQA**KKKK** derived from the Xenopus protein nucleoplasmin (28,31,97). Non-classical NLS sequences contain charged/polar residues distributed among non-polar residues (98).

Nuclear localization signal peptide requirements

Nuclear import is the key requirement which a NLS peptide has to fulfill in order to successfully mediate nuclear uptake of DNA. However, NLS peptides have to be introduced into the cytoplasm of the targeted cell, prior to nuclear import assessment. The introduction of NLS peptides can be accomplished by microinjection into the cytoplasm, expression in cells, administration to the cytoplasm, with or without the assistance of transfection reagents, or to isolated nuclei (99-101). Upon introduction, nuclear import of NLS peptides has to be quantitatively and qualitatively analyzed.

Western blot analysis and fluorescent microscopy can be used for the qualitative and quantitative analysis of the nuclear uptake. Flow cytometry, on the contrary, can only be used for the assessment of the quantitative nuclear uptake.

Evaluation of nuclear localization signal peptides

NLS peptides were evaluated on the ability to mediate nuclear import and expression of their DNA cargo into the target cell. In order to perform this evaluation, the data obtained from the nuclear import analysis methods and the transfection studies were examined.

Similar to DNA condensing and cell-penetrating peptides, NLS peptides can be evaluated but not compared. Head-to-head comparison of the peptides is complex due to

the differences in the experimental conditions. For example, the differences in the DNA concentrations or the NLS peptide/DNA ratio units or the cell lines used in the transfection studies. In addition, the utilization of various approaches for the introduction of NLS peptide/DNA particles into the cell target and for the assessment of the quantitative and qualitative nuclear import hinder direct comparison of the NLS peptides.

Evaluation of nuclear localization signal peptides on nuclear import and transgene expression

The number of the studies on NLS peptides is relatively small compared to the available studies on DNA condensing and cell-penetrating peptides. The majority of these studies have been scanned and several NLS peptides have been selected. More specifically, NLS peptides were examined for their ability to enter the nucleus of the target cell. They were also examined for their ability to mediate nuclear transport of DNA cargo, leading to its expression. NLS peptides which were not tested for DNA delivery into the nucleus can be found on **Table 6** of the appendix. In contrast, peptides coupled to cationic lipids or polymers were included in the selection since this combination constitutes an ordinary approach for the introduction of the NLS peptide/DNA condensates inside the target cells.

A total of 13 NLS peptides were evaluated on quantitative nuclear entry, with or without a DNA cargo, and on transfection efficiency. More specifically, when the nuclear uptake or the transfection efficiency of a NLS peptide was higher compared to that of other, nuclear transport deficient NLS peptides, it was evaluated as high (+). In the case that it was higher compared to naked DNA, it was evaluated as moderate (+/-). Lastly, there were peptides which displayed nuclear uptake or tranfection but no controls were utilized and others which showed low or no nuclear uptake and transfection. An overview of these peptides can be found on **Table 3**.

Eight out of the 13 selected peptides were the SV40 T large antigen NLS peptide and its derivatives. The majority of these peptides were not tested for quantitative cellular uptake. However, only the SV40 tetramer was tested for nuclear transport, with or without a DNA cargo, and transfection. NLSV404 was able to mediate nuclear transport of conjugated albumin. Its nuclear transport ability was observed in comparison with the transport of the deficient mutant cNLS sequence. NLSV404 was also shown to efficiently condense and protect plasmid DNA. The resulting NLSV404/DNA complexes were transfected into HeLa S6 cells, together with cNLS/DNA condensates. It was found that NLSV404 could induce rapid nuclear accumulation of pDNA. Except from HeLa S6 cells, NLSV404 was able to transfect other cell lines, such as Cos7 and 16HBE14o-, and its transfection efficiency was significantly higher compared to that of cNLS and pLL (102). Except from NLSV404, only two peptides, the SV40 PKKKRKV and the SV40 derivative PKKKRKVEDPYC, showed higher transfection efficiency as compared to deficient NLS peptides. However, that increase was not significant (103,104).

Concerning the remaining selected NLS peptides, the bipartite Ku70-NLS appeared to be the most favorable. Ku70-NLS was localized in the nucleus upon electroporetion of mammalian cells. Its nuclear localization activity was compared to the active mutant s1Ku70-NLS, which was also localized in the nucleus, and the transport deficient mutant s2Ku70-NLS, which was localized in the cytosol. The transgene expression of Ku70-NLS and s1Ku70-NLS

was compared to that of the monopartite NLS peptides. BEAS-2B cells were transfected with NLSV404, Tat_2 and their nuclear transport deficient analogues cNLS and Tat_2M1. The transfection efficiency of Ku70-NLS and s1Ku70-NLS was significantly higher than that of Tat_2 and NLSV404 (105).

