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Rational Design and Characterisation of a Linear Cell Penetrating Peptide for Non-Viral Gene Delivery --Manuscript Draft--

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Abstract:	<p>The design of a non-viral gene delivery system that can release a functional nucleic acid at the intracellular destination site is an exciting but also challenging proposition. The ideal gene delivery vector must be non-toxic, non-immunogenic, overcome extra- and intra-cellular barriers, protect the nucleic acid cargo from degradation with stability over a range of temperatures. A new 15 amino acid linear peptide termed CHAT was designed in this study with the goal of delivering DNA with high efficiency into cells in vitro and tissues in vivo. Rational design involved incorporation of key amino acids including arginine for nucleic acid complexation and cellular uptake, tryptophan to enhance hydrophobic interaction with cell membranes, histidine to facilitate endosomal escape and cysteine for stability and controlled cargo release. Six linear peptides were synthesised with strategic sequences and amino acid substitutions. Data demonstrated that all six peptides complexed pDNA to produce cationic nanoparticles less than 200 nm in diameter, but not all peptides resulted in successful transfection; indicating the influence of peptide design for endosomal escape. Peptide 4, now termed CHAT, was non-cytotoxic, traversed the plasma membrane of breast and prostate cancer cell lines, and elicited reporter-gene expression following intra-tumoural and intravenous delivery in vivo. CHAT presents an exciting new peptide for the delivery of nucleic acid therapeutics.</p>

Rational peptide design

DNA packaging and nanoparticle formation

Endocytosis and endosomal escape

In vitro gene expression

In vivo gene expression

Rational Design and Characterisation of a Linear Cell Penetrating Peptide for Non-Viral Gene Delivery

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Abstract

The design of a non-viral gene delivery system that can release a functional nucleic acid at the intracellular destination site is an exciting but also challenging proposition. The ideal gene delivery vector must be non-toxic, non-immunogenic, overcome extra- and intra-cellular barriers, protect the nucleic acid cargo from degradation with stability over a range of temperatures. A new 15 amino acid linear peptide termed *CHAT* was designed in this study with the goal of delivering DNA with high efficiency into cells *in vitro* and tissues *in vivo*. Rational design involved incorporation of key amino acids including arginine for nucleic acid complexation and cellular uptake, tryptophan to enhance hydrophobic interaction with cell membranes, histidine to facilitate endosomal escape and cysteine for stability and controlled

cargo release. Six linear peptides were synthesised with strategic sequences and amino acid substitutions. Data demonstrated that all six peptides complexed pDNA to produce cationic nanoparticles less than 200 nm in diameter, but not all peptides resulted in successful transfection; indicating the influence of peptide design for endosomal escape. Peptide 4, now termed *CHAT*, was non-cytotoxic, traversed the plasma membrane of breast and prostate cancer cell lines, and elicited reporter-gene expression following intra-tumoural and intravenous delivery *in vivo*. CHAT presents an exciting new peptide for the delivery of nucleic acid therapeutics.

Key Words: Gene Delivery; Linear Peptides; Cell Penetrating; Nucleic Acids; Non-viral

1. Introduction

Gene therapy requires an efficient vector that is non-toxic, non-immunogenic and can traverse the biological barriers to deliver the genetic material to the target site [1]. Peptide-based non-viral vectors have distinct advantages which include: (i) ease of synthesis, (ii) biocompatibility and (iii) low toxicity [2]. Cell Penetrating Peptides (CPPs) are short peptides designed to cross the cell membrane and deliver various cargo, such as nucleic acids, to a range of cell types [3,4]. Some of the first CPPs identified were natural peptides including the Penetratin peptide (RQIKIYFQNRRMKWKK), derived from the third helix of the homeodomain of Antennapedia [5], and the TAT peptide (GRKKRRQRRR), derived from the human immunodeficiency-1 virus (HIV-1) [6]. Both types of CPPs are characterised by regions of basic amino acids (R or K) and an α -helical conformation, where the amine of an amino acid forms a hydrogen bond with carbonyl group which is repeated every four residues; characteristics responsible for potent cellular penetration [7].

However, correlating the design of α -helical amphipathic peptides to functionality is inherently difficult as there is a fine tipping point where lysis and cytotoxicity can occur [8]. Therefore, there is a need to design simpler linear peptides that are easily synthesised, less expensive and with the right design can be just as effective. The success of CPPs such as oligoarginines (e.g. R8) indicates that an amphipathic nature is not essential for cell penetration [9]. In the case of Penetratin, it is the cationic nature rather than the helical structure that is responsible for cell penetration [10]. Arginine rich peptides (ARPs) have superior characteristics over other basic residues for nucleic acid binding and cell penetration as a result of the guanidinium moiety in the arginine amino acid side chain and are the cationic residue of choice [9-12].

Hydrophobicity is also essential for promoting interactions between the CPP and the lipid cell membrane [11]. For example, the hydrophobic counterion pyrenebutyrate has been shown to

enhance the transport of oligoarginines across lipid bilayers; increasing cellular uptake of R9 4-fold in HeLa cells [12]. Tryptophan is a strongly hydrophobic residue included in the design of synthetic peptides CADY (GLWRALWRLLRSLWRLWRA) and CADY2 (GLWWRLWWRLRSWFRLWFRA), which have been shown to penetrate cell membranes [13]. The authors attribute the cellular uptake mechanism utilised by CADY largely to the action of aromatic tryptophan, which directly interacts with cell membrane components. As a result, CADY delivered siRNA targeting GAPDH to U2OS human osteosarcoma cells with a resultant 80% knockdown of GAPDH at both the mRNA and protein levels [14]. Therefore, tryptophan residues were considered in the design of the linear peptides in this study.

Endosomal escape is also a critical factor in design of an effective CPP. Histidine-rich peptides are usually endosomolytic in nature and can facilitate endosomal escape through the proton sponge or 'flip-flop' effects [15]. For example, TAT peptides are typically trapped in the endosome but the addition of 10 histidine residues to TAT increased transgene expression up to 7000-fold in the human glioma cell line U251 [16]. Once the peptide-cargo has escaped the endosome, it is essential that the nucleic acid is released and studies have shown that the strong electrostatic forces may prevent this [17]. To circumvent this, cysteine residues have been employed to confer controlled intracellular cargo release through reduction of disulphide bonds in the intracellular environment [18]. It has been reported that the intracellular concentration of glutathione (GSH), which is three orders of magnitude higher than in the extracellular compartment, influences the reduction of disulphide bonds formed by cysteine residues [19]. The addition of cysteine residues has also been credited for improved transfection efficiency. Lo et al report the linear peptide CH₅TATH₅C produced up to 1,000-fold higher levels of transgene expression when compared to TAT-10H peptide containing no cysteine residues [16]. Similar effects were shown by Mann et al, as transfection efficiency was increased by

several orders of magnitude when cysteine residues were added to arginine- and histidine-containing peptides [20]. Thus, the inclusion of cysteine residues in a linear CPP is an important design criterion.

In this study, linear peptides were rationally designed to be as cost-effective and efficient as possible. Criteria for peptide design included: (i) limiting peptide sequence length without compromising cell penetration or nucleic acid binding; (ii) inclusion of six arginine residues, the minimum number of residues required for cell uptake [21,22]; (iii) inclusion of tryptophan residues to improve hydrophobic interaction with cell membranes; (iv) insertion of histidine residues at the core, for pH dependent endosomal escape; (v) inclusion of cysteine residues positioned at each terminal to enhance stability and cargo release once inside the cell.

Six peptides were systematically designed, according to the above criteria, in pairs to allow direct comparison and analysis of functionality based on specific amino acid sequence differences. The sequences of the novel linear peptides are shown in Table 1. The peptides were assessed for functionality and compared with the successful pH responsive amphipathic RALA peptide [23–32], which enabled the selection of the most promising peptide candidate, termed *CHAT* for further studies. The *CHAT*/pDNA nano-complexes were further characterised with respect to physiochemical properties, *in vitro* cellular delivery and *in vivo* functionality. The nano-complexes were also tested for functionality and stability post-lyophilisation which is critical in the development of a new cost-effective non-viral vector for gene therapy applications.

