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Cationic amphipathic histidine-rich peptides for gene delivery

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Abstract

Besides being a useful tool in research, gene transfer has a high potential as treatment for a variety of genetic and acquired diseases. However, in order to enable a gene to become a pharmaceutical, efficient and safe methods of delivery have to be developed. We recently found that cationic amphipathic histidine-rich peptide antibiotics can efficiently deliver DNA into mammalian cells. Our lead compound, LAH4 (KKALLALALHHLAHLALHLALALKKA), demonstrated in vitro transfection efficiencies comparable to those of commercially available reagents. Synthesis and evaluation of LAH mutants provided evidence that the transfection efficiency depends on the number and positioning of histidine residues in the peptide as well as on the pH at which the in-plane to transmembrane transition takes place. Moreover, recent results suggest that binding of the DNA complexes to the plasma membrane is mediated by heparan sulfate proteoglycans and that anionic phospholipids may be involved in the endosomal destabilization process. Finally, we also describe in this review the rationale that led to the development of LAH4 as a DNA carrier as well as the biophysical methods that have allowed us to propose a model which could explain the way this peptide destabilizes the endosomal bilayer.

Keywords: Amphipathic peptide; Gene transfer; Membrane destabilization; Antibiotic; Solid-state NMR

1. Introduction

Nucleic acid delivery has an extremely high potential in a wide variety of applications, including basic research, therapies for genetic and acquired diseases and in vaccination. However, DNA expression cassettes as well as other nucleic acid-based drugs such as siRNAs and antisense oligonucleotides enter into mammalian cells with low efficiencies. It is thus necessary to develop techniques able to efficiently deliver these bioactive compounds in order to enable full exploitation of their therapeutic potential.

One delivery approach that has been developed exploits the properties and tropisms of viruses. This is currently the most widely used system for gene delivery, particularly in clinical trials. All other approaches, collectively termed "non-viral gene delivery systems" try to mimic the efficiency of viral vectors by artificial means [1]. These approaches are essentially of two sorts: the first one uses physical methods to deliver DNA into cells. Among these techniques we find for example electroporation [2] and hydrodynamic DNA injection [3]. These approaches are more useful for in vivo applications than for in vitro experiments. The second approach consists in using structurally well-defined synthetic compounds, which are most often cationic. A large variety of molecules have been used, ranging from polypeptides such as polylysine [4], to synthetic polymers [5] and cationic lipids [6]. These molecules share several functions that are essential for transfection: besides packaging the DNA, they also protect it from nucleases and usually form positively charged particles which efficiently enter the cell after binding proteoglycans on the outer face of the membrane [7]. The carrier probably also facilitates the escape of the DNA complexes from intracellular vacuolar compartments into the cytosol.

Peptides are mostly used as auxiliary agents in combination with other gene transfer systems, in particular cationic polymers such as polylysine, providing either a cell targeting moiety [8], a membrane destabilization activity [9] or a nuclear localisation function [10]. A strategy that emerged in the mid-90s is the use of multifunctional peptides. Peptides such as KALA [11],

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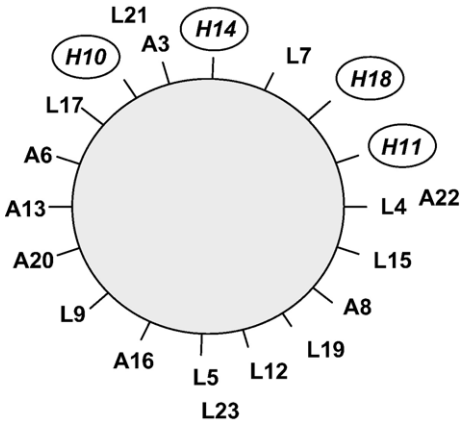

Fig. 1. Helical wheel diagram for LAH4 (residues 3–23). The helical wheel projections allow to visualize the distribution of hydrophobic and polar residues with respect to the helical axis. The angle of the positively charged helix face (histidine residues are in italic), at pH 5, for the LAH4 peptide is 100°.

ppTG20 [12], Hel 11-7 [13] and Vpr52–96 [14] at the same time bind DNA and destabilize membranes—two properties that seem required for efficient gene transfer of dividing cells. Interestingly, the two former peptides combine a positively charged lysine or arginine stretch required for DNA binding and an amphipathic membrane-destabilizing domain deriving from the fusogenic peptides GALA [11] and JTS-1 [15]. These transfecting peptides have a strong propensity for a α-helical conformation that positions the lysines or arginines on one face of the helix—thus, conferring amphiphilic properties.

