(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 November 2014 (20.11.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/184584 A1

(51) **International Patent Classification**:
C07K 14/00 (2006.01)

(21) **International Application Number**:
PCT/GB2014/051519

(22) **International Filing Date**:
16 May 2014 (16.05.2014)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
1308860.4 16 May 2013 (16.05.2013) GB

(71) **Applicant**: **KING'S COLLEGE LONDON** [GB/GB]; An Institute Incorporated by Royal Charter of Strand, London, Greater London WC2R 2LS (GB).

(72) **Inventors**: **MASON, James**; King's College London, Institute of Pharmaceutical Science, Franklin-Wilkins Building, 150 Stamford Street, London Greater London SE19NH (GB). **BANSAL, Sukhi**; King's College London, Institute of Pharmaceutical Science, Franklin-Wilkins Building, 150 Stamford Street, London Greater London SE19NH (GB). **ABBATE, Vincenzo**; King's College London, Institute of Pharmaceutical Science, Franklin-Wilkins Building, 150 Stamford Street, London Greater London SE19NH (GB).

(74) **Agent**: **WITHERS & ROGERS LLP**; 4 More London Riverside, London, Greater London SE1 2AU (GB).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

[Continued on next page]

(54) **Title**: DELIVERY PEPTIDES

(57) **Abstract**: There is provided a cationic amphipathic helical peptide for binding to and delivering a cargo molecule into a cell, the peptide comprising five or more 2,3-diaminopropionic acid (Dap) residues. In particular, the peptides can be used to deliver proteins and nucleic acids into mammalian cells. Preferably, the peptide comprises six Dap residues and these may ne methylated.

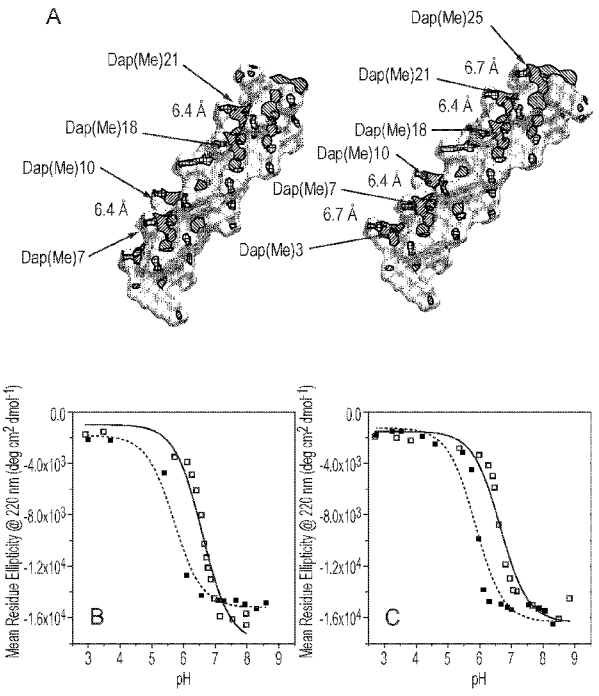


FIG. 1

WO 2014/184584 A1

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

Delivery Peptides

Field of the Invention

The present invention relates to peptides which can be used to deliver molecules into cells, in particular, mammalian cells. The molecules that can be delivered include proteins and nucleic acids.

Background to the Invention

The great therapeutic potential of RNA interference (RNAi) is threatened by waning interest from pharma in developing small interfering RNA (siRNA) based drugs (1). The root cause of much of the frustration regarding the development of RNAi based therapies is the inability to successfully deliver siRNA to target tissues (1). Further, gene knockdown experiments that are routinely performed in standard cell lines are often difficult to transfer to more biologically relevant model systems including primary and/or suspension human cell types.

Of the numerous non-viral delivery systems conceived to overcome this hurdle (2), we have focussed our attention on cationic amphipathic peptides containing pH responsive residues that are capable of harnessing the changes in pH associated with endocytosis and endosomal acidification to promote release of cargo to the cell cytosol (3,4). These peptides are capable of delivering plasmid DNA and siRNA to mammalian cell lines in vitro with both high efficacy and low associated toxicity (3-6), are proven to deliver antisense oligonucleotides to patient derived primary fibroblasts (7) and have also been successfully applied in vivo to deliver protein based vaccines adjuvanted with Toll-like receptor 9 agonist CpG oligonucleotide (8). The observation that pH responsive peptides have such properties has led us to consider how their delivery efficacy can be improved to enable delivery to suspension cell lines and primary cells and hence widen the scope of potential in vivo applications. Though sharing many properties with other polycationic delivery systems, the pH responsive peptides have an additional functionality which leads to a dramatic increase in gene delivery efficacy when compared with analogous peptides comprising cationic residues, such as ornithine or lysine, which are not sensitive to pH changes in the desired range (9-11). This behaviour is linked to pH dependent changes in conformation in solution (11), topology (12), disordering activity (9,13) in the target endosomal membrane and nucleic acid binding affinity (13,14).

To understand the role of the pH response in determining delivery efficacy, the behaviour during endosomal acidification must be considered. Acidification of the endosomes causes a dramatic change in the charge state of the carrier peptides as it switches from a nominal charge state of +5 to either +9 or +11, depending on the number of pH responsive residues that have been incorporated. Since electrostatic interactions determine much of the binding affinity of the peptide for the nucleic acid, the

affinity for each peptide for the cargo increases (13, 14) even though the actual effect on the complex is a release of peptides (14). Since the complex is fully saturated with peptide at neutral pH, all possible binding opportunities between nucleic acid and peptide are fulfilled. When the cationic charge on the peptide molecules doubles during endosomal acidification, only half of the bound peptides can now be accommodated by the nucleic acid as the charge state of the nucleic acid and the number of possible electrostatic interactions remains unchanged (14). Increasing the complement of pH responsive residues should enhance peptide release from peptide-nucleic acid complexes and consequent endosomal membrane disordering and delivery. However, a further hurdle exists since the peptides may exist either as monomers or small aggregates in aqueous solutions (15). Coulombic interactions, that exist between the protonated histidine residues when the peptide adopts an α-helix conformation in its self-associated state, constitute such a barrier that the protonation of the histidine residues and concomitant dissociation of the peptide into the monomeric and membrane accessible form may occur at a pH that is not achievable in endosomes when the histidine content is increased (13). Increasing the histidine content also reduced the size of siRNA/peptide complexes and this, together with the more acidic response, altered the uptake route of this cargo leading to much poorer than expected delivery (6).

Dap rich peptides respond at a higher pH than histidine containing analogues (11) but still respond in a range that could be exploited to drive endosomal release and this suggests a hypothesis: the same Coulombic interactions that acidified the pH response of histidine enriched peptides to a point that they were unable to act in the endosomes can be used to optimise the pH response of Dap enriched peptides (Fig. 1A). Increasing the number of Dap residues will have the added benefit that the change in nominal charge state during acidification would be increased (+5 to +11 rather than +9) and more peptides theoretically released to afford much better endosomal escape and nucleic acid delivery. The inventors further hypothesised that the observed higher toxicity of Dap in its primary amine form (11) would be mitigated by interrupting inter-molecular hydrogen bonding networks through alkylation of Dap side chain amino groups.

Having prepared peptides to test these hypotheses, the inventors monitored the pH dependent conformational switches of a series of Dap rich peptides with varying number of pH responsive elements and N-alkylation using CD spectroscopy in aqueous solution. The strongly beneficial effect of these modifications on both peptide mediated plasmid DNA and siRNA transfer is shown for a range of adherent cell lines including A549 adenocarcinomic human alveolar basal epithelial cells, MCF-7 human breast cancer cells, human umbilical vein endothelial cells (HUVEC) and differentiated THP-1 cells. Dap rich peptides with a tuned pH response are, in particular, also highly effective at mediating siRNA transfer to suspension monocyte THP-1 cells.

WO 2013/001041 discloses peptides primarily containing four histidine residues (referred to as LAH4). This document suggests that the histidine residues could be changed for other residues. However, the only relevant data in this document which relates to peptides in which the four histidine residues have been swapped for four lysine residues (LAK4) show that such peptides do not provide any effective transduction. In addition, this document discloses a peptide containing eight histidine residues (LAH8) but this peptide also does not provide any effective transduction.

Summary of the Invention

In a first aspect of the invention, there is provided a cationic amphipathic helical peptide for binding to and delivering a cargo molecule into a cell, the peptide comprising five or more 2,3-diaminopropionic acid (Dap) residues.

In a preferred embodiment, there is provided a cationic amphipathic helical peptide for binding to and delivering a cargo molecule into a cell, the peptide comprising six 2,3-diaminopropionic acid (Dap) residues.

The peptide is cationic meaning that it is positively charged. The peptide is positively charged so that it can bind to a cargo molecule which can be delivered into a cell. These cargo molecules are preferably negatively charged. Suitable molecules include peptides and nucleic acids such as DNA and RNA. In some embodiments, the cargo molecule may be a siRNA.

The peptide should be positively charged at neutral pH (i.e. a pH of about 7). In order for the peptide to be positively charged, it should contain one or more amino acids which have a positively charged group on the side chain of the amino acid at neutral pH. Any suitable positively charged amino acids can be used, including those which are naturally occurring and non-naturally occurring. For example, amino acids with a positively charged group on the side chain at neutral pH include lysine, arginine, ornithine and 2,4-diaminobutyric acid inter alia. Preferably, the peptide comprises one or more positively charged amino acid residues selected from lysine, arginine and ornithine. The peptide may comprise one or more positively charged amino acid residues selected from lysine and arginine. In some embodiments, the peptide comprises one or more lysine residues. The skilled person will appreciate that where multiple positively charged residues are contained in the peptide, they may be the same or different residues.

The peptide may comprise a plurality of positively charged (at neutral pH) amino acid residues. For example, the peptide may comprise 2, 3, 4, 5, 6, 7 or 8 positively charged amino acid residues such as lysine, arginine, ornithine and 2,4-diaminobutyric acid. In some embodiments, the peptide may comprise 3, 4, 5, 6 or 7 positively charged amino acid residues such as lysine, arginine and ornithine.

In further embodiments, the peptide may comprise 3, 4, 5 or 6 positively charged amino acid residues such as lysine, arginine and ornithine. The peptide may comprise 3, 4 or 5 positively charged amino acid residues such as lysine, arginine and ornithine. In particular embodiments, the peptide may comprise 4 positively charged amino acid residues such as lysine, arginine and ornithine. The positively charged amino acid residues may be selected from lysine and arginine. In some embodiments, the plurality of positively charged amino acid residues are all lysine residues.

The positively charged residues may be located close to or at one or both ends of the peptide. This means that the molecule to be delivered into a cell will be bound to the peptide at one or both ends of the peptide.

The positively charged residues may be located close to or at one end of the peptide. When they are located at the end of the peptide, the amino acid residues at the C-terminal or N-terminal end will be positively charged residues. For example, 2 or 3 lysine residues may be located at one end of the peptide.

When the positively charged residues are located close to an end of the peptide, this means that there may be 1 or 2 non-positively charged amino acid residues (and preferably not negatively charged amino acid residues) at the end of the peptide followed by the positively charged amino acid residues. For example, the residues at one end of the peptide may be Z-lysine-lysine, where Z is at the C- or N-terminal end of the peptide and is a non-positively charged amino acid residue (and preferably not a negatively charged amino acid residue).

In some embodiments, another amino acid residue may be present within the positively charged residues so that the positively charged residues are not contiguous. For example, the sequence of residues may be lysine-Z-lysine, where Z is a non-positively charged amino acid residue (and preferably not a negatively charged amino acid residue). This may occur when the positively charged residues are at the end of the peptide or close to the end of the peptide.

The skilled person will appreciate that some amino acids which are not charged at neutral pH may be positively charged under acidic conditions. The peptide may comprise such amino acid residues. However, these are not taken into account for determining whether the peptide is cationic as this should be measured at neutral pH (i.e. about 7).

The overall charge state of the peptide at neutral pH is preferably between +3 and +7. This is calculated by subtracting the number of negatively charged groups in the peptide from the number of positively charged groups in the peptide. In some embodiments, the overall charge state of the peptide

at neutral pH is between +4 and +6. In particular embodiments, the overall charge state of the peptide at neutral pH is +5.

When calculating the overall charge state of the peptide, it is necessary to take into account the NH_3^+ group at the N-terminus of the peptide. This is positively charged at neutral pH so that it will add an extra charge to the overall charge state of the peptide as there is no compensating negative charge at the C-terminus. Therefore, a peptide containing four lysine residues will have an overall charge state of +5 at neutral pH, the five positive charges coming from the four lysine residues and the NH_3^+ group at the N-terminus of the peptide.

The peptide comprises an amphipathic helix which means that it comprises a helix having a hydrophobic surface and a hydrophilic surface. These surfaces are generally formed by hydrophobic and hydrophilic side chains of the amino acid residues. Preferably, the helix is an α-helix.

The 2,3-diaminopropionic acid (Dap) residues in the peptide are polar meaning they are hydrophilic. Therefore, the Dap residues are preferably located along one side (or face) of the helix to form the hydrophilic surface. The hydrophilic surface may comprise additional hydrophilic amino acid residues such as serine, threonine, asparagine and glutamine. The hydrophilic surface may contain one or two non-hydrophilic amino acid residues without affecting the hydrophilic nature of the surface. For example, the hydrophilic surface may comprise a hydrophobic residue such as alanine or leucine.

When the Dap residues are located along one side of the helix, a Dap residue will generally appear every third or fourth residue in the amino acid sequence in view of the fact that a helix normally contains 3.6 amino acids per turn. However, a Dap residue may not appear at every turn in the peptide so there may be a larger gap before the next Dap residue appears in the sequence. This is likely to be roughly a multiple of 3.6 so, for example, there may be a gap of 6 or 7 amino acids in the sequence between Dap residues if a turn is missed. When the peptide comprises six Dap residues, the six Dap residues are preferably positioned in two groups of three on the hydrophilic face of the helix. The two groups may have a gap between them. For example, the peptide may have three Dap residues which appear every third or fourth residue with a gap of about 6 or 7 residues before another three Dap residues which appear every third or fourth residue.

The hydrophobic surface of the helix is formed by hydrophobic amino acid residues. Suitable hydrophobic residues which can be used in an α-helix are well known to those skilled in the art and include naturally occurring and non-naturally occurring amino acids. For example, suitable hydrophobic residues include alanine, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine and tryptophan. In some embodiments, the hydrophobic surface comprises residues selected from

alanine, leucine and valine. The hydrophobic surface may comprise residues selected from alanine and leucine.

In some embodiments, the peptide does not comprise any histidine residues.

The characteristics of the α-helix in peptides are well known and it is well within the capabilities of a skilled person to select appropriate hydrophobic and hydrophilic amino acid residues in order to design a peptide which can form an α-helix and which has a hydrophobic surface and a hydrophilic surface. For example, the position of the amino acid side chains can be visualised using a helical wheel representation (see Mount DM (2004). Bioinformatics: Sequence and Genome Analysis (2 ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. ISBN 0-87969-712-1). Further, the skilled person would be well aware of amino acids which should not be used in the peptide as they are not conducive to the formation of an α-helix. For example, the peptide should preferably not include proline in the helical portion of the peptide. Further, the peptide should preferably not include glycine as this may induce flexibility destabilising the helix conformation.

The peptide comprises Dap residues. At neutral pH, the amine group on the side chain is uncharged. At acidic pHs, the amine group becomes protonated. This causes the overall charge of the peptide to increase. Preferably, the pKa of the peptide is between 5 and 6.8 so that the Dap residues become protonated at a pH between 5 and 6.8. It has been found that this provides the most effective delivery of the cargo molecule into the cell. In some embodiments, the pKa of the peptide is between 5 and 6 so that the Dap residues become protonated at a pH between 5 and 6. When the peptide is contained in an endosomal membrane, protonation of the peptide acts to destabilise the membrane so that the cargo molecule carried by the peptide is released into the cell.

The overall hydrophobicity of the peptide helps to determine the pH at which the Dap residues in the peptide will become protonated. Therefore, the hydrophobicity can be altered by changing some of the hydrophobic amino acid residues in the peptide to tune the pH response of the peptide.

In use, a molecule such as a siRNA is bound to the peptide and it is administered to a cell. The peptide with siRNA cargo is taken up by the cell by endocytosis into an endosome. This endosome is acidified which causes protonation of the Dap residues and an increase in the overall charge of the peptide. This has the effect of increasing delivery of the cargo molecule, e.g. siRNA, into the cell.

