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RESEARCH PAPER

Design and Evaluation of Histidine-Rich Amphipathic Peptides for siRNA Delivery

Bérangère Langlet-Bertin • Christian Leborgne • Daniel Scherman • Burkhard Bechinger • A. James Mason • Antoine Kichler

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ABSTRACT

Purpose Short linear peptides have a high potential for delivering various drugs with therapeutic potential, including nucleic acids. Recently, we have shown that the cationic amphipathic histidine-rich peptide LAH4 (KKALLALALHHLAH LALHLALALKKA) possesses high plasmid DNA delivery capacities. Since such peptides are thought to efficiently disrupt endosomal membranes, we have tested their ability to deliver small interfering RNA (siRNA) into mammalian cells.

Methods Using a human cell line stably transfected with a *luciferase*-encoding expression vector, we have evaluated the ability of LAH4 and five derivatives thereof to deliver siRNAs and silence gene expression.

Results The six peptides are all efficient siRNA delivery vehicles whose efficiency in mediating gene silencing in 911-Luc cells was greater than that of commercially available compounds including Lipofectamine, DOTAP and polyethylenimine. In addition, by using the proton pump inhibitor bafilomycin A1, we show that efficient siRNA delivery to the cytosol requires acidification of the endosomes.

Conclusions The LAH4 histidine-rich cationic amphipathic peptides represent an interesting and promising family of compounds for siRNA delivery.

KEY WORDS amphipathic helix · cationic peptides · gene therapy · histidine · RNA interference

INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved process that inhibits gene expression by targeting messenger RNA (mRNA). Small interfering RNA (siRNA) strands complementary to the targeted mRNA, are the key to the interference process (1,2). The siRNAs are RNA duplexes commonly composed of two 21-mer oligonucleotides with a 19 nt complementary and a 2 nt single-stranded overhang at each 3′ end (1,2). These dsRNA molecules can down-regulate the expression of a target mRNA molecule in a sequence-specific manner, making them an attractive new class of drugs with broad potential for the treatment of diverse human diseases, including *inter alia*, cancers, genetic disorders, infections, and neurodegenerative diseases (3–5). In spite of siRNA therapeutic perspectives, their delivery remains a major technical hurdle, because siRNAs are highly anionic molecules, which do not cross cell membranes and physiological barriers.

While viruses are efficient delivery vehicles for nucleic acids in human cells, concerns have been raised regarding their immunogenicity and toxicity. Hence, a range of supposedly safer, non-viral vectors is being developed (6). The efficiency of such non-viral vectors is less satisfactory

B. Langlet-Bertin · C. Leborgne · A. Kichler
Genethon, 1bis rue de l'Internationale
BP60, 91002 Evry, France

D. Scherman · A. Kichler (✉)
UMR 8151 CNRS-U1022 INSERM, Université René Descartes
Chimie Paristech
Paris, France
e-mail: akich@genethon.fr

B. Bechinger · A. J. Mason
Faculté de Chimie, Institut Le Bel
4 rue Blaise Pascal
67000 Strasbourg, France

A. J. Mason
Pharmaceutical Science Division, King's College London
150 Stamford Street
London, UK

however, and efficient but safe delivery vehicles remain highly sought after.

Peptides have an interesting potential as non-viral nucleic acids delivery vehicles, not only because they are biodegradable but also due to their reduced size as well as the facility of product characterization and large-scale production. Recently, we have shown that the cationic amphipathic peptide LAH4 and its derivative LAH4-L1 (Table I) possess DNA delivery capacities which are comparable or even higher than those of commonly used commercial products (7–9). Efficient plasmid delivery requires vector-DNA self-assembly, protection of DNA from extracellular nucleases, and promotion of DNA cellular uptake, cytosolic release and trafficking to the nucleus for gene expression. Since delivery of siRNA to the RNA-induced silencing complex (RISC) in the cytoplasm is sufficient for activity (1,2), there are consequently fewer barriers to be overcome than for plasmid DNA transfection. Therefore, considering the high transfection efficiency of LAH4 and derivatives and, in particular, their membrane-disrupting behaviour at acidic pH (8), we were interested in investigating the potential of this class of peptides to deliver siRNAs into mammalian cells. Here, using human embryonic retinoblasts (911 cell line) as a model system or a variant, stably transfected with a luciferase-encoding expression vector (911-Luc), we compare the ability of a range of derivatives of LAH4 and LAH4-L1 to mediate either DNA delivery or specific knock-down of gene expression by monitoring luciferase activity. We show that the peptides are all efficient siRNA delivery vehicles and that their efficiency is higher than that of Lipofectamine, DOTAP and polyethylenimine (PEI). In addition, by using bafilomycin A1, we were able to show that efficient siRNA delivery requires endosome acidification. Finally, having identified peptide modifications that promote siRNA delivery, we investigate salt and buffer conditions as well as nucleic acid packaging techniques that enhance delivery.

MATERIALS AND METHODS

Materials

CMV-Luc (7.6 kb) is an expression plasmid encoding the firefly *luciferase* gene under the control of the human cytomegalovirus (CMV) immediate-early promoter. All the peptides were prepared by automated solid-phase synthesis on Millipore 9050 or ABI 431 synthesizers using fmoc chemistry. The peptides were stored at −80°C as a 1 mg/ml solution in water. The human embryonic retinoblasts (cell line 911) stably transfected with a luciferase-encoding expression vector (911-Luc) were kindly given by Dr. C. Le Bec (Genethon, France). The two small double-stranded RNAs, siRNA-Luc and siRNA-GFP, were synthesised by Sigma-Aldrich. Lipofectamine was obtained from Invitogen; DOTAP and the branched PEI of 25 kDa were from Sigma-Aldrich.

