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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 4091–4097
Bioorganic & Medicinal Chemistry

Novel cationic lipophilic peptides for oligodeoxynucleotide delivery

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Received 18 December 2006; revised 26 March 2007; accepted 26 March 2007
Available online 30 March 2007

Abstract—In search of new oligodeoxynucleotide (ODN) delivery agents, we evaluated novel peptides derived from core peptide H-GLRILLLKV-OH (CP). CP is a fragment designed from the T-cell antigen receptor (TCR) α-chain transmembrane sequence. CP was able to enter cells including T-cells and inhibited interleukin-2 (IL-2) production. To examine the effect of increased lipophilicity on cellular uptake and activity of CP, a lipoamino acid (2-aminododecanoic acid) was incorporated into peptide CP resulting in 2-aminodecanoyl-CP (LP). The toxicity of CP and LP was assessed by measuring the haemolytic activity. Neither compound caused any haemolysis of red blood cells. We have also compared the biological activities of the CP and LP. Using a T-cell antigen presentation assay, the more lipophilic LP caused greater inhibition of IL-2 production than the parent CP in the antigen stimulated T-cells. The LP also showed increased permeability than CP in the Caco-2 cell assay. We utilised the enhanced cell permeability property of LP in oligodeoxynucleotide ODN1 delivery. Isothermal titration calorimetry (ITC) suggested that CP and LP complex with ODN1 in a 12:1 (CP:ODN1) and 15:1 (LP:ODN1) ratio. These complexes were then transfected into human retinal pigment epithelial cells. The level of transfection was measured by the decreased production of the protein human vascular endothelial growth factor (hVEGF). The results revealed greater transfection efficiency for both CP and LP (47%, 55% more inhibition) compared to commercially available transfection agent cytofectin GSV™. These results suggested that the CP and particularly its lipophilic analogue LP have the potential to be used as oligodeoxynucleotide delivery systems.

1. Introduction

Gene therapy as a new paradigm for the treatment of a number of medical conditions has been receiving increasing attention since its application over a decade ago.[1] Many experimental approaches have been exploited in oligodeoxynucleotide (ODN) delivery. These include the use of phosphoramidate analogues,[2] lipofectin,[3] liposomes,[4] Sendai virus fusion proteins[5] and complexation of ligands to induce endocytosis.[6] At the present time, recombinant viral vectors are the most efficient.[7] However, because of safety issues they have limited application.[8] The challenge of searching for a better ODN delivery agent still continues.

Non-viral gene delivery agents must negotiate a multitude of barriers to be effective.[9–12] They must bind to DNA, target DNA to specific cells, escape cytoplasmic degradation and reach the nucleus. Cationic peptides are known to interact with DNA mainly by ionic interactions, forming stable DNA–peptide complexes.[13] We have shown that one such cationic peptide termed core peptide (CP) which is a fragment designed from the T-cell antigen receptor (TCR) α-chain transmembrane sequence. It has the ability to enter cells and reach the cell nucleus.[14] In another study, Cameron et al.[15] have shown an improved ability to deliver genes in cell culture using cationic lipids when compared with commercially available reagents such as lipofectin. By combining the two approaches, we designed a lipophilic derivative of CP, referred to as LP, by attaching a lipo-α-aminocarboxylic acid (LAA) (2-aminododecanoic acid) to the N-terminus of the parent CP and tested its efficacy in ODN transfection.

Keywords: Lipoamino acids; Oligonucleotide delivery; Modulated T-cell function.
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doi:10.1016/j.bmc.2007.03.078

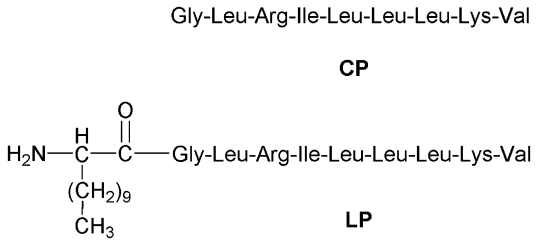

Figure 1. Structure of the core peptide **1** (CP) and lipid conjugated core peptide **2** (LP).