Table 1: Paired linear peptide sequences and design rationale

Peptide name	Sequence	Molecular weight (Da)	Design Rationale
Pep1	CRRRWHHHHHWRRRC	2219.50	<ul style="list-style-type: none"> Histidine residues at the core of the peptide sequence flanked by arginine. Two tryptophan residues included for enhanced interaction with cell membranes. Tryptophan residues at different positions to assess optimal sequence and positioning.
Pep2	CWRRRHHHHHRRRWC	2219.50	
Pep3	CHRHRHRWHRHRHC	2170.48	<ul style="list-style-type: none"> Arginine and histidine residues arranged in either alternating or block sequence to assess impact on functionality. Arginine block positioned at the core of the sequence, flanked by histidine at each end which contrasts to positioning in Pep1 and 2. Tryptophan positioned in the middle to assess positioning which contrasts the positioning in Pep1 and 2.
Pep4 (CHAT)	CHHHRRRWRRRHHC	2170.48	
Pep5	CRRRRWRRRRC	1660.01	<ul style="list-style-type: none"> Histidine removed from Pep5 to assess if endosomal escape is possible without Histidine residues Pep6 designed to include two glutamic acid residues reported to be involved in endosomal escape mechanisms in amphipathic peptides
Pep6	CEHHRRRWRRRHHC	2154.43	

2. Methods

2.1 Materials

2.1.1 Peptides

All peptides were produced by solid state synthesis (fluorenylmethoxycarbonyl (Fmoc) chemistry) (Biomatik, Canada) and supplied as a lyophilised powder that required reconstitution before use. Peptides were supplied in the acetate salt form and >95% purity. Following cleavage from the resin the desired products were purified by and confirmed by using Reverse Phase High Performance Liquid Chromatography (RPHPLC).

2.1.2 Plasmid DNA

pEGFP-N1 was purchased from Clontech (USA) and pCMV-Red Firefly Luc (pLuc) was purchased from Oxford Genetics (UK). Plasmid DNA (pDNA) was propagated in MAX Efficiency® DH5α™ Competent Cells (Life Technologies, UK), purified using PureLink® HiPure Plasmid Filter Maxiprep Kit (Life Technologies, UK) and quantified by UV absorption at 260 nm.

2.1.3 Cell lines

MCF-7 and MDA-MB-231 breast cancer cells (ATCC, USA) were maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, UK) supplemented with 10% foetal calf serum (FCS). DU145 and PC-3 prostate cancer cells were maintained as monolayers in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FCS. When cells reached approximately 80% confluency, they were passaged and were not used for experimental protocols beyond a passage number of 20. Cells were maintained in an incubator at 37°C with 5% CO₂ atmosphere and subjected to mycoplasma testing routinely. All *in vitro* cell experiments were carried out at 37°C in a 5% CO₂ incubator. All cell lines were

authenticated by short tandem repeat (STR) profiling, which was conducted by the respective supplier.

2.1.4 Animals

BALB/c SCID mice were purchased from Charles River Laboratories (UK). The animals were housed in an open facility at 21°C and 50% humidity with food and water *ad libitum*. Experimental protocols were compliant with the UK Scientific Act of 1986 and covered by the Department of Health, Social Services and Public Safety project license number 2794.

2.2. Methods

2.2.1 Design and Synthesis of Novel Peptides

Six novel peptides were designed to produce a peptide that could condense pDNA into nanoparticles and transfect cells with a short simplistic linear design, to reduce production complexity and costs. Arginine residues were included in various sequences to provide DNA complexation and facilitate cellular uptake. Without an amphipathic, fusogenic nature the linear design may be hindered by the lack of endosomal escape mechanism. Therefore, histidine residues have been included to assess the effect on endosomal escape. Cysteine residues have also been included for controlled intracellular release. Peptides were designed in pairs and amendments were made to the peptide sequences, including amino acid substitutions and deletions (Table 1). Maestro 3D structure prediction software (version 12.2.012, Schrodinger, Germany) and the Protparam tool were then used to predict the possible properties of the peptides.

2.2.2 Formulation of Peptide/pEGFP-N1 complexes

pEGFP-N1 pDNA was complexed by each peptide according to a range of N:P ratios (the molar ratio of positively charged nitrogen atoms in the peptide to negatively charged

phosphates in the pDNA backbone) via electrostatic interactions. According to the N:P ratio, a quantity of peptide was added to 1 µg of pDNA in aqueous solution and the final volume was adjusted to 50 µL with molecular grade water. Samples were then incubated at room temperature for 30 min to allow the formation of the nanoparticles. All nanoparticles were used immediately following their preparation unless otherwise stated.

2.2.3 Size and zeta potential analysis of nanoparticles

Peptide/pDNA complexes were prepared at a range of N:P ratios to complex 0.5 µg pEGFP-N1 as described in section 2.2.2. A Malvern Zetasizer Nano ZS instrument with DTS software (Malvern Instruments, UK) was used to measure the mean hydrodynamic particle size of nanoparticles dispersed in H₂O by Dynamic Light Scattering (DLS) at 20°C followed by measurement of zeta potential using Laser Doppler Velocimetry.

2.2.4 Transfection with peptide nanoparticles delivering pEGFP-N1

MCF-7 and MDA-MB-231 breast cancer cells were seeded in 96-well tissue culture plates at a density of 2.25×10^4 and 2.5×10^4 cells per well respectively, and PC-3 and DU145 prostate cancer cells were seeded at a density of 2.5×10^4 and 2×10^4 cells per well respectively. Cells were allowed to adhere overnight at 37°C with 5% CO₂, before being conditioned for 2 h in OptiMEM (Invitrogen, UK) prior to transfection. 50 µL of Peptide/pDNA nanoparticles containing 0.5 µg pEGFP-N1 was added to the appropriate wells and cells were incubated with the nanoparticles for 4 h, before being replaced with complete media. Endosomal escape was assessed by adding 1 mM chloroquine (Sigma-Aldrich, UK) immediately before transfection, giving a final concentration of 10 µM chloroquine in the nanoparticle transfection mix.

2.2.5 *Fluorescence microscopy*

To facilitate qualitative analysis of pEGFP-N1 expression correlating to transfection efficiency, cells were visualised and imaged 48 h following transfection under fluorescent light using an EVOS FL Cell Imaging System (Life Technologies, UK).

2.2.6 *Flow cytometric analysis of transfection efficiency*

Transfected cells were trypsinised and resuspended in 2% formaldehyde in PBS and kept refrigerated at 4°C until analysis. A FACScalibur system (BD Bioscience, UK) was used for the detection of green fluorescent protein (GFP) expressing cells.

2.2.7 *Gel retardation assay*

CHAT/pDNA complexes were prepared at a range of N:P ratios to complex 0.5 µg pEGFP-N1 before incubation at room temperature for 30 min as described in section 2.2.2. Samples were then electrophoresed through a 1% agarose gel containing ethidium bromide (EtBr) with Tris-acetate (TAE) running buffer at 100 V for 60 min. Mobility of pDNA in the gel was visualised by ethidium bromide staining under UV light using a Multispectrum Bioimaging System (UVP, UK). ImageJ software (version 1.52a, NIH, USA) was used to perform densitometric analysis on gel retardation gel images to quantify fluorescent intensity from each band correlating to amount of pDNA in each sample.

2.2.8 *Complexation of pDNA*

CHAT/pDNA complexes were prepared at a range of N:P ratios to complex 0.5 µg pEGFP-N1 as described in 2.2.2. Quant-iT™ PicoGreen® Reagent (Life Technologies, UK) was diluted 1:200 in TAE buffer and 50 µL was added to each sample. Sample fluorescence was analysed by excitation at 480 nm and the fluorescence emission intensity measured at 520 nm using a FLUOstar Omega Multimode Plate Reader (BMG Labtech, UK).

2.2.9 Transmission electron microscopy (TEM)

CHAT/pDNA complexes were prepared with 1 µg pEGFP-N1 as described in section 2.2.2 and total volume was reduced to 30 µL to increase the concentration. Nanoparticles were loaded onto a carbon coated copper 400 mesh grid (TAAB Laboratories, UK) and allowed to dry. Following drying, the samples were stained with UranylLess (EMS Microscopy Academy, USA) at room temperature for 1 min, washed with molecular grade water and allowed to dry again. Nanoparticles were imaged using a JEM-1400Plus Transmission Electron Microscope (Jeol, USA) at an accelerating voltage of 120 kV.