The side chain of the Dap residues may be unmodified so that it is a primary amine, i.e. the side chain is $-CH_2-NH_2$. However, in some embodiments, the amine group of one or more of the Dap residues may be alkylated. The amine group may be alkylated such that an alkyl group replaces one of the

hydrogen atoms to form a secondary amine, i.e. the side chain is -CH_2-NHR, where R is the alkyl group. Alternatively, the amine group may be alkylated such that an alkyl group replaces both of the hydrogen atoms to form a tertiary amine, i.e. the side chain is -CH_2-NRR', where R and R' are alkyl groups. The alkyl group may be a C_{1-6} alkyl. Preferably, the alkyl group is methyl, ethyl or propyl. More preferably, the alkyl group is methyl or ethyl. In particular embodiments, the alkyl is methyl such that the Dap residue can be methylated or dimethylated.

It is possible for the Dap residues of the peptide to have different modification statuses, i.e. one or more Dap residues may be unmodified, one or more Dap residues may be alkylated once and/or one or more Dap residues may be alkylated twice. However, preferably, the Dap residues all have the same modification status, i.e. all the Dap residues are unmodified, all the Dap residues are alkylated once or all the Dap residues are alkylated twice.

It has been found that alkylation of the Dap residues helps to reduce the toxicity of the peptide.

Where a Dap residue is modified twice, i.e. dialkylated, each alkyl group may be the same or different. For example, the Dap residue may be dimethylated. Alternatively, the Dap residue may have one methyl group and one ethyl group. Preferably, where the Dap residue is modified twice, both alkyl groups are the same.

The peptide comprises five or more Dap residues. Preferably, the peptide has 5, 6, 7 or 8 Dap residues. Particularly preferred is a peptide having six Dap residues (i.e. only six Dap residues).

The peptide may be between 20 and 50 amino acids in length. In some embodiments, the peptide may be between 20 and 40 amino acids in length. In other embodiments, the peptide may be between 20 and 35 amino acids in length. In further embodiments, the peptide may be between 20 and 30 amino acids in length.

In some embodiments, the peptide may be polymerised. For example, two or more peptides could be joined together with a linker between the peptides.

In particular embodiments, the peptide comprises one of the following sequences:

1) KKXLL AXLLX LLALL ALXLL XALKX K (SEQ ID NO. 1); or

2) KKXLL AXALX ALLAL LAXLA XALKX K (SEQ ID NO. 2),

wherein X represents a Dap residue which can be modified or unmodified, and wherein the leucine, alanine and lysine residues may be switched with another amino acid with similar properties, i.e. a conservative substitution. For example, the positively charged lysine may be switched with another

positively charged amino acid such as arginine. The hydrophobic leucine may be switched for another hydrophobic amino acid such as alanine or valine. The hydrophobic alanine may be switched for another hydrophobic amino acid such as leucine or valine.

In some embodiments, up to 15 amino acids, up to 10 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids or 1 amino acid selected from the leucine, alanine and lysine residues may be switched with another amino acid with similar properties.

In further embodiments, the peptide comprises one of the following sequences:
1) KKXLL AXLLX LLALL ALXLL XALKX K (SEQ ID NO. 1); or
2) KKXLL AXALX ALLAL LAXLA XALKX K (SEQ ID NO. 2),
wherein X represents a Dap residue which can be modified or unmodified.

In some embodiments, the peptide consists of one of the following sequences:
1) KKXLL AXLLX LLALL ALXLL XALKX K (SEQ ID NO. 1); or
2) KKXLL AXALX ALLAL LAXLA XALKX K (SEQ ID NO. 2),
wherein X represents a Dap residue which can be modified or unmodified.

The peptide may be made in any suitable way and a skilled person would be capable of producing such peptides. For example, the peptide could be produced using chemical synthesis.

In a second aspect of the invention, there is provided a cationic amphipathic helical peptide for binding to and delivering a molecule into a cell, the peptide comprising four 2,3-diaminopropionic acid (Dap) residues, wherein at least one of the amine groups on one of the Dap residues is alkylated.

The amine group may be alkylated such that an alkyl group replaces one of the hydrogen atoms to form a secondary amine, i.e. the side chain is $-CH_2-NHR$, where R is the alkyl group. Alternatively, the amine group may be alkylated such that an alkyl group replaces both of the hydrogen atoms to form a tertiary amine, i.e. the side chain is $-CH_2-NRR'$, where R and R' are alkyl groups. The alkyl group may be a C_{1-6} alkyl. Preferably, the alkyl group is methyl, ethyl or propyl. More preferably, the alkyl group is methyl or ethyl. In particular embodiments, the alkyl is methyl such that the Dap residue can be methylated or dimethylated.

It is possible for the four Dap residues of the peptide to have different modification statuses, i.e. one or more Dap residues may be unmodified, one or more Dap residues may be alkylated once and/or one or more Dap residues may be alkylated twice, as long as at least one Dap residue is alkylated. However,

preferably, the four Dap residues all have the same modification status, i.e. all the Dap residues are alkylated once or all the Dap residues are alkylated twice.

Where a Dap residue is modified twice, i.e. dialkylated, each alkyl group may be the same or different. For example, the Dap residue may be dimethylated. Alternatively, the Dap residue may have one methyl group and one ethyl group. Preferably, where the Dap residue is modified twice, both alkyl group are the same.

The other features described above for the first aspect of the invention are equally applicable to the second aspect of the invention.

Rather than having four Dap residues, the peptide may have 5, 6, 7 or 8 Dap residues. Preferably, the peptide has 6 Dap residues.

In particular embodiments, the peptide comprises one of the following sequences:
1) KKLAX ALXLL ALLWL XLAXA LKKA (SEQ ID NO. 3); or
2) KKALL AXALX LLALL ALXLA XALKK A (SEQ ID NO. 4),
wherein X represents a Dap residue, and wherein the leucine, alanine, lysine and tryptophan residues may be switched with another amino acid with similar properties, i.e. a conservative substitution. For example, the positively charged lysine may be switched with another positively charged amino acid such as arginine. The hydrophobic leucine may be switched for another hydrophobic amino acid such as alanine or valine. The hydrophobic alanine may be switched for another hydrophobic amino acid such as leucine or valine.

In some embodiments, up to 15 amino acids, up to 10 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids or 1 amino acid selected from the leucine, alanine, lysine and tryptophan residues may be switched with another amino acid with similar properties.

In further embodiments, the peptide comprises one of the following sequences:
1) KKLAX ALXLL ALLWL XLAXA LKKA (SEQ ID NO. 3); or
2) KKALL AXALX LLALL ALXLA XALKK A (SEQ ID NO. 4),
wherein X represents a Dap residue.

In some embodiments, the peptide consists of one of the following sequences:
1) KKLAX ALXLL ALLWL XLAXA LKKA (SEQ ID NO. 3); or
2) KKALL AXALX LLALL ALXLA XALKK A (SEQ ID NO. 4),
wherein X represents a Dap residue.

Also provided is a pharmaceutical composition comprising a peptide as described above bound to a cargo molecule and one or more pharmaceutically acceptable excipients. The cargo molecule may be a peptide or nucleic acid such as DNA or RNA. In one embodiment, the cargo molecule is a nucleic acid.

The pharmaceutical composition may be in the form of a dry powder prepared by spray drying or spray freeze drying. Preferably, the dry powder is prepared by spray drying. The dry powder formulation may be suitable for inhalation.

The pharmaceutical composition may comprise mannitol as carrier.

In a further aspect of the invention, there is provided a peptide described above for use in therapy.

Also provided is the peptide described above for use in delivering a cargo molecule to a cell, preferably a mammalian cell such as a human cell. The cargo molecule may be a peptide or nucleic acid such as DNA or RNA. In some embodiments, the cargo molecule may be a siRNA.

In a particular embodiment, there is provided a peptide described above for use in delivering a cargo molecule to cells of the lung.

Brief Description of the Figures

The invention will now be described in detail, by way of example only, with reference to the following figures:

Figure 1. Manipulating the pH response of Dap rich peptides. A surface representation of LADap(Me)4-L1 (left) and LADap(Me)6-L1 modelled as α-helices that likely exist in an oligomeric state at neutral to basic pH in suspension (**A**). Coulombic interactions potentially exist between the two pairs or two triads of ionisable Dap(Me) residues located close to each other on one surface of the amphipathic α-helix and will be greater for LADap(Me)6-L1. The change in CD at 220 nm for four peptides in aqueous suspension as detected by far-UV CD spectroscopy is plotted as a function of pH; LADap4-L1 (white boxes) and LADap6-L1 (black boxes) (**B**), LADap(Me)4-L1 (white boxes) and LADap(Me)6-L1 (black boxes) (**C**). Increasing the complement of either the primary amine Dap (**B**) or the N-methylated Dap(Me) (**C**), from four to six residues, causes a notably more acidic conformational response.

Figure 2. Nucleic acid transfer activity to various cell lines. Transfection of MCF-7 or A549 cells with a luciferase reporter gene by Lipofectamine 2000™, LAH4-L1 and a variety of Dap and N-methyl-Dap rich peptides (**A**). Specific knockdown of GAPDH in A549 (**B**) and MCF-7 (**C**) cells mediated by four Dap or Dap(Me) rich peptides or Lipofectamine 2000™ benchmarks and assessed by western blot 72 hours post transfection. Cells were transfected with peptide/siRNA complexes containing GAPDH siRNA (+) or negative control siRNA (-) and β-actin served as an internal control for equal protein loading and off target effects. Densitometry results are shown as an average of three independently repeated experiments (**D**). The effect of 0, 25 or 50% bronchoalveolar lavage fluid (BALF) on siRNA transfection of A549 cells is shown by Western Blot (**E**) and its densitometry (**F**). (*) and (+) $p < 0.05$ improvement relative to LAH4-L1 and LADap4-L1 respectively.

Figure 3. Specific knockdown of GAPDH in HUVEC cells mediated by four Dap or Dap(Me) rich peptides or Lipofectamine 2000™ benchmark and assessed by western blot either 72 or 48 hours post transfection for cells transfected with 100 or 150 nM siRNA respectively (**A**). Cells were transfected with peptide/siRNA complexes containing GAPDH siRNA (+) or negative control siRNA (-) and β-actin served as an internal control for equal protein loading and off target effects. Densitometry results are shown as an average of three independently repeated experiments (**B**). (*) $p < 0.05$ improvement relative to LAH4-L1. Toxicity of non viral vectors to HUVEC cells was assessed using the MTT assay 24 hrs after a 4 hr incubation in serum free media (**C**). Vectors were prepared as for siRNA transfection experiments with 150 nM siRNA and a peptide to siRNA weight ratio of 10:1.

Figure 4. Specific knockdown of GAPDH in differentiated, adherent macrophage (A) or suspension monocyte (**B**) THP-1 cells mediated by four Dap or Dap(Me) rich peptides or Lipofectamine 2000™ or siPORT benchmarks and assessed by western blot 72 hours post transfection. Cells were transfected with peptide/siRNA complexes containing GAPDH siRNA (+) or negative control siRNA (-) and β-actin served as an internal control for equal protein loading and off target effects. Densitometry results are shown for as an average of three independently repeated experiments (**C**). (+) and (*) $p < 0.05$ improvement relative to LAH4-L1 and Lipofectamine 2000™ or siPORT benchmarks respectively. Toxicity of non viral vectors to adherent macrophage or suspension monocyte THP-1 cells was assessed using the MTT assay 24 hrs after a 4 hr incubation (**D**). Vectors were prepared as for siRNA transfection experiments and the peptide to siRNA weight ratio was 10:1 in all cases.

Figure 5. Live cell confocal imaging reveals differential localisation of LAH4-L1 or LADap(Me)6-L1 peptide/siRNA complexes (10:1 w/w) in HUVEC with reference to lysosomes. The effect of treatment with chlorpromazine and 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 HUVEC containing siRNA delivered by LAH4-L1 (A,

B, C) and LADap(Me)6-L1 (D, E, F) are shown in the absence (A,D) or presence of chlorpromazine (B, E) or nystatin treatment (C, F) 24 hours after transfection. Scale bar = 20 μm.

Figure 6. Overview of the synthetic route for obtaining three different peptides from a single assembled resin-peptide sequence. The N-terminus and lysine residues were Boc protected. Dap (X) is Dde protected and sequential treatment with three different combinations of reagent provides either non-methylated-, N-methylated- or N,N-dimethylated-Dap containing cationic amphipathic peptides.

Figure 7. DNA transfection of A549 cells mediated by pH responsive peptides in optiMEM-1 or 50% BALF. Each well of a 24-well plate contained 1 μg of luciferase plasmid DNA. Lipofectamine™ 2000 was used as control. Transfection efficiency and protein level were evaluated at 48 h post-transfection. Transfection efficiency expressed as relative light unit (RLU)/mg protein. Bars shown as mean ± standard deviation (n = 6). Significance difference was determined using one way ANOVA analysis followed by Bonferroni's post test. *$p < 0.05$, ***$p < 0.001$.

Detailed Description of the Invention

Example 1 - Manipulating the pH response of 2,3-diaminopropionic acid rich peptides to mediate highly effective and non-toxic gene silencing

SUMMARY

Cationic amphipathic pH responsive peptides possess high in vitro and in vivo nucleic acid delivery capabilities and function by forming a non-covalent complex with cargo, protecting it from nucleases, facilitating uptake via endocytosis and responding to endosomal acidification by being released from the complex and inserting into and disordering endosomal membranes. We have designed and synthesised peptides to show how Coulombic interactions between ionizable 2,3-diaminopropionic acid (Dap) side chains can be manipulated to tune the functional pH response of the peptides to afford optimal nucleic acid transfer and have modified the hydrogen binding capabilities of the Dap side chains in order to reduce cytotoxicity. When compared with benchmark delivery compounds, the peptides are shown to have low toxicity and are highly effective at mediating gene silencing in adherent MCF-7 and A549 cell lines, primary human umbilical vein endothelial cells and both differentiated macrophage-like and suspension monocyte-like THP-1 cells.

MATERIAL AND METHODS

Materials - The peptides comprising natural amino acids (Table 1) were purchased from Pepceuticals Ltd (Nottingham, UK) as desalted grade. Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Boc-Lys(Boc)-OH•DCHA, NovaSyn TGR Resin with a modified Rink linker and (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) (HCTU)) Fmoc-Dap(ivDde)-

OH was purchased from Merck BioSciences, NovaBiochem, (Nottingham, UK) and AnaSpec (Fremont, CA). Peptide synthesis grade dimethylformamide (DMF), Oxyma, diisopropylcarbodiimide, 2,4,6-collidine and hydroxybenzotriazole were purchased from Aldrich. All other reagents were analytical grade or better.

Peptide design - A series of eleven cationic amphipathic peptides was used in the present study (Table 1) based upon either the most potent nucleic acid delivery peptide generated over previous studies, LAH4-L1 (9) or a shortened version, LAH (11). Designed to adopt an amphipathic α-helix in membranes, when bound to nucleic acids and in a self-associated state at high pH in aqueous media, the histidine residues will segregate on one face and, in an idealised helical wheel representation, present an angle of 80°. Here, we have studied the effect of replacing histidine with Dap and its methylated derivatives to determine their effect on the pH response of the peptide. The three shortened peptides comprising 24 amino acids are an evolution of our previous work (11) and are designed to show the effect of N,N-di-methylation of the four Dap residues on the biophysical and gene delivery properties. To evaluate the potential for modified peptides to provide significant and substantial improvements in gene silencing capabilities, a further eight peptides were conceived using the LAH4-L1 sequence as a template. Two derivatives of this peptide with four pH responsive residues were prepared; one comprising Dap (LADap4-L1) and one comprising N-methylation of Dap (LADap(Me)4-L1). Our previous work (11) suggested that peptides comprising four Dap residues only would have a pH response that is a little high to function optimally as a pH switch during endocytosis. However, further work indicates that increasing the number of pH responsive residues from four to six can cause a much more acidic pH response when the hydrophobicity of the peptide is adjusted accordingly (13). The final series of peptides therefore is based on a LAH6-L1 peptide, which has been obtained by replacing two alanine residues with histidines, rearranging the three C-terminal residues and adjusting the hydrophobicity of the peptide by replacing two further alanines with leucines. The resulting peptide has an average hydrophobicity of 0.02 on the Eisenberg (15) scale and can be compared with LAH4-L1 at 0.05, LAH at 0.027 and is slightly less hydrophobic than the LAH6 peptides in the previous study (13). The series comprising six pH responsive amino acids is analogous to the series described above comprising four residues with the exception that two di-methyl Dap derivatives (LADap(Me_2)6-L1 and the more hydrophilic LADap(Me_2)6-A1 were successfully prepared.