In this review, we discuss the rationale that led to the development of a new family of transfection agent and we present a model that could explain how these peptides function.

2. Antimicrobial peptides

At present, more than 800 different antimicrobial peptides have been described—the list is available at the Antimicrobial Sequences Database (http://www.bbcm.units.it/~tossi/pag1.htm). These peptides are produced in many tissues and cell types of a variety of invertebrate, plant and animal species, including humans [16–20]. They can be classified into several groups on the basis of their origin, activity spectrum or structure. However, since similar structural patterns are shared by peptides from different organisms, a classification based on amino acid composition and structure is the most useful [16]. 5 subgroups can be identified [21]: (i) anionic peptides, (ii) linear cationic α-helical peptides, (iii) cationic peptides enriched for specific amino acids, (iv) anionic and cationic peptides that contain cysteine and form disulphide bonds, and (v) anionic and cationic peptide fragments of larger proteins.

Notably, the linear cationic α-helical peptide subgroup is one of the most important (containing almost 300 peptides; http://www.bbcm.units.it/~tossi/pag1.htm) and these peptides have the broadest spectrum of activity [22]. Typically, they have a size ranging from 10 to 50 amino acid residues, present an overall net positive charge due to excess arginine and lysine residues and they contain a significant number of hydrophobic amino acids. In aqueous solutions, many of these peptides are disordered, but in the presence of membranes, all or part of the molecule adopts a α-helical conformation. Edmundson helical wheel analysis shows that such a helix is often amphipathic with the polar and hydrophobic amino acid side chains separated on opposite faces of the helix (see for example Fig. 1). Proton-decoupled ^{15}N solid-state NMR [23], ATR-FTIR [24], fluorescence [25] and CD spectroscopy [26] on oriented samples indicate that peptides such as magainins are oriented with their helix long axis parallel to the membrane surface.

The precise mechanisms of how the peptides exert their biological activity have not been completely elucidated. The peptides are, however, likely to first be attracted by the negative charges that exist on the outer face of Gram-negative (lipopolysaccharide) and Gram-positive (lipoteichoic acid) bacteria. The cytotoxic activity results then either from their detergent-like properties or after the peptides have reached intracellular targets [17,18,20].

3. Transfection activity of LAH peptides

3.1. LAH4

By taking into consideration the above-mentioned properties of antibacterial peptides, in particular of the linear cationic α-helical ones, we reasoned that most of them correspond exactly to what could be the requirements for a "good" peptidic DNA carrier, namely: the peptide should (i) be relatively short, (ii) present cationic residues which allow electrostatic interactions with DNA but with a limited positive charge density, (iii) be soluble in aqueous solutions and, (iv) be able to interact with and destabilize membranes.

We tested our hypothesis by evaluating the DNA condensing and transfecting capacities of LAH4 and derivatives thereof. LAH4 (Table 1) was initially designed as a model peptide to study the membrane disturbing properties and interaction contributions that determine the membrane topology of α-helical amphipathic antibacterial peptides [17,27,28]. When considering the primary sequence of the peptide, LAH4 is characterized by the presence of two lysines at each end of the peptide which, besides ensuring good solubility in aqueous environments, allows for electrostatic interactions with DNA. In addition to these cationic residues, LAH4 also contains hydrophobic amino acids – alanines and leucines – as well as 4 histidines. The latter residues are of interest since they may allow, thanks to the imidazole group, which has a pK_a of about 6.0, the buffering, and subsequently disruption, of endosomal vesicles [5,29].

Table 1
Sequence of the LAH1–LAH5 series

Peptide	Sequence
LAH1	KKLALALALALHALALALALKKA
LAH2	KKLAHLALALALGLALAHLAKKA
LAH3	KKALALGLHLAHLALHLALALKKA
LAH4	KKALLALALHHLAHLALHLALALKKA
LAH5	KKALLALALHHLAHLAHHLALALKKA

The secondary structure of LAH4 has been investigated using circular dichroism [28]. At acidic pH, when the histidines are charged, the peptide exhibits a mostly random coil conformation. At neutral pH, a helical conformation is induced with the helical content at pH 7.5 calculated to be 39%. As observed with other antimicrobial peptides such as magainins, CD spectroscopy indicates that addition of phospholipid membranes induces a helical conformation in LAH4. The helix content of LAH4 at pH 5.5 increases from 26% in the absence of membranes to 55% and 70% in the presence of POPC or POPG liposomes, respectively [28].