2.2.10 Serum stability

CHAT/pDNA complexes were prepared at N:P ratio 12 as described in section 2.2.2. Peptide complexes were incubated for 1, 2, 3, 4, 5 and 6 h at 37°C in the presence and absence of 10% foetal calf serum. Subsequently, sodium dodecyl sulfate (SDS) (Sigma-Aldrich, UK) was added (10%) to de-complex the pDNA from CHAT. Following incubation for 10 min, samples were electrophoresed through a 1% agarose gel containing ethidium bromide with Tris-acetate running buffer at 100 V for 60 min and visualised using a Multispectrum Bioimaging System (UVP, UK).

2.2.11 Temperature stability

CHAT/pDNA complexes were prepared at N:P ratio 12 as described in section 2.2.2. Particle size was measured at various temperatures using a Nano ZS Zetasizer with DTS software (Malvern Instruments, UK).

2.2.12 Stability over time

CHAT/pDNA complexes were prepared at N:P ratio 12 as described in section 2.2.2. Complexes were stored at room temperature for 0, 7, 14, 21 and 28 days before the particle size and zeta potential were measured as described in section 2.2.3.

2.2.13 Cellular uptake pathway studies

To investigate cellular entry of CHAT/pDNA complexes, prior to transfection as described in Section 2.2.4, cells were pre-treated with either 10 μ M Chlorpromazine (Sigma-Aldrich, UK), 200 μ M Genistein (Sigma-Aldrich, UK), 5 mmol/L methyl- β -cyclodextran (M β CD) (Sigma-Aldrich, UK) or stored at 4°C in OptiMEM for 2 h before transfection. Transfection efficiency was then analysed by flow cytometry using FACScalibur system (BD Bioscience, UK) as described in Section 2.2.6.

2.2.14 Microscopic analysis of intracellular delivery of pDNA

pDNA was fluorescently labelled with the Cy3 fluorophore (Mirus, USA) according to manufacturer's instructions. MDA-MB-231 cells seeded at 4×10^4 per well were then transfected with nanoparticles as described in section 2.2.4. 4 h post-transfection cells were fixed and permeabilised with 4% formaldehyde and 0.1% Triton-X (Sigma, UK) for 20 min. Cells were then stained with Fluorescein (FITC)-phalloidin (Life Technologies, UK) at room temperature for 15 min and mounted onto a microscope slide with Fluoroshield mounting medium containing DAPI nuclear stain (Life Technologies, UK). Cells were then imaged using a TSC SP5-Leica Microsystems confocal microscope (Leica, UK) and analysed using LAS AF Lite Software (Leica, UK).

2.2.15 Cell viability

Cell viability was evaluated by **AlamarBlue Cell Viability Assay (Invitrogen, UK)**. MDA-MB-231 and PC-3 cells were seeded at a density of 1.5×10^4 cells per well and MCF-7, DU145 cells seeded at 1.2×10^4 cells per well, and allowed to adhere in a 96 well plate. Cells were transfected with CHAT/pDNA as described in section 2.2.4. CellTiter reagent was added to each well 24 h post-transfection and incubated for 2 h. Absorbance was read at 490 nm using a BioTek Powerwave XS plate reader (BioTek, UK) and any background absorbance was

subtracted from readings. Cell viability was expressed as a percentage of the untreated control where the untreated control is considered to be 100% viable.

2.2.16 Lyophilisation of CHAT/pDNA complexes

CHAT/pDNA complexes were prepared at N:P 8, 10 and 12 and transferred into a glass lyophilisation vial with the cryoprotectant Trehalose at a concentration of 2.5 wt% and frozen at -40°C for 1 h. Subsequently the samples were subjected to primary drying at -40°C and 60 mTorr for 24 h and a secondary drying program of -30°C and 120 mTorr for 3 h, -30°C and 190 mTorr for 3 h, -25°C and 190 mTorr for 3 h and 20°C and 190 mTorr for 6 h.

2.2.17 Haemolysis assay

Ovine whole blood (TCS Biosciences Ltd, UK) was centrifuged at 400 g for 20 min to separate erythrocytes. Erythrocytes were washed twice using citrate phosphate buffer at either pH 5.5 or pH 7.4, centrifuged at 400 g for 20 min, and supernatant discarded. Citrate phosphate buffer solutions were prepared with 0.1 M citric acid ($\text{C}_6\text{H}_8\text{O}_7$) and 0.2 M disodium hydrogen phosphate (Na_2HPO_4). Erythrocytes were then re-suspended in citrate phosphate buffer solution (pH 5.5 and pH 7.4) at a concentration of 1×10^8 cells/mL. CHAT/pEGFP-N1 complexes were prepared at N:P 12, and added to erythrocyte suspension at each pH, and incubated at 37°C for 30 min under constant shaking at 100 rpm. Following incubation, erythrocytes were centrifuged at 400 g for 5 min, and the supernatant collected. Absorbance of supernatant was measured using a Nanodrop 2000 spectrophotometer (ThermoScientific, USA) at 541 nm. A solution of 1% Triton X-100 was used as a positive control, representing 100% haemolysis. Samples treated only with the citrate phosphate buffer at each pH served as negative controls. Percentage haemolysis was calculated as a percentage relative to the positive control.

2.2.18 *In vivo functionality*

pLuc was labelled with Cy5 fluorophore (Mirus Bio, USA) at 1:0.5 w/v%. CHAT/pLuc-Cy5 complexes containing 20 µg pDNA at N:P 8 were lyophilised and reconstituted before being administered via injection directly into the tumour site of BALB/c SCID mice bearing subcutaneous 4T1 breast cancer xenografts on the dorsal flank. As a commercial control in vivo PEI (PolyPlus, UK) was used to synthesise PEI/pLuc-Cy5 complexes at 2:1 ratio. Additionally, N:P 8 CHAT/pLuc complexes containing 50 µg pDNA were delivered intravenously via tail vein injection to BALB/c SCID mice bearing subcutaneous MDA-MB-231 breast cancer xenografts on the dorsal flank. Whole-body bio-imaging was carried out 24 h after treatment. Mice were administered intraperitoneally with 150 mg/kg D-Luciferin potassium salt (GoldBio, USA) in PBS 10-20 min prior to imaging, as per manufacturer's instructions. Under gaseous anaesthetic of isoflurane, mice were imaged using the InVivo Xtreme Preclinical Optical/X-ray imaging system (Bruker, USA). Mice were then sacrificed and the lungs, liver, kidneys, spleen, brain and tumour were harvested for *ex vivo* analysis.

2.2.19 *Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)*

mRNA was extracted from harvested organs by phase separation using TRIzol Reagent (Life Technologies, UK) as per manufacturer's instructions. Reverse transcription was carried out using a TC-312 thermal cycler (Techne, UK) with cycles set at 25°C for 5 min, 37°C for 90 min and 70°C for 10 min to convert mRNA to cDNA. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) analysis was conducted to probe for luciferase transgene expression using 96-well plates (Roche, UK). Primers used in qRT-PCR were designed using Primer-Blast tool available on the National Centre for Biotechnology Information website (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and purchased from Integrated DNA Technologies (IDT, UK). Primer efficiency (between 1.9 and 2.1) and specificity was confirmed through the generation of standard curves from serially diluted samples and melt

curve analyses. Primer sequences as follows: Luciferase Forward 5' TCGTGACTTCCCATTGCCA 3', Luciferase Reverse 5' GGGATGATCTGGTTGCCGAA 3', β -actin Forward 5'AGGAGTACGATGAGTCCGGC 3', β -actin Reverse 5' AAAACGCAGCTCAGTAACAGTC 3'. For each primer set the volumes required to give triplicates of 9 μ L/well for each sample were calculated based on the following volumes: 5 μ L SYBR Green (Roche, UK), 3 μ L of H₂O and 1 μ L of primer (IDT, UK). Subsequently, 1 μ L of sample cDNA was added to appropriate wells to give a final volume of 10 μ L/well. qRT-PCR was carried using a Lightcycler 480 II (Roche, UK). A 'touchdown' qRT-PCR protocol was used where denaturation was conducted at 95°C for 20 sec followed by annealing starting at 67°C for 10 sec which was reduced by 3°C every cycle for four cycles. A further 45 cycles of denaturation (95°C for 20 sec), annealing (55°C for 10 sec) and extension (72°C for 10 sec) were carried out. Ct values generated were used to quantify luciferase expression relative to β -actin using the $\Delta\Delta$ Ct method. Gene expression levels were calculated and expressed in terms of fold change relative to untreated control.