We hypothesized that CP and its lipidic analogue LP might be potential ODN delivery agents based on the idea that they possess cationic surfaces (two-positive net charge), being capable of complexing with negatively charged oligodeoxynucleotides (ODN). Furthermore, LAAs and their homo-oligomers, the lipopeptides, represent a class of compounds which combine structural features of lipids with those of amino acids and peptides.[16] The LAAs possess a high degree of membrane-like character, which facilitates their crossing of biological membranes into cells, and they have the additional effect of protecting labile compounds from nuclease digestion.[17,18] These properties may be advantageous for delivering ODN into cells.

The aim of this study was to investigate the applications of CP/LP (Fig. 1) as ODN delivery agents to deliver an anti-sense ODN1[19–23] containing a sequence DS085 - targeting both human vascular endothelial growth factor (hVEGF) upstream of the translation initiation codon and capable of inhibiting its expression. In addition, we looked at the degree of CP/LP that caused inhibition of IL-2 production in the antigen stimulated T-cells using a T-cell antigen presentation assay. Furthermore, we report the cell permeability of these peptides in the Caco-2 cell model and the ODN1 binding ratios using isothermal titration calorimetry (ITC). We also examined the toxicity of these synthetic peptides in an erythrocyte haemolysis study.

2. Results and discussion

2.1. Chemical synthesis

Core peptide (CP) and the lipoamino acid modified CP lipopeptide (LP) were successfully synthesised on the solid support using Boc strategy and HBTU/DIPEA coupling reagents on preloaded Val-PAM resin (Table 1).

Table 1. Mass spectral data and yields for the peptides CP and LP

Peptide	Mass	MS (*m*/*z*)	Yield (%)
1 CP	1024.36	1025.4 (MH^+) 513.4 ($M2H^+/2$) 342.4 ($M3H^+/3$)	81
2 LP	1221.89	1222.4 (MH^+) 611.8 ($M2H^+/2$) 408.2 ($M3H^+/3$)	75

Table 2. Haemolysis induced by SDS, CP and LP after 30 min at 37 °C ($n = 3$)

	(mM)	Haemolysis (%)	SD
SDS	50	0.60	0.33
	200	1.31	1.10
	1000	29.82	1.48
CP	50	1.13	0.63
	200	−0.36	0.09
	1000	1.79	1.01
LP	50	0.93	0.46
	200	0.54	0.39
	1000	0.78	0.34

2.2. Haemolysis

Since both peptides CP and LP are charged, they have the potential to damage the cell membrane, hence we have examined the cell toxicity of these peptides. The erythrocyte haemolysis model has been widely used to determine the membrane toxicity.[24] SDS, an anionic surfactant was used as a positive control. The haemolysis caused by SDS increased with concentration, as reported previously.[25,26] The haemolytic activity of peptide CP and LP was less than 2% at 1 mM concentration while SDS presented a toxic level of almost 30% at the same concentration (Table 2). Since the haemolysis of these peptides was minimal, they were suitable to use in cell models including the T-cell, Caco-2 cell and retinal pigment endothelium transfection models.

2.3. Inhibition of IL-2 production in T-cell assay

Previously, we showed that the peptide CP modulated T-cell function resulting in inhibition of IL-2 production in vitro.[14] To investigate the effect of the lipid modified LP on T-cell function, the activity of CP was compared with the activity of LP in the antigen presentation assay.[27] The assay was performed on six separate occasions using three concentrations of CP and LP (5, 10 and 25 μM) each concentration tested in triplicate. Figure 2 represents the average percentage of IL-2 production in presence of CP or LP in various concentrations. The results for CP showed inhibition of IL-2 production which was similar to our previous study.[14] Similarly, LP showed a consistent dose titration inhibition of T-cells that was significantly greater than the similar activity of CP.