Finally, Edmundson helical wheel analysis shows that the α-helix is amphipathic with the polar and hydrophobic amino acid side chains separated on opposite faces of the helix (Fig. 1).

3.2. DNA condensing and transfecting properties of LAH4

Gel mobility shift assays showed that peptides encompassing 4 lysines and 1 to 5 histidines (LAH1–LAH5, Table 1) are able to efficiently complex plasmid DNA [30]. The complete DNA retardation was observed for all the peptides at a w/w peptide/DNA ratio of 2.5/1. This corresponds to 0.59 peptide per bp or expressed as +/− charge ratio to 1.5. Notably, no differences of DNA complexation capacities were expected between the LAH1–LAH5 peptides since all of them present 5 positive charges at neutral pH.

Evaluation of the transfection efficiency was performed on several cell lines by using an expression cassette encoding the reporter gene luciferase. The results showed that the highest activity was obtained with the peptides having at least 4 histidine residues [30]. The efficiency of LAH4 was also compared to that of the branched PEI of 25 kDa and the monocationic lipid DOTAP on four different cell lines. The results indicate that the peptide has transfection capacities that equal those of these two commercially available transfection agents.

3.3. Synthesis and evaluation of LAH4 mutants

The synthesis of mutant peptides is interesting since in addition to offering a better insight of what the requirements are to obtain a high transfection efficiency, it can also lead to the development of even more efficient compounds. A peptide of 26 amino acids, such as LAH4, has 26^{20} possible combinations of only the standard L-amino acids and hence the possibilities of peptide optimization are immense.

We synthesized a variety of peptides and tested their capacity to complex DNA and transfect cells (Table 2 ; [30]). From their evaluation, several conclusions can be drawn: (i) the ability of peptides to complex DNA is not solely linked to the global charge of the peptide suggesting that structural requirements need to be fulfilled (histatin-5 for example did not complex well DNA; Table 2), (ii) a comparison of results obtained with LAH4 and H4-LAK4 show that the histidine residues have to be positioned in the core of the peptide, (iii) the position of the histidine residues within the polar face is important but there is a certain tolerance on the detailed location [30].

4. Mechanism

4.1. Cell binding

In order to promote cell entry of bioactive drugs such as DNA, one can take advantage of two different strategies: the first one consists in associating the drug with a carrier able to favor cell penetration by a process referred to as non-specific adsorptive endocytosis. The second one consists in coupling a ligand that is recognized by a receptor expressed at the cell surface to the carrier. This latter approach will not be detailed here because it is beyond the scope of this review.

Most non-viral DNA carriers have their optimal transfection activity when the particles present an overall net positive charge (i.e., excess of cationic charges with respect to the DNA phosphate). This may be explained by the fact that cationic charges allow binding of the complexes to anionic lipids or heparan sulfate proteoglycans (HSPGs) which may be present on the cell surface. However, if LAH4 behaves similarly to other tested peptides – like oligoarginines – then it is likely that the initial attraction to the cell surface is mediated not by anionic lipids but by proteoglycans [31]. In fact, it was shown for polylysine-based [7] and lipid-based DNA complexes [32] that the absence of HSPGs severely impairs the transfection efficiency. Based on this observation, we performed binding experiments on wild-type (CHO-K1) and mutant proteoglycan deficient Chinese hamster ovary cells (A-745 cells; [33]) with a fluorescently labelled LAH4 peptide. Preliminary data suggest that the absence of HSPGs results in a significantly reduced binding of the peptide to the cell surface (AK, unpublished results). Further investigations are, however, needed in order to fully characterize the binding partner(s) of LAH4/DNA complexes. Our and others experiments seem to suggest that anionic lipids do not have a significant role in the binding affinity of cationic complexes to eukaryotic cells, however

Table 2
Histidine-rich peptides

Peptide	Sequence	Retardation assay (μg peptide[a])	Transfection efficiency[b]
LAH4	KKALLALALHHLAHLALHLALALKKA	2.5	1000
Histatin-5	DSHAKRHHGYKRKFHEKHHSHRGY	>50[c]	1<
H4-LAK4	HHALLALALKKLAKLALKLALALHHA	1	20

[a] The indicated amount of peptide is the minimal amount required to retard migration of 1 μg of plasmid DNA during agarose gel electrophoresis.
[b] We gave the value 1000 to the luciferase activity obtained with LAH4. Transfection experiments were performed on HepG2 cells.
[c] Some of the DNA was retarded but not all.

anionic lipids do perform an important role in mediating transfection of cationic complexes as we show below.

