Published in final edited form as:

J Control Release. 2012 March 10; 158(2): 293–303. doi:10.1016/j.jconrel.2011.11.024.

Effective endogenous gene silencing mediated by pH responsive peptides proceeds via multiple pathways

Jenny. K.W. Lam^{a,b}, Wanling Liang^b, Yun Lan^{a,b}, Poulami Chaudhuri^a, Michael Y.T. Chow^b, Katarzyna Witt^a, Laila Kudsiova^a, and A. James Mason^{a,*}

^aInstitute of Pharmaceutical Science, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH

^bDepartment of Pharmacology & Pharmacy, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong

Abstract

Cationic amphipathic histidine rich peptides possess high plasmid DNA and siRNA delivery capabilities. To further understand the pH responsive siRNA delivery process and evaluate the capabilities of such peptides we have investigated their ability to mediate specific silencing of endogenous GAPDH gene activity in MCF-7 and A549 cells and compared this with plasmid DNA delivery. A substantial and selective reduction of both GAPDH activity and expression was achieved using pH responsive peptide vectors, which compared favourably with that mediated by commercially available non-viral vectors in terms of efficacy and toxicity. Furthermore, by comparing the efficacy of both gene delivery and silencing mediated by a series of such peptides, their sensitivities to known inhibitors of endocytotic processes, and their route of uptake via confocal live cell imaging, we show that both plasmid DNA and siRNA are internalised via endocytosis. However siRNA entry facilitated by LAH4-L1, proceeds via a cholesterol dependent mechanism, in contrast to DNA transfer which is associated with clathrin dependent endocytosis. Furthermore, using peptides that respond at increasingly acidic pH, we demonstrate that the route of entry for the siRNA that ultimately mediates silencing is peptide specific and while some pH responsive peptides promote the escape of labelled siRNA from endosomes, others may promote entry via alternative mechanisms.

Keywords

pH responsive peptides; endocytosis; gene silencing; GAPDH; siRNA

INTRODUCTION

Nucleic acids, including plasmid DNA for gene therapy and small interfering RNA (siRNA) for RNA interference (RNAi), have considerable therapeutic potential. Double stranded RNA 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 [1-3]. However, their delivery to human cells remains a major technical hurdle. While viruses are efficient delivery vehicles for nucleic acids in human cells, concerns have been raised regarding their immunogenicity

*Corresponding author: Institute of Pharmaceutical Science, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH; Tel: + 44 207 848 4813; Fax: + 44 207 848 4800; james.mason@kcl.ac.uk.

and toxicity. Hence a range of non-viral vectors are being developed which are expected to have fewer safety concerns [4]. Many such vectors enter human cells using endosomal pathways but, while uptake of the complexes can be readily promoted, escape of the complexes from endosomes is often observed to be poor and is a considerable limiting factor in their use. Therefore the efficiency of such non-viral vectors may not be sufficient for their development as general delivery systems for therapeutic applications, despite recent *in vivo* delivery successes using cationic amphipathic peptides [5], and highly efficient but safe delivery vehicles remain highly sought after. Incorporating pH sensitive imidazole groups, when designing non-viral vectors, has proven to be an effective strategy for increasing the transfection efficiency of compounds that deliver their cargo to mammalian cells via endocytosis [6]. Our own work has focused on using amphipathic α -helical peptides incorporating pH sensitive residues [7-15]. The histidine residues in the vector peptide LAH4 are uncharged at neutral pH but when the pH of the endosomal lumen drops, the side-chains become protonated, large numbers of peptides are released from the nucleic acid-peptide complex [7] and are capable of adopting a conformation and alignment in the membrane that induces membrane disorder [8-11]. Hence, pH induced changes in the peptide charge and nucleic acid binding affinity during endosomal acidification enhance the ability of the complex to escape the endosome and reach the cell cytosol and/or eventually, nucleus.

While the mechanisms underlying effective and efficient plasmid DNA delivery have been explored in some detail [7, 9-13], only recently have we reported that such peptides are also effective at delivering siRNA and mediating gene silencing in 911 cells stably expressing a *Luciferase* gene as a reporter [15]. Since the pH responsive peptide delivery system does not incorporate a nuclear localization sequence, the ability of this system in future to mediate gene transfer *in vivo*, where cells are slowly dividing, may be limited in the absence of a means of promoting trafficking of DNA to the nucleus for expression. In contrast, delivery of siRNA to the RNA-induced silencing complex (RISC), located in the cytoplasm, is sufficient for RNAi activity and hence, with fewer barriers to be overcome, the prospects for a siRNA delivery system that incorporates potent endosomal release capabilities are greater. To better assess the potential of this system, we have evaluated the capabilities of a series of histidine containing peptides, that respond at differing pH, to mediate effective and efficient endogenous gene silencing in MCF-7 human breast cancer and A549 adenocarcinomic human alveolar basal epithelial cells. Using confocal live cell imaging in conjunction with a study of peptide mediated GAPDH silencing in the presence of known inhibitors of endocytic processes we have identified the major mechanisms of siRNA transfer. We are able to show that not only does the mechanism of siRNA transfer differ from that of plasmid DNA but that multiple routes for siRNA transfer are available, depending on the properties of the vector peptide.

MATERIALS AND METHODS

Materials

The peptides (Table 1) were purchased from either EZBiolab (Carmel, IN, USA) or Pepceuticals Ltd (Nottingham, UK) as desalted grade and used as provided. HPLC analysis indicated there were no notable differences in the purity of the synthetic product. Dulbecco's modified eagle medium (DMEM), Opti-MEM 1 reduced serum medium, antibiotic-antimycotic liquid, fetal bovine serum (FBS), Lipofectamine 2000™, CellLight™ ER-GFP BacMam 2.0 and LysoTracker Green DND-26 were purchased from Invitrogen (CA, USA). GenMute™ was obtained from SignaGen Laboratories (Ijamsville, MD, USA). The luciferase assay system was purchased from Promega (Madison, WI, USA). KDalert™ GAPDH assay kit was purchased from Ambion (Austin, TX, USA). GelRed™ nucleic acid stain was purchased from Biotium (Hayward, CA, USA). GAPDH primary antibody was

purchased from Santa Cruz Biotechnology (CA, USA). Amersham ECLTM Western Blotting detection reagents were purchased from GE Healthcare (Amersham, UK). Plasmid DNA (gWIZTM Luciferase) was purchased from Aldevron (Fargo, ND, USA). siRNAs (Silencer®/Silencer® Select GAPDH positive control siRNA and Silencer®/Silencer® Select negative control siRNA) were purchased from Ambion (Austin, TX, USA) and have been extensively tested by the manufacturers, showing minimal off-target effects. Fluorescently labelled siRNA (siGLO cyclophilin B control siRNA) was purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). All other reagents were analytical grade or better.

Particle Size Measurement

Peptide/DNA or peptide/siRNA complexes were prepared at various weight ratios. Nucleic acids solution and peptide solution were prepared separately in distilled water or 150 mM NaCl. Equal volumes of nucleic acids and peptide solution were mixed together to give a final volume of 50 µl containing 1 µg of nucleic acids. After leaving for 30 minutes at room temperature, the hydrodynamic diameter of the complexes was measured by Photon Correlation Spectroscopy (DelsaTMNano C, Beckman Coulter, CA, USA).