Peptide synthesis - NovaSynTGR resin with a modified Rink amide linker (0.22 mmol/g) was used for peptide synthesis on a 1 mmol scale. Fmoc deprotection was achieved with 20% piperidine in DMF (v/v) (2 x 7 min) and acylation of Fmoc protected amino acids was achieved using HCTU and collidine in the molar ratio (1:0.98:2) in peptide synthesis grade DMF using a fourfold excess for 1 hour under a nitrogen atmosphere. Exceptionally, Fmoc Dap(Dde)OH was incorporated using a six

fold excess or with DCI and OXYMA in a six fold excess. The acylation of Fmoc-Dap(Dde)-OH was extended for a minimum of six hours. A Boc group was incorporated at the N-terminus to avoid undesired alkylation of the N-terminal lysine. Successful acylations and deprotections were confirmed by trinitrobenzene sulphonic acid (TNBS) test. Complete deprotection of Dde protecting groups was achieved by incubation of the resin in 2% hydrazine/DMF (v/v) (2 x 30 min) and confirmed following a small scale cleavage and analytical RP-HPLC and MALDI–TOF mass spectrometry.

Solid-phase N-methylation of DAP residues - Following removal of the Dde protecting group, Resin bound LADap4-L1 (200 mg, 44 μmol) was treated with 2-nitrobenzene sulfonyl chloride (2-Nbs-Cl) (117 mg, 528 μmol) and collidine (106 mg, 880 μmol) in 3 ml DMF for 3 hours. The resin was washed with DMF and dichloromethane (DCM) before 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene (MTDB) (821 mg, 528 μmol) and methyl 4-nitrobenzene sulfonate (MSNBC) (191 mg, 880 μmol) were added in 4 ml DMF for 45 minutes and the resin washed again thoroughly with DMF and DCM. 20 eq. 1,8-diazabicyclo [5,4,0]undec-7-ene (DBU) (134 mg, 880 μmol) and 2-mercaptoethanol (ME) (138 mg, 1.76 mmol) were added in 4 ml DMF and a distinct yellow colour was noted. After 45 minutes the resin bound peptide was washed thoroughly with DMF, DCM and MeOH and dried under vacuum.

Synthesis of NN-dimethylated Dap containing peptides - For the synthesis of LADap(Me_2), Fmoc-Dap(Me_2)-OH was incorporated in the presence of HOBt/DIPCDI in a 1:1 (v/v) DMF:DMSO mixture. However, extensive recoupling was necessary to achieve complete acylation as indicated by TNBS resin tests. For the synthesis of LADap(Me_2)6-L1 and LADap(Me_2)6-A1 a solid-phase reductive amination approach was undertaken by adapting a previously described method (16). Resin bound LADap6-L1 (22 μmol) was swollen in THF (0.5 ml) and treated with 37% aqueous formaldehyde (50 μl, 556 μmol) and 100 μl of a 1:1 (v/v) water:acetic acid solution. After 15 minutes sodium cyanoborohydride (200 μl, 1M in THF) was added. The reaction was shaken for 3 hours, and then washed with THF, water, MeOH, DCM and THF. The above procedure was repeated once more and the mixture incubated overnight. Finally the resin was washed as described above and dried in vacuo.

Cleavage and purification - The dried peptide resins were treated with TFA/phenol/H_2O/triisopropylsilane (87.5/5/5/2.5) for two – three hours before filtration into an excess of ice cold diethyl ether. The precipitate was centrifuged into a pellet and washed in an excess of fresh ice cold diethyl ether; this was repeated three times and finally the precipitated peptide was dissolved in in 0.1% TFA and freeze-dried. Purification for both purchased and in house synthesised peptides was performed using water/acetonitrile gradients using either a Waters Symmetry™ C8, 5 μm, 7.8 x 100 mm column or a Waters SymmetryPrep™ C8, 7 μm, 19 x 300 mm column.

Table 1. Sequences of LAH or LADap derivatives used in this study. pH responsive residues are marked in bold. The pK_α quoted for the peptides is the midpoint of the main conformational transition detected in solution using far-UV circular dichroism.

Peptide	Sequence	Length	pK_α at 37°C
LAH	KKLA**H**AL**H**LLALLWL**H**LA**H**ALKKA-NH_2 (SEQ ID NO. 5)	24	5.33[a]
LADap	KKLA**X**AL**X**LLALLWL**X**LA**X**ALKKA-NH_2 (SEQ ID NO. 3)	24	6.22 ± 0.16
LADap(Me_2)	KKLA**X**AL**X**LLALLWL**X**LA**X**ALKKA-NH_2 (SEQ ID NO. 3)	24	6.80 ± 0.11
LAH4-L1	KKALLA**H**AL**H**LLALLAL**H**LA**H**ALKKA-NH_2 (SEQ ID NO. 6)	26	5.29 ± 0.25
LADap4-L1	KKALLA**X**AL**X**LLALLAL**X**LA**X**ALKKA-NH_2 (SEQ ID NO. 4)	26	6.64 ± 0.08
LADap(Me)4-L1	KKALLA**X**AL**X**LLALLAL**X**LA**X**ALKKA-NH_2 (SEQ ID NO. 4)	26	6.62 ± 0.04
LAH6-L1	KK**H**LLA**H**LL**H**LLALLAL**H**LL**H**ALK**H**K-NH_2 (SEQ ID NO. 7)	26	4.45 ± 0.25
LADap6-L1	KK**X**LLA**X**LL**X**LLALLAL**X**LL**X**ALK**X**K-NH_2 (SEQ ID NO. 1)	26	5.67 ± 0.17
LADap(Me)6-L1	KK**X**LLA**X**LL**X**LLALLAL**X**LL**X**ALK**X**K-NH_2 (SEQ ID NO. 1)	26	5.77 ± 0.09
LADap(Me_2)6-L1	KK**X**LLA**X**LL**X**LLALLAL**X**LL**X**ALK**X**K-NH_2 (SEQ ID NO. 1)	26	n.d.
LADap(Me_2)6-A1	KK**X**LLA**X**AL**X**ALLALLA**X**LA**X**ALK**X**K-NH_2 (SEQ ID NO. 2)	26	n.d.

RESULTS

Peptide synthesis - The solid phase syntheses of short peptides containing a single monomethylated, dimethylated and trimethylated Dap have previously been reported and the synthesis of Dap has been achieved by ring opening reaction of β-lactones derived from serine (16). Furthermore the 2-nitrobenzenesulphonyl group has been utilised for the mono alkylation of the side chain amines using both solution and solid phase (21). Reductive amination protocols have been utilised for the synthesis of alkylated amine side chains again both in solution and on the solid phase (22-24). However, whilst the amino acids derivatives are readily accessible and can be incorporated into model peptides, their incorporation into complex peptides is more challenging. Incorporation of Fmoc Dap(Boc)OH into the peptides described was straightforward, however the incorporation of Fmoc Dap(Me_2) was problematic with incomplete acylations and required extended reaction times. A wide range of

activation chemistries was investigated in order to optimise the reaction conditions. The best conditions were activation with DIC and HOBt in a mixture of DMSO and DMF. In this work, we describe a "three-birds in one-pot" solid-phase approach where we obtained three different peptides, namely non-methylated-, N-methylated- and N,N-dimethylated-Dap containing cationic amphipatic peptides from a single assembled resin-peptide sequence. The synthetic route is summarized in Scheme 1 in **Fig. 6**. Solid phase reactions were carried out by the synthesis of the hexa-Dap(Dde) peptide and successful removal of the Dde group to afford the hexaDap-amino peptide. Subsequently, sulphonylation with 2-nitrobenzene sulphonyl chloride followed by methylation with methyl 4-nitrobenzene sulphonate and removal of the protecting group using mercaptoethanol and base afforded the hexa-N-monomethylated-Dap containing peptide. The hexa-N,N-dimethylDAP peptide was obtained by reductive amination of the hexaDap-amino peptide with formaldehyde and sodium cyanoborahydride. The synthesised peptides were purified by preparative HPLC and characterised by MALDI-TOF MS. To our knowledge, this is the first example of multiple and selective methylation of a complex peptide using a solid-phase approach. The orthogonal strategy could be applied to any peptide sequence and further expanded to obtain diverse methylations by employing multiple and orthogonal protecting groups.

NN-Dimethylation of Dap has a dramatic effect on cytotoxicity – Though not apparent for LAH, in our previous study LADap was observed to cause some cytotoxicity during transfection (11), which we suspected could be ascribed to the four Dap primary amines. Therefore, we first investigated whether reducing hydrogen-bonding opportunities at the Dap side chain amine would improve its tolerance by cells in culture. Alkylation of the Dap primary amine may however also impact on the pK_a. Predictions for the side chain pK_a obtained using Marvin View 5.1.4 for three model compounds based on 2-acetoamido-3-aminopropanamide show that the free amine is predicted to have the lowest pK_a with the NN-dimethyl derivative having a pK_a approximately 0.6 units higher. Measurement of side chain pK_a in a peptide undergoing a conformational transition associated with a change in aggregation state is hindered by the large amounts of material required for potentiometric analyses and the unfavourable line broadening observed in NMR for self-associated peptides (11). Instead, we have observed the cooperative, pH responsive, change of conformation of cationic amphipathic peptides in aqueous solution using far-UV CD. LADap and LADap(Me_2) adopt an α-helix conformation when dissolved in neutral or slightly basic aqueous solution and, when titrated with acid, an increasingly disordered conformation is observed. The CD intensity at 220 nm is considered indicative of α-helix content and can be plotted as a function of pH. The midpoint of this conformational transition, pK_α, has been observed to be closely related to the side chain pK_a when this latter information has been tractable and is an important determinant of nucleic acid transfer efficacy as it describes the transition from a self-associated form which is the trigger for effective disruption of the endosomal membrane (12, 25). Although other interactions may be expected to contribute to the pK_α when compared with the pK_a, an

increase of the same magnitude (~0.6 pH units) is seen in both the pK_α for the Dap rich peptide and the pK_a for the model compound following dimethylation (**Table 2**).

The pK_α of LADap(Me_2) is raised by N,N-dimethylation and is likely to be too high to substantially aid endosomal release, dimethylation nevertheless has beneficial effects on the nucleic acid transfer properties of the resulting peptide. Delivery of luciferase reporter gene to adenocarcinomic human alveolar basal epithelial A549 cells indicates that both Dap rich peptides are effective at mediating gene transfer but, when considered in terms of luciferase activity per mg of protein, their performance is substantially inferior to that of Lipofectamine 2000™ (4:1; volume to weight DNA) and LAH4-L1. However, the protein content of the cells, commonly used to calibrate the amount of specific luciferase activity, can be misleading. When the protein content of the wells are plotted and compared with that of untreated cells, a clearer picture emerges indicating that peptide-mediated nucleic acid transfer causes dose dependent cytotoxicity. Transfected cells would normally be expected to have a higher protein content than untreated cells, as is observed for LAH4-L1 and LADap(Me_2) at low peptide doses, and hence significant reductions in cell protein content are a strong indicator of cytotoxicity. LADap is notably more toxic to A549 cells when compared with the dimethylated LADap(Me_2). Accordingly, the transfection efficacies of LADap and Lipofectamine 2000™ (4:1) in this experiment are substantially overstated. When luciferase activity is plotted per well, not only is the robust and non-toxic transfection efficacy of LAH4-L1 evident, offering a 13.0 fold improvement over Lipofectamine 2000™, but also the advantages of LADap(Me_2) over LADap are clear.

The pH response of Dap rich peptides can be tuned – Using LAH4-L1 as a template, we investigated the effect of replacing histidine residues with either Dap or N-methyl Dap on the cooperative, pH dependent, conformational response in solution. Furthermore, we tested whether this conformational response could be tuned by increasing the Dap complement in the peptide, and consequently increasing the Coulombic interactions expected between Dap or Dap(Me) residues located close to each other in space (**Fig. 1A**). At the same time, we mitigated the expected reduction in hydrophobicity by increasing the number of leucine residues at the expense of alanine residues. The overall aim was to obtain peptides with the appropriate hydrophobicity to afford favourable interactions with nucleic acids and membranes, switch from a nominal charge of +5 to +11 during endosomal acidification and have a conformational transition between pH 5 and pH 6.

Far-UV CD spectra were obtained for LAH4-L1, LAH6-L1 and the Dap and mono-methyl Dap containing analogues in aqueous Tris amine buffer at various pH. The pH dependent conformational responses, reflected in the CD intensity at 220 nm, were plotted and revealed the effect of incorporating either four or six Dap or mono-methyl Dap residues in the peptide primary sequence (**Fig. 1**; **Table 2**). For LAH4-L1, the $pK_\alpha = 5.29 \pm 0.25$ and is in agreement with previous work while

pK_α = 4.45 ± 0.25 for LAH6-L1 is substantially higher than that reported recently for LAH6 peptides of greater hydrophobicity (12). The response of LADap4-L1 at 6.64 ± 0.08 is substantially more basic than the histidine-containing analogue. N-methylation of the Dap side chain did not alter the pH response significantly (**Fig. 1B/C**). Notably however the two peptides containing six Dap or six N-methyl Dap residues had a much more acidic response when compared with the analogues comprising only four such residues with midpoints for the conformational transition at pH = 5.67 ± 0.17 and 5.77 ± 0.09 respectively, a drop of ≈ 1 pH unit. Taken together this indicates that, when located close together in space, increasing the number of pH responsive residues can indeed be used to tune the pK_α.

The pH response of the six N,N-dimethyl-Dap containing peptide, LADap(Me_2)6-L1, was not determined as this peptide was found to be insoluble in aqueous media. The removal of hydrogen-bonding opportunities from the Dap side chain had a substantial effect on the peptide hydrophobicity, as suggested by the large increase in the peptide retention time when analysed by HPLC. A further six N,N-dimethyl-Dap variant, LADap(Me_2)6-A1 was prepared in an attempt to circumvent this by reducing the hydrophobicity by substituting four leucines with alanine. While a small improvement in solubility was noted and the HPLC retention time reduced, this peptide remained insufficiently soluble for biophysical studies. LADap(Me_2)6-L1 was however soluble in 50% trifluoroethanol and a far-UV CD spectrum was obtained which indicated the desired α-helix conformation would theoretically be obtainable if it could be delivered to the endosomal membrane.

We have previously linked the membrane disordering capabilities of pH responsive peptide to their nucleic acid transfer capabilities (9) and that the midpoint of the pH dependent peptide induced membrane disordering pK_{mem} reflects the contributions of both peptide and membrane composition to the pH dependent membrane activity (13). The incorporation of chain deuterated lipids such as POPC-d31 or POPS-d31 along with cholesterol in lipid bilayers designed to mimic the endosomal membrane allows the effect of pH dependent changes in peptide behaviour on either zwitterionic or anionic lipids to be monitored. ^2H spin echo NMR spectra of multi lamellar vesicles are characterised by a series of quadrupolar splittings that correspond to the deuterated groups which are located at increasing depth in the membrane and which decrease with magnitude towards the hydrophobic core of the membrane where groups are more disordered. When order parameters are averaged over the whole acyl chain and plotted as a function of pH, the effect of pH responsive peptides can be observed. As observed previously for histidine rich peptides (13), both LADap(Me)4-L1 and LADap(Me)6-L1 effectively increase the disorder of such membranes at acidic pH with a substantially more basic response detected for both peptides in anionic membranes compared with zwitterionic membranes. Interestingly, in contrast with histidine rich peptides (13), LADap(Me)6-L1 consistently responds at a more basic pH when compared with LADap(Me)4-L1. This may be related to the exothermic heat of binding resulting from electrostatic interactions between peptide and lipids that develop at the

membrane surface during protonation that has been observed to be particularly strong for Dap (26). Nevertheless, both peptides are expected to be capable of disordering their target membranes at pH that are readily achievable during endocytosis.