2.4. Caco-2 cell experiments

The cellular uptake of the peptide CP and its lipophilic analogue LP were examined using the Caco-2 cell model. This human colonic adenocarcinoma cell line is widely used in drug adsorption studies. It forms confluent monolayers and differentiates to cells with an enterocyte-like morphology under standard condition.[28] Caco-2 cell studies revealed that the permeability coefficient P_{app} of ^{14}C-mannitol was $5.55 \pm 0.61 \times 10^{-7}$ cm/s (control). In the presence of 1% DMSO in HBSS, the P_{app} for CP and the lipid modified derivative LP were both relatively low, $4.16 \pm 1.43 \times 10^{-8}$ and $1.1 \pm 0.23 \times$

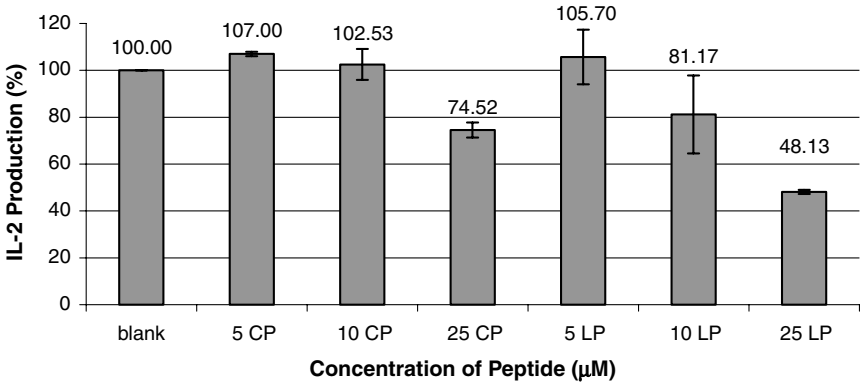

Figure 2. Average percentage IL-2 production in presence of CP and LP. All samples were in 0.25% DMSO. There is dose-dependent inhibition of LP on T-cell activation.

Table 3. Caco-2 cell apparent permeability P_{app} (cm/s) ($n = 3$)

	P_{app} (cm/s)
CP	$0.42 \pm 0.14 \times 10^{-7}$
LP	$1.10 \pm 0.23 \times 10^{-7}$
^{14}C-Mannitol	$5.55 \pm 0.61 \times 10^{-7}$

10^{-7} cm/s, respectively. Therefore, the transport of LP through the Caco-2 monolayers was 2.6× faster than CP. Peptides CP and particularly LP had low solubility in aqueous solution. Since 1% DMSO had no influence on the integrity of the cell monolayers, we used this solution to solubilise the peptides. To further examine the toxicity of the compounds, transepithelial electrical resistance of the monolayers was monitored before and after the experiments. The transepithelial electrical resistance showed no significant reduction, indicating the non-toxic nature of the compounds (Table 3).

2.5. Isothermal titration calorimetry experiments

Isothermal titration calorimetry experiments were performed to monitor the formation of complexes between peptides CP and LP with ODN1. Figure 3a and b shows the sequential heat release (μcal/s) while titrating ODN 1 with peptides CP and LP. The results indicated a molar ratio of 12:1 for CP:ODN1 and 15:1 for LP:ODN1 for the final complex formation. The higher molar ratio required for LP:ODN1 compared to CP:ODN1 was probably due to the shielding effect of the long lipid chains of LP to the ODN charged binding site. The intermolecular hydrophobic interactions between LP might also have contributed to this effect.