Gel retardation assay

Peptide/siRNA complexes were prepared at various ratios with 0.2 µg siRNA in 10 µl TAE buffer. After leaving the complexes for 30 minutes at room temperature, the samples were loaded into a 2% w/v agarose gel containing GelRedTM nucleic acids stain. Gel electrophoresis was run in TAE buffer at 100 V for 20 minutes and the gel was visualized under the UV illumination. For nuclease protection assay, the complexes were prepared as described above. After 30 minutes, the complexes were incubated with 80 ng of RNase A for 30 minutes at 37°C to allow degradation of unprotected siRNA. The activity of RNase was then stopped by the addition of 1 µl of RNase inhibitor. 5 µl of 2 mM SDS was added to the samples to release the protected siRNA. The resulting mixtures were incubated at room temperature for 1 hour. The samples were then loaded into a 2% w/v agarose gel and electrophoresis was run as described above. Untreated siRNA, SDS treated siRNA and RNase treated siRNA were used as controls.

Cell Culture

A549 and MCF-7 cells were obtained from ATCC (Manassas, VA, USA). The cells were maintained at 5% CO₂, 37°C in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. The cells were subcultured once weekly.

Cytotoxicity assay

A549 and MCF-7 cells were seeded on 24 well plates at a density of 1×10^5 cells per well. Various amount of peptides, Lipofectamine 2000TM and GenMuteTM were added to the cells in Opti-MEM I reduced serum medium. After 4 hours of incubation at 37°C, the cells were washed with PBS and MTT solution (0.8 mg/ml) was added. After 2 hours of incubation, the insoluble formazan was dissolved in isopropanol for 2 hours at 4°C. Cell viability was assessed by measuring the absorbance at 570 nm. The results were expressed as percentage of the A_{570nm} for cells treated with peptides against that obtained for untreated cells.

DNA transfection

A549 and MCF-7 cells were transfected with peptide/DNA complexes at 1 µg luciferase/well in 24-well plates. The complexes were prepared in Opti-MEM I reduced serum medium or DMEM medium supplemented with 10% FBS. After 4 hours of incubation at 37°C, the cells were washed with PBS and fresh DMEM supplemented with 10% FBS were added to

the cells. After 48 hours, the luciferase expression was detected using the luciferase assay system according to the manufacturer's protocol. The cells were transfected with Lipofectamine 2000™ under the same condition. For transfection study involving inhibitors, the experiments were carried as described above except the cells were pre-treated with chlorpromazine (10 µg/ml) or nystatin (25 µg/ml) for 30 minutes before the addition of the peptide/DNA complexes. The inhibitors were also present in the medium during the 4 hours incubation with the complexes. The results were expressed as relative light unit produced per mg of protein. Results representative of three independent repeated experiments are shown.

siRNA transfection

A549 and MCF-7 cells, were transfected with peptide/siRNA complexes containing 1 pmol GAPDH siRNA or negative control siRNA/well in 96-well plates with 5000 cells per well giving a final siRNA concentration of 10 nM. The complexes were prepared in Opti-MEM I reduced serum medium. After 4 hours of incubation at 37°C, the cells were washed with PBS and fresh DMEM supplemented with 10% FBS were added to the cells. After 48 hours, the GAPDH expression was detected using KDaAlert™ GAPDH assay kit according to the manufacturer's protocol. The cells were transfected with GenMute™ under the same condition. The results were expressed as percentage of GAPDH expression compared between the GAPDH siRNA and negative control siRNA delivered by the same peptide. Results representative of three independent repeated experiments are shown. For western blot analysis, MCF-7 cells were transfected with peptide/siRNA complexes containing 50 pmol GAPDH siRNA or negative control siRNA/well in 6-well plates, with 6×10^4 cells per well giving a final siRNA concentration of 50 nM. The complexes were prepared in Opti-MEM I reduced serum medium or DMEM medium supplemented with 10% FCS. After 4 hours of incubation at 37°C, the cells were washed with PBS and fresh DMEM supplemented with 10% FCS were added to the cells. After 72 hours, the cells were washed with cold PBS and lysed. The cell extracts containing 20 µg of protein were loaded into a 10% SDS-polyacrylamide gel and electrophoresis was run at 150V for 60 minutes. After the proteins were resolved, they were transferred into a nitrocellulose membrane which was blocked in 5% non-fat dry milk for 2 hours with shaking. The membrane was washed and incubated with primary antibody overnight at 4°C on shaking. After rinsing the membrane was incubated with horseradish peroxidase conjugated secondary antibody for 2 hours at room temperature. The bound secondary antibodies were detected with ECL™ Western blotting detection reagents. For Western blot assay involving inhibitors, the experiments were carried as described above except the cells were pre-treated with chlorpromazine (10 µg/ml), nystatin (25 µg/ml) or chloroquine (100 µM) for 30 minutes before the addition of the peptide/siRNA (10:1, w/w) complexes. The inhibitors were also present in the medium during the 4 hours incubation with the complexes. For all Western Blot experiments, densitometry was performed using GelEval software (Version 1.35) and is reported as an average of three independent repeats.

Confocal live cell imaging

MCF-7 cells were seeded on a 35mm Mattek glass bottom culture dish (Mattek Corp. Ashland, MA) with 1×10^5 cells per well and incubated for overnight. For early endosomes labelling, the cells were transduced with CellLight™ ER-GFP BacMam (Rab5a as marker) in complete medium at a density of 10-30 viral particles per cell. The cells were further incubated for 20 hours before imaging. LAH4-L1/siRNA or LAH6-X1L-W/siRNA complexes containing 2.5 µg of siGLO (Cy3-siRNA) prepared in 1ml of Opti-MEM I were added to the cells which were incubated for 30 minutes. The cells were then washed with warm PBS twice and replaced with fresh serum supplemented DMEM. The cells were imaged at 1 hour, 4 hours, 16 hours and 20 hours post-transfection. At 5 minutes prior to

imaging, the medium was replaced with Opti-MEM I. For lysosomes labelling, LysoTracker Green DND-26 (50 nM) was added to the cells 2 minutes before imaging. During live cell imaging, the cells were incubated at 37°C and 5% CO₂ in a heated Perspex box. For confocal imaging involving inhibitors, the experiments were carried out as described above except the cells were treated with nystatin (25 µg/ml) for 30 minutes before the addition of and during the incubation with the complexes. Confocal laser scanning microscopy (Carl Zeiss LSM 510 Meta/AxioCam system, Germany) with argon laser (488nm) and HeNe laser (543nm) was used to visualize the cells. The microscope was equipped with Plan-Apochromat 64x/1.4 oil DIC objective lens. The images were analysed with Zeiss LSM Image Browser (Version 4.2.0.121).

Statistical Analysis

Statistical analysis was by ANOVA with Bonferonni post-hoc *t*-test, one tailed.