Cell Culture

Dulbecco's modified Eagle medium (DMEM; Gibco-BRL) was supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% of fetal calf serum (FCS; HyClone). We used human embryonic retinoblasts (cell line 911) for DNA transfection assays. For the siRNA experiments, we made use of 911-Luc cells which were cultured in the presence of 0.1 mg/ml of geneticin.

siRNA and DNA Retardation Assay

siRNA and DNA binding were studied by means of an agarose gel retardation assay. Briefly, siRNA or plasmid DNA (1 μg) and increasing amounts of peptide were each diluted in 25 μl of 150 mM NaCl and mixed. After an incubation of 15 min at room temperature, samples (20 μl) were electrophoresed through either a 1.3% (for siRNA) or

Table I Sequence of the Peptides

Peptide	Length	Charge at neutral pH	Sequence	Average Hydrophobicity[a] (*H*)	Hydrophobic moment (*μH*)
LAH4	*26*	*+5*	*KKALLALALHHLAHLALHLALALKKA*	*1.28*	*1.35*
LAH4-Leu	26	+5	KKALLAL**L**LHHL**L**HALLHL**L**LALKKA	2.53	1.92
LAH4-L1	*26*	*+5*	*KKALLAHALHLLALLALHLAHALKKA*	*1.28*	*1.79*
LAH4-L1-R	26	+5	**RR**ALLAHALHLLALLALHLAHAL**RR**A	1.26	1.79
LAH4-L1-Leu	26	+5	KKALLAH**L**LHLLLAL**L**LHL**L**HALKKA	2.53	2.68
LAH4-L1-F	26	+5	**FF**KKLAHALHLLALLALHLAHALKKA	1.72	2.75

[a] Hydrophobicity and hydrophobic moment calculated using the CCS scale (13). Hydrophobic moment assumes ideal α-helical structure throughout the length of the peptide.

1% (for plasmid DNA) agarose gel using Tris-borate-EDTA buffer. Nucleic acids were visualised after SYBR Safe (Invitrogen) staining.

Centrifugation Assay

3.75 µg of either siRNA or plasmid DNA were mixed with 22.5 µg of LAH4-L1-F (peptide/nucleic acids w/w ratio of 6) in 125 µl of 150 mM NaCl. After incubating for 20 min at room temperature, the amount of nucleic acids and peptide was quantified by measuring, from an aliquot, the OD (260 and 280 nm) and by a Bradford protein assay. Complexes were then centrifuged for 10 min at 14,000g. The nucleic acids and peptide that remained in the supernatant were quantified. As a control, we performed the same assay using naked plasmid DNA and siRNA as well as the peptide alone.

Transfection of Plasmid DNA

100,000 cells were plated in 24-well plates (Costar) one day before transfection. The desired amount of peptide and 4 µg of plasmid DNA were each diluted in 100 µl of 150 mM NaCl and gently mixed. Of note, DNA complexes were generated by using varying peptide/DNA w/w ratios. After 15 min of incubation, the mixture was diluted with serum-free medium to a final volume of 1 ml; 0.5 ml of the transfection mixture was then put in each well of the duplicate. After incubation for 3 h at 37°C, the medium was replaced with fresh medium containing 10% serum. Luciferase activity was assayed 41 h after transfection. These experiments were performed in duplicate. The transfection efficiency is expressed as light units/10 s/mg protein, and the values are the means of the duplicates.

Transfection of siRNAs

To evaluate the capacity of the different peptides to introduce siRNAs into mammalian cells, we made use of 911 cells that have been stably transfected with a CMV-luciferase plasmid (911-Luc). Typically, we measured 1.6×10^7 light units/10 s/mg of protein. The siRNA that was used to knock down luciferase, Luc-sense: 5′-CGUACG CGGAAUACUUCGATT-3′; Luc-antisense: 5′-UCGAA GUAUUCCGCGUACGTT-3′, has been previously described (10). The siRNA used to target the eGFP mRNA has been previously reported by Caplen *et al.* (11): eGFP-sense: 5′-GCAAGCUGACCCUGAAGUUCAU-3′; eGFP-antisense: 5′-GAACUUCAGGGUCAGCUUGCCG-3′. The transfection protocol was similar to that used to deliver plasmid DNA: briefly, 911-Luc cells were plated in 24-well plates the day before transfection. The siRNA was diluted in 150 mM NaCl (unless otherwise stated) before addition of the cationic peptide. After 20 min of incubation, the mixture was diluted with serum-free culture medium. The final concentration of the siRNA in the well was 50 nM unless otherwise stated. The transfection mixture was then deposited in each well of the duplicate. After a 3 h incubation period, the medium was replaced with DMEM supplemented with 10 % FCS, and luciferase expression was evaluated about 30 h post-transfection. The siRNA delivery efficiency was expressed as percent of luciferase remaining after treatment as compared to non-treated cells (=100%).

The siRNA transfection experiments involving bafilomycin A1 (final concentration of 185 nM) were conducted as described above except that the drug was added after dilution of the complexes with DMEM, just before addition of the transfection medium to the cells.

In the experiments using carrier DNA, the siRNA and the DNA from salmon sperm were first mixed before the transfection reagent was added. Of note, the amount of peptide was adjusted in such a way that the peptide/nucleic acids w/w ratio was similar to that of the peptide/siRNA complexes.

Luciferase Assay

For luciferase activity, cells were harvested in 250 µl lysis buffer (8 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, 1% Triton X-100, 15% glycerol, and 25 mM Tris-phosphate buffer pH 7.8). The cell lysate was then transferred into Eppendorf tubes and centrifuged for 5 min at 10,000 g to pellet debris. Luciferase light units were measured in a 96-well plate format with a luminometer (Perkin Elmer) from an aliquot of the supernatant (50 µl). The measurements were done over 10 s after automatic injection of 100 µl assay buffer (lysis buffer without Triton X-100 supplemented with 2 mM ATP) and 100 µl of luciferin solution (167 µM in water; Molecular Probes). Luciferase background was subtracted from each value, and the protein content of the transfected cells was measured by Bradford dye-binding using the BioRad protein assay. Statistical analysis was performed using ANOVA with Bonferroni post hoc t-test, one-tailed.