4.2. Cell entry

One approach which provides insights into the intracellular trafficking of DNA complexes consists of using drugs which interfere with cellular processes. A proton pump inhibitor such as bafilomycin A1 for example, not only gives information about the role of endosomal acidification during the transfection process but can also indicate whether DNA particles enter the cell through an endocytic pathway [34]. LAH4-mediated transfection in the presence of bafilomycin A1 was significantly lower as compared to non-treated cells [30]. Since histidines are required to obtain maximal transfection with LAH peptides and since the p*K* values of the four histidine imidazole groups of LAH4, determined in the presence of dodecylphosphocholine micelles, are 5.4, 5.8, 5.9 and 6.0 [27], the results suggest an important role of histidine protonation. Moreover, the results indicate that at least some of the complexes enter through endocytosis. Whether the complexes enter through clathrin-mediated endocytosis as it is the case for Vpr for example [35] remains to be shown.

4.3. Endosomal escape

Solid-state NMR has proven to be an excellent means of studying peptide–lipid interactions. Spectral features in such studies are sensitive to the chemical environment, orientation with respect to an external magnetic field and the motions of membrane components that carry an NMR active nucleus. Oriented membrane samples have been used extensively in this laboratory to study peptides inserted into membrane mimicking lipid bilayers. Specific ^{15}N labelling of the LAH4 peptide and subsequent incorporation into hydrated phospholipid bilayers allows the ^{15}N chemical shift anisotropy (CSA) to be determined which, for an α-helical peptide, gives a direct measure of the helix tilt angle [36]. LAH4 has been shown to adopt an alignment parallel to the membrane surface at acidic pH, whilst at neutral pH it is transmembrane [27,37]. The midpoint of transition occurs at $pH_{50}=6.1$.

Antimicrobial peptides induce a variety of effects on biological membranes including causing the release of calcein from dye loaded vesicles, inducing lipid flip-flop and can, themselves, translocate from one side of the membrane to another [38,39]. The insertion of a peptide into a transmembrane orientation has been shown to be sufficient to induce lipid flip-flop [40] whilst the induction of anionic lipid flip-flop has been proposed as an important factor in the release of cationic complexes from endosomes [41]. Hence, the ability of LAH4 and derivatives to induce such effects may be crucial in determining the efficiency of transfection depending on the cell type under investigation.

To further probe the mechanism of LAH4 mediated DNA transfection we have shown using wide-line ^{31}P NMR of hydrated peptide–lipid powder samples that both magainin and LAH4 can induce large scale morphological changes in phospholipid bilayers at increasing concentrations [18,42]. A powder pattern that is commonly obtained for hydrated phospholipids bilayers is replaced by a sharp resonance representing an oriented bilayer which, depending on the ^{31}P chemical shift position would correspond to a micellar, a bicellar or a wormlike sheet phase [43,44]. This could provide a mechanism for the disruption of the endosomal membrane as LAH4 peptides are released from the cationic peptide/DNA complex on acidification of the endosomal lumen.

Further details of the mechanism of membrane disruption have been obtained from identifying the nature of the peptide–lipid interactions. Many membrane active peptides are cationic in nature and have been shown to interact with anionic lipids in bacterial membranes. Such anionic lipids are usually only present on the intracellular face of eukaryotic membranes although they can also be found on the external surface when cells become transformed [45]. The change in surface charge caused by the binding of a cationic peptide to an anionic membrane can also be measured using solid-state NMR methods. The concept of the "molecular voltmeter" where the quadrupolar splitting observed in wideline ^{2}H spectra of headgroup deuterated lipids has recently been extended to include ^{31}P and ^{14}N MAS methods [46,47] which are based on the known response of the chemical shift and quadrupolar coupling respectively, to a change in surface charge. Both these latter techniques are rather useful since they do not require stable isotope labelling as the NMR active nuclei comprise almost 100% of the natural isotope abundance, and under MAS conditions, resonances from different lipid types can be separated. Using this ^{31}P MAS technique we have shown that LAH4 does indeed interact preferentially with anionic lipids in mixed anionic/zwitterionic membranes and this interaction is enhanced at acidic pH when the four histidine residues become positively charged [42].