2.2.20 Statistical analysis

Unless otherwise stated, three independent experiments were conducted for analysis of data from the nanoparticle characterisation and the in vitro and in vivo studies. The results are presented as mean $\pm$ SEM (n = 3). Statistically significant differences were computed using either unpaired t-test or one-way analysis of variance and a p-value of ≤ 0.05 being considered as significant. Statistical posthoc tests for individual experiments are detailed in figure legends. Statistical analysis was performed using Prism 8.0 (GraphPad Software, CA, USA).

3. Results

3.1. Peptide design

Peptides were designed in pairs to facilitate direct comparison and analysis of functionality based on specific amino acid sequence differences (Table 1). The Protparam tool was used to compute the possible physical and chemical properties of each peptide and parameters such as molecular weight and number of positively charged residues were then used when calculating N:P ratios for each peptide. Maestro 3D structure modelling software was also used to predict the possible structure and allow visualisation of amino acid placement in each peptide (Figure 1).

Figure 1: Predicted 3D structure of novel peptides computed using Maestro software (Schrodinger). Amino acid key: yellow: cysteine, blue: arginine, green: tryptophan, turquoise: histidine, red: glutamic acid.

3.2. Initial screening of peptides physicochemical properties

Initial studies were conducted to assess if the novel peptides would complex pDNA to form nanoparticles suitable for cellular uptake. The gel retardation assay indicated that pEGFP-N1 was fully neutralised by all six linear peptides (S1 & S2). A complexation study confirmed these findings where increasing N:P ratio resulted in an increased complexation efficiency with each peptide (S3). Complexation above 70% with Pep3 and Pep4 was achieved from N:P 4 and by N:P 6 with Pep1, Pep2 and Pep5, but Pep6 never reached more than 60% complexation. DLS was used to characterise the complexes with regard to hydrodynamic size, zeta potential, PDI and particle count. The results show that pEGFP-N1 was complexed by each peptide into cationic nanoparticles at various N:P ratios and Pep1-4 produced particles with low PDI, as summarised in Table 2 (S4 & S5). However, Pep5 and Pep6 displayed higher PDI values and lower zeta potentials which may be attributed to differences in peptide sequence when compared to Pep1-4.

Table 2: Summary of nanoparticle characteristics with each novel peptide at N:P 12 following DLS analysis using a Malvern Zetasizer Nano ZS (n=3 +/-SEM).

Peptide	Size (nm)		Zeta potential (mV)		PDI
	Mean	SD	Mean	SD	
1	127.60	67.60	27.30	5.41	0.31
2	154.90	39.70	35.30	2.92	0.20
3	136.79	39.53	30.60	2.39	0.23
4	207.30	42.87	29.00	1.70	0.25
5	101.89	26.74	20.45	1.77	0.45
6	156.60	12.59	10.70	3.09	0.72

3.3. Functionality of novel peptides

To assess the functional ability of each peptide to deliver pDNA cargo to cells, nanoparticles at a range of N:P ratios with each peptide were prepared with pEGFP-N1 and delivered to MCF-7 and MDA-MB-231 breast cancer cells (S6). Figure 2 shows fluorescence microscope images of each peptide following transfection in both cell lines at N:P 12. Pep1-4 and Pep6 indicated successful transfection, but Pep5 was not any more efficient than the DNA only (S6E). In fact, the only peptide capable of producing transfection efficiencies comparable to RALA (30% in MCF-7 and 27% in MDA-MB-231) was Pep4, which at N:P 12 resulted in 30% and 23% transfection in MCF-7 and MDA-MB-231 cells respectively (Figure 2B).

Figure 2: In vitro functionality of novel linear peptides in MC-7 and MDA-MB-231 breast cancer cells. Cells were transfected for 4 h with peptide/pEGFP-N1 nanoparticles at N:P ratios

12 or RALA and incubated for a further 48 h before analyses. A) Fluorescent microscopy images captured with and EVOS FL Cell Imaging System to visualise the expression of the GFP (Scale bar = 400 μ m). B) Mean Fluorescence Intensity (MFI) value of GFP in each sample analysed with flow cytometry using a BD FACS Calibur C) Quantification of transfection efficiency analysed by flow cytometry using a BD FACS Calibur ($\pm$ SEM, n=3). D) Quantification of transfection efficiency in MCF-7 breast cancer cells in the presence or absence of endosomal escape agent chloroquine (10 μ M) analysed by flow cytometry using a BD FACS Calibur ($\pm$ SEM, n=3). Statistical analysis was carried out by One-Way ANOVA and Dunnett post-test (*; p-value <0.05, **; p-value <0.01, ***; p-value <0.001).

MCF-7 cells were transfected with nanoparticles in the presence or absence of chloroquine, a known endosomal disrupter. Higher transfection efficiency in the presence of chloroquine would indicate that the peptides cannot escape the endosome. In MCF-7 cells, transfection efficiencies with Pep4, Pep5 and Pep6 were not significantly different following the addition of chloroquine (Figure 2C), indicating that endosomal entrapment did not impede transfection. Statistically significant increases in transfection were observed following transfection with Pep1, Pep2 and Pep3 in the presence of chloroquine (*; p-value <0.05) indicating that each of these peptides did not escape the endosome in MCF-7 cells.

The initial screening of the peptides indicated that Pep4 fulfilled criteria with respect to physiochemical characteristics, cellular entry and endosomal escape. The sequence of Pep4 (CHHHRRRWRRRHHC) has given rise to the nomenclature of CHAT (Cysteine, **H**istidine, Arginine, **T**ryptophan) and all subsequent studies will be with this peptide.

3.4. Characterisation of CHAT/pDNA complexes

The agarose gel indicated charge neutralisation of pDNA from N:P ratio 4 onwards with no migration through the gel. Indeed, with higher N:P ratios the pDNA is less visible; indicative of a tighter condensation as confirmed with the densitometric analysis (Figure 3A & Figure 3B). The Picogreen assay indicated complexation of the pDNA at 90% from NP10 (Figure 3C). The nature of intercalated electrostatic interaction between peptide and DNA will always have <10% nucleic acid detectable even though it is fully complexed in the nanoparticle. TEM revealed the formation of nanoparticles at N:P 10 (Figure 3D) and with a Z-Average of 207.3 nm and a zeta potential of +29 mV (Figure 4). Figure 4C also indicates that over 99% of the nanoparticle population are nano-sized.

Figure 3: Physicochemical characteristics of CHAT. A) Agarose gel electrophoresis indicating mobility of pEGFP-N1 when combined at different N:P ratios with CHAT. B) Densitometry of relative fluorescence detected in the agarose gel with respect to characteristic DNA conformation bands (i.e. supercoiled, open circular/nicked or linear conformation) (n=3, $\pm$ SEM). C) DNA complexation efficiency assay using Quant-iT™ PicoGreen® Reagent indicating the efficiency of CHAT to complex DNA at a range of N:P ratios (n=3, $\pm$ SEM). D) Image of CHAT/pDNA complexes at N:P 10 taken using transmission electron microscopy (T.E.M.) at 40 k magnification (Scale bar = 200 nm).

Figure 4: Physicochemical characteristics of CHAT. A) Mean hydrodynamic size (Z-average) and zeta potential and B) Polydispersity index (PDI) and particle count (kilocounts per second) of nanoparticles prepared with pEGFP-N1 and CHAT at a range of N:P ratios measured using a Malvern Zetasizer Nano ZS instrument (n=3, $\pm$ SEM). C) Representative size distribution by intensity of CHAT/pEGFP-N1 nanoparticles at N:P 12.