MTT Assay

Cytotoxicity of the [cationic transfection agent/siRNA-Luc] complexes was evaluated by performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) assay. Briefly, 30 h after transfection, the cell culture medium was removed and replaced by medium containing 0.5 mg/ml MTT. After incubation at 37°C for 3h30, the medium was removed and 200 µl of DMSO was added to each well to dissolve the formazan crystals produced from the reduction of MTT by viable

cells. Absorbance was then measured at 570 nm. Untreated cells were used as control (=100% viability).

Association/Endocytosis of Transfection Agent/siRNA Complexes

Complexes were rendered fluorescent by using a fluorescently labelled siRNA-Luc (a Fitc group was conjugated to the 3′ end of the antisense strand; Sigma-Aldrich). After preparation of the complexes as described above, they were added to 911-Luc cells (final siRNA concentration = 50 nM). After 3 h of incubation at 37°C, cells were washed with cold phosphate-buffered saline (PBS), harvested in 1 mM EDTA in PBS and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, Grenoble, France).

RESULTS

We have previously reported that the cationic amphipathic histidine-rich peptide LAH4 possesses robust plasmid DNA transfection properties (7,12). More recently, we found that when reducing the size of the hydrophilic face of the helix from 100° (LAH4) to 80°, the transfection efficiency of the resulting peptide is enhanced (8).

In the present study, we investigated the effect of three different sequence alterations (Table I): 1) replacement of the four terminal lysines by arginine residues (LAH4-L1-R), 2) increase of the hydrophobicity of LAH4 and LAH4-L1 by replacing three alanines with leucines, and 3) addition of two phenylalanines at the N-terminus of LAH4-L1. These changes altered the overall hydrophobicity of each peptide and, under the assumption of a perfect α-helix in the membrane, also affected the hydrophobic moment, a vectorial property of amphipathic peptides (Table I) (13).

Gel Mobility Shift Assay

The capacity of the different peptides to complex plasmid DNA and siRNAs was investigated by performing a gel retardation assay. All of the peptides were able to inhibit the migration of DNA and siRNA, indicating that they complexed the nucleic acids efficiently (Table II). It is notable, however, that between 2.5 and 5 times more peptide is required to retard the migration of siRNAs when compared with plasmid DNA. This underscores the view that complex formation and interactions of the cationic peptides with plasmid DNA or a siRNA are different. A possible explanation may be that, in contrast to DNA, siRNA cannot be condensed, and the peptide-RNA complex formation therefore lacks cooperativity (14).

A gel mobility shift assay does not allow for quantification of the free and bound peptide. To study this issue in more detail, we performed a centrifugation assay with LAH4-L1-F, the most promising peptide investigated here (see below). For this experiment, we used a peptide/nucleic acids w/w ratio of 6, which allows complete retardation of siRNA (Table II) and which is efficient for both DNA (Fig. 1) and siRNA (Fig. 2B) transfection. After centrifugation of the complexes for 10 min at 14,000 g, the remaining nucleic acids and peptide in the supernatant were quantified. The results show that neither plasmid DNA nor siRNA were present in the supernatant. However, in good agreement with the gel mobility shift assay, we found that 52% of the peptide was present when the assay was performed with plasmid DNA. Also in agreement with the retardation assay, a much lower amount of LAH4-L1-F (16.5%) was found in the supernatant when siRNA complexes were centrifuged.

Table II Gel Mobility Shift Assay

Peptide	DNA[a]	siRNA[a]
LAH4	2.5 μg	8 μg
LAH4-Leu	2 μg	5.3 μg
LAH4-L1	2.5 μg	8 μg
LAH4-L1-R	2 μg	8 μg
LAH4-L1-Leu	1.5 μg	8 μg
LAH4-L1-F	2 μg	5.3 μg

[a] Amount of peptide required to completely inhibit agarose gel migration of 1 μg of either plasmid DNA or siRNA.

Plasmid DNA Transfection

Next, we transfected human embryonic retinoblasts (911 cells) using increasing amounts of peptide mixed with a constant

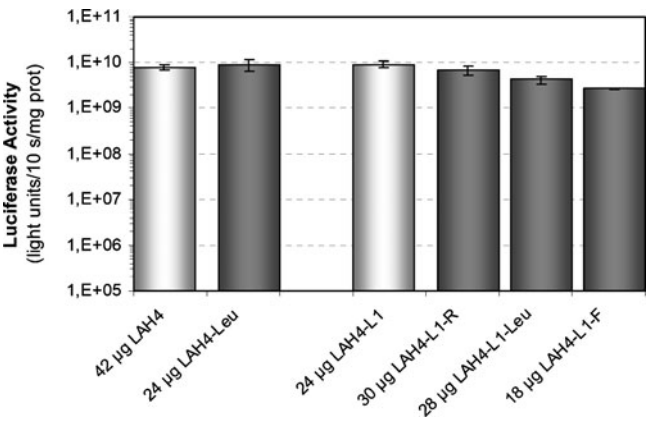

Fig. 1 DNA transfection efficiency of LAH derivatives. Increasing amounts of peptide were mixed with a constant amount of luciferase reporter plasmid (4 μg per duplicate), and the complexes were incubated for 3 h with 911 cells in a serum-free media. Culture medium was replaced by a fresh one containing 10% serum, and luciferase expression was assayed 41 h later. For each peptide, the condition where the highest transfection efficiency was obtained is shown.