The tuned pH responsive peptides have improved nucleic acid transfer capabilities – The ability of the peptides to mediate nucleic acid transfer was first assessed by monitoring the delivery of luciferase reporter gene to both A549 and MCF-7 human breast cancer cells (**Fig. 2A**). Lipofectamine 2000™ was used here as a benchmark and at an optimised volume to weight DNA ratio of 2:1 to minimise toxicity seen in the earlier experiments. LAH4-L1 was effective at mediating luciferase expression in both cell types but was somewhat inferior to Lipofectamine 2000™ when used at the optimised ratios. Consistent with the high pK_α and the greater expected toxicity of the Dap side chain free amine, LADap4-L1 was consistently less effective than LAH4-L1 in mediating delivery to both cell types. However, N-methylation of the Dap side chain led to a substantial improvement in delivery with LADap(Me)4-L1 providing a 4.1 and 2.0 fold improvement over LADap4-L1 and LAH4-L1 respectively for MCF-7 cells ($p < 0.05$) and efficacy that matched that of LAH4-L1 for A549 cells. Increasing the Dap or N-methyl Dap content in the peptide and the concomitant acidification of the conformational response led to a further increase in peptide mediated luciferase expression with the N-methylated peptide again offering the better performance. LADap(Me)6-L1 provided an 8.0 or 19.7 fold improvement over LADap4-L1 and a 2.1 or 2.6 fold improvement over Lipofectamine 2000™ for MCF-7 or A549 cells respectively which was also significant ($p < 0.05$).

The abilities of the peptides to successfully mediate specific silencing of GAPDH expression were then assessed in A549 cells (**Fig. 2B**), MCF-7 cells (**Fig. 2C**), HUVEC cell (**Fig. 3A**), THP-1 cells differentiated into macrophages (**Fig. 4A**) and undifferentiated THP-1 cells in suspension (**Fig. 4B**). Specific reductions in expression of GAPDH were assessed by densitometry of Western Blots where the effects of administering siRNA targeting GAPDH was compared with that of non-targeting siRNA on the expression of GAPDH as endogenous reporter and β-actin as internal toxicity/specificity control (**Fig. 2D, 3B, 4C**). Lipofectamine 2000™ was used as a benchmark for A549 cells, MCF-7 cells, HUVEC cells and differentiated, adherent THP-1 cells but was unsuited for use with suspension THP-1 cells where the majority of cells were killed and insufficient protein for Western Blots obtained; siPORT was used as benchmark in its place and, for comparison, also with differentiated THP-1 cells. For A549 and MCF-7 cells, all five peptides mediated effective reductions in GAPDH expression but no significant improvements over LAH4-L1 or Lipofectamine 2000™ were observed (**Fig. 2B-D**).

To test the suitability of the peptides for future pulmonary delivery applications in vivo the ability of the peptides to mediate silencing of GAPDH expression in A549 cells was repeated in the presence of

bronchoalveolar lavage fluid (BALF) as a model for airway surface liquid (ASL) (**Fig. 2E/F**). As shown in the Western blot (**Fig. 2E**) and subsequent densitometry (**Fig. 2F**), increasing the percentage of BALF in the transfection medium caused a clear dose dependent reduction in gene silencing efficacy. This was most evident for LADap4-L1 with 50% BALF causing substantial attenuation of the GAPDH silencing. Whereas for LADap(Me)6-L1 the effect of BALF was minor and an effective reduction of GAPDH expression by 70.6% was maintained. Increasing the number of Dap or Dap(Me) residues from four to six conferred substantial and significant ($p < 0.05$) protection from the inhibitory effects of BALF although any enhanced gene silencing attributable to N-methylation of Dap under these conditions was not significant.

The ability of the peptides to mediate silencing of GAPDH expression was then tested in adherent, primary HUVECs. These hard to transfect cells required much higher siRNA concentrations with as much as 150 nM siRNA necessary for notable specific silencing to be observed (**Fig. 3A**). Nevertheless densitometry of the gel (**Fig. 3B**) indicates the peptides performed well in comparison with the Lipofectamine 2000™ benchmark, with LADap(Me)6-L1 again mediating the most effective gene silencing and offering a significant improvement on the LAH4-L1 template peptide ($p < 0.05$). Importantly, the highly effective gene silencing afforded by the pH responsive peptides did not come at the expense of high cytotoxicity (**Fig. 3C**). For Lipofectamine 2000™, only 70.9 ± 4.1% cell viability was retained after treatment whereas each of the pH responsive peptides offered an improvement ($p < 0.05$) with cell viability remaining between 80.8 and 91.5%. Interestingly, while tuning the pH response by increasing the Dap or Dap(Me) content from four to six residues did not have a clear effect on GAPDH silencing efficacy, a possible beneficial effect on reducing cytotoxicity was detected with LADap(Me)6-L1 causing less cytotoxicity than LADap(Me)4-L1 ($p < 0.05$) and LADap6-L1 causing less than LADap4-L1 ($p = 0.059$).

The peptides were effective at mediating specific silencing of GAPDH expression in both differentiated, adherent, macrophage and suspension, monocyte THP-1 cells (**Fig. 4A-C**). For differentiated, adherent, macrophage THP-1 cells, effective silencing of GAPDH expression was observed only for Dap or Dap(Me) rich peptides when six of these residues were incorporated in the peptides with four residues conferring much poorer siRNA transfection capabilities (**Fig. 4C**). Both LADap6-L1 and LADap(Me)6-L1, along with the original LAH4-L1 template, outperformed siPORT when delivering siRNA at 100 nM but not at 50 nM while the performance of Lipofectamine 2000™ was quite variable. For the THP-1 cells cultured in suspension as a model for monocytes, siRNA was administered at either 20 or 40 nM, in three independently repeated experiments, and a significant ($p < 0.05$) improvement over LAH4-L1 and siPORT was observed (**Fig. 4C**). When siRNA was administered at 20 nM, two Dap rich peptides had an improved performance while at the higher concentration all of the five peptides outperformed siPORT. Interestingly, N-methylation of the Dap

sidechain caused a substantial and significant ($p < 0.05$) improvement in GAPDH silencing whether the peptides contained four or six pH responsive units; LADap(Me)6-L1 was the best performing peptide reducing GAPDH expression by 92.3 ± 3.7 %.

The viability of either suspension monocyte or differentiated macrophage THP-1 cells treated with the peptide and benchmark vectors was tested using the colorimetric MTT assay in the presence and absence of siRNA cargoes (**Fig. 4D**). Differentiated, adherent, macrophage THP-1 cells tolerated the vectors reasonably well but significant reductions in viability ($p < 0.05$) of between 19 and 34% were observed, with the presence or absence or cargo having little effect (**Fig. 4D**). siPORT was much better tolerated by differentiated THP-1 cells and when formulated with siRNA no significant reductions in viability were observed however this vector performed poorly as an siRNA transfection agent. In contrast, suspension, monocyte THP-1 cells were much less sensitive to the peptide vectors (**Fig. 4D**) and although reductions in viability were significant for all but LADap6-L1, their magnitude was substantially lower. Lipofectamine 2000™ was highly toxic to the suspension THP-1 cells, confirming the observation noted when trying to use it as a benchmark in the corresponding siRNA delivery experiments.

Finally the route of uptake of peptide/siRNA complexes was monitored using live cell confocal microscopy with siRNA labelled with Cy3 and acidic compartments, including mid to late endosomes as well as lysosomes, labelled with LysoTracker® DND-26 (**Fig. 5**). The entry of peptide/siRNA complexes to HUVECs mediated by either LAH4-L1 (**Fig. 5A-C**) or LADap(Me)6-L1 (**Fig. 5D-F**) was monitored 24 hours after transfection allowing the effect of chlorpromazine (**Fig. 5B/E**) or nystatin (**Fig. 5C/F**) pre-treatment on uptake to be assessed. Both peptides mediated effective uptake of Cy3-labelled siRNA with substantial co-localisation of red siRNA with green intracellular compartments (**Fig. 5A/D**), particularly for LADap(Me)6-L1. Pre-treatment with chlorpromazine had little effect on delivery to HUVECs mediated by either peptide (**Fig. 5B/E**) in contrast with nystatin, which notably affected delivery by both peptides but in different ways. For delivery mediated by LAH4-L1, pre-treatment with nystatin caused an apparent increase in co-localisation of siRNA containing complexes with acidic compartments (**Fig. 5C**). Whereas, the same treatment lead to a substantial reduction in uptake, with very little co-localisation detected, when delivery was mediated by LADap(Me)6-L1 (**Fig. 5F**).

DISCUSSION

Since the histidine rich predecessors of the Dap rich peptides presented here have been successfully used to deliver a wide variety of cargoes, without prior conjugation, both in vivo and in an ex vivo study, we were interested to improve the capabilities of pH responsive peptides such that not only fibroblasts and other adherent cell lines, but also suspension cell lines and primary cells become

tractable using this technology with robust gene silencing maintained in more challenging conditions. The pH responsive peptides are notable in that they are capable of not only binding non-covalently and condensing nucleic acids, preventing their degradation by endogenous nucleases and promoting cellular uptake, but are also able to promote escape from endosomes. The endosomal escape mechanism is likely to be distinct from that of the proton sponge hypothesis that has been established for other poly-cationic molecules although this may play a role (27-30); as analogous peptides, which do not undergo pH dependent conformational changes, are much less effective at delivering nucleic acid cargo (11). Our earlier observation, that Dap rich peptides might respond to pH changes in a range that could be exploited to drive endosomal release, suggested that enhanced delivery capabilities could be obtained if the pH response could be tuned. In practice, the Dap rich peptides behave similarly to their histidine templates and the pH response, when the number of Dap residues is increased from four to six, is acidified to the same extent as that achieved in the histidine containing analogues. In contrast with the histidine rich predecessors, the tuning of the pH response in Dap rich peptides is reflected in the improved delivery of plasmid DNA to A549 and MCF-7 cells and leads to substantial improvements in siRNA delivery either under more challenging conditions or to more challenging cell types.

While little difference between pH responsive peptides was noted in delivery of siRNA to either MCF-7 or A549 cells, substantial improvements in delivery of siRNA to the latter were observed when transfection was performed in the presence of lung surfactant containing bronchoalveolar lavage fluid (BALF) and mediated by Dap rich peptides with a tuned pH response. With pulmonary siRNA delivery an attractive route for the treatment of a wide variety of diseases affecting the airway, pH responsive peptides have been formulated as dry powders in a parallel study (31). Transfections in the presence of BALF are designed to model the likely barrier to transfection presented by airway surface liquid (ASL). ASL covers the epithelial cells along the respiratory tract and consists mainly of phospholipids and surfactant-associated proteins which may affect the stability of siRNA/peptide complexes and hence their delivery efficacy (32, 33). Though delivery of nucleic acids to A549 cells mediated by Lipofectamine 2000™ is robust in the presence of BALF, the transfection efficiency when delivery is mediated by histidine rich peptides is weak (31). Here we show that siRNA transfer in the presence of BALF is also weak for peptides containing only four Dap or Dap(Me) residues but when these are increased to six, good delivery efficacy is maintained. These results are in agreement with our earlier studies comparing plasmid DNA transfer (31) and highlight an advantage of tuning the pH response which is only manifested in more challenging delivery conditions. N-methylation of the Dap residues also offers a consistently observed enhancement in delivery efficacy.

The availability of cells derived from the human umbilical cord has played an important role in the development of vascular biology. HUVECs are endothelial cells that line the umbilical cord vein and

have provided a critical model that has enable breakthroughs in understanding cellular and molecular events that underpin a wide variety of disease processes (34) and are considered a hard to transfect cell. They require elevated levels of siRNA for noticeable gene silencing and this leads to substantial cytotoxicity when non-viral delivery systems are used. Lipofectamine 2000™ offers robust gene silencing under the conditions used in the present study, but this is accompanied by substantial cytotoxicity. Gene silencing in HUVECs by Lipofectamine 2000™ is matched by the Dap or Dap(Me) rich peptides, with LADap(Me)6-L1 the most effective. Furthermore, the gene silencing mediated by the peptides comes with a much lower cytotoxity cost which was itself reduced through tuning the pH response in the Dap(Me) rich peptides. HUVECs were also studied here using live cell confocal microscopy to better understand the source of the improved delivery efficacy. In our previous study we demonstrated that, in MCF-7 cells, the route of uptake for pH responsive peptide mediated siRNA delivery differs from that for DNA delivery with the former preferring clathrin independent endocytosis (6). Furthermore, while caveolae rather than clathrin dependent endocytosis was implicated in LAH4-L1 mediated siRNA delivery, increasing the histidine content in the delivery peptide precluded entry via this mechanism and was suggested to be an important contributory factor in the lower than expected delivery efficacy for such peptides (6). In the present study, chlorpromazine has little effect on uptake mediated by either LAH4-L1 or LADap(Me)6-L1, confirming that again clathrin mediated endocytosis does not provide the major route of uptake for effective delivery mediated by either peptide. Blocking caveolae dependent endocytosis of LAH4-L1/siRNA complexes did not prevent uptake but led to a much greater co-localisation with acidic compartments, most likely lysosomes. This suggests that, analogous to polyplex uptake (40), when complexes can enter via both clathrin and caveolae dependent endocytosis, blocking the caveolae pathway may channel complexes to the lysosomal compartments for degradation. Blocking caveolae dependent endocytosis did not trigger the same increase in co-localisation of complexes and lysosomes when delivery was mediated by LADap(Me)6-L1. This could reflect either an inability of such complexes to enter via clathrin dependent endocytosis, when the caveolae dependent pathway is blocked, or a much greater ability to escape from acidic compartments. In either case, with uptake mediated by both LAH4-L1 and LADap(Me)6-L1 sensitive to blockage of caveolae dependent endocytosis, the live-cell confocal imaging study indicates that uptake mediated by peptides containing six Dap(Me) residues is likely to proceed in a manner distinct from that of analogues containing six histidine residues and suggests another important factor that contributes to their increased siRNA delivery efficacy.

The human monocytic leukaemia cell line THP-1 is widely used as a model to probe either monocyte or macrophage biology (35). A substantial and useful improvement in siRNA delivery to macrophage THP-1 cells also accompanied the tuning of the pH response in Dap or Dap(Me) rich peptides but no improvement over the LAH4-L1 template peptide was shown.

THP-1 monocytes are of considerable interest since a wide variety of diseases may benefit from monocyte directed, siRNA based interventions. In particular, recent work has shown that silencing the chemokine receptor CCR2 in inflammatory monocytes prevents their accumulation in sites of inflammation in a mouse model (36). The beneficial impact of this effect was demonstrated in models of atherosclerosis, coronary artery occlusion, diabetes and tumour growth (36). Numerous other targets including, inter alia, Egr-1 for Alzheimer's disease (37), hepcidin for anaemia of chronic disease (38) and WEE1 for myeloid and lymphoid leukaemia (39) have been suggested following studies on THP-1 cells and effective and non-perturbing delivery agents to such cells are therefore highly sought after. While a large number of non-viral siRNA delivery systems have been developed and are widely used to mediate delivery to adherent cell lines, fewer options are available for those working with suspension cell lines where typically either lipid based transfection agents, such as Lipofectamine 2000™, or electroporation are used (35) and, while an optimized lipid nanoparticle has been successfully deployed to mediate silencing of CCR2 in an inflammatory monocyte mouse model (36), there remains a need to develop non-toxic agents that can routinely, robustly and specifically effect gene silencing in monocytes both in vitro and in vivo.

Tuning the pH response of the Dap or Dap(Me) rich peptides had less impact on delivery to monocyte THP-1 cells than that observed for macrophage THP-1. Instead, the highly efficient gene silencing mediated by LADap(Me)4-L1 and LADap(Me)6-L1 rather demonstrated the benefits of N-methylation of the Dap side chain. These two peptides are attractive candidates for further development for delivery to suspension cells since they surpassed the two benchmark delivery compounds. The relatively high cytotoxicity observed for Lipofectamine 2000™ precluded its use for siRNA delivery to monocyte THP-1 cells, with insufficient material recovered for Western blot experiments. In contrast, siPORT was much gentler but suffered from poor gene silencing efficacy.

In conclusion, using a global approach for on-resin, multiple and selective N-methylation of peptides, we prepared a family of peptides to show that overcoming the charge/self-association barrier by replacing histidine with Dap or Dap(Me) and tuning the pH dependent conformational response, by increasing the number of Dap or Dap(Me) residues, substantially improves the delivery capabilities of pH responsive peptides. N-methylation of the Dap residues notably increased gene silencing in monocyte THP-1 cells. The improvements were sufficient that the pH responsive peptides outperform the benchmark liposomal delivery agents and are able to mediate highly effective gene silencing with low associated toxicity, suggesting that that they will be adaptable for in vivo siRNA delivery.