To understand this interaction in further detail we have incorporated chain deuterated lipids into mixed membranes and observed the effect on the quadrupolar splittings throughout the lipid acyl chain as a probe of the chain order. Wideline ^{2}H NMR of such systems provides information on the dynamic structure of the lipid acyl chains [48] and can identify membrane disruption or peptide binding to specific membrane constituents [49]. We found that in a lipid bilayer composed of phosphatidylcholine, phosphatidylserine and cholesterol LAH4 caused a significant reduction of lipid acyl chain order for deuterated phosphatidylserine at acidic pH but not at neutral pH. By contrast, when deuterated phosphatidylcholine was used as a reporter at both acidic and neutral pHs no such effect was observed [42]. These observations are consistent with a model whereby LAH4 inserts into the membrane and inserts preferentially in the vicinity of anionic lipid head groups. The degree of membrane destabilization was assessed for a number of LAH4 isomers and it was discovered that this was related to the ability of the same isomers to mediate transfection of a variety of both primary and transformed cell lines [42]. These results indicate that anionic lipids play an important role in the release mechanism of the LAH4/DNA complex.

4.4. Proposed model

On the basis of our results, we can propose the following model for the way LAH4 acts (Fig. 2). LAH4/DNA complexes that lead to the highest reporter gene expression are those generated at a w/w peptide/DNA transfection ratio of 6/1 [30]. Translated in a molar ratio, it gives 1.42 LAH4/bp and expressed as +/− charge ratio, it gives 3.6. This ratio is, however, likely to be overestimated since not all the peptides bind to DNA (unpublished results). With an overall net positive charge, one can hypothesize that the complexes bind to the cell membrane via non-specific electrostatic interactions. The binding sites on the cell surface are probably sulfated proteoglycans, as suggested by experiments done with HSPGs deficient cells. After binding, non-specific endocytosis allows for cell entry of DNA particles as well