Table 3. Overview of NLS peptides for DNA delivery

Name	Sequence	Nuclear import (peptide)	Nuclear import (particle)	Transfection efficiency	Ref.
Ku70-NLS	C-KVTKRKHGAAGAASKRPK-G-KVTKRKHGAAGAASKRPK	+	ND	+	(105)
NLS/NL	H-CKKKSSSDDEATADSQHSTPPKKKRKVEDPKDFPSELLS	ND	+	+	(106)
NLSV404	$(PKKKRKVG)_4C$	+	+/-	+	(102)
SV40	KKPNKKKRKE	ND	ND	-	(107)
SV40	βACGAGPKKKRKV	ND	ND	Yes	(108)
SV40 derivative	PKKKRKVEDPYC	ND	ND	+	(103)
BICP27	RPRRPRRRPRRR	ND	+	+	(109)
NLS (SV40)	CGGPKKKRKVG-NH_2	ND	Yes	Yes	(110,111)
SV40	PKKKRKV	ND	+	Yes	(112)
SV40	PKKKRKV	ND	ND	+/-	(113)
PNA-NLS	PKKKRKV	ND	ND	+/-	(114)
M9	GNQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGYGGC	ND	+	+	(115)
NLS-CTHD	GYGPKKKRKVGGC	ND	ND	+	(104)

(+)= high, (+/-)= moderate, (-)= low, Yes= no controls was utilized, ND= non-defined

Discussion

DNA condensing peptides, cell-penetrating peptides (CPP) and nuclear localization signal (NLS) peptides were evaluated on their functionality, in order to select the ones which can be successfully used as carriers for nucleic acid delivery.

DNA condensing peptides were evaluated on DNA condensation and protection. From a total of about 80 synthetic and natural peptides, 23 were evaluated as best scoring. These peptides displayed the ability to tightly condense and fully protect plasmid DNA at low peptide/DNA ratio units (Table 1). The synthetic peptides R15 (44), pR6, pK6 and pK12 (53) could also mediate *in vitro* DNA delivery. However, the majority (82%) was unable to deliver DNA into cells. With the assistance of delivery systems or lysosomotropic agents, several peptides could induce membrane translocation and delivery of their DNA cargo into the target cell.

CPPs were evaluated on quantitative cellular uptake and on transfection efficiency. The ability of a total of 27 peptides to mediate cellular uptake and transfection of their nucleic acid cargo was compared to the respective controls, utilized in the *in vitro* experiments (Table 2). The comparison revealed that the transfection efficiency of the majority of the peptides was similar to that of the respective controls. However, there were several peptides which exhibited higher, but non-significant, transfection efficiency: ppTG1, ppTG20 (74), MPG (81), LAH4 (82) and Vpr(55-91) (83), all implicated in pDNA delivery. Merely 2 peptides, R15 (44) and Pep-3 (84), implicated in pDNA and PNA delivery respectively, displayed significantly higher transfection efficiency as compared to that of commercially available transfection reagents. Moreover, 30 additional CPPs were able to enter target cells. However, they were not tested for DNA delivery (Table 5).

NLS peptides were evaluated on nuclear import and transfection efficiency. The number of the NLS peptides that could mediate nuclear import of their DNA cargo was relatively small (Table 3). Ku70-NLS (105) and NLSV404 (102), in particular, were the only evaluated NLS peptides which displayed significantly higher transfection efficiency than that of nuclear transport deficient NLS peptides. In addition, there were peptides which were localized in the nucleus but they were not tested for nuclear delivery of DNA (Table 6).

In conclusion, there were peptides of each category which could efficiently function. However, only several peptides showed encouraging *in vitro* results concerning their ability to mediate nucleic acid delivery alone. These peptides mainly belong to the CPP category, since DNA condensing and NLS peptides need the assistance of delivery systems in order to be internalized and deliver DNA cargo into cells. The *in vivo* testing of these CPPs is essential for their potential use as carriers for nucleic acid delivery in patients. However, only a minority has been tested *in vivo* including R15 (85), ppTG1, ppTG20 (74), MPG-8 (77) and rPOA (93). R15 (85) and rPOA (93), showed significantly higher transfection efficiency, upon administration to mice, as compared to that of controls and naked siRNA respectively. Unfortunately, none of these peptides has reached clinical application. A single peptide as a carrier for nucleic acid delivery has not yet been proved to be successful and it becomes evident that the integration of different functionalities into one particle is essential.