RESULTS

Size of complexes

The size of peptide/nucleic acid complexes comprising either siRNA or DNA and each of the three peptides were determined at peptide to nucleic acid ratios related to the *in vitro* transfection experiments (Fig. 1). Complexes were prepared in either distilled water or 150 mM NaCl, as a simple model for the Earle's Balanced Salt Solution [16] on which most cell culture media are based. The complexes are cationic at all peptide to nucleic acid weight ratios with the lowest weight ratio (5:1) corresponding to a charge ratio of between 2.6 and 2.9 and the greatest weight ratio (20:1) corresponding to between 10.3 and 11.4. Hence the complexes vary between being weakly or very strongly cationic. When complexes were prepared in water, particles of between 90 and 300 nm in diameter are produced whereas complexes prepared in 150 mM NaCl were much larger with particle sizes ranging between 250 and 9000 nm. In general, the sizes obtained for siRNA containing complexes in water (Fig. 1A) were larger than their plasmid DNA counterparts (Fig. 1B) prepared at the same peptide to nucleic acid weight ratios though these differences were small and often not significant.

In contrast, when prepared in 150 mM NaCl the siRNA/peptide complexes comprising either LAH6-X1L or LAH6-X1L-W were as much as ten times smaller than the corresponding plasmid DNA/peptide complexes, above an apparent threshold weight ratio of 10:1 (Fig. 1 B/D). When complexes were prepared using the LAH4-L1 sequence, the sizes of siRNA/peptide and DNA/peptide complexes were not significantly different.

The effect of the Cy3 fluorescent label on particle size was also assessed in both distilled water and 150 mM NaCl (Supp. Fig. 1). A notable increase in peptide/siRNA particle size was observed in distilled water for all peptides when Cy3 was incorporated. This effect was less apparent when the complexes were studied in 150 mM NaCl, with the qualitative differences described for Cy3 free particles retained. At the 10:1 peptide to siRNA weight ratio, the LAH4-L1 and LAH6-X1L complex sizes were unaffected by the presence of the dye; the complexes formed with LAH6-X1L-W were significantly larger in the presence of dye ($p < 0.05$) but remained substantially smaller ($p < 0.05$) than the corresponding particles containing LAH4-L1.

Gel Retardation Assay

The peptides were tested for their abilities to bind with and protect siRNA from nuclease degradation using gel retardation assay (Fig. 2). For the binding study, the disappearance of siRNA band indicated that LAH4-L1, LAH6-X1L and LAH6-X1L-W all showed ability to

bind and form complexes with siRNA. For nuclease protection study, when the naked siRNA was subjected to RNase activity, the siRNA band disappeared. This indicated the enzymatic degradation of unprotected siRNA from RNase. The recovery of siRNA band after the complexes were challenged with RNase indicated that all three peptides offered nuclease protection to siRNA from 10:1 w/w ratio.

MTT assay

The viability of cells in the presence of increasing amounts of either Lipofectamine 2000™ or peptide vectors was assessed using the colorimetric MTT assay (Supp. Fig. 2). The assay was performed both in the presence and absence of nucleic acid cargo. Peptide concentrations varying from 5 to 20 µg per well (10-40 µg/ml) were chosen as these correspond to the peptide concentrations screened when delivering 1 µg plasmid DNA. Only 0.67 µg or 13.3 ng siRNA are required per well in, respectively, the 6 and 96 well plates. This gives corresponding peptide concentrations in siRNA transfection experiments of between 0.665 and 10.05 µg/ml and hence the conditions chosen for testing cell viability represent the upper limit for peptide in either DNA or siRNA transfection experiments. The A549 cells were more sensitive to the presence of either lipid or peptide vectors when compared with MCF-7 cells where peptides caused significant ($p < 0.05$) reductions in cell viability only at higher concentrations while others did not lead to significant toxicity. Reductions in cell viability due to peptide were less in all cases than that caused by Lipofectamine 2000™ which was administered in the same amount as required for optimal transfection.

GAPDH silencing in A549 cells

The ability of the LAH4-L1 peptide, the peptide from previous studies with the most effective gene transfer ability, to mediate specific silencing of GAPDH activity in A549 adenocarcinomic human alveolar basal epithelial cells was probed by monitoring the production of NADH in cell lysates obtained 48h after transfection according to the KDalert GAPDH assay kit. The fluorescence emission intensity at 590 nm produced by such lysates is shown for cells transfected with either Silencer® Select siRNA targeting the GAPDH enzyme or a negative control Silencer® Select siRNA (Fig. 3A). Growth and stability of A549 cells in the 96 well plates was variable and contributed to variation in the measured enzyme activities, yet specific silencing of GAPDH activity with only 10 nM siRNA was readily apparent and reproducible. When GAPDH activity is represented as a proportion of the activity remaining for the negative control (Fig. 1B), the ability of the LAH4-L1 peptide to effectively and specifically mediate GAPDH activity silencing is clear. In transfection experiments, whether siRNA or plasmid DNA, a fixed amount of nucleic acid is titrated with increasing amounts of peptide vector to establish an optimum. A clear threshold of peptide to 10 nM siRNA (15:1, w/w) is required for effective silencing of GAPDH activity. Below this threshold, silencing was not significant whereas, at peptide to siRNA ratios of 15:1 (w/w) and above, specific reductions in activity of at least 69.5% were obtained. This reduction in activity rose to 81.6% at the highest peptide to siRNA ratio. An apparent non-specific reduction in GAPDH activity is observed when siRNA delivery is mediated by Lipofectamine 2000™.

Transfection efficiency of peptides against MCF-7 and A549 cells (comparison of plasmid DNA and siRNA-GAPDH activity)

The efficacy of three peptides in mediating specific silencing of GAPDH activity in MCF-7 cells was investigated using the KDalert™ kit as above (using 10 nM Silencer® rather than Silencer® Select siRNA) (Supp. Fig. 3A). All three peptides were capable of mediating reductions in GAPDH activity though the peptide to siRNA ratios required for optimal silencing varied considerably. LAH4-L1 consistently mediated effective silencing of

GAPDH activity. It was one of two peptides that was capable of matching the performance of GenMute™, reducing GAPDH activity by 70.9%, and the only peptide that offered significant ($p < 0.05$) improvements over Lipofectamine™2000. Specific reductions in GAPDH activity in A549 cells mediated by peptides matched that obtained with GenMute™ and exceeded that obtained with Lipofectamine 2000™ which performed poorly in comparison with its efficacy towards MCF-7 cells (Supp. Fig. 3A/B).

The abilities of the three peptides to mediate effective delivery of *luciferase* reporter gene to the same MCF-7 cells were also assessed (Supp. Fig. 3C). The three peptides had robust gene transfer capabilities, outperforming the Lipofectamine 2000™ positive control ($p < 0.05$ at all peptide/DNA ratios). Transfection experiments comparing the DNA transfection efficacy of the three peptides were also performed using A549 cells and comparable results were obtained (Supp. Fig. 3D). The DNA transfection abilities of the peptides, when delivering *luciferase* reporter gene to MCF-7 cells, were sensitive to the presence of serum in the cell culture medium with notable reductions occurring in practically all conditions (Supp. Fig. 3C). These reductions were of a lower magnitude when compared with those observed for A549 cells (Supp. Fig. 3D) and, consistent with previous work [11], complexes with a greater amount of peptide were more resistant to the effects of serum. LAH6-X1L-W, in particular, resisted the effects of serum at the highest peptide concentration tested.