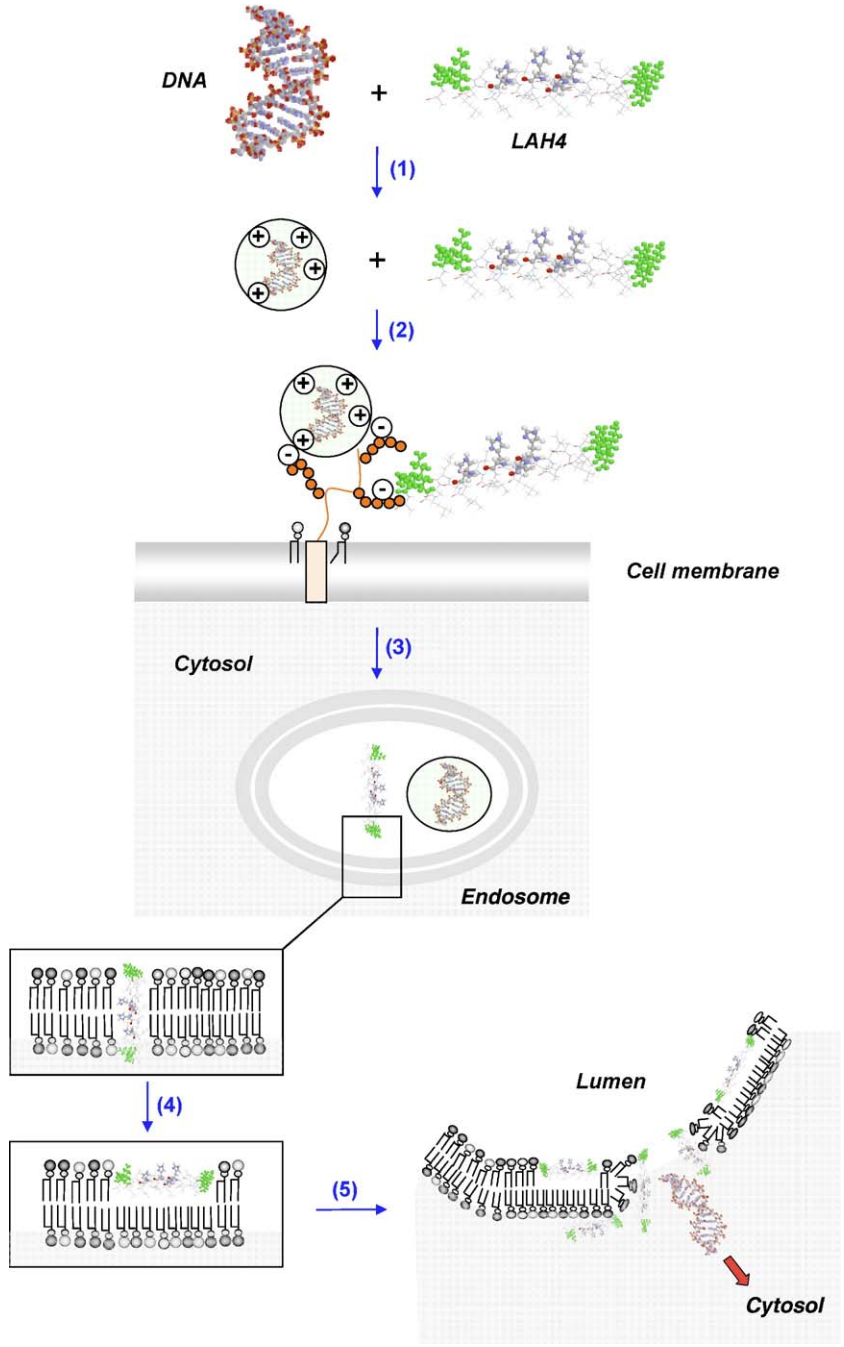

Fig. 2. Schematic figure showing the key steps of LAH4 mediated DNA transfection of eukaryotic cells. (1) Addition of LAH4 (lysine residues are in green and histidines are in bold) to plasmid DNA at a 1.4 LAH4/bp ratio generates positively charged DNA complexes (green sphere) but leaves also a proportion of non-complexed peptide. (2) Particles and peptides interact with the cell membrane, preferentially with proteoglycans, via non-specific ionic interactions. (3) The DNA complexes as well as free peptide enter the cell through endocytosis. At neutral pH, LAH4 is either associated with DNA or it adopts a transmembrane orientation. (4) During acidification of the endosome, histidine residues become protonated and LAH4 adopts an in-plane alignment. (5) Membrane destabilization occurs and it is followed by the release of DNA (+LAH4?) into the cytosol.

as of free LAH4. In the environment of the early endosome, when the pH is still above 6, LAH4 adopts a transmembrane orientation. During acidification of the endosome, the histidine residues become protonated and LAH4 presents an overall net charge of +9 instead of +5. This event probably frees a significant amount of LAH4 from DNA. In the acidic environment of late endosomes, LAH4 adopts an in-plane orientation, interacts with negatively charged lipids and, if the concentration of peptide is high enough, this is followed by a membrane destabilization that allows DNA and/or DNA/LAH4 to enter the cytosol. Exactly how membrane permeabilisation occurs remains to be determined. Possible mechanisms include detergent-like [17] or flip-flop [41] induced destabilization.

5. Conclusion

We developed a biotechnologically feasible approach for gene delivery using synthetic cationic amphipathic peptides containing a variable number of histidine residues. Gene transfer to different cell lines in vitro was achieved with an efficiency comparable to commercially available reagents. It should, however, be kept in mind that the gap between viral and non-viral vectors is still significant. Indeed, not only do recombinant viruses need fewer gene copy numbers to achieve the same efficiency as non-viral vectors but they are also capable of efficiently transducing various primary cells such as dendritic cells or stem cells which have a therapeutical potential. Thus, current use of synthetic carriers in clinical trials is hampered by their low efficiency as well as by their cytotoxicity—induced either by their low biodegradability or due to the high concentration of complexes required for gene transfer. Taken together, the existing transfection agents are useful tools for fundamental research but in order for them to become also useful in clinical trials, it will be necessary to precisely understand the transfection process as we are beginning to do with LAH4. This in turn will allow the development of approaches able to overcome each identified limiting step.

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