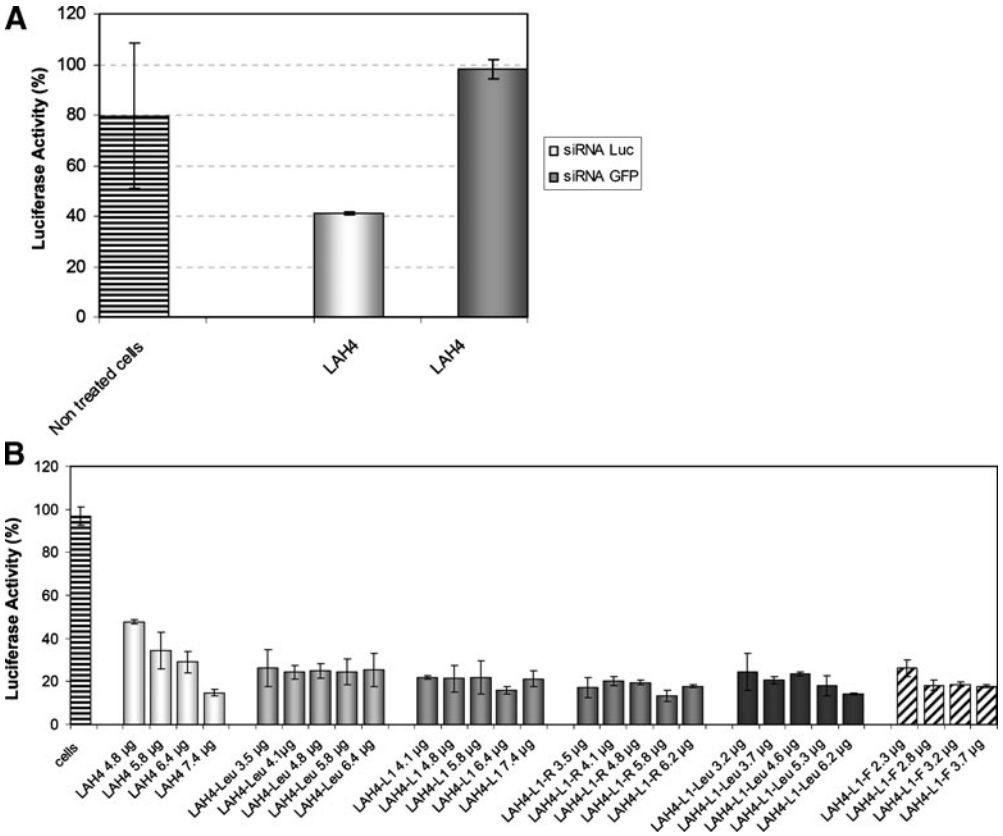

Fig. 2 Specific luciferase silencing mediated by LAH peptides. (**A**). Luciferase activity is assayed after delivery of either 50 nM siRNA (=0.23 μg siRNA/well) targeted against luciferase or, as a control, green fluorescent protein (GFP) using the LAH4 peptide at the w/w peptide/siRNA ratio of 10.5/1 (4.8 μg peptide/0.46 μg siRNA/duplicate). The significance of the specific knock-down was tested by ANOVA (LAH4 $p = 0.002$). (**B**) Comparison of the ability of each of the LAH4 derivatives to mediate luciferase knock-down (the indicated amounts of peptide were used with 0.46 μg of siRNA).

amount of plasmid encoding for luciferase (4 µg/duplicate). The optimal expression achieved and the corresponding peptide amount are shown for each derivative (Fig. 1). The results show that within a factor of three, all five peptides allowed for the same expression level (Fig. 1), although, of the six peptides, LAH4-L1-F had the lowest efficiency for delivery of plasmid DNA.

siRNA Delivery

To evaluate the capacity of the different peptides to introduce siRNAs into mammalian cells, we made use of 911 cells that have been stably transfected with a CMV-luciferase plasmid (911-Luc). Typically, we measured 1.6×10^7 light units/10 s/mg of protein. The siRNA that was used to knock down luciferase has been previously described (10). As a control and to identify specific gene knock-down, we performed a transfection assay with siRNAs targeting the eGFP gene (11). A preliminary experiment conducted with LAH4 showed that this peptide was able to efficiently deliver siRNA into 911-Luc cells (Fig. 2A). As expected, delivery of siRNA targeting the GFP gene did not result in a decrease of the luciferase expression.

Next, we tested four to five different concentrations for each peptide and measured the knock-down efficiency. As shown in Fig. 2B, all the peptides allowed for a reduction of about 80% of the luciferase levels. It was noticed that while an efficient knock-down was obtained with low amounts of LAH4-L1-F, higher amounts of LAH4 were required to obtain the same effect. The toxicity of the peptide/siRNA complexes on 911-Luc cells after transfection was estimated by performing a MTT colorimetric assay. The results indicate that the cytotoxicity of the peptide-based complexes is low; the cell viability is as high, or even higher, than when using transfection agents such as Lipofectamine (Fig. 3).

Comparison with Other Transfection Agents

Of the six peptides tested, LAH4-L1-F consistently performed well, particularly at lower peptide concentrations. Therefore, although the differences between the six peptides were small, we used this derivative to perform a comparison with commercially available compounds. We transfected 911-Luc cells with 50 nM of siRNA (=0.23 µg siRNA/well) using increasing amounts of LAH4-L1-F, DOTAP (a monocationic lipid), Lipofectamine (a mixture of a polycationic lipid and DOPE) and the branched PEI of 25 kDa (B-PEI). Of note, preliminary siRNA delivery experiments were performed with each of the transfection reagents in order to determine the N/P range in which the compound was active. The results shown in Fig. 4A indicate that the peptide is more efficient than the three other formulations at mediating knock-down of luciferase expression in 911 cells. Maximal gene silencing is obtained at