Silencing of GAPDH gene expression

In addition to observing the suppression of GAPDH activity 48 h after transfection of siRNA, the expression of GAPDH was also assessed 72 h after transfection (dynamic study of GAPDH expression available for MCF-7 cells, Supp. Fig. 4) with 50 nM Silencer® siRNAs using a Western blot (Fig. 4). The intensity of bands corresponding to GAPDH are compared with those corresponding to actin, allowing the specific nature of the gene expression silencing to be confirmed or non-specific effects of siRNA transfection to be identified. Specific reductions in GAPDH protein are mediated by all three peptides tested as well as by the GenMute™ and Lipofectamine 2000™ control. In each case where a positive siRNA for GAPDH has been delivered, the corresponding band becomes either much fainter or disappears entirely while bands corresponding to actin remain of constant intensity. This can be contrasted with the bands for GAPDH when a negative siRNA has been delivered. Comparing densitometry readings for GAPDH bands corresponding to positive or negative siRNA delivery and using the intensity of the actin bands to normalise the protein content from each well, specific reductions in GAPDH of $41.7 \pm 8.6\%$ are mediated by GenMute™, which are exceeded by those mediated by LAH6-X1L and LAH4-L1 ($p < 0.05$) but not LAH6-X1L-W, with a maximum reduction of $66.0 \pm 7.7\%$ obtained with LAH6-X1L (Fig. 4B). Lipofectamine 2000™ mediated very robust, specific silencing of GAPDH expression in MCF-7 cells (Fig. 4A/B) but caused a notably large number of the adherent cells to detach following treatment. Hence, due to the apparent non-specific reduction of GAPDH activity in A549 cells mediated by Lipofectamine 2000™ and its higher observed toxicity when compared with GenMute™, the latter was used as the sole positive control/commercial benchmark for the remainder of the study. Similar siRNA transfection experiments were performed with FBS included in the medium during the transfection process (Fig. 4C) as was a more detailed characterisation of LAH4-L1 mediated reductions in expression incorporating a dose response experiment using MCF-7 cells and a comparative experiment with A549 cells (Supp. Fig. 5). As with the DNA transfection experiments performed above, the presence of serum limited the ability of the three peptides tested, as well as GenMute™ to mediate effective reductions in GAPDH protein detectable 72 h after transfection. As with previous work [11], the effects of serum on peptide mediated nucleic acid transfer appear to be cell line specific hence a detailed investigation of this effect is beyond the scope of the present study. Nevertheless, LAH4-L1 and LAH6-X1L

continued to exceed the performance of GenMute™ ($p < 0.05$), which was not capable of mediating significant reductions in GAPDH expression in the presence of serum.

Effect of inhibitors on plasmid DNA and siRNA delivery

The effects on both DNA and siRNA transfection efficacy of pre-treating the cells with known inhibitors of endosomal pathways was assessed using again either the *Luciferase* plasmid or 50 nM GAPDH siRNA transfection assays with GAPDH expression silencing assessed by Western blot (Fig. 5). Pre-treatment with chlorpromazine, an inhibitor of clathrin mediated endocytosis, led to a dramatic and significant reduction in luciferase expression in both A549 (Fig. 5A) and MCF-7 (Fig. 5B) cells. In MCF-7 cells, luciferase activity was reduced to below 5% of that of untreated cells when either LAH4-L1 or LAH6-X1L-W were used to mediate transfection (Fig. 5B). In contrast, pre-treatment with nystatin, an inhibitor of caveolae mediated endocytosis had more diverse effects. Significant ($p < 0.05$) but more modest reductions in luciferase activity were observed in A549 cells when LAH4-L1 or LAH6-X1L-W were used to mediate transfection (Fig. 5A) and in MCF-7 cells when LAH4-L1 was used (Fig. 5B). However, transfection mediated by LAH6-X1L was unaffected in either cell line (Fig. 5A/B) as was transfection mediated by LAH6-X1L-W in MCF-7 cells (Fig. 5B).

The effect of various inhibitors on pH responsive peptide mediated silencing of GAPDH expression was assessed in a similar manner (Fig. 5C/D). Treatment with bafilomycin A1 during peptide mediated siRNA transfection caused cytotoxic effects that prevented sufficient material being recovered for Western Blot analysis. A 5 hour treatment of both cell lines with inhibitors indicated a modest but significant cytotoxic effect for chloroquine and chlorpromazine (Supp. Fig. 6). These cytotoxic effects were controlled by using the intensity of the actin bands to normalize the expression of GAPDH. Based on densitometry of three independent repeats, only nystatin was observed to have a significant effect ($p < 0.05$) on the transfection efficiency mediated by LAH6-X1L (Fig. 5D). Only a modest and non-significant reduction in silencing of GAPDH expression was observed following pre-treatment with chlorpromazine while, interestingly a substantial enhancement was observed following pre-treatment with nystatin. The sensitivity of LAH4-L1 mediated silencing of GAPDH expression, to inhibitors was also assessed. The sensitivity of LAH4-L1 and LAH6-X1L to nystatin in particular differed, with a notable and significant ($p < 0.05$) reduction in transfection efficiency observed for LAH4-L1 contrasting with the enhancement observed for LAH6-X1L (Fig. 5D). Therefore, not only were the effects of inhibitors on GAPDH expression peptide specific, the effect of inhibitors on peptide mediated siRNA transfection differ from those where DNA is the cargo.

Confocal live cell imaging

Entry of fluorescently labelled siRNA to both A549 (Supp. Fig. 7) and MCF-7 cells (Fig. 6/7) was tracked using live cell confocal imaging. For MCF-7 cells, Cy-3 labelled siRNA appears red and is located in all images in discrete clusters within the cell while two separate endosome labelling approaches were used. First, a Rab5a targeting sequence fused to emerald GFP was used to mark early clathrin-coated endosomes (Fig. 6). Secondly, LysoTracker® Green DND-26 was used to label cellular compartments with low internal pH which may include mid to late endosomes as well as lysosomes (Fig. 7). When red siRNA is co-localised with green intracellular compartments a yellow to orange colour is produced. Representative images obtained at 1 hour (Fig. 6A/B), 4 hours (Fig. 6C/D) and 16 hours (Fig. 6E/F) indicate that little or no co-localisation of siRNA with Rab5a containing early endosomes occurs even at the earliest time point. This indicates that either all the siRNA has entered and escaped the endosomes within 1 hour or that clathrin independent uptake mechanisms predominate. For the experiment where mid to late endosomes are labelled,