3.5. Stability of CHAT/pDNA complexes

The CHAT/pDNA nanoparticles were stable over a 28-day time course and a range of temperatures (4°C – 40°C) (Figure 5). DNA integrity was confirmed via gel electrophoresis following incubation in 10% serum for 6 h (Figure 5C). Lyophilised CHAT/pLuc nanoparticles showed decrease in the nanosized range from 99.1% to 97.5% compared to fresh nanoparticles, which was not statistically significant (Figure 5D & Figure 5E).

Figure 5: Stability of CHAT/pDNA complexes at N:P 10. A) Mean hydrodynamic size of and zeta potential of CHAT/pEGFP-N1 nanoparticles assessed across a 28 day time period (n=3, $\pm$ SEM). B) Mean hydrodynamic size and PDI of CHAT/pEGFP-N1 nanoparticles measured using a temperature trend function on the Malvern Zetasizer Nano ZS (n=3, $\pm$ SEM). C) Stability of CHAT/pEGFP-N1 in the presence of foetal calf serum up to 6 hours. D) Comparison of Z-average of freshly prepared and lyophilised CHAT/pDNA NPs (n=3, $\pm$ SEM), E) Size distribution of lyophilised CHAT/pDNA at N:P10.

3.6. *In vitro* efficacy of CHAT/pDNA nanoparticles

To determine functionality in other cell lines, CHAT/pEGFP-N1 nanoparticles were used to transfect prostate cancer cell lines DU145 and PC-3. At N:P 12, the transfection efficiency was 30% in DU145 and 25% in PC-3 cells, which is comparable to our established delivery peptide RALA efficacies of 33% and 20% respectively (Figure 6A & Figure 6B). Orthogonal sectioning and confocal microscopy confirmed intracellular and nuclear delivery of Cy3 labelled pDNA 4 h post-transfection in MDA-MB-231 cells (Figure 6C). The alamarBlue assay revealed 90% cellular viability in MCF-7 and 4T1 cells with almost 100% viability in PC-3, MDA-MB-231 and DU-145 cells (Figure 6D), and was significantly higher (*; p-value <0.05) than that of Lipofectamine (S8). Erythrocytes were used to interrogate the lytic activity on cellular membranes of the CHAT peptide at both physiological pH of 7.4 and endosomal conditions with a pH of 5.5. (Figure 7A). Membrane lysis rose from 39% at a pH 7.4 to 100% at pH 5.5, indicative of pH responsiveness.

Figure 6: In vitro functionality CHAT. A) Light fluorescent microscopy images following transfection of DU145 and PC-3 prostate cancer cells with nanoparticles comprising pEGFP-N1 and CHAT compared to RALA. B) Quantification of transfection efficiency in prostate cancer cells analysed by flow cytometry at 4% gating using a BD FACS Calibur (n=3, $\pm$ SEM). C) Confocal microscopy images and orthogonal sectioning following transfection of MDA-MB-231 breast cancer cells with CHAT at N:P 12 delivering Cy3 labelled pDNA at 4 h post-transfection taken with a TSC SP5-Leica Microsystems confocal microscope (Leica, UK and analysed using LAS AF Lite Software (Leica, UK). D) Cell viability of MCF-7 and MDA-MB-231 breast cancer cells and DU145 and PC-3 prostate cancer cells following transfection with CHAT/pEGFP-N1 at NP 6 (n=3, $\pm$ SD).

Transfections were completed in MCF-7 and MDA-MB-231 cells pre-treated with various cellular uptake pathway inhibitors including chlorpromazine (inhibitor of clathrin-mediated endocytosis), genistein (inhibitor of caveolae mediated inhibition) and M β CD (cholesterol inhibitor, affecting clathrin and caveolae mediated uptake). At 4°C nanoparticles may only enter the cell through direct translocation, since endocytosis, an energy-dependent process, is inhibited at this temperature [33,34]. Therefore, cellular uptake was also investigated at 4°C to investigate if non-energy-dependent direct internalisation pathways were involved (Figure 7B). In MCF-7 cells, uptake was reduced with each of the inhibitors and significantly reduced at 4°C (**; p-value <0.01). In MDA-MB-231 cells, transfection was reduced significantly under all conditions with most significance in the chlorpromazine and genistein treated cells (***; p-value <0.001).

Figure 7: In vitro and in vivo functionality of CHAT. A) Transfection efficiency of CHAT/pEGFP-N1 complexes assessed in MCF-7 and MDA-231 breast cancer cells following pre-treatment with endosomal escape and endocytosis inhibitors to determine possible cellular uptake pathway. Statistical analysis was carried out by One-Way ANOVA and Dunnett post-

test (*; p-value <0.05, **; p-value <0.01, ***; p-value <0.001). B) Haemolytic activity of CHAT/pEGFP-N1 on erythrocytes at pH 5.5 and pH 7.4 (n=3± SEM), statistical analysis was carried out by unpaired t-test (*; p-value <0.05, **; p-value <0.01, ***; p-value <0.001). C) Representative fluorescent dorsal images captured using a Bruker InVivo Xtreme Preclinical Optical/X-ray imaging system 24 h following intratumoural administration of CHAT/pLuc-Cy5 (left) and PEI/pLuc-Cy5 (right) complexes to mice bearing 4T1 breast cancer xenografts. D) Representative fluorescent ventral images 24 h following intratumoural administration of CHAT/pLuc-Cy5 (left) and PEI/pLuc-Cy5 (right) complexes to mice bearing 4T1 breast cancer xenografts. E) Quantification of luciferase gene expression in the brain, lungs, liver, kidney, spleen, and tumour following systemic administration of CHAT/pLuc via tail vein injection, analysed using qRT-PCR. Total RNA was extracted from organs, reverse transcribed and luciferase expression relative to housekeeper (β -actin) analysed using qRT-PCR. Gene expression is expressed as fold difference in relative to untreated control (n=3, $\pm$ SEM).

3.7. *In vivo* functionality of CHAT/pDNA nanoparticles

Functionality of lyophilised CHAT/pLuc was confirmed *in vivo* in 4T1 and MDA-MB-231 breast cancer xenograft models. Intratumoural delivery of Cy-5 labelled complexes showed similar presence of fluorescence in CHAT/pLuc N:P 8 and commercial transfection agent PEI/pLuc N:P 2 with total fluorescence of 2.72×10^7 and 2.78×10^7 a.u. 24 h post injection, respectively (Figure 7C and D). Gene delivery following systemic delivery of CHAT/pLuc was also confirmed with qRT-PCR 48 h post-injection. The greatest gene expression was found in the liver (10-fold), lungs (8-fold), kidney (8.5-fold) and tumour (5-fold) compared to untreated controls (Figure 7D).

4. Discussion

The complex design and difficulty in predicting functionality of alpha helical amphipathic peptides led to development of more simplistic peptide designs. The success of arginine-rich peptides such as oligoarginines (e.g. R8) indicates that an amphipathic nature is not essential for cell penetration [35]. Here, six linear peptides were designed to interrogate the importance of histidine, tryptophan and arginine residues in a 15 amino acid sequence. The compaction of pDNA by cationic species such as arginine in the peptides can be attributed to gradual shrinking of the pDNA coil and is followed by pDNA collapse in a continuous process [36]. This continual process can be evidenced in the gel retardation assays and encapsulation assays in the supplementary data (S1-S3). The sequence and theoretical net charge of Pep1 – Pep4 is similar resulting in minimal variation in neutralisation and complexation profiles. The different sequence and resultant net charge of Pep5 (no histidine residues) and Pep6 (two anionic glutamic acid residues replacing two histidine) is evident in the gel retardation and complexation assay, where neutralisation of pDNA occurs at a higher N:P ratio, and in the case of Pep6 a lower maximum complexation efficiency of 70%. Each of the linear peptides condensed the pDNA into nanoparticles with a size (<200 nm) and charge ($>+20$ mV) suitable for cellular entry. Again, the differences in Pep5 and Pep6 are evidenced here, with high PDI values and lower zeta potential due to the lower peptide net charge. This lower net charge, ultimately reduced the DNA binding capacity of Pep5 and Pep6, and consequently nanoparticle formation was impaired.