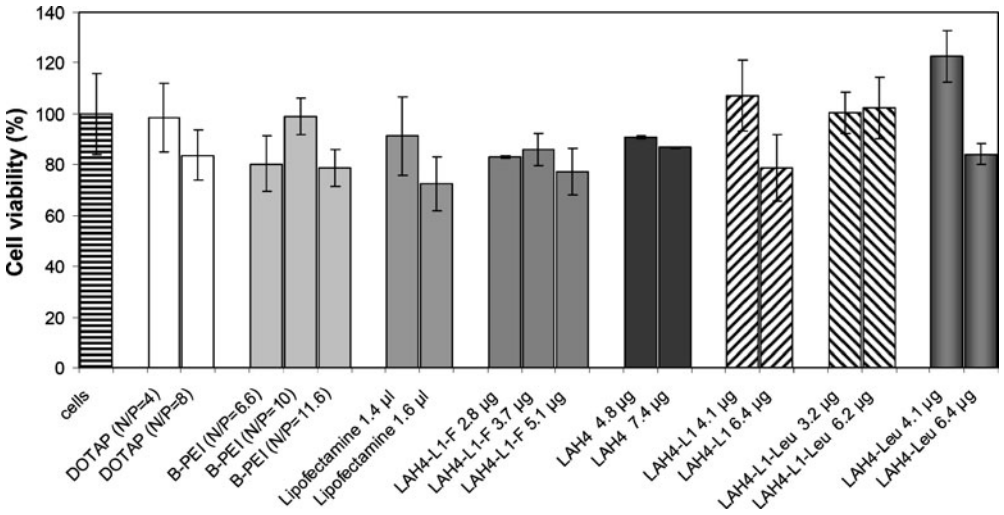

Fig. 3 Toxicity of a range of non-viral vectors to 911-Luc cells as determined by an MTT assay. The [transfection agent/siRNA] complexes were generated as described in the experimental section, and the MTT assay was performed 30 h after transfection. The indicated amounts of vector were used with 0.46 μg of siRNA-Luc.

transfection agent/siRNA ratios which are higher than those usually required for efficient DNA transfection. This is in good agreement with the results previously published with cationic agents such as DOTAP (15) and PEI (16).

By using a fluorescently labelled siRNA, it is possible to quantify, using flow cytometry, the amount of nucleic acid that is associated with or endocytosed by the cells. We assessed binding of the labelled complexes to 911- Luc cells

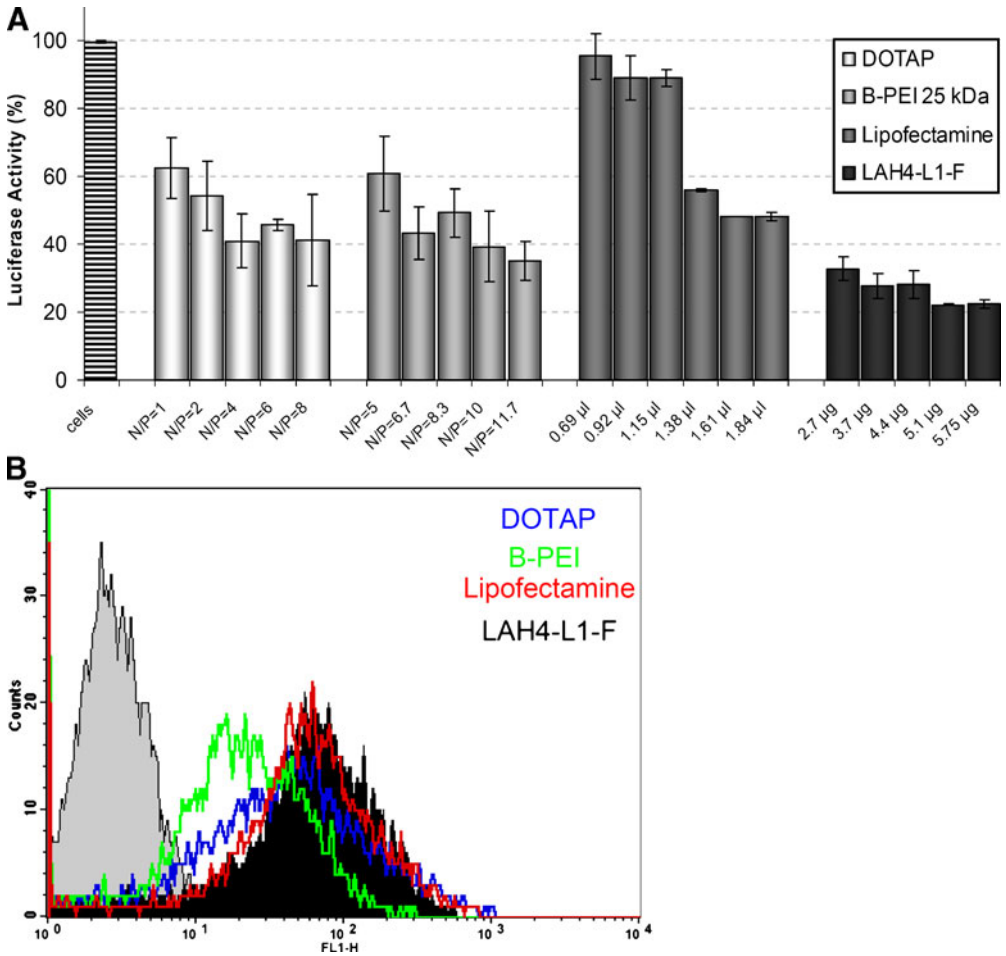

Fig. 4 The specific luciferase knock-down efficiency of LAH4-L1-F against 911-Luc cells is compared with that of other commonly used non-viral siRNA delivery vehicles (**A**). The optimum knock-down mediated by the peptide is significantly greater than that mediated by Lipofectamine ($p < 0.001$), DOTAP ($p < 0.10$) and B-PEI ($p < 0.10$). The indicated amounts of vector are given for 0.46 μg of siRNA-Luc (0.23 μg/well). (**B**) Association of fluorescently labelled siRNA (50 nM) with 911-Luc cells after 3 h of incubation.

after 3 h of incubation. As illustrated in Fig. 4B, the results show that noticeably more siRNA associates with the cells when formulated with the peptide LAH4-L1-F as when using the non-peptidic vectors DOTAP and B-PEI. Lipofectamine, however, allowed for an association/endocytosis which was almost as high as when using the peptide.