An alternative approach for the utilization of peptides in nucleic acid delivery is their combination with delivery systems. Peptides of every peptide category have been coupled to a variety of carriers. For instance, octaarginine (R8) and octalysine (K8) have been coupled

to liposomes (116,117), while SV40 has been coupled to PEG/PEI conjugates (118). However, CPPs were the most frequently studied peptide category. Especially peptide Tat has been extensively studied *in vitro*, as it has been coupled to many different delivery systems in order to facilitate their delivery. These systems include liposomes (119-122), micelles (123), gold particles (124,125), PEG-PEI conjugates (60,126) and quantum dots (127). Even though several of these delivery systems have been tested *in vivo* (128,129), none of them has been in clinical studies yet. Taken together, there is a need for an alternative, effective strategy, which will potentially provide efficient peptide-based nucleic acid carriers.

Rational design is the principal strategy for the production of peptide-based gene carriers. It is focused on the optimization of distinct steps of the process of cellular entry, however, without analyzing the influence that this optimization might have on other steps of this process. For instance, when the stability of a vector in the extracellular environment is optimized, the possible effect of this optimization on its dissociation inside the target cells is not considered. In order to avoid such problems, the utilization of a random but integrative approach which describes the correlation between the intracellular barriers and the effect of the characteristics of vectors on those barriers is necessary (130).

The fusion of a variety of single functional peptides for the production of recombinant polypeptides constitutes a possible approach. For example, a DNA condensing peptide, two CPPs and a NLS peptide were combined in order to design a novel biopolymer, which could condense pDNA, induce membrane translocation and deliver its cargo into the nucleus of target cells (131). The design and production of such polypeptides can be achieved through the concept of directed evolution.

The directed evolution strategy concerns the selection of polypeptides, with functional characteristics, ideal for efficient gene transfer. Firstly, a collection of variable functional peptides has to be fused in different combinations in order to construct a gene library which encodes for multifunctional recombinant polypeptides. The polypeptides can be recombinantly expressed, purified and subsequently selected after high-throughput screening. The most functional and efficient polypeptides can be finally obtained after several cycles of high-throughput screening. In conclusion, the key feature which makes this approach advantageous is its random, combinatorial nature.

Conclusion

Even though the number of functional DNA condensing, cell-penetrating and nuclear localization signal peptides is relatively big, there were only several peptides able to efficiently mediate delivery of nucleic acids *in vitro*. In addition, a small number of CPPs was tested *in vivo*, but without reaching clinical application. Even the coupling of these peptides to different delivery systems, have not offered an efficient gene carrier yet. As a result, there is a need for an alternative approach. Taking advantage of the concept of directed evolution, it is possible to combine single peptides in order to design and select functional and efficient recombinant polypeptides, which can be potentially used in nucleic acid delivery.