Transfection studies showed poor efficiency in both MCF-7 and MDA-MB-231 cells for all of the linear peptides, with the exception of Pep4 (Figure 2). Differences in transfection efficiency between the peptides highlights the effect of sequence on CPP functionality. Pep1 and Pep2 were designed to have arginine residues at the edges of the peptide chain, flanked by two

tryptophan residues, with histidine residues at the centre. Pep2 showed slightly increased transfection efficiency overall compared to Pep1 (S6A & S6B) indicating a more central tryptophan positioning within the sequence may be of benefit. Studies have reported problems with toxicity when excessive tryptophan residues are present [37], which might lead to less efficient internalisation if the peptide becomes inserted too deeply into the lipid membrane [38]. Therefore, in Pep3 and Pep4, the number of tryptophan was reduced to one, which is positioned at the centre of each sequence. The inclusion of hydrophobic tryptophan residues in the novel peptides is proposed to enhance cell surface adsorption; aiding membrane translocation [39]. Futaki et al evaluated the effect of introducing a hydrophobic moiety to arginine-rich CPPs. Comparison of the transfection efficiency of N-terminally acylated oligoarginines with variable numbers of arginine residues and acyl chains, found stearyl-octaarginine (STR-R8) to have the highest transfection efficiency; complexing with pDNA yielding a transfection efficacy comparable to that of Lipofectamine, with no significant cytotoxicity [40]. In the case of these linear peptides, the central tryptophan residue, may provide the hydrophobicity required for enhanced interaction with cell membranes.

Transfection studies demonstrated the need for histidine to facilitate endosomal escape. Histidine is widely accepted as an efficient endosomal escape facilitator through the 'proton sponge effect' [41]. The histidine position in the peptide sequence had an impact on endosomal escape functionality. Histidine positioned at the centre and interleaved throughout the sequence as with Pep1, Pep2 and Pep3 renders the escape capabilities of histidine redundant (Figure S7). Pep3 had histidine and arginine interleaved along the sequence but Pep4 had a block of arginine residues on each side of the central tryptophan with the histidine residues at the edge of the sequence. From the transfection data it is clear that the composition of Pep4 with histidine blocks at each terminal, flanking a central arginine block is best for transfection and concurs

with other studies with CH₂R₄H₂C [42] CH₄R₉H₃C [20] and CH₅TATH₅C [16]. Endosomal escape was not expected with Pep5, which contains no histidine residues, as it is generally recognised that ARPs do not facilitate endosomal escape independently [43]. The replacement of two histidine residues with glutamic acid did not enhance endosomal escape in Pep6. Glutamic acid is credited as responsible for fusogenic activity in various peptides but inclusion in Pep6 resulted only in reducing the overall cationicity of the peptide thereby reducing the proton sponge potential of histidine. In the case of Pep4, effective endosomal escape occurred in MCF-7, but significant increases in transfection were observed following addition of chloroquine with all peptides in MDA-MB-231 cells (S7). The positioning of histidine at each terminus is critical in the CHAT sequence for endosomal escape but this is also cell line dependent.

CHAT complexed pDNA with high efficiency into size-suitable nanoparticles from N:P 2, indicative of the strong cationic nature of the peptide, owing to the inclusion of several arginine and histidine residues (Figure 3 & Figure 4). This electrostatic interaction between the peptide and nucleic acid allows for nanoparticle self-assembly and thus a formulation process which is simple, quick and cost efficient. Particle size is important for cellular uptake and a size <200 nm is cited as optimal for endocytosis and this was confirmed via TEM [44]. The CHAT/pDNA nanoparticles were stable over time and temperature and lyophilisation did not affect the nanoparticles in terms of size and PDI, which is important for scale up and storage considerations (Figure 5). The lyophilised nanoparticles remained stable for 28 days, which is important in the context of further development, as clinical applications of cationic complex non-viral gene carriers are usually hindered by instability [45].

Intra-nuclear delivery was confirmed with confocal microscopy, however, a proportion of Cy3-

pDNA can be visualised in the cytosol, even 4 h post-transfection. Given the evidence of partial endosomal escape in MDA-MB-231 cells in the chloroquine study, it is possible that some of the Cy3-pDNA is trapped within endosomes unlike in MCF-7. Krasowska et al have reported that metastatic MDA-MB-231 cells were much more endocytic (and exocytic) than weakly metastatic MCF-7 cells [46]. Such dominant endocytic processes in MDA-MB-231 cells may therefore heavily influence the cellular uptake patterns of CHAT. It is possible that the robust endocytic routes in the metastatic MDA-MB-231 cells are more difficult to escape than in MCF-7 cells. Nevertheless, impressive transfection efficiency with CHAT was observed in a further two cell lines, namely DU145 and PC-3 prostate cancer cells (Figure 6A & Figure 6B), indicating that CHAT has potential for delivery to a many cells lines.

Endocytosis, including macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis by various CPPs, has been demonstrated and extensively studied [47]. Cellular uptake studies in both MCF-7 and MDA-MB-231 cells indicate that no single endocytic pathway seems to be the obvious route of entry (Figure 7A), and it is likely that more than one uptake pathway is utilised by CHAT, which is similar to the findings with CH₄R₉H₃C [20]. Despite the complexity, the dominance of the endosomal clathrin and caveolae routes is evident; highlighting how differences in cell lines can impact transfection ability. Moreover, the interaction of ARPs with cells to facilitate uptake may be dependent on the composition of the cell membrane. Syndecans, a family of ubiquitously expressed transmembrane proteoglycans, are reported to facilitate uptake of cationic CPPs (penetratin, R8 and TAT) [48]. Syndecan-4 particularly binds and mediates transport of cationic CPPs through the plasma membrane into K562 erythroleukemia cells [49]. In MDA-MB-231 cells, syndecan-1 and -2 are predominant along with serglycin, which are expressed at low levels in less aggressive cells such as MCF-7. In addition, syndecan-2 also seems to suppress syndecan-4 in the more

aggressive cells [50]. It is possible that differences in transfection observed between MDA-MB-231 and MCF-7 cells may be due to a difference in proteoglycans, specifically syndecan expression on the cell membrane. Increased syndecan-4 may result in enhanced transfection, and could explain the higher transfection levels observed in MCF-7 cells when compared to MDA-MB-231 cells, which express lower levels of syndecan-4.

Lack of toxicity is a major prerequisite for any delivery system because patient safety is always of paramount importance. The main feature of cell penetrating peptides is to cross cell membranes at low concentrations without causing significant membrane damage. ISO 10993-5 standards describe that cell viability $\leq 70\%$ is cytotoxic and categorise levels of toxicity as follows: 80%–60% weak cytotoxicity; 60%–40% moderate and below 40% strongly cytotoxic [51]. Cell viability studies in both breast and prostate cancer cell lines demonstrated that CHAT elicited no toxic effect (Figure 6D), and was significantly better (*; p-value < 0.05) when compared to commercial transfection reagent Lipofectamine (S8). This highlights the innocuous nature of CHAT and agrees with studies investigating similar linear peptides $\text{CH}_2\text{R}_4\text{H}_2\text{C}$ [42] $\text{CH}_4\text{R}_9\text{H}_3\text{C}$ [20] and $\text{CH}_5\text{TATH}_5\text{C}$ [16], which are all reported to be non-toxic. The pH-dependent haemolytic activity exhibited by CHAT is important to ensure minimal toxic effects during cellular uptake, while allowing for nucleic acid release following endosomal entrapment. This activity of CHAT is similar to H5WYG; a histidine-rich peptide which permeabilises cell membranes in a weakly acidic medium, following protonation of the histidine residues. In the presence of H5WYG, 97% of HeLa cells are permeabilised at pH 6.2 within 15 min, 50% at pH 6.8 and less than 5% at pH 7.1 [41]. The histidine amino acids in the CHAT sequence are likely to become protonated in the endosome, facilitating endosomal escape in a similar way to H5WYG.