representative images, obtained between 22 and 23 hours post-transfection, are shown (Fig. 7). After around 20 hours, differences in the distribution of the siRNA clusters according to the peptide used to mediate transfection are more notable (Fig. 7A/B). For LAH6-X1L, in contrast with earlier time points, there is some evidence for co-localisation between red siRNA and green acidic compartments. Most of the compartments appear green, however a number of yellow compartments can be detected (Z-section available for 20 hour time point as supplementary material) with red clusters intermingled with, but separate from, acidic compartments. This suggests that much of the siRNA has been taken up into acidic compartments but may now have either escaped or been degraded at this later stage. The few red clusters apparent at this later time point could therefore represent quantities of siRNA that have successfully escaped from either late endosomes or lysosomes. MCF-7 cells containing LAH4-L1/siRNA particles have a very low level of co-localisation at all time points and can be compared with LAH6-X1L at 20 hours after transfection (Fig. 7A/C). Instead, notable clusters of red siRNA can be observed within the cell but not confined to acidic intracellular compartments (Fig. 7A). This suggests that beyond 20 hours post-transfection, LAH4-L1/siRNA particles have either already successfully escaped from endosomes or entered the cell via an alternative mechanism. Similar results were obtained using A549 cells though with dextran-alexa-fluo used to label endosomes. Again, a much greater degree of co-localisation between siRNA and endosomes was observed when transfection was mediated by LAH6-X1L compared with LAH4-L1 (Supp. Fig. 7). Nystatin was the only inhibitor tested to significantly affect the reduction of GAPDH expression mediated by either peptide and hence the effect of nystatin pre-treatment on the intracellular distribution of siRNA in MCF-7 cells was also investigated (Fig. 7B/D). Little or no obvious effect of nystatin on LAH6-X1L mediated delivery of Cy-3 labelled siRNA was observed (Fig. 7D) with the distribution resembling that of the untreated cells. In contrast nystatin had a strong effect on the distribution of Cy-3 labelled siRNA delivered by LAH4-L1 (Fig. 7B). Whereas clusters of red label dissipate over time in untreated MCF-7 cells (Fig. 7A), in nystatin pre-treated cells, large numbers of red clusters appear trapped close to the internal boundaries of the cells. This is consistent with nystatin strongly disrupting the usual distribution of siRNA in the cell mediated by LAH4-L1 but not LAH6-X1L.

DISCUSSION

The potential of RNAi to be used in future therapeutic applications has reinvigorated research in the use of non-viral vectors to deliver nucleic acids safely and effectively to cells. The longer duration of a therapeutic effect observed in slowly dividing cells coupled with the cytosolic, as opposed to nuclear, location of action are considerable advantages for siRNA over DNA when considering their potential for therapeutic applications. However, at least two factors constitute increased challenges for siRNA over DNA delivery. Although it might be expected that cationic lipids, polymers and peptides that have been used to deliver plasmid DNA effectively will also perform similarly when called upon to deliver siRNA, the fundamental distinctions between DNA and siRNA may require a re-evaluation of the requirements of a non-viral delivery system [17]. In particular, following results obtained with lipidic [18] or polyethylenimine [19] delivery systems, it has been suggested that RNA, a stiffer molecule than DNA, may not condense in the same manner when complexed with a cationic agent and that the resultant particles may be too large for cellular entry via clathrin mediated endocytosis with a consequent reduction in gene silencing ability [17]. Furthermore, siRNA is potentially more prone to degradation by serum nucleases and hence the need for effective encapsulation of the nucleic acid may be more acute [17]. Understanding the specific requirements for siRNA delivery systems, in particular understanding the route of cellular uptake is key to their further development and adoption in future therapies.

Among non-viral vectors used for siRNA delivery, cationic cell-penetrating peptides (CPP) have attracted increasing attention. Sharing many similarities with antimicrobial peptides, they are able to interact with, disorder and cross cell membranes while binding to poly-anionic targets including nucleic acids [20]. One approach to using CPPs for siRNA delivery is to chemically conjugate oligonucleotides to the peptide ensuring that any peptide that enters the cell will necessarily be accompanied by the therapeutic molecule. Some doubts have been raised however about the abilities of such peptides to mediate translocation in the absence of non-conjugated helper peptides [21]. The ability of peptides, that are non-covalently bound to an oligonucleotide, to mediate nucleic acid transfer has however been demonstrated, most notably using the primary amphipathic peptide MPG [22]. MPG comprises a hydrophobic domain derived from the fusion sequence of HIV gp41 and a hydrophilic domain derived from the nuclear localisation sequence of SV40 T-antigen [22]. This peptide has been used successfully to deliver both plasmid DNA and siRNA to mammalian cells *in vitro* [23, 24] and indeed to primary cells and *in vivo* [5]. Disagreement on the route of delivery exists, since data that supports both endocytosis dependent [23] and independent uptake have been reported [24]. Taking this approach a stage further, a synthetic peptide with secondary amphipathicity has been reported recently that forms stable, non-covalent complexes with siRNA and has potent siRNA transfer capabilities, mediating delivery into both primary and suspension cell lines [25]. This peptide, CADY, comprises 20 residues combining aromatic tryptophan residues and cationic arginine residues that are segregated in two separate surfaces when the peptide adopts α -helix conformation in membranes or when bound to siRNA [26]. Again, it was reported that CADY and its siRNA cargo enters cells via a mechanism that is independent of the major endosomal pathway [25].

The present results confirm that the pH responsive amphipathic peptides described here are effective at binding siRNA non-covalently and delivering it to a variety of human cell lines, mediating potent gene silencing *in vitro*. High levels of specific silencing of GAPDH activity and expression in both cell lines tested, coupled with low toxicity, compare favourably with the two commercially available non-viral vectors. The potent silencing mediated by the pH responsive peptides suggests that, as with plasmid DNA delivery [8-14], complexes may indeed be taken up into endocytic pathways and that endosomal acidification aids the escape of siRNA to the cytosol. However, in light of the recent study where the similarly sized and amphipathic but non-pH responsive CADY peptide was reported to deliver siRNA via an endocytosis independent mechanism [25] and considering the possibility that siRNA/peptide complexes might be too large to enter via clathrin dependent endocytosis, the main endocytic pathway, we have employed histidine rich peptides that have a differing response to pH changes in order to identify whether a single or multiple pathway(s) exist. In particular, we investigated the relative size of DNA/peptide or siRNA/peptide complexes, characterised the effect of inhibitors on peptide mediated silencing of GAPDH expression and observed the entry of fluorescently labelled siRNA via live cell confocal imaging.

The comparison of the relative sizes of the peptide/siRNA and peptide/DNA complexes indicated that the nature of both the peptide and cargo determines the size of complexes prepared in 150 mM NaCl. In water, a strong electrostatic interaction is expected between the cationic peptide and anionic nucleic acids, leading to the formation of small and tight complexes. In the presence of salt, counter ions are expected to weaken the electrostatic interaction by shielding the effective charge of peptides and nucleic acids. Consistent with this, larger and more loose complexes were formed when the ionic strength of the environment is increased. The small molecular weight of siRNA allowed the formation of far smaller complexes, when either LAH6-X1L or LAH6-X1L-W were used in place of LAH4-L1, when compared with the corresponding DNA containing complexes. The greater

condensation induced by the LAH6 peptides may lead to such peptide/siRNA complexes being taken up by differing routes to those used by peptide/DNA complexes or LAH4-L1/siRNA complexes.