2.6. DNA delivery studies

The effectiveness of the peptide–ODN1 complexes were tested and compared in their ability to transfect retinal pigment endothelial cells (D-407). Cytofectin–ODN1 complex was used as a standard. Cytofectin is a commercially available lipoplex transfection agent containing a 'helper-lipid' such as dioleylphosphatidylethanolamine (DOPE), and it can deliver both ODNs and

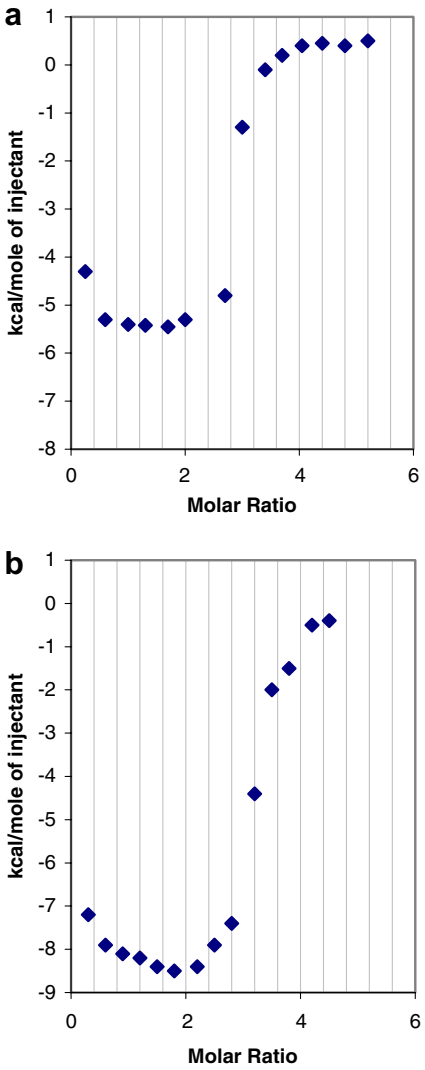

Figure 3. (a) Isothermal titration calorimetry curve of the complex formation between CP and ODN1. (b) Isothermal titration calorimetry curve of the complex formation between LP and ODN1. Peptides were dissolved in 1% DMSO, 35% acetonitrile solution.

plasmids. The transfection efficacy was indirectly measured by the reduction of hVEGF expression, due to

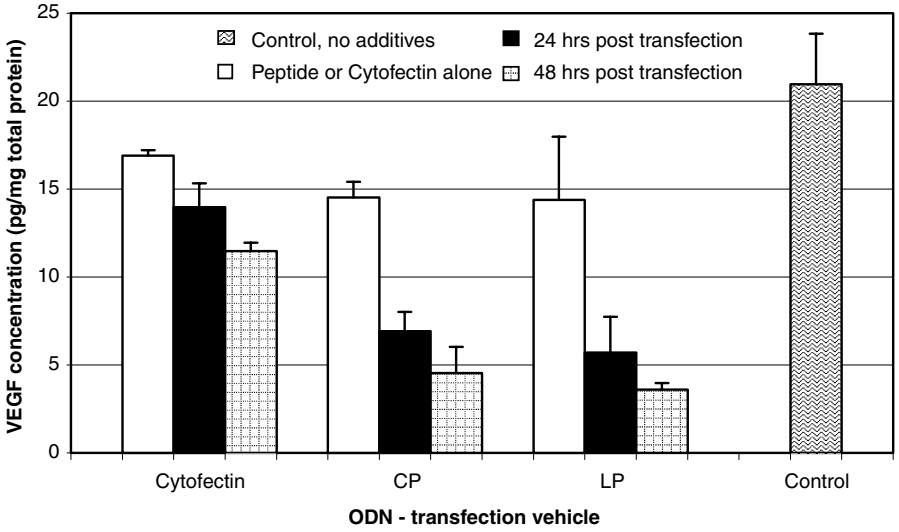

Figure 4. ELISA for hVEGF concentration after 24 and 48 h in conditioned media of transfection vehicles (cytofectin–ODN1 complex, CP-ODN1 complex, and LP-ODN1 complex, means ± SD, and control).