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Appendix

Table 4: Overview of DNA condensing peptides

Name	Sequence	DNA condensation	DNA protection	Reference
Arginine-9	R9	ND	ND	(44)
Arginine-10	R10	ND	ND	(44)
Arginine-11	R11	ND	ND	(44)
Arginine-12	R12	ND	ND	(44)
Arginine-13	R13	ND	ND	(44)
Arginine-14	R14	ND	ND	(44)
RV	R1V6	+/-	ND	(55)
RV	R2V6	+	ND	(55)
RV	R4V6	++	ND	(55)
Branched	$(KK)_2$KGGC	--	ND	(45)
Branched	$(KKK)_2$KGGC	+/-	ND	(45)
Branched	$(KWK)_2$KGGC	--	ND	(45)
Branched	$(KWKK)_2$KGGC	+/-	ND	(45)
Branched	$(KWKKK)_2$KGGC	+	ND	(45)
Branched	$(RR)_2$KGGC	--	ND	(45)
Branched	$(RRR)_2$KGGC	+/-	ND	(45)
Branched	$(RWRR)_2$KGGC	+	ND	(45)
Branched	(OOO)2KGGC	+/-	ND	(45)
Branched	(OOOO)2KGGC	++	ND	(45)
R6	CHR6HC	+	++	(53)
K6	CHK6HC	+/-	+	(53)
K12	CHK6H2K6HC	+/-	+	(53)
P2	SPKRSPKRSPKR	-	--	(48)
H9	KTPKKAKKP	-	ND	(39)
AlkCWKn	AlkCWK3	-		(43)
	AlkCWK8	+/-	ND	(43)
	AlkCWK13	+	ND	(43)
AlkCWKn dimer	DiCWK3	-	ND	(43)
	DiCWK8	+/-	ND	(43)
	DiCWK13	+	ND	(43)
AlkCWKn	AlkCWK18	-	+/-	(54)
CWKn	CWK18	+	+/-	(54)
McKenzie II	CWK17C	+	+/-	(54)
McKenzie V	CWK4CK3CK3CK4C	+/-	++	(54)
McKenzie 1	AlkCWK18	ND	-	(46)
McKenzie 2	AlkCWK8	ND	ND	(46)
McKenzie 3	CWK17C	ND	+/-	(46)
McKenzie 4	CK2C	--	ND	(46)
McKenzie 5	CK4C	-	ND	(46)
McKenzie 6	CK6C	+	ND	(46)
McKenzie 7	CK8C	+	+/-	(46)
McKenzie 8	CKCKC	+/-	ND	(46)
McKenzie 9	CK2CK2C	+/-	ND	(46)
McKenzie 10	CK3CK3C	+	ND	(46)
McKenzie 13	CHK6HC	++	ND	(46)
McKenzie 14	CHK3HK2HC	++	ND	(46)
McKenzie 15	C(HK)4C	++	ND	(46)
McKenzie 16	CHKHCH2KHC	++	ND	(46)
Salmon protamine	MPRRRRSSSRPVRRRRRPRV SRRRRRRGGRRRR	+	+	(49)

Table 5: Selection of CPPs with the highest quantitative cellular uptake

Name	Sequence	Reference
PenArg	RQIRIWFQNRRMRWRR	(132)
BF2d (from Buforin 2)	TRSSRAGLQWPVGRVHRLLRKGGC	(133)
MG2d	GIGKFLHSAKKWGKAFVGQIMNC	(133)
ProAntpHD	RQPKIWFPNRRKPWKK	(134)
C45D18	DTWPGVEALIRILQQLLFIHFRIGCQH	(135)
RSV-A6	KRIPNKKPGKKT	(136)
RSV-A7	KRIPNKKPGKK	(136)
RSV-A8	KRIPNKKPKK	(136)
RSV-A9	RRIPNRRPRR	(136)
PTD-4	YARAAARQARA	(137)
maize gamma-zein	$(VRLPPP)_3$	(138)
IX	QLALQLALQALQAALQLA	(139)
Ribotoxin L3 Loop	GNGKLIKGRTPIKFGKADCDRPPKHSQNGMGK	(140)
pVEC	LLIILRRRIRKQAHAHSK	(141)
Penetratin and analogues	RQIKIWFQNRRMKWKK	(142)
Pep-1	KETWWETWWTEWSQPKKKRKV	(143)
SynB5	RGGRLAYLRRRWAVLGR	(144)
Tat_{48-60}	GRKKRRQRRRPPQ	(38)
Tat_{49-57}	RRRQRRKKR	(145)
PACAP	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK	(146)
NrTP1, NrTP5	YKQCHKKGGKKGSG, ykqchkkGGkkGsG	(147)
MCo, kB1	GGVCPKILKKCRRDSDCPGACICRGNGYCGSGSD, GLPVCGETCVGGTCNTPGCTCSWPVCTRN	(148)
iMP	inlkalaalakkil	(149)
S41	CVQWSLLRGYQPC	(150)
SAP(E)	VELPPPVELPPPVELPPP	(151)
C105Y	CSIPPEVKFNKPFVYLI	(152)

Table 6: NLS peptides mediating nuclear localization

Name	Sequence	Reference
N-Myc, p53	PPQKKIKS, PQPKKKP	(153)
HSV-1 ICP27	ARRPSCSPERHGGKVARLQPPPTKAQPA	(154)
NLS of the M protein	KKGKKVTFDKLERKIRR	(155)
MEQ	RRRKRNRDAARRRRKQ(101)(101)(101)(101)(101)	(101)
PFV IN	RVARPASLRPRWHKPSTVLKVLNPR	(156)
$TWIST^{NLS}$	^{37}RKRR^{40}, ^{73}KRGKK^{77}	(157)