In order to understand the potentially differing mechanisms observed for siRNA uptake mediated by histidine rich peptides, we have selected peptides for the present study that respond at differing pH. In a parallel study, we have investigated the pH response of a range of histidine rich peptides, including those studied here (Iacobucci *et al*, unpublished results). Interestingly, we find that the effect of increasing the histidine content in the peptide leads to a considerable depression of the pK value for the main transition from α -helix to disordered conformations in solution. The value obtained for LAH4-L1 is 5.10 ± 0.15 whereas that for LAH6-X1L is 3.43 ± 0.01 . This indicates that the two peptides may differ considerably in their response to endosomal or even lysosomal acidification, escaping to the cytosol at different time points and consequently having differing sensitivities to inhibitors.

A recent study of the effectiveness of using inhibitors to study endocytic pathways concluded that inhibitors, including chlorpromazine, showed poor specificity for distinct endocytic pathways, reduced cell viability and were highly cell line dependent [27]. Nevertheless their use may be informative particularly when used, as here, under the same conditions on the same cell line to compare either cargo or related peptide vectors. Chloroquine has a positive effect on transfection mediated by polylysine [28], but has been shown previously [13] to have no effect on LAH4 mediated DNA transfection and here, no effect on pH responsive peptide mediated siRNA transfection is observed.

Chlorpromazine is considered to be an inhibitor of clathrin mediated endocytosis [29] and is shown here to substantially reduce delivery of plasmid DNA to MCF-7 cells. Although chlorpromazine is used as an inhibitor of one endocytic pathway only, this result is in agreement with previous work where sensitivity of gene transfer to the specific inhibitor of vacuolar-type H⁺-ATPase, bafilomycin A1, indicates endosomal acidification is required for effective delivery of cargo [10, 14]. A total sensitivity to bafilomycin A1, observed for pH responsive peptide LAH4-L1 mediated silencing of luciferase activity in 911 cells, suggests that endosomal acidification is also required for effective delivery of siRNA [15]. Here, cellular toxicity induced by bafilomycin A1 treatment prevented investigation of its effect on delivery. Notably however, inhibition of peptide mediated GAPDH expression with chlorpromazine is not significant when either the LAH4-L1 or LAH6-X1L peptides are used. This may implicate clathrin independent endocytic pathways in the uptake of peptide/siRNA complexes.

Confocal microscopy images of live cells taken at various time points were used to track the fate of fluorescently labelled cargo. When a Rab5a targeting label was used to mark clathrin derived endosomes, little or no co-localisation was observed when delivery was mediated by either peptide. Similarly, little or no co-localisation was observed for cargo and acidic compartments at early time points. Caveosomes are negative for staining with Rab5a and have a neutral luminal pH [33]. This adds further support to a clathrin-independent uptake mechanism for LAH4-L1 mediated uptake of siRNA, and implicates caveolae mediated endocytosis.

Caveolae are non-coated, smooth, flask-shaped vesicles of the plasma membrane and are smaller than clathrin-coated vesicles with a diameter of 50-90 nm. Nystatin is often used as an inhibitor of caveolae mediated endocytosis since their maintenance is dependent on cholesterol to which polyene antibiotics, such as nystatin, bind [30]. Nystatin has no inhibitory effect on the delivery of plasmid DNA to MCF-7 cells mediated by two of the three peptides tested here though a significant ($p < 0.05$) but modest inhibitory effect is

observed when delivery is mediated by LAH4-L1. Nystatin had a far more notable effect on the uptake of peptide/siRNA complexes. For LAH4-L1, a substantial and significant ($p < 0.05$) reduction in peptide mediated silencing is observed as is a notable effect on the intracellular distribution of labelled siRNA, implicating caveolae, rather than clathrin, mediated endocytosis for uptake of LAH4-L1/siRNA particles as opposed to LAH4-L1/DNA complexes. For LAH6-X1L however, instead of a reduction in peptide mediated silencing of GAPDH expression, treatment with nystatin modestly enhances silencing mediated by the peptide ($p < 0.05$). This enhancement may be accounted for by yet to be characterised, effects of formation of pores or through sequestration of cholesterol into the pore structure induced by nystatin challenge [31, 32] which may facilitate direct transfer across the plasma membrane providing an alternative mechanism for siRNA delivery.

Taken together, the relative particle sizes of peptide/DNA and peptide/siRNA complexes, the responses to inhibition of endocytic pathways and the live cell confocal imaging suggest that the bulk of LAH4-L1 mediated siRNA uptake occurs via mechanisms that are similar to, but distinct from those responsible for DNA delivery. Clathrin mediated endocytosis is almost exclusively used for uptake of peptide/DNA complexes. In contrast, cholesterol dependent, and hence potentially also caveolae dependent, endocytosis appears to be favoured when peptide/siRNA complexes enter the cell. Given the relative sizes of the complexes and the caveolae (50-90 nm), uptake of peptide/siRNA complexes by this route may seem even less likely than uptake via clathrin mediated endocytosis, where a maximum size for clathrin coated vesicles of 150 nm is generally accepted [35]. However, studies on the uptake of fluorescent latex beads of defined size indicate that entry of particles as large as 500 nm is dependent on cholesterol [36] and cholesterol-dependent and caveolae dependent endocytosis may not therefore be synonymous. Interestingly, this study showed that above a threshold of 200 nm, the predominant pathway for internalisation shifted from clathrin mediated to a cholesterol dependent process [36].

The differential intracellular distribution of siRNA and the differing effects of nystatin on silencing and uptake mediated by LAH4-L1 and LAH6-X1L suggests the latter mediates uptake by alternative mechanisms. A comparison of the effects of endocytic inhibitors on DOTAP/DNA lipoplex and PEI/DNA polyplex uptake indicated that polyplex uptake can proceed via both clathrin and caveolae mediated endocytosis [34]. Polyplexes that enter via clathrin mediated endocytosis are targeted to the lysosomal compartment for degradation and hence did not contribute to the observed transfection which was solely attributed to polyplexes that enter via caveolae mediated endocytosis [34]. By analogy, since LAH6-X1L responds to changes in acidity only at rather low pH, this peptide may promote escape from lysosomes thus enabling clathrin-mediated endocytosis as a viable alternative when caveolae mediated pathways are inhibited. The experiments with inhibitors and the live cell confocal imaging are therefore consistent with two models for siRNA uptake mediated by peptides such as LAH6-X1L, which form smaller particles and whose conformational response is at a much more acidic pH. Direct transfer of siRNA across the membrane may be facilitated by the peptide. Alternatively, siRNA/LAH6-X1L complexes are internalised by either clathrin or caveolae mediated endocytosis and processed to lysosomes with peptide/siRNA being able to escape from these compartments only at a very late stage.

Since both LAH4-L1 and LAH6-X1L peptides can effectively mediate silencing of GAPDH expression, the present results indicate that multiple routes for pH responsive peptide mediated siRNA delivery exist and differ from those used for peptide mediated DNA delivery. Since both endocytosis dependent and independent uptake routes have been suggested for related amphipathic, but non pH responsive, peptides [23-25], this area provides scope for further optimisation of the uptake and delivery efficiency of such systems. Considering the recent success when using primary amphipathic cell penetrating

peptides to deliver therapeutic siRNA *in vivo* [5], further development and adaptation of pH responsive secondary amphipathic peptides, focussing on the route of uptake, will be of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Medical Research Council (NIRG G0801072/87482 to AJM), the Wellcome Trust (VIP Award to AJM) and the University of Hong Kong Seed Funding Programme (JKWL). JKWL was the holder of a Maplethorpe Postdoctoral Fellowship of the University of London. JKWL and AJM thank Ms Mia So (Department of Pharmacology and Pharmacy, The University of Hong Kong) for her help in DNA transfection study, Mr Tony Chan (Department of Anatomy, The University of Hong Kong) and Ms Jing Guo (Faculty Core Facility, LKS Faculty of Medicine, The University of Hong Kong) for their assistance in the live cell confocal imaging experiment. We are grateful to Dr Antoine Kichler for critical reading of the manuscript.