Inhibition of Endosomal Acidification

The plasmid DNA transfection efficiency of cells by LAH4 has been shown to be significantly reduced when endosomal acidification is inhibited by the H^+-ATPase inhibitor bafilomycin A1 (7). This result indicated that protonation of the imidazole groups of the histidine residues plays an important role in the transfection process and in particular for endosomal escape. Similar experiments conducted with siRNA and with the two template peptides (LAH4 and LAH4-L1) resulted in a severe reduction of the knock-down efficiency (Fig. 5). This demonstrates that an efficient delivery of siRNAs by such peptides requires acidification of the endosomes. In analogy to the conclusions from a detailed biophysical analysis of the LAH4/DNA complexes at different pH (17), this acidification probably leads to the following sequence of events: protonation of the histidines, which increases the net charge of the peptide from +5 to +9. This liberates a major fraction of peptides from the siRNAs, which are then free to interact with and destabilize the endosomal membrane (17). An additional process that might be involved in endosomal escape is the proton sponge effect. Indeed, the imidazole group, which has a pKa of about 6, can induce the buffering of the endosomal vesicles, and this in turn, like in the case of PEI (18–20), should cause osmotic swelling and subsequent endosome disruption.

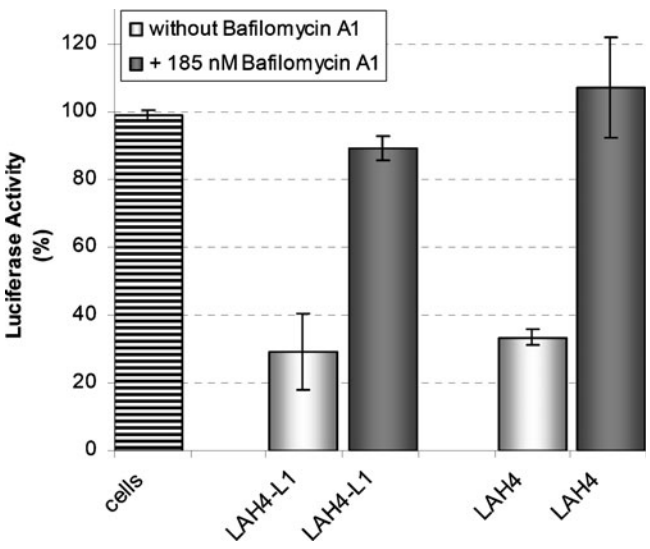

Fig. 5 Comparison of specific luciferase knock-down in 911-Luc cells mediated by the two histidine-rich template peptides in the absence or presence of bafilomycin A1. LAH4 and LAH4-L1 were used at a peptide/siRNA w/w ratio of 10.5, and the final concentration of siRNA was of 50 nM (4.8 μg of peptide/0.46 μg siRNA/duplicate). Bafilomycin A1 abolishes peptide-mediated knock-down of gene expression ($p < 0.05$).

Optimization of the siRNA Delivery

We then evaluated the siRNA concentration dependence of the knock-down by using the LAH4-L1-F peptide as transporter system. The results shown in Fig. 6A indicate that at 25 nM of siRNA, the luciferase levels are already reduced by more than 70%.

We next asked whether the presence of salt influences the efficiency of siRNA delivery. Therefore, we compared the activity of complexes generated either in water, 150 mM NaCl or PBS. The results shown in Fig. 6B indicate that for the four peptides that were tested, the highest efficiency is obtained when complexes were prepared in water. The least efficient formulations were those which were generated in PBS. These results indicate that salt composition and concentration plays a role during complex formation.

We previously hypothesized—and to some extent demonstrated—that the activity of compounds with endosome-buffering capacities, such as PEIs (21) and LAH4 (12), requires a threshold amount of transfection agent. In fact, we have been able in these earlier studies to keep the transfection efficiency constant, although with low amounts of reporter gene, by adding an excess of transfection agent (i.e. increasing the w/w ratio in favour of the transfection agent). Alternatively, we had efficiently transfected cells using low amounts of reporter gene when keeping the amount of complexes high by mixing the plasmid of interest with salmon sperm DNA.

In the present assays, the amount of siRNA that is used is significantly lower than that for the plasmid transfection experiments, i.e., 0.23 µg siRNA/well as compared to 2 µg plasmid/well.

We therefore asked whether addition of carrier DNA to the siRNA could increase the overall efficiency. To address this point, we generated complexes with 50 nM of siRNA (0.23 µg/well), which were mixed with increasing amounts of carrier DNA. Similar trends were observed for both LAH4 (Fig. 7A) and LAH4-L1-F (Fig. 7B), namely that the addition of salmon sperm DNA slightly increased the knock-down efficiency. The effect of carrier DNA was the highest when adding 2.5- to 5-fold the amount of siRNA. These results are in good agreement with the recent report of Rhinn and colleagues, who demonstrated that addition of carrier DNA to siRNA lipoplexes (cationic lipid/siRNA complexes) leads to more efficient complexes to transfer siRNA at low concentration (22).