<u>References for Background to Invention and for Example 1</u>

1. Ledford, H. (2010) Nature 468, 487.
2. Li, S.D. & Huang, L. (2006) Gene Ther. 13, 1313-1319.

3. Kichler, A., Leborgne, C., März, J., Danos, O. and Bechinger, B. (2003) Proc. Natl. Acad. Sci. USA 96, 1564-1568.

4. Kichler, A., Mason, A.J. and Bechinger, B. (2006) Biochim. Biophys. Acta 1758, 301-307.

5. Langlet-Bertin, B., Leborgne, C., Scherman, D., Bechinger, B., Mason, A.J. and Kichler, A. (2010) Pharm. Res. 27, 1426-1436.

6. Lam J.K.W., Liang, W., Lan, Y., Chaudhuri, P., Chow, M.Y.T., Witt, K., Kudsiova, L. and Mason, A.J. (2012) J. Control. Release 158, 293-303.

7. Gerard, X., Perrault, I., Hanein, S., Silva, E., Bigot, K., Defoort-Delhemmes, S., Rio, M., Munnich, A., Scherman, D. Kaplan, D., Kichler, A. and Rozet, J-M. (2012) Molecular Therapy – Nucleic Acids 1, e29.

8. Zhang, T.T., Kang, T.H., Ma, B., Xu, Y., Hung, C-F. and Wu, T-C. (2012) Vaccine 30, 784-793.

9. Mason, A.J., Martinez, A., Glaubitz, C., Danos, O., Kichler, A. and Bechinger, B. (2006) FASEB J. 20, 320-322.

10. Mason, A.J., Leborgne, C., Moulay, G., Martinez, A., Danos, O., Bechinger, B. and Kichler, A. (2007) J. Control. Release 118, 95-104.

11. Lan, Y., Langlet-Bertin, B., Abbate, V., Vermeer, L.S., Kong, X., Sullivan, K., Leborgne, C., Scherman, D., Hider, R.C., Drake, A.F., Bansal, S., Kichler, A. and Mason, A.J. (2010) ChemBioChem 11, 1266-1272.

12. Vogt, T.C.B. and Bechinger, B. (1999) J. Biol. Chem. 274, 29115-29121.

13. Iacobucci, V., Di Giuseppe, F., Bui, T.T., Vermeer, L.S., Patel, J., Scherman, D., Kichler, A., Drake, A.F. and Mason, A.J. (2012) Biochim. Biophys. Acta 1818, 1332-1341.

14. Prongidi-Fix, L., Sugawara, M., Bertani, P., Raya, J., Leborgne, C., Kichler, A. and Bechinger, B. (2007) Biochemistry 46, 11253-11262.

15. Eisenberg, D., Weiss, R.M., Terwilliger, C.T. and Wilcox, W. (1982) Faraday Symp. Chem. Soc. 17, 109–120.

16. Kretsinger, J.K. and Schneider, J.P. (2003) J. Am. Chem. Soc. 125, 7907-7913.

17. Davis, J.H. (1983) Biochim. Biophys. Acta 737, 117-171.

18. Schäfer, H., Mädler, B. and Volke, F. (1995) J. Magn. Reson. 116, 145-149.

19. Sternin, E., Bloom, M., and MacKay, A.L. (1983) J. Magn. Reson. 55, 274-282.

20. Seelig, A. and Seelig, J. (1974) Biochemistry 13, 4839-4845.

21. Miller, S.C. and Scanlan, T.S. (1998) J. Am. Chem. Soc. 120, 2690-2691.

22. Brown, E.G. and Nuss, J.M. (1997) Tetrahedron Lett. 38, 8457-8460.

23. Bui, C.T., Rasoul, F.A., Ercole, F. Pham, Y. and Maeji, N.J. (1998) Tetrahedron Lett. 39, 9279-9282.

24. Gordon, D.W. and Steele, J. (1995) Bioorg. Med. Chem. Lett. 5, 47-50.

25. Marquette, A., Mason, A.J. and Bechinger, B. (2008) J. Pept. Sci. 14 488-495.

26. Hoernke, M., Schwieger, C., Kerth, A. and Blume, A. (2012) Biochim. Biophys. Acta 1818, 1663-1672.

27. Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B. and Behr, J.P. (1995) Proc. Natl. Acad. Sci. 92, 7297-7301.

28. Behr, J.P. (1997) Chimia International Journal for Chemistry 51, 34-36.

29. Pack, D.W., Hoffman, A.S., Pun, S. and Stayton, P.S. (2005) Nature Reviews Drug Discovery 4, 581-593.

30. Sonawane, N.D., Szoka Jr, F.C. and Verkman, A.S. (2003) J. Biol. Chem. 278, 44826-44831.

31. Liang, W., Kwok, P.C.L., Chow, M.Y.T., Tang, P., Mason, A.J., Chan, H-K., Lam, J.K.W. Formulation of pH responsive peptides as inhalable dry powders for pulmonary delivery of nucleic acids. (in revision)

32. Sanders, N., Rudolph, C., Braeckmans, K., De Smedt, S.C., Demeester, J. (2009) Adv. Drug Deliv. Rev. 61, 115-27.

33. Goerke, J. (1998) Biochim. Biophys. Acta. 1408, 79-89.

34. Park, H-J., Zhang, Y., Georgescu, S.P., Johnson, K.L., Kong, D. Galper, J.B. (2006) Stem Cell Rev. 2, 93-102

35. Maeß, M.B., Buers, I., Robenek, H. and Lorkowski, S. (2011) Cold Spring Harb. Protoc. doi:10.1101/pdb.prot5612

36. Leuschner, F. et al. (2011) Therapeutic siRNA silencing in inflammatory monocytes in mice. Nature Biotech. 29, 1005-1010.

37. Giri, R.K., Rajagopal, V., Shahi, S., Zlokovic, B.V. and Kalra, V.K. (2005) Am. J. Cell. Physiol. 289, C264-C276.

38. Theurl, I., Theurl, M., Sifert, M., Mair, S., Nairz, M., Rumpold, H., Zoller, H., Bellmann-Weiler, R., Niederegger, H., Talasz, H. and Weiss, G. (2008) Blood 111, 2392-2399.

39. Tibes, R., Bogenberger, J.M., Chaudhuri, L., Hagelstrom, R.T., Chow, D., Buechel, M.E., Gonzales, I.M., Demuth, T., Slack, J., Mesa, R.A., Braggio, E., Yin, H.H., Arora, S. and Azorsa, D.O. (2012) Blood 119, 2863-2871.

40. Rejman, J., Bragonzi, A., Conese, M. (2005) Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes, Mol. Ther. 12, 468–474.

Example 2 - Formulation of pH responsive peptides as inhalable dry powders for pulmonary delivery of nucleic acids

SUMMARY

Nucleic acids have the potential to be used as therapies or vaccines for many different types of disease but delivery remains the most significant challenge to their clinical adoption. pH responsive peptides

containing either histidine or derivatives of 2,3-diaminopropionic acid (Dap) can mediate effective DNA transfection in lung epithelial cells with the latter remaining effective even in the presence of lung surfactant containing bronchoalveolar fluid (BALF), making this class of peptides attractive candidates for delivering nucleic acids to lung tissues.

1. INTRODUCTION

Nucleic acid therapy has the potential to treat a wide range of diseases affecting the airways including cystic fibrosis (CF) [1-3], lung cancer [4-6] and inflammatory diseases such as chronic obstructive respiratory disease (COPD) and asthma [7-10]. Inhalation is a desirable route of administration to deliver therapeutic nucleic acids to the lungs because of its non-invasive nature and lower endonuclease activity in the airways compared with the blood serum. In addition, direct application of therapeutic agents to the target site can minimize systemic adverse effects. Delivery however remains the biggest hurdle to nucleic acid therapy. Viral vectors are highly effective nucleic acid delivery agents, but the risk of insertional mutagenesis [11, 12] and high immunogenicity [13] have led researchers to seek safer alternatives. Non-viral vectors generally have a better safety profile compared with their viral counterparts but their transfection efficiency is often less than satisfactory for use in the clinic and more effective vectors for pulmonary delivery remain highly sought after.

Intracellular barriers pose a major challenge to nucleic acid delivery. Non-viral vectors usually enter cells through endocytosis [14] and, once inside cells, they are transported into the endosomes and eventually the lysosomes where acidification occurs and the degradative enzymes are activated. To ensure good transfection efficiency, therapeutic nucleic acids must be able to escape from the endosomes or lysosomes before degradation take place, or bypass the endosomal pathway completely. In our group, pH responsive peptides containing histidine or 2,3-diaminopropionic acid (Dap) as pH responsive elements are being investigated to deliver nucleic acids [15, 16]. These two structurally similar pH responsive peptides (LAH series and LADap series) are cationic amphipathic peptides and each peptide contains four or six pH responsive residues. They can form non-covalent complexes with nucleic acids and promote endosomal escape. In an acidic environment, peptides are released from the complexes and change their conformation enabling membrane destabilising activity. Subsequently, nucleic acids are released from the endosomal/lysosomal compartments into the cytoplasm [15, 17]. Our previous study has demonstrated that the pH responsive peptides are capable of mediating both highly efficient DNA or siRNA transfection in mammalian cell lines [15, 17] (Abbate et al., unpublished). Others have shown that LAH4 peptides can effectively deliver nucleic acids to patient derived primary fibroblasts [18] and facilitate intracellular delivery in vivo by way of subcutaneous injection of protein-based vaccines adjuvanted with Toll-like receptor 9 agonist CpG oligonucleotide (CpG) to generate enhanced CD8+ T cell immune responses and antitumor effects [19]. With proven efficacy in delivery to a variety of cultured, primary cells and tumour cells together with the first

example of in vivo tolerance and efficacy we were interested to develop a pH responsive peptide-based formulation that is suitable for effective nucleic acid delivery by pulmonary administration.

Besides intracellular barriers, a further obstacle to pulmonary DNA delivery is the presence of airway surface liquid (ASL) which covers the epithelial cells along the respiratory tract [20]. This layer of liquid consists mainly of phospholipids and surfactant-associated proteins [21] which may affect the stability of the DNA complexes, and hence the delivery efficiency.

In this study, the DNA delivery efficiency of LAH and LADap pH responsive peptides were evaluated on human lung epithelial cells (A549) with bronchoalveolar lavage fluid (BALF), obtained from rats, used as model to study the effect of ASL on transfection efficiency.

2. MATERIALS AND METHODS

2.1 Materials - LAH4-L1 peptide (> 70% purity) was purchased from ChinaPeptide (Shanghai, China) and used as provided. For other peptides, their detailed synthetic procedures and characterisation are described above in Example 1. The sequences of LAH or LADap peptides used in this study are shown in Table 2. Plasmid DNA (gWIZ™ Luciferase) was purchased from Aldevron (Fargo, ND, USA). Mannitol (Pearlitol 160C) was purchased from Roquette (Lestrem, France). Dulbecco's modified eagle medium (DMEM), Opti-MEM 1 reduced serum medium, antibiotic-animycotic liquid, fetal bovine serum (FBS) and Lipofectamine™ 2000 were purchased from Invitrogen (CA, USA). The luciferase assay system was purchased from Promega (Madison, WI, USA). GelRed™ nucleic acid stain was purchased from Biotium (Hayward, CA, USA). All other reagents and solvents were purchased from Sigma (Poole, UK) and were of analytical grade or better.

2.2 Cell Culture - A549 (human lung adenocarcinoma epithelial cells) were obtained from ATCC (Manassas, VA, USA). The cells were maintained at 5% CO_2, 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 twice weekly.

2.3 Preparation of peptide/DNA complexes - Peptide/DNA complexes were prepared at 10:1 ratio (w/w). Peptide solution and DNA solution were prepared separately in ultrapure water. Equal volumes of peptide solution and DNA solution were mixed to give peptide/DNA complexes with 0.02 mg/mL DNA concentration. The mixture was allowed to incubate for 30 min at room temperature before further processing.

2.4 Bronchoalveolar lavage fluid (BALF) collection - Bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea of rats (Sprague-Dawley) and washing the airway with 1 mL of PBS. The collected BALF was centrifuged and the supernatant was collected and stored on ice before

use. All animal works were approved by the committee on the use of live animals in teaching and research (CULATR), the laboratory animal unit, The University of Hong Kong.

2.5 In vitro transfection in culture plate in or in TSI setup - A549 cells were transfected with peptide/DNA complexes at 1 μg DNA per well or dry powder formulations equivalent to 1 μg DNA per well in 24-well plates in Opti-MEM I reduced serum medium or 50% BALF (v/v). After 4 h of incubation at 37°C, the cells were washed with PBS. Fresh DMEM supplemented with 10% FBS were added to the cells. At 48 h post-transfection, the luciferase expression was detected using the luciferase assay system according to the manufacturer's protocol. The cells were also transfected with Lipofectamine™ 2000 under the same condition.

2.6 Statistical Analysis - Statistical testing was performed by Prism software (version 5.0d) and the data were analysed by one-way ANOVA followed by Bonferroni's post test.

3. RESULTS

3.1 DNA transfection mediated by pH responsive peptides in the presence of BALF - The DNA transfection efficiency of pH responsive peptides was assessed in A549 cells in OptiMEM-1 reduced serum medium or in the presence of 50% BALF (Fig. 7). In general, LADap peptides performed substantially better than the LAH peptides. Within the LAH series, LAH4-L1 had the highest transfection efficiency (7.9×10^4 RLU/mg protein) in OptiMEM-1 reduced serum medium. Among all the peptides tested, LADap(Me)6-L1 had the highest transfection efficiency (1.1×10^6 RLU/mg protein) and performed significantly better than Lipofectamine™ 2000 ($p < 0.05$). In the presence of 50% BALF, there was a significant reduction of transfection efficiency with all the peptides as well as with Lipofectamine™ 2000. The biggest reduction in transfection was observed with LAH4-L1 (35-fold reduction), and the lowest reduction of transfection was observed with LADap6-L1 (2-fold reduction). However, LADap(Me)6-L1 retained the highest transfection efficiency (2.8×10^5 RLU/mg protein) and performed significantly better than Lipofectamine™ 2000 ($p < 0.001$) with both LADap(Me)6-L1 and Lipofectamine™ 2000 suffering from an approximate 4-fold reduction in transfection efficiency after the addition of 50% BALF. For reasons of cost and availability, LAH4-L1 was used as a representative of the pH responsive peptide class for the formulation studies.

4. DISCUSSION

In this work, we aimed to develop a peptide-based DNA delivery system for pulmonary delivery via inhalation. Previous studies have already demonstrated that pH responsive peptides are effective in mediating DNA transfection in a variety of mammalian cell lines [15]. Here we further investigate the suitability of the pH responsive peptides for delivering DNA to airway tissues. Since the ASL

covering the airway epithelial cells represents an early barrier for DNA delivery following pulmonary administration, we examined the effect of ASL on DNA transfection of the peptides using BALF collected from rats as model. Inhibited of luciferase plasmid delivery by BALF was observed with all the peptides tested as well as with Lipofectamine™ 2000. This could be attributed to the presence of negatively charged proteins and surfactants in BALF, which may alter the overall charges of DNA complexes, leading to the reduction of interaction between DNA complexes and surfaces, or causing premature dissociation of the non-covalent peptide/DNA complexes [24]. In general, peptides containing six pH responsive residues were found to be more effective in resisting the deleterious effect of BALF as has been observed previously when similarly assessing the affect of serum [36]. In particular, delivery mediated by LADap6-L1 and LADap(Me)6-L1 were the least affected by the presence of BALF and these peptides provided the highest overall transfection efficiency in A549 cells. LADap(Me)6-L1 continued to outperform Lipofectamine™ 2000 in the presence of BALF, and this, together with its low associated cytotoxicity (Abbate et al., unpublished data), makes it a very promising candidate for lung delivery.