the presence of ODN1, by a well-established ELISA-system (Cytelisa™ Human VEGF).[19] The results of the ELISA-assay (Fig. 4) showed that both complexes reduced the hVEGF levels significantly compared to standard cytofectin GSV™. While cytofectin inhibited only 8–25%, CP and LP inhibition were 55%, 70%, and 63%, 76%, respectively, at 24, 48 h. This can be translated to 47–51% more inhibition than the commercial cytofectin. In comparing CP and LP, the LP–ODN1 complex showed a greater reduction of the hVEGV-level compared to CP–ODN1 complex after 24 and 48 h. Statistical analysis using one way ANOVA with a Tukey's post hoc test with a 95% confidence limit revealed that both CP and LP possessed a significant difference ($P = 0.035$) at post 24 and 48 h. These complexes were highly efficient in transfecting human retinal pigment endothelial cells. This could possibly be explained by an enhanced protection against nuclease attack of CP within the cell by the linked LAA-chain.

3. Conclusion

We have described the synthesis, toxicity, cell permeability and in vitro biological activity of CP and its lipidic analogue LP. Biological activities were assessed using T-cell antigen presentation assay and experiments of ODN1 transfection into human retinal pigment epithelial cells. Both CP and LP were non-toxic to red blood cells at the concentration between 50 μM and 1 mM. By attaching a LAA to CP, we produced a compound of higher activity than that of the parent peptide. In the T-cell antigen presentation assay, LP inhibited IL-2 production in T-cells greater than CP by 35.4% at 25 μM. This result was reflected by the increased permeation of LP compared to CP in the Caco-2 cell monolayer model. We also discovered that CP, a fragment of TCR, was able to bind to ODN1 to form a stable complex and deliver the ODN1 into cells to inhibit hVEGF expression significantly. In addition, the attachment of a lipid moiety to CP enhanced the hVEGF expression inhibition to 76% after 48 h, which is 6% more superior than the parent CP.

The mechanism of CP/LP delivery of ODN1 is still unclear. The presence of positive charges, arginine, and lysine, might allow the binding of DNA. We speculate that the CP might be a ligand for cell-surface receptor that initiated endocytosis to deliver ODN1 into cells, similar to the report from Bachmann.[29] The potential of these compounds as new transfection/gene delivery agents is under continuing investigation.

4. Experimental

4.1. Peptide synthesis

2-Aminododecanoic acid and its Boc-protected intermediate was synthesised by literature methods[16] and used as a racemic mixture. Peptide CP and peptide LP were synthesised using standard solid phase synthetic methods (Fig. 1).[30] Boc-Val-PAM resin (0.736 mmol/g, 993 mg) was swelled in dimethylformamide (DMF) in a sintered glass peptide synthesis vessel for 90 min. Boc group was removed with trifluroacetic acid (TFA). An activation mixture consisting of Boc-amino acid (3 equiv per mol amino-group), HBTU (2-(1*H*-benzotriazole-1-yl)-1,3,3-tetramethyluronium hexafluoro phosphate, 0.5 M in DMF, 3 equiv) and diisopropylethylamine (DIAE) (0.442 mL, 4 equiv) was shaken for 15 min. Negative ninhydrin reaction (5 min.) showed nearly quantitative coupling ($\geqslant$99.98%) and the Boc protecting group was subsequently removed using 100% TFA. Between all manipulations the resin was washed thoroughly with DMF. Coupling and deblocking of Boc protecting groups were done in an analogous manner for peptide CP and LP. Upon completion of the synthesis and removal of the terminal Boc groups, the resin was washed with DMF, methanol and DCM.

The resin was dried to constant weight over KOH in vacuo. The peptides were cleaved from the resin using HF and *p*-cresol as scavenger. The cleaved peptide was precipitated using diethyl ether, redissolved in acetonitrile/water 60:40 with 0.1% TFA and lyophilised to afford a white amorphous powder.