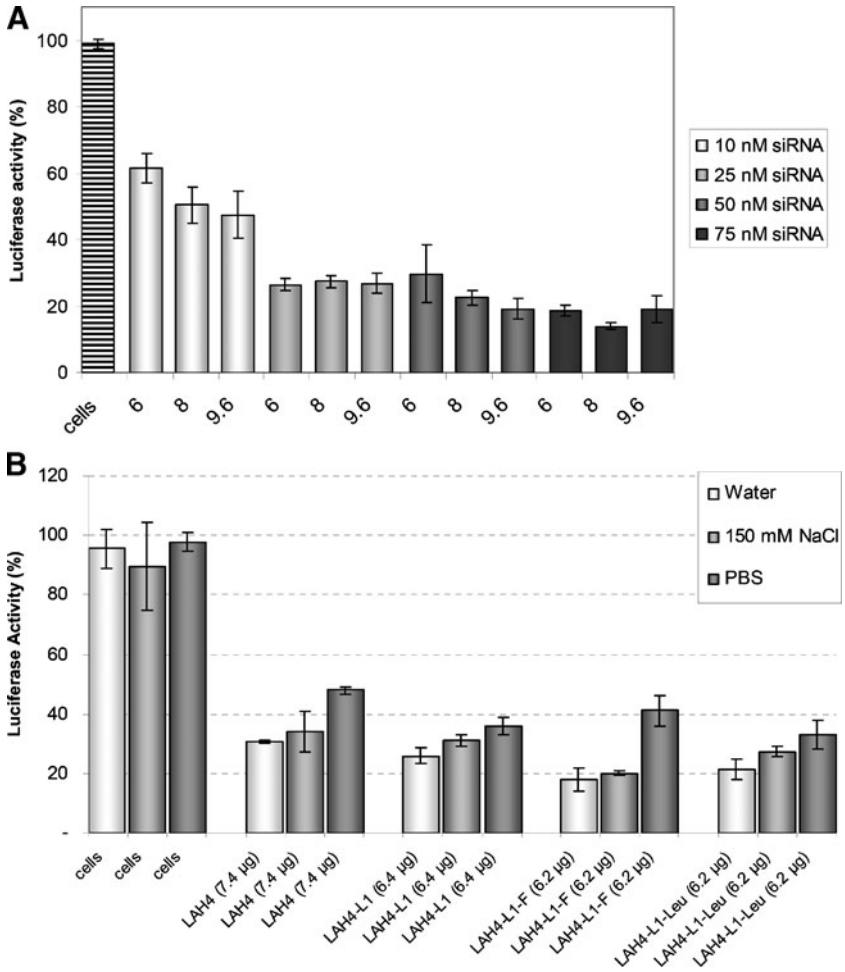

Fig. 6 (**A**) The effect of decreasing the amount of siRNA on the luciferase knock-down efficiency mediated by LAH4-L1-F. The vector/siRNA w/w ratios which were used are indicated in the figure. (**B**) The effect of varying the ionic content of the complexes on the luciferase knock-down efficiency of 50 nM siRNA delivered by LAH4 peptides (the indicated amounts of peptide are for 0.46 μg of siRNA). Using PBS instead of water significantly reduces knock-down efficiency mediated by LAH4 ($p = 0.00864$) and LAH4-L1-F ($p = 0.0694$), while the same trend is observed for all four peptides.

DISCUSSION

Cell-penetrating peptides (CPP) have been developed into important tools in biomedical research and for pharmacological applications to transfer proteins, drugs and DNA across cellular membranes. The use of CPPs carries big hopes also for gene-silencing approaches where small interfering RNA (siRNAs) have to cross the cytoplasmic membranes. Poly-arginines (polyR) are commonly used sequences in current applications, and this class of peptides in most cases uses endosomal pathways. Unfortunately, it is often observed that the siRNA-polyR complexes are taken up into cells but that endosomal escape is a limiting factor. As a consequence, other compounds have been designed, such as the 27 residue MPG peptide (GALFLGFLG AAGSTMGAWSQPKKKRKV), which contains a hydrophobic domain derived from the fusion sequence of HIV gp41 and a hydrophilic domain derived from the nuclear localization sequence of SV40 T-antigen (23). The same group recently described an amphipathic peptide named CADY for the delivery of siRNA ("Ac-GLWRALWRLL RSLWRLLWRA-cysteamide") (24). The design of this compound was based on the DNA transfection peptide PPTG1 (25).

A strategy which has proven to favour the endosomal escape of plasmid DNA consists in the coupling of histidine residues to the transfection agent. Indeed, Midoux and colleagues demonstrated that the transfection efficiency of a polylysine partially substituted with histidyl residues was dramatically increased compared to the non-modified polymer (26,27). The rationale for this modification was based on the fact that histidyl residues become cationic upon protonation of the imidazole groups at pH below 6.0. Thus, the imidazole ring confers a PEI-like proton sponge activity to the agent to which it is coupled. Based on these results, branched histidine-lysine (HK) peptides have been designed for the delivery of nucleic acids, including siRNAs (28). We, on the other hand, focused our work on short linear cationic amphipathic peptides containing various numbers of histidine residues. Among the peptides that have been evaluated, the 26-residues-long peptides LAH4 and LAH4-L1 exhibited the highest DNA transfection activities, which were comparable with or even more potent than those of the commercially available PEI (7,8). Considerable