REFERENCES

- [1]. Karagiannis TC, El-Osta A. RNA interference and potential therapeutic applications of short interfering RNAs. *Cancer Gene Ther.* 2005; 12:787–795. [PubMed: 15891770]
- [2]. Dallas A, Vlassov AV. RNAi: A novel antisense technology and its therapeutic potential. *Med. Sci. Monit.* 2006; 12:RA67–RA74. [PubMed: 16572063]
- [3]. Barik B. Silence of the transcripts: RNA interference in medicine. *J. Mol. Med.* 2005; 83:764–773. [PubMed: 16028076]
- [4]. Li SD, Huang L. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. *Gene Ther.* 2006; 13:1313–1319. [PubMed: 16953249]
- [5]. Crombez L, Morris MC, Dufort S, Aldrian-Herrada S, Nguyen Q, McMaster G, Coll J-L, Heitz F, Divita G. Targeting cyclin B1 through peptide-based delivery of siRNA prevents tumour growth. *Nucleic Acids Res.* 2006; 37:4559–4569. [PubMed: 19483097]
- [6]. Midoux P, Pichon C, Yaounac J-J, Jaffrès P-A. Chemical vectors for gene delivery: a current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carriers. *Br. J. Pharmacol.* 2009; 157:166–178. [PubMed: 19459843]
- [7]. Prongidi-Fix L, Sugawara M, Bertani P, Raya J, Leborgne C, Kichler A, Bechinger B. Self-promoted cellular uptake of peptide/DNA transfection complexes. *Biochemistry.* 2007; 46:11253–11262. [PubMed: 17848098]
- [8]. Mason AJ, Gasnier C, Kichler A, Prévost G, Aunis D, Metz-Boutigue M-H, Bechinger B. Enhanced membrane disruption and antibiotic action against pathogenic bacteria by designed histidine-rich peptides at acidic pH. *Antimicrob. Agents Chemother.* 2006; 50:3305–3311. [PubMed: 17005809]
- [9]. Mason AJ, Martinez A, Glaubitz C, Danos O, Kichler A, Bechinger B. The antibiotic and DNA transfecting peptide LAH4 selectively associates with, and disorders, anionic lipids in mixed membranes. *FASEB J.* 2006; 20:320–322. [PubMed: 16352649]
- [10]. Kichler A, Mason AJ, Bechinger B. Cationic amphipathic histidine-rich peptides for gene delivery. *Biochim. Biophys. Acta.* 2006; 1758:301–307. [PubMed: 16540079]
- [11]. Mason AJ, Leborgne C, Moulay G, Martinez A, Danos O, Bechinger B, Kichler A. Optimising histidine rich peptides for efficient DNA delivery in the presence of serum. *J. Control. Release.* 2007; 118:95–104. [PubMed: 17254661]
- [12]. Kichler A, Leborgne C, Danos O, Bechinger B. Characterisation of the gene transfer process mediated by histidine-rich peptides. *J. Mol. Med.* 2007; 85:191–201. [PubMed: 17111132]
- [13]. Kichler A, Leborgne C, März J, Danos O, Bechinger B. Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. *Proc. Natl. Acad. Sci. USA.* 2003; 96:1564–1568. [PubMed: 12563034]

- [14]. Lan Y, Langlet-Bertin B, Abbate V, Vermeer LS, Kong X, Sullivan K, Leborgne C, Scherman D, Hider RC, Drake AF, Bansal S, Kichler A, Mason AJ. Incorporation of 2,3-diaminopropionic acid in linear cationic amphipathic peptides produces pH sensitive vectors. *ChemBioChem*. 2010; 11:1266–1272. [PubMed: 20480482]
- [15]. Langlet-Bertin B, Leborgne C, Scherman D, Bechinger B, Mason AJ, Kichler A. Design and evaluation of histidine-rich amphipathic peptides for siRNA delivery. *Pharm. Res.* 2010; 27:1426–1436. [PubMed: 20393870]
- [16]. Bryant JC. Earle's balanced salt solution: Preparation of the saline. *Tissue Culture Association Manual*. 1975; 1:185–187.
- [17]. Gary DJ, Puri N, Won Y-Y. Polymer-based siRNA delivery: perspectives on the fundamental and phenomenological distinctions from polymer-based DNA delivery. *J. Cont. Rel.* 2007; 121:64–73.
- [18]. Spagnou S, Miller AD, Keller M. Lipidic carriers of siRNA: differences in the formulation, cellular uptake, and delivery with plasmid DNA. *Biochemistry*. 2004; 43:13348–13356. [PubMed: 15491141]
- [19]. Grayson ACR, Doody AM, Putnam D. Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery *in vitro*. *Pharm. Res.* 2006; 23:1868–1876. [PubMed: 16845585]
- [20]. Henriques ST, Melo MN, Castanho MARB. Cell-penetrating peptides and antimicrobial peptides: how different are they? *Biochem. J.* 2006; 399:1–7. [PubMed: 16956326]
- [21]. Turner JJ, Jones S, Fabani MM, Ivanova G, Arzumano AA, Gait MJ. RNA targeting with peptide conjugates of oligonucleotides, siRNA and PNA. *Blood Cells Mol. Diseases*. 2007; 38:1–7.
- [22]. Morris MC, Vidal P, Chaloin L, Heitz F, Divita G. A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucleic Acids Res.* 1997; 25:2730–2736. [PubMed: 9207018]
- [23]. Simeoni F, Morris MC, Heitz F, Divita G. Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. *Nucleic Acids Res.* 2003; 31:2717–2724. [PubMed: 12771197]
- [24]. Veldhoen S, Laufer SD, Trampe A, Restle T. Cellular delivery of small interfering RNA by a non-covalently attached cell-penetrating peptide: quantitative analysis of uptake and biological effect. *Nucleic Acids Res.* 2006; 34:6561–6573. [PubMed: 17135188]
- [25]. Crombez L, Aldrian-Herrada G, Konate K, Nguyen QN, McMaster GK, Brasseur R, Heitz F, Divita G. A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells. *Mol. Ther.* 2009; 17:95–103. [PubMed: 18957965]
- [26]. Konate K, Crombez L, Deshayes S, Decaffmeyer M, Thomas A, Brasseur R, Aldrian G, Heitz F, Divita G. Insight into the cellular uptake mechanism of a secondary amphipathic cell-penetrating peptide for siRNA delivery. *Biochemistry*. 2010; 49:3393–340. [PubMed: 20302329]
- [27]. Vercauteren D, Vandenbroucke RE, Jones AT, Rejman J, Demeester J, De Smedt SC, Sanders NN, Braeckmans K. The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Mol. Ther.* 2010; 18:561–569. [PubMed: 20010917]
- [28]. Erbacher P, Roche AC, Monsigny M, Midoux P. Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/lactosylated polylysine complexes. *Exp. Cell Res.* 1996; 225:186–194. [PubMed: 8635511]
- [29]. Wang LH, Rothberg KG, Anderson RG. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J. Cell Biol.* 1993; 123:1107–1117. [PubMed: 8245121]
- [30]. Rothberg KG, Ying Y-S, Kamen BA, Anderson RGW. Cholesterol controls the clustering of the glycopospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. *J. Cell Biol.* 1990; 111:2931–2938. [PubMed: 2148564]
- [31]. Récamier KS, Hernández-Gómez A, González-Damián J, Ortega-Blake I I. Effect of membrane structure on the action of polyenes: I. nystatin action in cholesterol- and ergosterol-containing membranes. *J. Membrane Biol.* 2010; 237:31–40. [PubMed: 20872217]