In vivo functionality of CHAT was confirmed following direct delivery and detection of fluorescence in subcutaneous 4T1 tumours (Figure 7C). Similarly, Tanaka et al demonstrated the *in vivo* potential of STR-CH₂R₄H₂C for delivery of siVEGF. Following intra-tumoural delivery of STR-CH₂R₄H₂C/siVEGF complexes, tumour growth was significantly reduced in ICR mice bearing S-180 sarcoma xenografts [42]. Although transfection rate of CHAT/pDNA nanoparticles at N:P 12 in 4T1 cell line was low at 10% (data not shown), the nanoparticles were internalised and transfected the tumours following both intratumoural and intravenous injections, which supports a statement that *in vitro* results do not always translate in the *in vivo* setting [52]. The intracellular delivery of CHAT/pLuc-Cy5 was comparably with *in vivo* PEI (Figure 7C and D), which provides an opportunity for a simple, highly localised gene therapy system with *in vivo* efficiency comparable to that of a commercial transfection agent. To date no group researching linear peptides has investigated systemic delivery, which is essential for the treatment of advanced disseminated disease. Therefore, this study measured the distribution of bioluminescence following systemic delivery of CHAT/pLuc. A 10-fold increase in luciferase expression in the lungs, liver and kidney, and a 5-fold increase was detected in the tumour following qRT-PCR analysis (Figure 7E). Nanoparticle accumulation in RES organs (liver, lung, and spleen) has been previously reported by Zhao et al [52]. This is also similar finding to the studies conducted with RALA peptide and other cationic peptides such as MPG-8, where nanoparticles favoured highly vascularised regions [23,53,54].

It is possible that the inclusion of more histidine residues may improve transfection power, and a deeper understanding of cell line variability with CPPs is required. Furthermore, modification of CHAT with a targeting moiety and/or stealth molecule may ensure specificity and enhance *in vivo* stability for systemic application. Future work will focus on functionalising CHAT/pDNA complexes to optimise this delivery system for *in vivo* gene delivery

applications, through masking of the cationic charge of the peptide to reduce recognition by RES in the systemic circulation, which is critical so that off-target transgene expression is avoided.

5. Conclusion

As a consequence of rational peptide design, a novel linear peptide termed CHAT has been developed for gene delivery. CHAT mediated packaging and delivery of pDNA, resulting in successful transfection in a range of cell lines *in vitro* and reporter-gene expression *in vivo* in 4T1 and MDA-MB-231 breast xenograft models. CHAT holds potential as a low-cost, easy formulated delivery system for the administration of nucleic acid therapeutics. In addition, this rational and systematic peptide design and characterisation process has highlighted the importance of peptide sequence on functionality and will serve to inform future peptide design approaches.

6. References

1. De Laporte L, Cruz Rea J, Shea LD. Design of modular non-viral gene therapy vectors. *Biomaterials*. 2006;27(7):947–54.
2. Han K, Yang J, Chen S, Chen J-X, Liu C-W, Li C, et al. Novel gene transfer vectors based on artificial recombinant multi-functional oligopeptides. *Int J Pharm*. 2012;436(1–2):555–63.
3. Heitz F, Morris MC, Divita G. Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br J Pharmacol*. 2009;157:195–206.
4. Deshayes S, Morris MC, Divita G, Heitz F. Cell-penetrating peptides: tools for intracellular delivery of therapeutics. *Cell Mol Life Sci*. 2005;62(16):1839–49.
5. Derossi D, Joliot A, Chassaing G, Prochiantz A. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem*. 1994;269:10444–50.
6. Vives E, Brodin P, Lebleu B. A Truncated HIV-1 Tat Protein Basic Domain Rapidly Translocates through the Plasma Membrane and Accumulates in the Cell Nucleus. *J Biol Chem*. 1997;272(25):16010–7.
7. Mann A, Thakur G, Shukla V, Ganguli M. Peptides in DNA delivery: current insights and future

- directions. *Drug Discov Today*. 2008;13(3–4):152–60.
8. Kim S, Hyun S, Lee Y, Lee Y, Yu J. Nonhemolytic Cell-Penetrating Peptides: Site Specific Introduction of Glutamine and Lysine Residues into the α -Helical Peptide Causes Deletion of Its Direct Membrane Disrupting Ability but Retention of Its Cell Penetrating Ability. *Biomacromolecules*. 2016;17(9):3007–15.
 9. Nakase I, Takeuchi T, Tanaka G, Futaki S. Methodological and cellular aspects that govern the internalization mechanisms of arginine-rich cell-penetrating peptides. *Adv Drug Deliv Rev*. 2008;60(4–5):598–607.
 10. Scheller A, Oehlke J, Wiesner B, Dathe M, Krause E, Beyermann M, et al. Structural requirements for cellular uptake of α -helical amphipathic peptides. *J Pept Sci*. 1999;5:185–94.
 11. Jones AT, Sayers EJ. Cell entry of cell penetrating peptides: tales of tails wagging dogs. *J Control Release*. 2012;161(2):582–91.
 12. Guterstam P, Madani F, Hirose H, Takeuchi T, Futaki S, El Andaloussi S, et al. Elucidating cell-penetrating peptide mechanisms of action for membrane interaction, cellular uptake, and translocation utilizing the hydrophobic counter-anion pyrenebutyrate. *Biochim Biophys Acta*. 2009;1788(12):2509–17.
 13. Kurzawa L, Pellerano M, Morris MC. PEP and CADY-mediated delivery of fluorescent peptides and proteins into living cells. *Biochim Biophys Acta*. 2010;1798(12):2274–85.
 14. Crombez L, Aldrian-Herrada G, Konate K, Nguyen QN, McMaster GK, Brasseur R, et al. A new potent secondary amphipathic cell-penetrating peptide for siRNA delivery into mammalian cells. *Mol Ther*. 2009;17(1):95–103.
 15. Mason AJ, Leborgne C, Moulay G, Martinez A, Danos O, Bechinger B, et al. Optimising histidine rich peptides for efficient DNA delivery in the presence of serum. *J Control Release*. 2007;118(1):95–104.
 16. Lo SL, Wang S. An endosomolytic Tat peptide produced by incorporation of histidine and cysteine residues as a nonviral vector for DNA transfection. *Biomaterials*. 2008;29(15):2408–14.
 17. Mann A, Thakur G, Shukla V, Singh AK, Khanduri R, Naik R, et al. Differences in DNA condensation and release by lysine and arginine homopeptides govern their DNA delivery efficiencies. *Mol Pharm*. 2011;8(5):1729–41.
 18. Bauhuber S, Hozsa C, Breunig M, Göpferich A. Delivery of nucleic acids via disulfide-based carrier systems. *Adv Mater*. 2009;21(32–33):3286–306.
 19. Lushchak V. Glutathione homeostasis and functions: potential targets for medical interventions. *J Amino Acids*. 2011;2012.
 20. Mann A, Shukla V, Khanduri R. Linear short histidine and cysteine modified arginine peptides constitute a potential class of DNA delivery agents. *Mol Pharm*. 2014;11(3):683–96.
 21. Schmidt N, Mishra A, Lai GH, Wong GCL. Arginine-rich cell-penetrating peptides. *FEBS Lett*. 2010;584(9):1806–13.
 22. Mitchell DJ, Kim DT, Steinman L, Fathman CG, Rothbard JB. Polyarginine enters cells more