TABLES

Table 2. Sequences of LAH or LADap peptides used in this study. pH responsive components are highlighted in bold. X = Dap, X_1 = N-methyl Dap

Peptide	Sequence
LAH4-L1	KKALLA**H**AL**H**LLALLAL**H**LA**H**ALKKA-NH_2 (SEQ ID NO. 6)
LAH6-X1L-W	K**H**KLL**H**LL**H**LLALLWL**H**LL**H**LLK**H**K-NH_2 (SEQ ID NO. 8)
LAH6-X1-L	K**H**KLL**H**LL**H**LLALLAL**H**LL**H**LLK**H**K-NH_2 (SEQ ID NO. 9)
LADap4-L1	KKALLA**X**AL**X**LLALLAL**X**LA**X**ALKKA- NH_2 (SEQ ID NO. 4)
LADap(Me)4-L1	KKALLA**X_1**AL**X_1**LLALLAL**X_1**LA**X_1**ALKKA- NH_2 (SEQ ID NO. 6)
LADap6-L1	KK**X**LLA**X**LL**X**LLALLAL**X**LL**X**ALK**X**K- NH_2 (SEQ ID NO. 1)
LADap(Me)6-L1	KK**X_1**LLA**X_1**LL**X_1**LLALLAL**X_1**LL**X_1**ALK**X_1**K- NH_2 (SEQ ID NO. 1)

References for Example 2

[1] Sinn PL, Anthony RM, McCray PB, Jr. Hum Mol Genet. 2011;20:R79-86.

[2] Zhang L, Button B, Gabriel SE, Burkett S, Yan Y, Skiadopoulos MH, et al. PLoS Biol. 2009;7:e1000155.

[3] Johnson LG, Olsen JC, Sarkadi B, Moore KL, Swanstrom R, Boucher RC. Nat Genet. 1992;2:21-5.

[4] Moon C, Oh Y, Roth JA. Clin Cancer Res. 2003;9:5055-67.

[5] Vachani A, Moon E, Wakeam E, Albelda SM. Am J Respir Cell Mol Biol. 2010;42:385-93.

[6] Sumimoto H, Yamagata S, Shimizu A, Miyoshi H, Mizuguchi H, Hayakawa T, et al. Gene Therapy. 2005;12:95-100.

[7] Al-Jamal R, Wallace WA, Harrison DJ. Expert Opin Biol Ther. 2005;5:333-46.

[8] Huang HY, Chiang BL. Curr Opin Mol Ther. 2009;11:652-63.

[9] Factor P. Mol Ther. 2003;7:148-52.

[10] Parry-Billings M, Ferrari N, Seguin R. Curr Opin Investig Drugs. 2010;11:1276-85.

[11] Dave UP, Jenkins NA, Copeland NG. Science. 2004;303:333-.

[12] Kohn DB, Sadelain M, Glorioso JC. Nature Reviews Cancer. 2003;3:477-88.

[13] Bessis N, GarciaCozar FJ, Boissier MC. Gene Therapy. 2004;11:S10-S7.

[14] Khalil IA, Kogure K, Akita H, Harashima H. Pharmacol Rev. 2006;58:32-45.

[15] Lam JK, Liang W, Lan Y, Chaudhuri P, Chow MY, Witt K, et al. J Control Release. 2012;158:293-303.

[16] Lan Y, Langlet-Bertin B, Abbate V, Vermeer LS, Kong X, Sullivan KE, et al. Chembiochem : a European journal of chemical biology. 2010;11:1266-72.

[17] Langlet-Bertin B, Leborgne C, Scherman D, Bechinger B, Mason AJ, Kichler A. Pharm Res. 2010;27:1426-36.

[18] Gerard X, Perrault I, Hanein S, Silva E, Bigot K, Defoort-Delhemmes S, et al. Molecular Therapy Nucleic Acids. 2012;1:e29.

[19] Zhang TT, Kang TH, Ma B, Xu Y, Hung CF, Wu TC. Vaccine. 2012;30:784-93.

[20] Sanders N, Rudolph C, Braeckmans K, De Smedt SC, Demeester J. Adv Drug Deliv Rev. 2009;61:115-27.

[21] Goerke J. Biochim Biophys Acta. 1998;1408:79-89.

[22] Roesnecker J, Naundorf S, Gersting SW, Hauck RW, Gessner A, Nicklaus P, et al. Journal of Gene Medicine. 2003;5:49-60.

[23] Duncan JE, Whitsett JA, Horowitz AD. Human Gene Therapy. 1997;8:431-8.

[24] Ernst N, Ulrichskotter S, Schmalix WA, Radler J, Galneder R, Mayer E, et al. J Gene Med. 1999;1:331-40.

Claims

1. A cationic amphipathic helical peptide for binding to and delivering a cargo molecule into a cell, the peptide comprising five or more 2,3-diaminopropionic acid (Dap) residues.

2. The peptide of claim 1, wherein the amphipathic helix of the peptide comprises a hydrophobic surface and a hydrophilic surface.

3. The peptide of claim 2, wherein the Dap residues are located along one face of the helix to form the hydrophilic surface.

4. The peptide of claim 2 or claim 3, wherein the hydrophobic surface comprises residues selected from alanine, leucine and valine.

5. The peptide of any preceding claim, wherein the pKa of the peptide is between 5 and 6 so that the Dap residues become protonated at a pH between 5 and 6.

6. The peptide of any preceding claim, wherein the Dap residues are unmodified.

7. The peptide of any one of claims 1 to 5, wherein the amine group on the side chain of the Dap residues is alkylated.

8. The peptide of claim 7, wherein the amine group on the side chain of the Dap residues is methylated.

9. The peptide of claim 7, wherein the amine group on the side chain of the Dap residues is dimethylated.

10. The peptide of any preceding claim, wherein the peptide comprises a plurality of positively charged amino acid residues at pH 7 located close to or at one or both ends of the peptide for binding a cargo molecule.

11. The peptide of any preceding claim, wherein the overall charge state of the peptide at pH 7 is between +4 and +6.

12. The peptide of any preceding claim, wherein the peptide is between 20 and 30 amino acids in length.

13. The peptide of any preceding claim, wherein the peptide comprises one of the following sequences:

1) KKXLL AXLLX LLALL ALXLL XALKX K (SEQ ID NO. 1); or

2) KKXLL AXALX ALLAL LAXLA XALKX K (SEQ ID NO. 2),

wherein X represents a Dap residue, and wherein up to three amino acids selected from the leucine, alanine and lysine residues may be switched with another amino acid with similar properties.

14. The peptide of any preceding claim, wherein the peptide comprises one of the following sequences:

1) KKXLL AXLLX LLALL ALXLL XALKX K (SEQ ID NO. 1); or

2) KKXLL AXALX ALLAL LAXLA XALKX K (SEQ ID NO. 2),

wherein X represents a Dap residue.

15. The peptide of claim 1 having six Dap residues, wherein the amphipathic helix of the peptide comprises a hydrophobic surface and a hydrophilic surface, wherein the six Dap residues are located along one face of the helix to form the hydrophilic surface and the hydrophobic surface is formed of hydrophobic amino acid residues, wherein the pKa of the peptide is between 5 and 6 so that the Dap residues become protonated at a pH between 5 and 6, and wherein the amine group on the side chain of the Dap residues is methylated.

16. A cationic amphipathic helical peptide for binding to and delivering a molecule into a cell, the peptide comprising four 2,3-diaminopropionic acid (Dap) residues, wherein at least one of the amine groups on one of the Dap residues is alkylated.

17. The peptide of claim 16, wherein the amphipathic helix of the peptide comprises a hydrophobic surface and a hydrophilic surface.

18. The peptide of claim 16, wherein the four Dap residues are located along one face of the helix to form the hydrophilic surface.

19. The peptide of claim 17 or claim 18, wherein the hydrophobic surface comprises residues selected from alanine, leucine and valine.

20. The peptide of any one of claims 16 to 19 preceding claim, wherein the pKa of the peptide is between 5 and 6.8 so that the Dap residues become protonated at a pH between 5 and 6.8.

21. The peptide of any one of claims 16 to 20, wherein the amine group on the side chain of the Dap residues is alkylated.

22. The peptide of claim 21, wherein the amine group on the side chain of the Dap residues is methylated.

23. The peptide of claim 21, wherein the amine group on the side chain of the Dap residues is dimethylated.

24. The peptide of any one of claims 16 to 23, wherein the peptide comprises a plurality of positively charged amino acid residues at pH 7 located close to or at one or both ends of the peptide for binding a cargo molecule.

25. The peptide of any one of claims 16 to 24, wherein the overall charge state of the peptide at pH 7 is between +4 and +6.

26. The peptide of any one of claims 16 to 25, wherein the peptide is between 20 and 30 amino acids in length.

27. The peptide of any one of claims 16 to 26, wherein the peptide comprises one of the following sequences:

1) KKLAX ALXLL ALLWL XLAXA LKKA (SEQ ID NO. 3); or

2) KKALL AXALX LLALL ALXLA XALKK A (SEQ ID NO. 4),

wherein X represents a Dap residue, and wherein up to three amino acids selected from the leucine, alanine, lysine and tryptophan residues may be switched with another amino acid with similar properties.

28. The peptide of any one of claims 16 to 26, wherein the peptide comprises one of the following sequences:

1) KKLAX ALXLL ALLWL XLAXA LKKA (SEQ ID NO. 3); or

2) KKALL AXALX LLALL ALXLA XALKK A (SEQ ID NO. 4),

wherein X represents a Dap residue.

29. A pharmaceutical composition comprising a peptide of any preceding claim bound to a cargo molecule and one or more pharmaceutically acceptable excipients.

30. The composition of claim 29, wherein the cargo is a peptide or a nucleic acid.

31. The composition of claim 29 or claim 30, wherein the composition is in the form of a dry powder which is suitable for inhalation.

32. A peptide according to any one of claims 1 to 28 or the pharmaceutical composition of any one of claims 29 to 31 for use in therapy.

33. A peptide according to any one of claims 1 to 28 or the pharmaceutical composition of any one of claims 29 to 31 for use in delivering a cargo molecule to a cell.

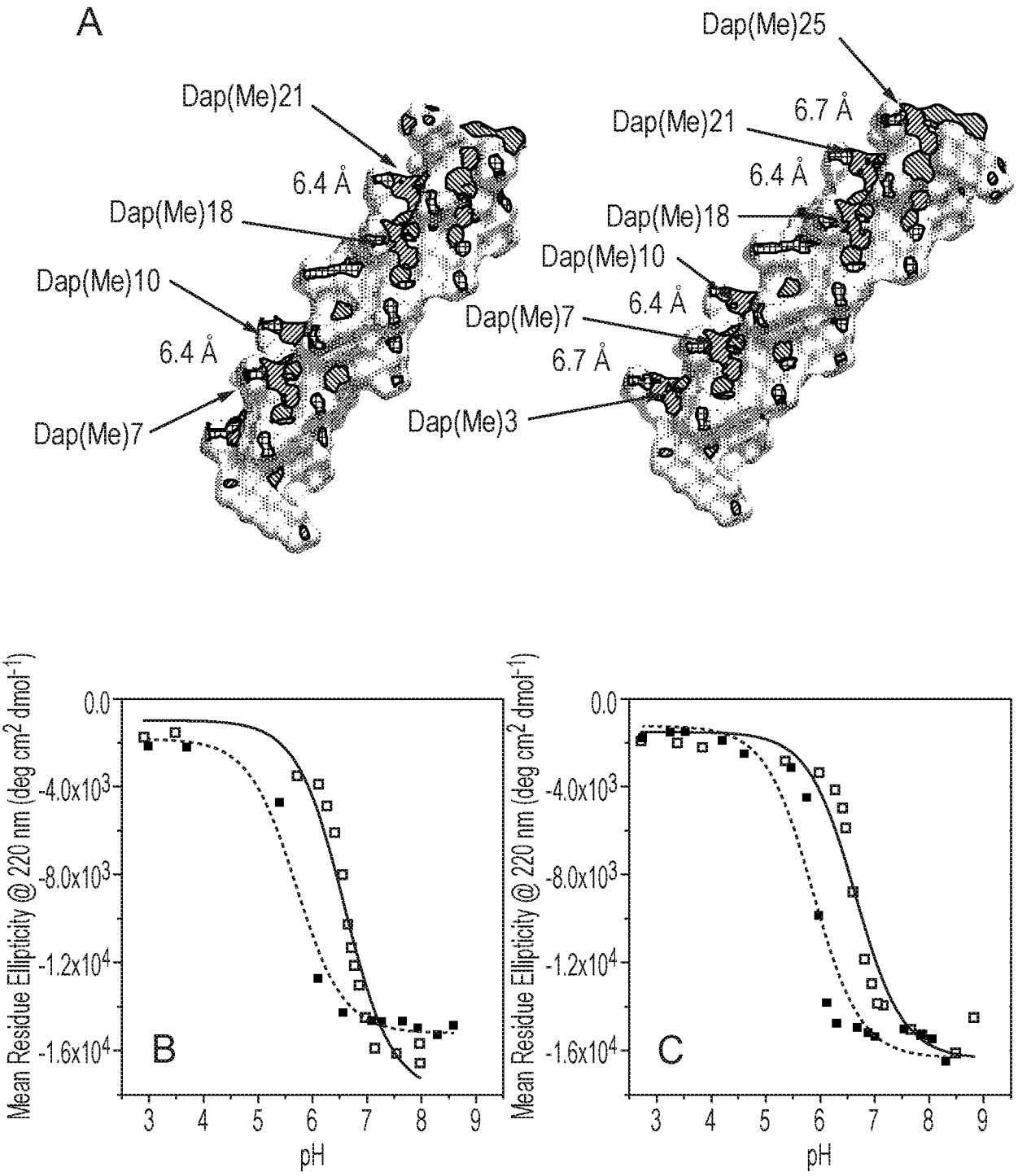
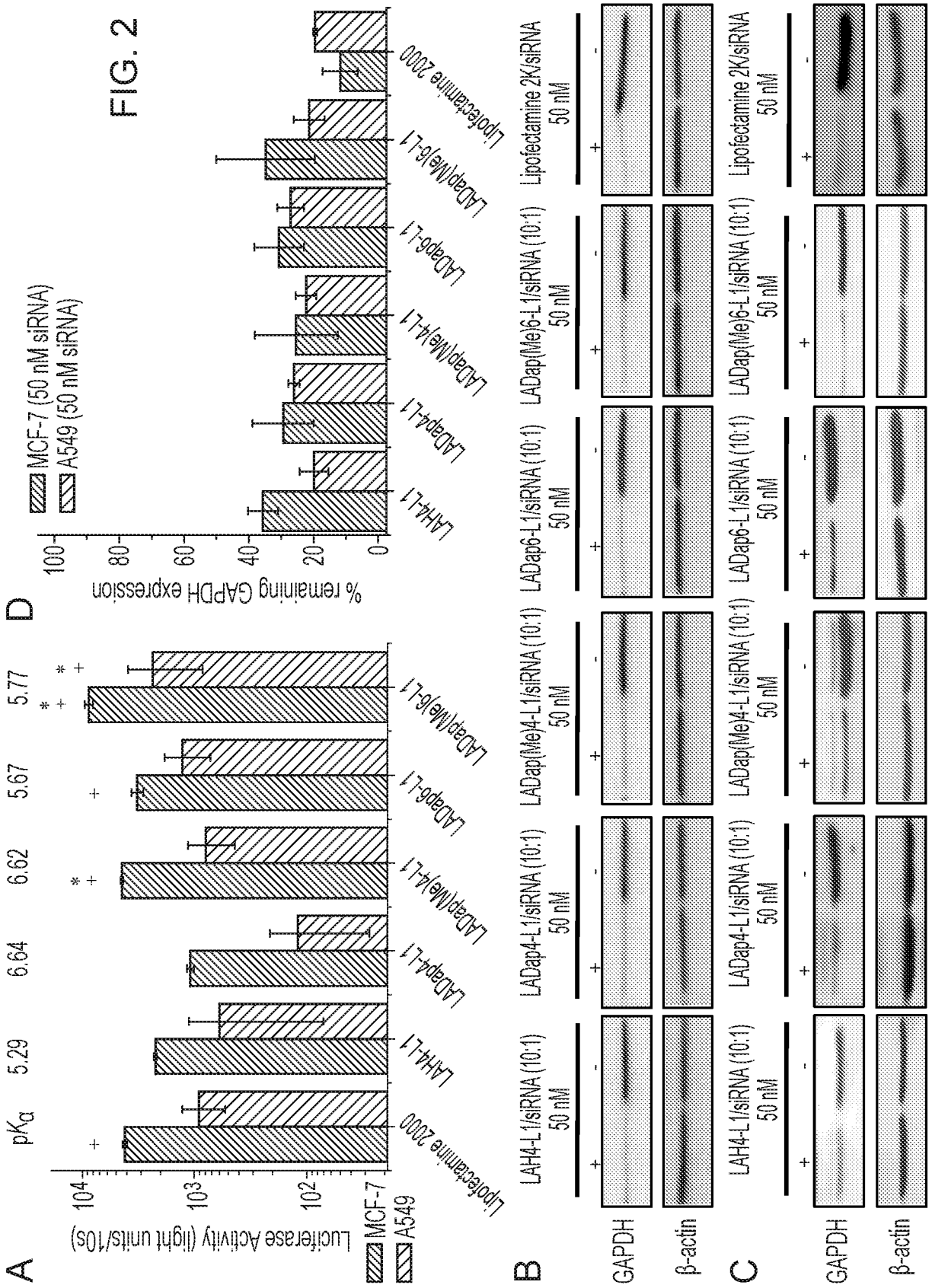