- [32]. González-Damián J, Ortega-Blake I. Effect of membrane structure on the action of polyenes II: nystatin activity along the phase diagram of ergosterol and cholesterol-containing POPC membranes. *J. Membrane Biol.* 2010; 237:41–49. [PubMed: 20871987]
- [33]. Parton RG, Simons K. The multiple faces of caveolae. *Nat. Rev. Mol. Cell Biol.* 2007; 8:185–194. [PubMed: 17318224]
- [34]. Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol. Ther.* 2005; 12:468–474. [PubMed: 15963763]
- [35]. Bishop NE. An update on non-clathrin-coated endocytosis. *Reviews in Medical Virology.* 1997; 7:199–209. [PubMed: 10398484]
- [36]. Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem. J.* 2004; 377:159–69. [PubMed: 14505488]

Figure 1.

Hydrodynamic diameter of peptide/siRNA (A, B) and peptide/DNA (C, D) complexes prepared in water (A, C) or 150 mM NaCl (B, D) measured by photon correlation spectroscopy (n=3). (* and +) $p < 0.05$ compared with equivalent DNA containing complex or LAH4-L1 complex respectively.

Figure 2.

Agarose gel retardation assay of peptide/siRNA binding and nuclease protection study. Peptide/siRNA complexes were prepared at different ratio (w/w). For nuclease protection assay, the complexes were incubated with ribonuclease A at 37°C for 30 minutes. The enzyme activity was stopped by ribonuclease inhibitor and the complexes were dissociated using 2 mM SDS. Untreated siRNA, siRNA treated with nuclease and siRNA treated with SDS were served as control. Electrophoresis was carried out at 100V for 20 minutes and the gel was stained with GelRed™.

Figure 3.

GAPDH activity silencing in A549 cells, mediated by the cationic histidine rich peptide LAH4-L1, expressed (A) as arbitrary fluorescence units with both positive and negative siRNA shown and (B) with positive siRNA expressed as a percentage of the negative siRNA control (specific knockdown). (*) $p < 0.05$ compared with negative control. The ratios (w/w) of 10 nM siRNA to peptide are given in parentheses. Experiment performed with Silencer Select siRNA (Human, Mouse, Rat).

Figure 4.

Silencer (Human, Mouse, Rat) GAPDH siRNA transfection in MCF-7 cells analysed by Western Blot. (A) Cells were transfected with GenMute™ or peptide/siRNA complexes containing GAPDH siRNA (+) or negative control siRNA (-). Protein analysis was carried out 72 hours after transfection. β -Actin served as an internal control for equal total protein loading. The bands were analysed by densitometry with the density of the GAPDH band normalised to that of the actin band of the corresponding sample (B). The ratio of 50 nM siRNA to peptide (w/w) is indicated in each case. The effect of including 10% FBS in the media on GAPDH siRNA transfection in MCF-7 cells analysed by Western Blot (C). The weight ratio of peptide to 50 nM siRNA is 10:1. (*) $p < 0.05$ or (+) $p < 0.1$ improvement compared with GenMute™ in the corresponding experiment.

Figure 5.

The effect of inhibitors on peptide mediated DNA and siRNA (50 nM) transfection. Luciferase activity following peptide mediated transfection in A549 (**A**) and MCF-7 (**B**) cells is shown for cells pre-treated with either chlorpromazine (10 μ g/ml) or nystatin (25 μ g/ml) relative to untreated cells. Silencing of GAPDH expression in MCF-7 cells, mediated by LAH6-X1L and LAH4-L1, following treatment with inhibitors is shown relative to silencing in untreated cells as a Western Blot (**C**) and as densitometry of three independent repeats (**D**). The weight ratio of peptide to nucleic acid is 10:1 for both DNA and siRNA transfection. (*) $p < 0.05$ compared with silencing in the absence of inhibitors.

Figure 6.

Live cell confocal imaging reveals differential localisation of LAH4-L1 or LAH6X1L peptide/siRNA complexes (10:1 w/w) in MCF-7 cells with reference to endosomes. Cy3-labelled siRNA appears red while GFP, transiently expressed in early endosomes using rab5a as a marker, appears green. Images of MCF-7 cells containing siRNA delivered by LAH4-L1 (**A, C, E**) and LAH6X1L (**B, D, F**) are shown at 1 hour (**A, B**), 4 hours (**C, D**) and 16 hours (**E, F**) after transfection, Scale bar = 20 μ m.

Figure 7.

Live cell confocal imaging reveals differential localisation of LAH4-L1 or LAH6-X1L peptide/siRNA complexes (10:1 w/w) in MCF-7 cells with reference to lysosomes. The effect of treatment with nystatin is also shown. Cy3-labelled siRNA appears red while LysoTracker® DND-26, administered 5 minutes prior to imaging, appears green and accumulates in cellular compartments with low internal pH. Images of MCF-7 cells containing siRNA delivered by LAH4-L1 (A, B) and LAH6-X1L-W (C, D) are shown in the absence (A,C) or presence of nystatin treatment (B,D) between 21 and 22 hours after transfection. Scale bar = 20 μ m.

Table 1

Sequences of LAH4 or LAH6 derivatives used in this study. Histidine residues are marked in bold

Peptide	Sequence	Peptide length (residues)	Average Hydrophobicity(<i>H</i>) [*]
LAH4-L1	KKALLA H ALHLLALLALH L AH A LKKA	26	0.050
LAH6-X1L	K H KLL H LLHLLALLAL H LLHLLK H K	25	0.024
LAH6-X1L-W	K H KLL H LLHLLALLWL H LLHLLK H K	25	0.028

^{*} As determined by the Eisenberg (1982) Consensus scale (Ile, 0.73; Phe, 0.61; Val, 0.54; Leu, 0.53; Trp, 0.37; Met, 0.26; Ala, 0.25; Gly, 0.16; Cys, 0.04; Tyr, 0.02; Pro, -0.07; Thr, -0.18; Ser, -0.26; His, -0.40; Glu, -0.62; Asn, -0.64; Gln, -0.69; Asp, -0.72; Lys, -1.1; and Arg, -1.8).