- efficiently than other polycationic homopolymers. *J Pept Res.* 2000;56:318–25.
23. McCarthy HO, McCaffrey J, McCrudden CM, Zholobenko A, Ali AA, McBride JW, et al. Development and characterization of self-assembling nanoparticles using a bio-inspired amphipathic peptide for gene delivery. *J Control Release.* 2014;189:141–9.
 24. Cole G, Ali AA, McCrudden CM, McBride JW, McCaffrey J, Robson T, et al. DNA vaccination for cervical cancer: Strategic optimisation of RALA mediated gene delivery from a biodegradable microneedle system. *Eur J Pharm Biopharm.* 2018;127:288–97.
 25. Udhayakumar VK, De Beuckelaer A, McCaffrey J, McCrudden CM, Kirschman JL, Vanover D, et al. Arginine-Rich Peptide-Based mRNA Nanocomplexes Efficiently Instigate Cytotoxic T Cell Immunity Dependent on the Amphipathic Organization of the Peptide. *Adv Healthc Mater.* 2017;6(13):1601412.
 26. Massey AS, Pentlavalli S, Cunningham R, McCrudden CM, McErlean EM, Redpath P, et al. Potentiating the Anticancer Properties of Bisphosphonates by Nanocomplexation with the Cationic Amphipathic Peptide, RALA. *Mol Pharm.* 2016;13(4):1217–28.
 27. McCrudden CM, McBride JW, McCaffrey J, McErlean EM, Dunne NJ, Kett VL, et al. Gene therapy with RALA / iNOS composite nanoparticles significantly enhances survival in a model of metastatic prostate cancer. *Cancer Nanotechnol.* 2018;9(5).
 28. McCrudden CM, McBride JW, McCaffrey J, Ali AA, Dunne NJ, Kett VL, et al. Systemic RALA/iNOS Nanoparticles: A Potent Gene Therapy for Metastatic Breast Cancer Coupled as a Biomarker of Treatment. *Mol Ther - Nucleic Acids.* 2017;6:249–58.
 29. Cole G, Ali AA, McErlean E, Mulholland EJ, Short A, McCrudden CM, et al. DNA vaccination via RALA nanoparticles in a microneedle delivery system induces a potent immune response against the endogenous prostate cancer stem cell antigen. *Acta Biomater.* 2019;96:480–90.
 30. Bennett R, Yakkundi A, McKeen H, McClements L, McKeogh T, Arthur K, et al. RALA-mediated delivery of FKBPL nucleic acid therapeutics. *Nanomedicine.* 2015;10(19):2989–3001.
 31. Mulholland EJ, Ali A, Robson T, Dunne NJ, McCarthy HO. Delivery of RALA/siFKBPL nanoparticles via electrospun bilayer nanofibres: An innovative angiogenic therapy for wound repair. *J Control Release.* 2019;316:53–65.
 32. Ali AA, McCrudden CM, McCaffrey J, McBride JW, Cole G, Dunne NJ, et al. DNA vaccination for cervical cancer; a novel technology platform of RALA mediated gene delivery via polymeric microneedles. *Nanomedicine.* 2016;13:921–32.
 33. Walrant A, Correia I, Jiao C-Y, Lequin O, Bent EH, Goasdoué N, et al. Different membrane behaviour and cellular uptake of three basic arginine-rich peptides. *Biochim Biophys Acta.* 2011;1808(1):382–93.
 34. Brock R. The uptake of arginine-rich cell-penetrating peptides: putting the puzzle together. *Bioconjug Chem.* 2014;25(5):863–8.
 35. Akitaya T, Seno A, Nakai T, Hazemoto N, Murata S, Yoshikawa K. Weak interaction induces an ON/OFF switch, whereas strong interaction causes gradual change: Folding transition of a long duplex DNA chain by poly-L-lysine. *Biomacromolecules.* 2007;8(1):273–8.

36. Rydberg HA, Matson M, Åmand HL, Esbjö EK, Norde B. Effects of Tryptophan Content and Backbone Spacing on the Uptake Efficiency of Cell-Penetrating Peptides. *Biochemistry*. 2012;51:5531–9.
37. Bechara C, Sagan S. Cell-penetrating peptides: 20 years later, where do we stand? *FEBS Lett*. 2013;587(12):1693–702.
38. Nakase I, Akita H, Kogure K, Gräslund A, Langel Ü, Harashima H, et al. Efficient intracellular delivery of nucleic acid pharmaceuticals using cell-penetrating peptides. *Acc Chem Res*. 2012;45(7):1132–9.
39. Futaki S, Ohashi W, Suzuki T, Niwa M, Tanaka S, Ueda K, et al. Stearilated arginine-rich peptides: A new class of transfection systems. *Bioconjug Chem*. 2001;12(6):1005–11.
40. Pichon C, Gonc C, Midoux P. Histidine-rich peptides and polymers for nucleic acids delivery. *Adv Drug Deliv Rev*. 2001;53:75–94.
41. Tanaka K, Anazawa TK, Gawa TO, Uda YS, Akashima YT, Ukuda TF, et al. A Novel, Bio-Reducible Gene Vector Containing Arginine and Histidine Enhances Gene Transfection and Expression of Plasmid DNA. *Chem Pharm Bull*. 2011;59(2):202–7.
42. El-Sayed A, Futaki S, Harashima H. Delivery of macromolecules using arginine-rich cell-penetrating peptides: ways to overcome endosomal entrapment. *AAPS J*. 2009;11(1):13–22.
43. Sharma R, Nisakar D, Shivpuri S, Ganguli M. Contrasting effects of cysteine modification on the transfection efficiency of amphipathic peptides. *Biomaterials*. 2014;35:6563–75.
44. El-Sayed A, Harashima H. Endocytosis of gene delivery vectors: from clathrin-dependent to lipid raft-mediated endocytosis. *Mol Ther*. 2013;21(6):1118–30.
45. Krasowska M, Grzywna ZJ, Mycielska ME, Djamgoz MBA. Fractal analysis and ionic dependence of endocytotic membrane activity of human breast cancer cells. *Eur Biophys J*. 2009;38(8):1115–25.
46. Copolovici DM, Langel K, Eriste E, Langel Ü. Cell-penetrating peptides: design, synthesis, and applications. *ACS Nano*. 2014;8(3):1972–94.
47. Letoha T, Keller-Pintér A, Kusz E, Kolozsi C, Bozsó Z, Tóth G, et al. Cell-penetrating peptide exploited syndecans. *Biochim Biophys Acta - Biomembr*. 2010;1798(12):2258–65.
48. Rapraeger AC, Ott VL. Molecular interactions of the syndecan core proteins. *Curr Opin Cell Biol*. 1998;10(5):620–8.
49. Theocharis AD, Skandalis SS, Neill T, Mulhaupt HAB, Hubo M, Frey H, et al. Insights into the key roles of proteoglycans in breast cancer biology and translational medicine. *Biochim Biophys Acta*. 2015;1855(2):276–300.
50. ISO 10993-5 Biological evaluation of medical devices - Part 5: Tests for cytotoxicity: in vitro methods. 3rd ed. International Standard ISO 10993-5. Geneva, Switzerland: International Organization for Standardization; 2009.
51. Liu Y, Tseng Y-C, Huang L. Biodistribution studies of nanoparticles using fluorescence imaging: a qualitative or quantitative method? *Pharm Res*. 2012;29(12):3273–7.

52. Blanco E, Shen H, Ferrari M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nat Biotechnol.* 2015;33(9):941–51.

Table 1

Peptide name	Sequence	Molecular weight (Da)	Design Rationale
Pep1	CRRRWHHHHHWRRRC	2219.50	<ul style="list-style-type: none">• Histidine residues at the core of the peptide sequence flanked by arginine.• Two tryptophan residues included for enhanced interaction with cell membranes.• Tryptophan residues at different positions to assess optimal sequence and positioning.
Pep2	CWRRRHHHHHRRRWC	2219.50	
Pep3	CHRRHRWHRHRHRC	2170.48	<ul style="list-style-type: none">• Arginine and histidine residues arranged in either alternating or block sequence to assess impact on functionality.• Arginine block positioned at the core of the sequence, flanked by histidine at each end which contrasts to positioning in Pep1 and 2.• Tryptophan positioned in the middle to assess positioning which contrasts the positioning in Pep1 and 2.
Pep4 (CHAT)	CHHHRRRWRRRHHC	2170.48	
Pep5	CRRRWRRRRC	1660.01	<ul style="list-style-type: none">•Histidine removed from Pep5 to assess if endosomal escape is possible without Histidine residues• Pep6 designed to include two glutamic acid residues reported to be involved in endosomal escape mechanisms in amphipathic peptides
Pep6	CEHHRRRWRRRHHC	2154.43	

Peptide	Size (nm)		Zeta potential (mV)		PDI
	Mean	SD	Mean	SD	
1	127.60	67.60	27.30	5.41	0.31
2	154.90	39.70	35.30	2.92	0.20
3	136.79	39.53	30.60	2.39	0.23
4	207.30	42.87	29.00	1.70	0.25
5	101.89	26.74	20.45	1.77	0.45
6	156.60	12.59	10.70	3.09	0.72

Figure 4

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