A
Dap(Me)21
6.4 Å
Dap(Me)18
Dap(Me)10
6.4 Å
Dap(Me)7
Dap(Me)25
Dap(Me)21
6.7 Å
6.4 Å
Dap(Me)18
Dap(Me)10
6.4 Å
Dap(Me)7
6.7 Å
Dap(Me)3
Mean Residue Ellipticity @ 220 nm (deg cm2 dmol-1)
0.0
-4.0x10^3
-8.0x10^3
-1.2x10^4
-1.6x10^4
B
3 4 5 6 7 8 9
pH
Mean Residue Ellipticity @ 220 nm (deg cm2 dmol-1)
0.0
-4.0x10^3
-8.0x10^3
-1.2x10^4
-1.6x10^4
C
3 4 5 6 7 8 9
pH

FIG. 1

FIG. 2


A
pKa 5.29 6.64 6.62 5.67 5.77
Luciferase Activity (light units/10s)
MCF-7
A549
LAH4-L1
LADap4-L1
LADap(Me)4-L1
LADap6-L1
LADap(Me)6-L1
Lipofectamine 2000
D
MCF-7 (50 nM siRNA)
A549 (50 nM siRNA)
% remaining GAPDH expression
B
LAH4-L1/siRNA (10:1) 50 nM
LADap4-L1/siRNA (10:1) 50 nM
LADap(Me)4-L1/siRNA (10:1) 50 nM
LADap6-L1/siRNA (10:1) 50 nM
LADap(Me)6-L1/siRNA (10:1) 50 nM
Lipofectamine 2K/siRNA 50 nM
GAPDH
β-actin
C
LAH4-L1/siRNA (10:1) 50 nM
LADap4-L1/siRNA (10:1) 50 nM
LADap(Me)4-L1/siRNA (10:1) 50 nM
LADap6-L1/siRNA (10:1) 50 nM
LADap(Me)6-L1/siRNA (10:1) 50 nM
Lipofectamine 2K/siRNA 50 nM
GAPDH
β-actin

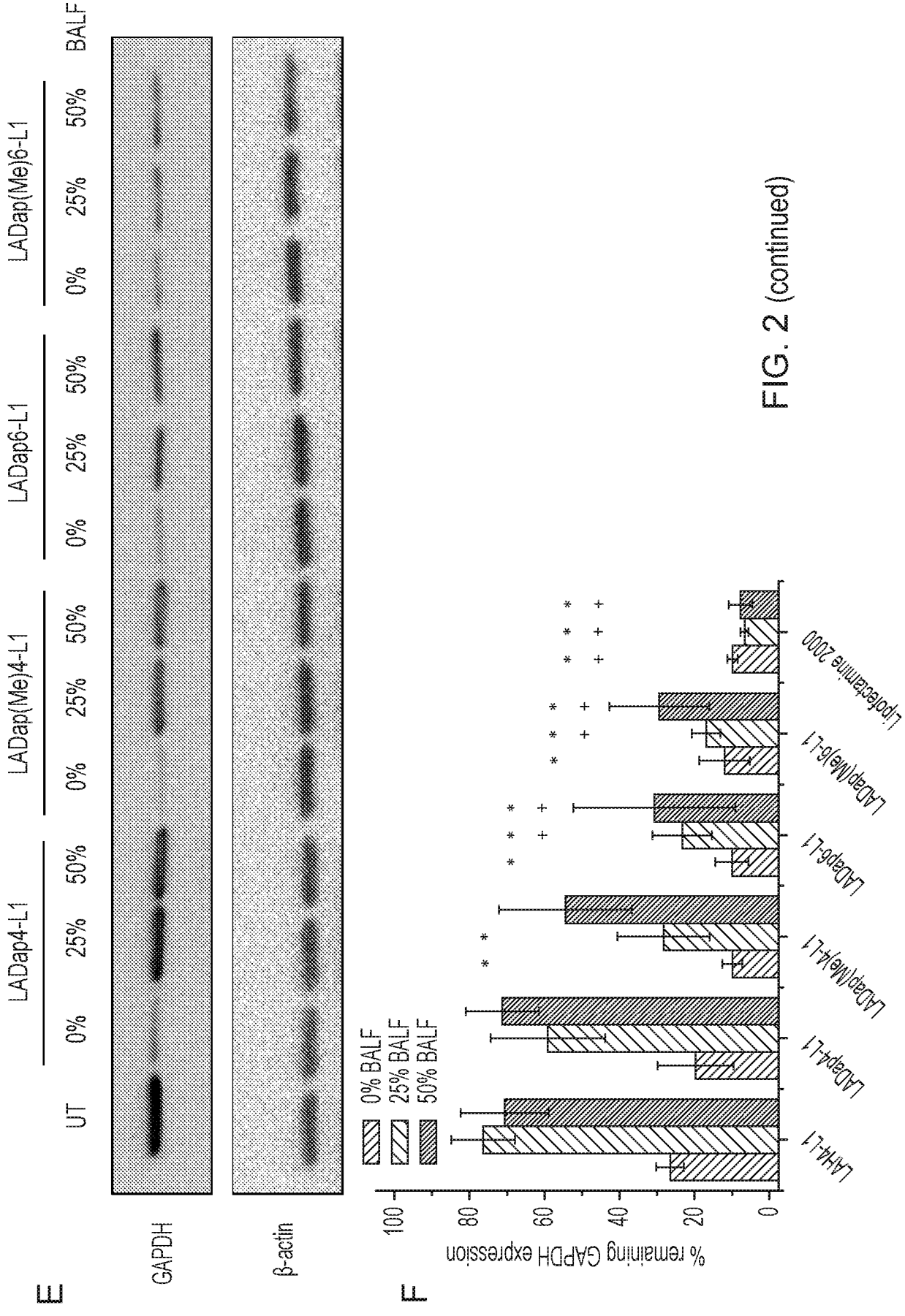


FIG. 2 (continued)

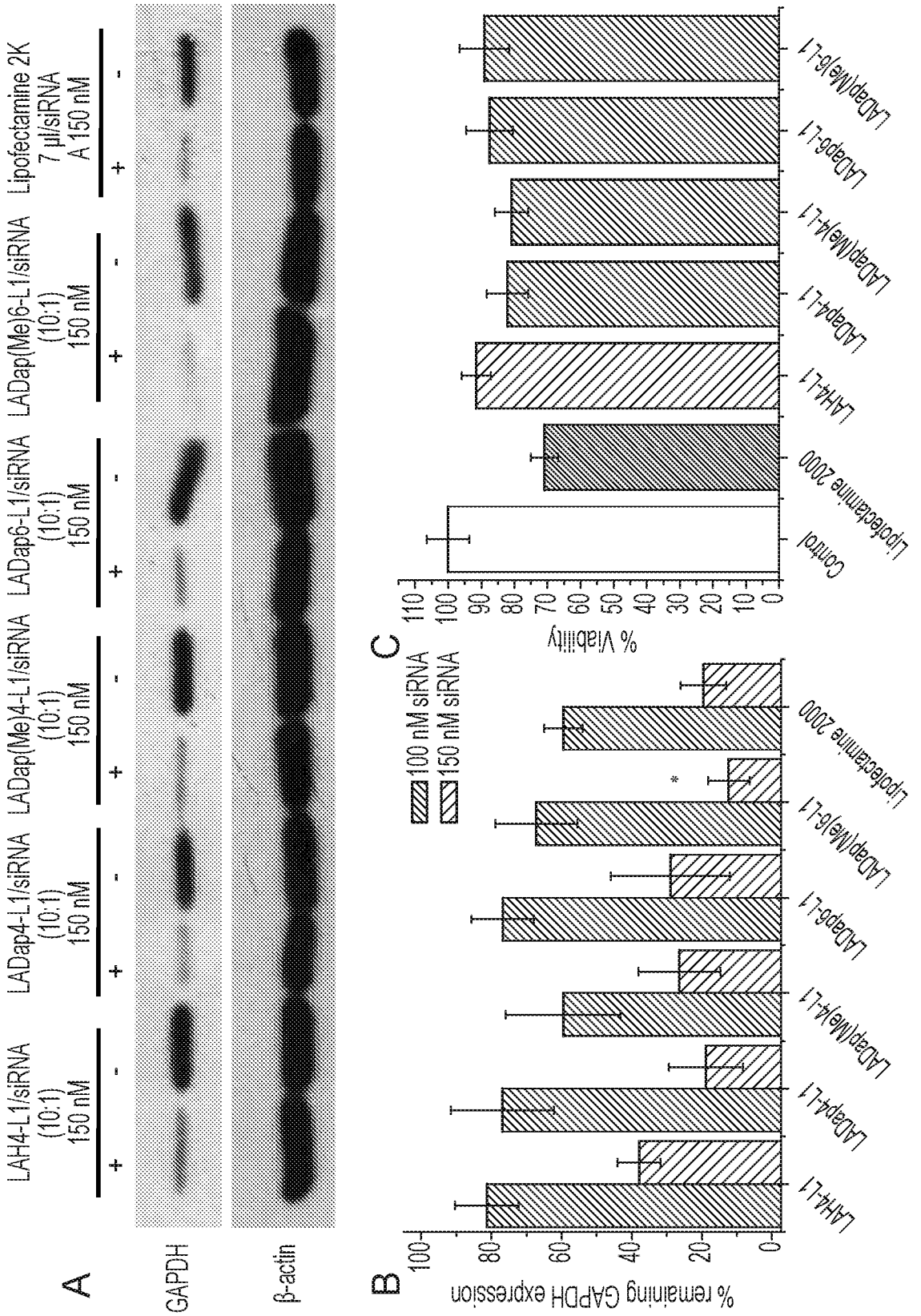

A
LAH4-L1/siRNA (10:1) 150 nM
LADap4-L1/siRNA (10:1) 150 nM
LADap(Me)4-L1/siRNA (10:1) 150 nM
LADap6-L1/siRNA (10:1) 150 nM
LADap(Me)6-L1/siRNA (10:1) 150 nM
Lipofectamine 2K 7 µl/siRNA A 150 nM
GAPDH
β-actin
B
% remaining GAPDH expression
100 nM siRNA
150 nM siRNA
LAH4-L1
LADap4-L1
LADap(Me)4-L1
LADap6-L1
LADap(Me)6-L1
Lipofectamine 2000
C
% Viability
Control
Lipofectamine 2000
LAH4-L1
LADap4-L1
LADap(Me)4-L1
LADap6-L1
LADap(Me)6-L1

FIG. 3

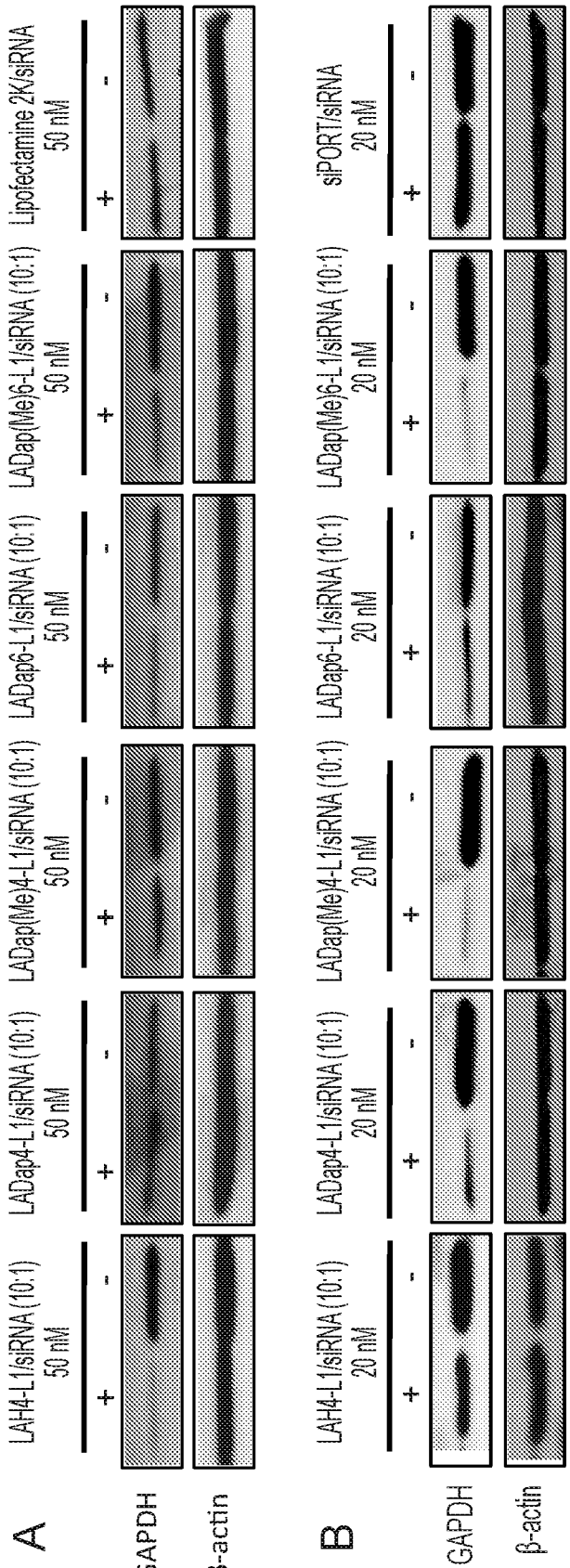

A
LAH4-L1/siRNA (10:1) 50 nM
LADap4-L1/siRNA (10:1) 50 nM
LADap(Me)4-L1/siRNA (10:1) 50 nM
LADap6-L1/siRNA (10:1) 50 nM
LADap(Me)6-L1/siRNA (10:1) 50 nM
Lipofectamine 2K/siRNA 50 nM
GAPDH
β-actin
B
LAH4-L1/siRNA (10:1) 20 nM
LADap4-L1/siRNA (10:1) 20 nM
LADap(Me)4-L1/siRNA (10:1) 20 nM
LADap6-L1/siRNA (10:1) 20 nM
LADap(Me)6-L1/siRNA (10:1) 20 nM
siPORT/siRNA 20 nM
GAPDH
β-actin