Analytical RP-HPLC (Agilent Zobrax SB-C18, 4.6 μm, 4.6×150 mm) was performed in order to optimise the appropriate gradient for the preparative HPLC and to identify the synthesised peptide (UV absorption at 214 nm). The solvent gradient was started with 0% acetonitrile, increasing to 50% acetonitrile at 5 min and increasing further to 100% acetonitrile for 15 min and finally decreasing steadily to 0% acetonitrile for 5 min at a constant flow of 1 mL per min. One-hundred milligrams of each crude peptide was preparative separated on RP-HPLC (Vydac TSK-GEL preparative C18 column, 10 μm, 25×250 mm) using an acetonitrile/water gradient and monitored by UV detection at a wavelength of 214 nm. The solvent gradient began with 20% acetonitrile, maintaining constant for 10 min, increasing to 50% acetonitrile within 10 min, maintaining for 40 min and increasing further to 100% acetonitrile within 15 min and keeping for 5 min. Gradient elution was finalised by decreasing to 20% acetonitrile within 5 min with a constant flow at 1 mL per min. The peptide containing fractions were collected and characterised by electrospray-MS (Perkin-Elmer Mass Spectrometer Sciex API3000 combined with a Shimadzu HPLC). The resulting peptide LP was used as a racemic mixture.

4.2. Erythrocyte haemolysis

Phosphate buffer solution (PBS), pH 7.4, Drabkin's agent and sodium dodecyl sulphate (SDS) were acquired from Sigma Chemical Co (Brisbane, Australia). The erythrocyte model was performed according to a modified Helenius method.[24] Rat blood was collected into heparinized tubes and centrifuged at 2200*g* for 10 min to isolate erythrocytes. The cells were washed 3× with PBS (centrifuged at 800*g*, 10 min) and resuspended in PBS to their original weight. It was stored at 4 °C and used in 3 h after collection. In a 48-well plate, 0.2 mL of the test samples were added to 0.2 mL of the prewarmed erythrocyte suspension and incubated for 30 min at 37 °C. Final peptide concentrations were 50 μM, 200 μM and 1 mM in triplicate. After incubation, the mixtures were spun at 2200 g for 2 min. Twenty microliters of the supernatants were added to 5 mL of Drabkin's agent. The tubes were mixed and covered for 30 min. The absorbance was recorded at 540 nm. The percentage of haemolysis was calculated as follows:

$$\%\ \text{Haemolysis} = \frac{\overline{\text{Abs}}_{\text{sample}} - \overline{\text{Abs}}_{-\text{ve}}}{\overline{\text{Abs}}_{+\text{ve}} - \overline{\text{Abs}}_{-\text{ve}}} \times 100\%$$

where Abs is the average UV absorbance, -ve is the negative control which indicated the level of spontaneous haemolysis (Abs of 20 μL supernatant taken from mixture comprised of 0.2 mL buffer and 0.2 mL erythrocytes). +ve is the positive control of maximum haemolysis (Abs of 20 μL of uncentrifuged mixture comprised of 0.2 mL buffer mixed with 0.2 mL erythrocytes).

4.3. T-cell antigen presentation assay

Cell line 2B4.11 is a murine T helper cell hybridoma that expresses a complete antigen receptor on the cell surface and produces IL-2 following antigen (cytochrome *c*) recognition[31]; an IL-2-dependent T-cell line (CTLL) for conventional biological IL-2 assays; and the B-cell hybridoma cell line LK35.2 (LK, I-E^k bearing) which acts as the antigen-presenting cell.[32] The hybridomas were grown in T-cell medium [RPMI 1640 medium containing 10% fetal calf serum (FCS), gentamycin (80 μg/mL), glutamine (2 mM) and mercaptoethanol (0.002%)]. The mouse T-cell 2B4.11 hybridoma (5×10^4) was cultured in microtiter wells with LK35.2 antigen-presenting B cells (5×10^4) and 50 μM pigeon cytochrome *c*. After 18–24 h, 100 μL of assay supernatant was removed and assayed for the presence of IL-2. Serial twofold dilutions of the supernatant in media were cultured with the IL-2-dependant T-cell line CTLL. After 16 h, the CTLL cells were pulsed with [^{3}H]-thymidine for 6 h, and IL-2 measurements were determined. CP and LP were tested in the antigen presentation assay at final concentrations of 5, 10, 25, and 50 μM all at a final DMSO concentration of 0.25%.