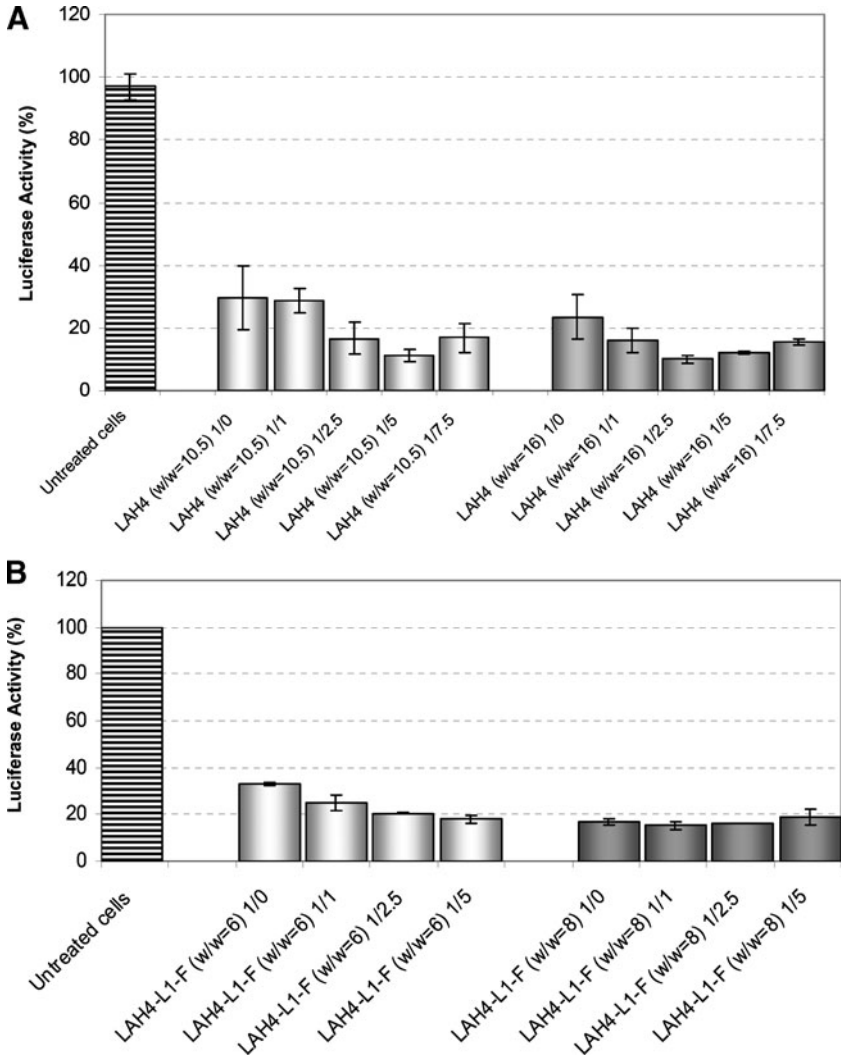

Fig. 7 Comparison of the effect of packaging 50 nM siRNA with salmon sperm DNA (siRNA/stuffer DNA w/w ratio of 1/0, 1/1, 1/2.5, 1/5 and 1/7.5) in a complex with LAH4 used at a w/w vector/nucleic acids ratio of 10.5 and 16 (**A**) and LAH4-L1-F used at a w/w vector/nucleic acids ratio of 6 and 8 (**B**).

information has been obtained concerning the LAH4-mediated plasmid DNA transfection activity; in particular, biophysical investigations have revealed a self-promoted endosomal uptake mechanism (7,8,17). In contrast, nothing was known about the capacity of LAH-derived peptides to deliver siRNAs.

Here, for the first time, we studied the potential of LAH4 and five derivatives thereof to deliver siRNA into human retinoblasts which stably expressed luciferase. Our results show that all six peptides efficiently deliver small dsRNA duplexes into human retinoblasts. Interestingly, by performing a transfection in the presence of bafilomycin A1—an antibiotic that has been shown to selectively inhibit vacuolar-type H+- ATPases (29,30)—we were able to show that efficient delivery of siRNA requires acidification of endosomes. This is in agreement with the results which have been previously obtained in plasmid DNA transfection experiments (7).

When compared with the three commercially available cationic delivery systems, Lipofectamine, DOTAP and PEI, LAH4-L1-F was found to be the most efficient agent. This increased efficiency may be related to the higher capacity of the peptide to favour association of the siRNA with the cells, as shown by the experiments conducted using a fluorescently labelled siRNA (Fig. 4B). Importantly, we used commercially available compounds for comparison, which are described as being efficient for the delivery of plasmid DNA. However, highly efficient DNA carriers are not necessarily similarly effective for siRNA delivery. Indeed, while the branched PEI of 25 kDa is recognized as being one of the most efficient plasmid DNA transfection agents, results about its efficiency for the delivery of siRNAs are less clear: depending on the study, B-PEI was found to be either efficient (16) or poorly efficient (31) for delivery of dsRNA duplexes. In any case, recent results show that the capacity of PEI to deliver siRNAs can be significantly increased by introducing various modifications. (31–33). Whether the present LAH peptides also compare favourably with the new generation of compounds based on PEI remains to be shown.

The reasons why some compounds that are efficient for the delivery of plasmid DNA are rather poor vectors for siRNA are unknown. However, at least two key differences between pDNA and siRNA delivery can be identified. First, while plasmid DNA needs to be delivered into the nucleus, the siRNA does not. Thus, pDNA delivery requires an interaction with the transfection agent which is sufficiently strong to ensure its safe transport across the cytoplasm and delivery into the nucleus. In contrast, to be effective, dissociation in the cytoplasm of the siRNA from the carrier is required. This might, for example, explain why addition of PEG chains to PEI—which reduces the stability of the complexes—increases the efficiency of siRNA-mediated knock-down (33). Second, the plasmid DNA, which usually is several kilo base pairs long, can be condensed into small particles by cationic compounds. This is not the case for the 21 bp long siRNA. Indeed, it has been shown that RNA segments shorter than 260 bp behave as rigid rods (34). Further work is required to evaluate which, if any, of these properties contributes most to determining the siRNA delivery efficiency of non-viral vectors in general and histidine-rich peptides in particular.

CONCLUSION

Taken together, the present work shows that peptides of the LAH4 family are able to efficiently deliver siRNAs *in vitro* into a human cell line. In the future, it will be important to study the siRNA/peptide interactions in more depth as well as the siRNA delivery process. This, in turn, will allow us to further optimize the efficiency of the peptides as an *in vitro* gene silencing tool and ultimately for *in vivo* siRNA delivery.

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