FIG. 4

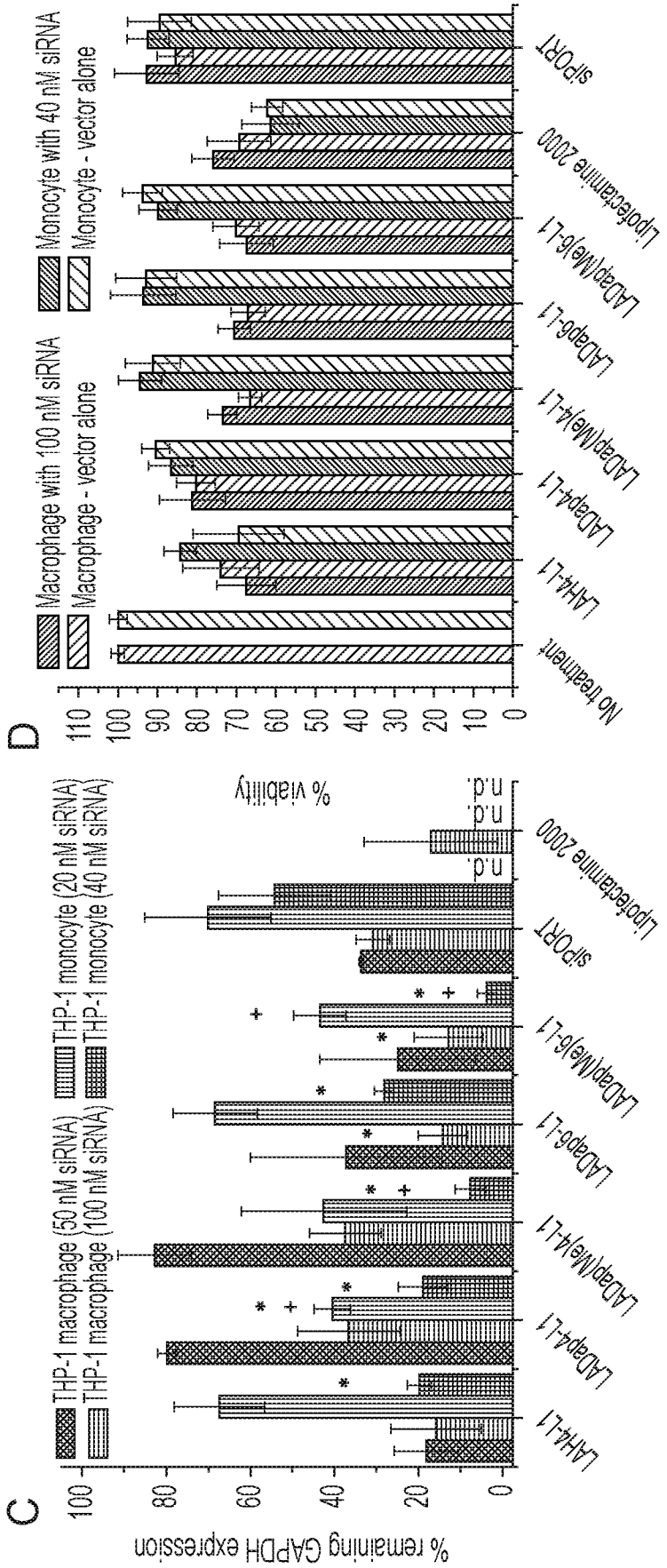


FIG. 4 (continued)

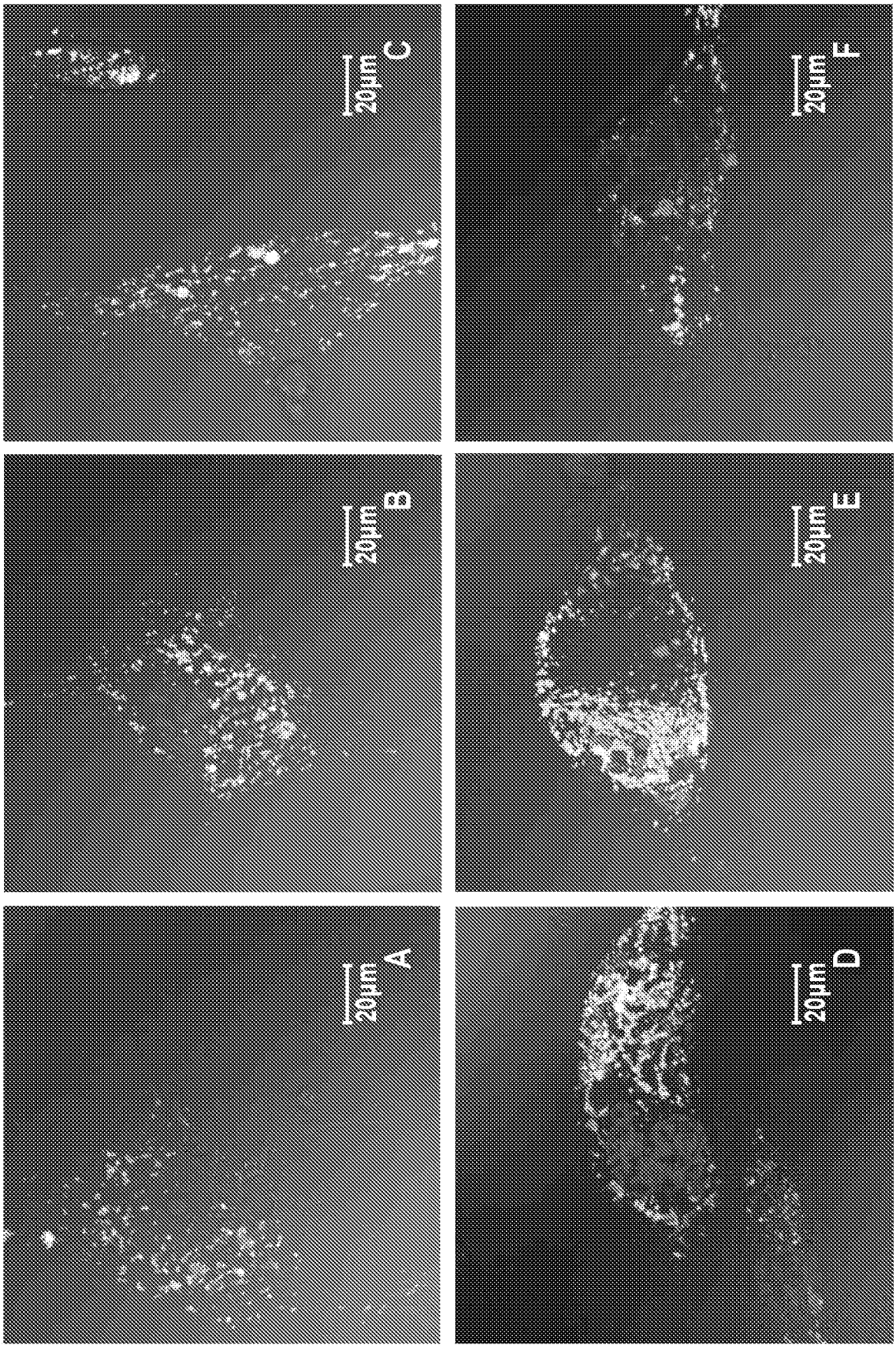


FIG. 5

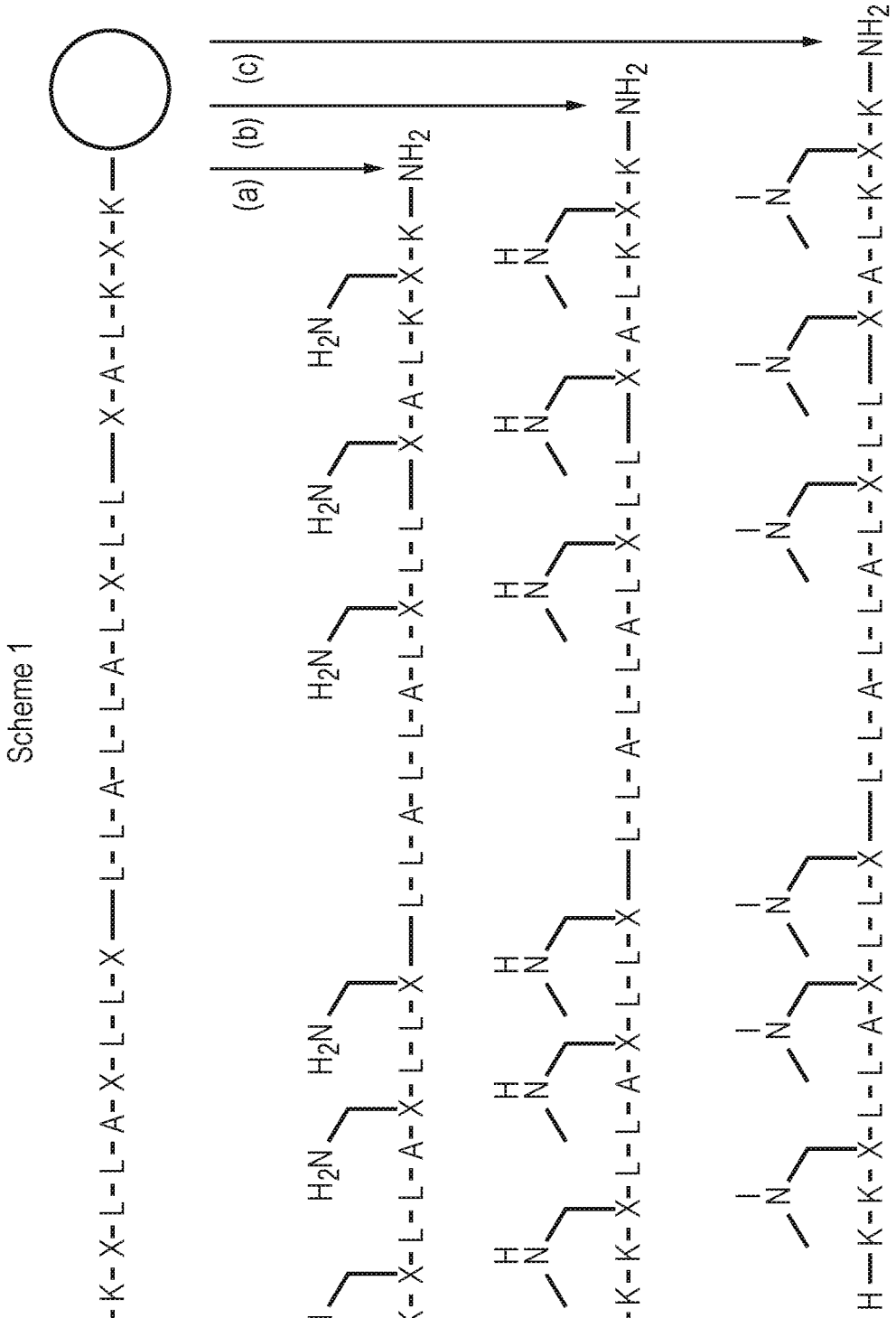

Scheme 1
(a)
(b)
(c)
where X - Dap(Dde)
(a) (i) N_2H_4, (ii) TFA
(b) (i) N_2H_4, (ii) 2-NBS-Cl, (iii) MSNBC, ME, (iv) TFA
(c) (i) N_2H_4, (ii) CH_2O, $NaCNBH_3$, (iii) TFA

FIG. 6

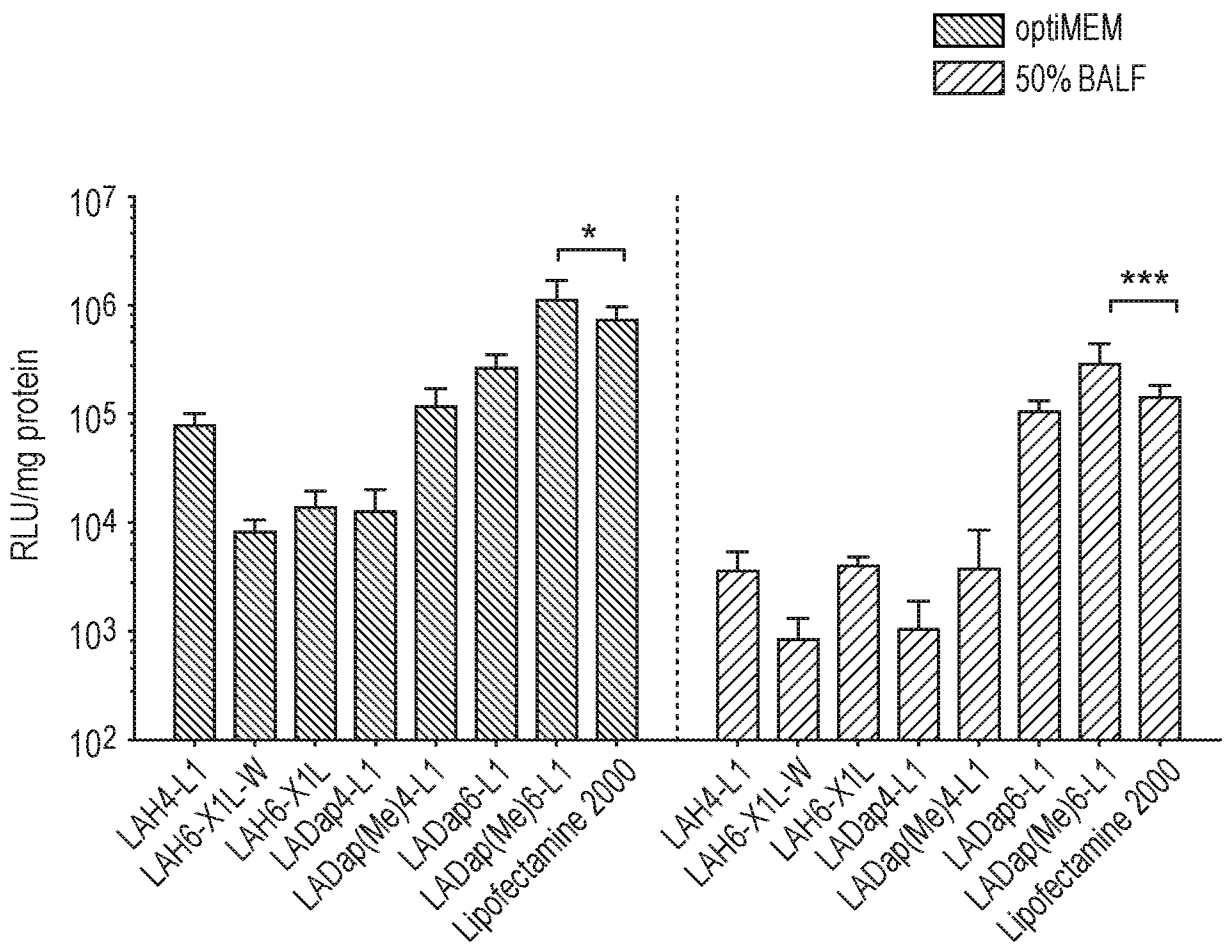

optiMEM
50% BALF
RLU/mg protein
10^7
10^6
10^5
10^4
10^3
10^2
*
***
LAH4-L1
LAH6-X1L-W
LAH6-X1L
LADap4-L1
LADap(Me)4-L1
LADap6-L1
LADap(Me)6-L1
Lipofectamine 2000
LAH4-L1
LAH6-X1L-W
LAH6-X1L
LADap4-L1
LADap(Me)4-L1
LADap6-L1
LADap(Me)6-L1
Lipofectamine 2000

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2014/051519

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YUN LAN ET AL: "Incorporation of 2,3-Diaminopropionic Acid into Linear Cationic Amphipathic Peptides Produces pH-Sensitive Vectors", CHEMBIOCHEM, vol. 11, no. 9, 14 June 2010 (2010-06-14), pages 1266-1272, XP55132470, ISSN: 1439-4227, DOI: 10.1002/cbic.201000073 the whole document	1-33

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 August 2014	20/08/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Luo, Xinmei

4

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JENNY K.W. LAM ET AL: "Effective endogenous gene silencing mediated by pH responsive peptides proceeds via multiple pathways", JOURNAL OF CONTROLLED RELEASE, vol. 158, no. 2, 1 March 2012 (2012-03-01) , pages 293-303, XP55132138, ISSN: 0168-3659, DOI: 10.1016/j.jconrel.2011.11.024 the whole document	1-15, 29-33
Y	MASON ET AL: "Optimising histidine rich peptides for efficient DNA delivery in the presence of serum", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 118, no. 1, 24 February 2007 (2007-02-24), pages 95-104, XP005903637, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2006.12.004 the whole document	1-15, 29-33
Y	PS STAYTON ET AL: "'Smart' delivery systems for biomolecular therapeutics", ORTHODONTICS AND CRANIOFACIAL RESEARCH, vol. 8, no. 3, 1 August 2005 (2005-08-01), pages 219-225, XP55133129, ISSN: 1601-6335, DOI: 10.1111/j.1601-6343.2005.00336.x the whole document	16-28
A	WO 2013/001041 A1 (GENETHON [FR]; FENARD DAVID [FR]; KICHLER ANTOINE [FR]; MARTIN SAMIA [) 3 January 2013 (2013-01-03) the whole document	1-33
X,P	VINCENZO ABBATE ET AL: "Manipulating the pH response of 2,3-diaminopropionic acid rich peptides to mediate highly effective gene silencing with low-toxicity", JOURNAL OF CONTROLLED RELEASE, vol. 172, no. 3, 1 December 2013 (2013-12-01), pages 929-938, XP55132558, ISSN: 0168-3659, DOI: 10.1016/j.jconrel.2013.09.033 the whole document	1-33

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2014/051519

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013001041 A1	03-01-2013	CA 2839633 A1	03-01-2013
		EP 2726497 A1	07-05-2014
		WO 2013001041 A1	03-01-2013