4.4. Caco-2 cell culture and permeability assay

^{14}C-mannitol (specific activity of 50 mCi/mmol) was purchased from Sigma Chemical Co (Brisbane, Australia). The radiochemical purity of the compounds used was >95%. All the medium components and reagents for cell culture were obtained from Gibco Life Technologies (Paisley, Scotland).

The Caco-2 cells were obtained from American Type Culture Collection. The cells were maintained in tissue culture flasks (Nunc, Roskilde, Denmark), and seeded at a cell density of approximately 5×10^5 cells/cm^2 into 6.5 mm Transwells® with polycarbonate membranes (0.4 μm pore size, Costar, Cambridge, USA). The culture media (Dulbecco's modified Eagle's medium, Gibco Paisley, Scotland) was supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 1% L-glutamine, 1% penicillin streptomycin solution. Fresh medium was replaced every second day, 0.1 mL on apical (AP) side and 0.6 mL on the basolateral side (BA). Cells with a passage number between 50 and 55 were used in the experiments. The integrity of the monolayers was monitored by measuring the transepithelial electrical resistance (TEER) and the transport of ^{14}C-mannitol. TEER was about 400–600 Ω/cm^2. At 30 days after seeding, the cells were rinsed with Hanks' balanced salt solution (HBSS) and equilibrated with 1% DMSO in HBSS in both AP side and BA side at 37 °C. Medium was poured off and placed in new wells containing 0.6 mL of 1% DMSO in HBSS. Two-hundred micromolar of test compounds were added onto the AP side. The inserts were transferred to new wells with 0.6 mL of buffer every 30 min over a period of 3 h in an incubator at 37 °C. Concentrations of CP and LP were determined

by liquid chromatography-mass spectrometry (LC-MS) (PE Sciex API 3000). The apparent permeability coefficient P_{app} (cm/s) was calculated as follows:

$$P_{app} = \frac{\Delta Q}{\Delta t}\frac{1}{AC_o}$$

where $\Delta Q/\Delta t$ is the permeability rate, A is the surface area of the monolayer, C_o is the initial concentration in the AP side.

4.5. ODN complex formation

Oligonucleotide ODN1 (5′-GAGCCGGAGAGGGAG CGCGA-targeting both human and rat VEGF upstream of the translation initiation codon) was purchased from Sigma–Aldrich (Brisbane, Australia). To manufacture complexes with the required molar charge ratio, ODN1 (0.5 mg/mL) was added to each peptide (1.5 mg/mL) mixed for 15 min, diluted with 250 μL sterile water and lyophilised. The optimal molar ratio (+/−) for the complex was 15:1 (peptide/ODN1).[33,34]

4.6. Isothermal titration calorimetry (ITC)

ITC experiments were performed in a MicroCal VP-ITC microcalorimeter. The oligonucleotide solution (1.3 μM) was placed in the sample cell and the peptide CP (303 μM), LP (283 μM), respectively, was dissolved in 1% DMSO, 35% acetonitrile aqueous solution and placed in the syringe. The cell temperature was maintained at 30 °C and the peptide was added to the solution of oligonucleotide using 25×4 μL injections, each injection was 4 min apart.

4.7. Transfection and hVEGF ELISA

Cells of human retinal pigment endothelium (RPE) origin, D407 were grown to 80% confluency in a 24-well plate and transfected with 1 μM (final concentration) of ODN1 in quadruplet sets using either cytofectin GSV™ (as per manufactures instructions) or the peptide/ODN1 complexes. The cells were grown under hypoxic conditions (5% CO_2/2% O_2) for 24/48 h after which the media was removed for analysis. Five-hundred microliters of media from each of the sample was placed in a microcon-30™ concentrator and centrifuged to a volume of 10 μL. Five-hundred microliters of phosphate buffer (pH 7.2) was then applied to wash the sample which was centrifuged to a final volume of 100 μL. The samples were then used in a sandwich ELISA assay as per the manufactures instructions (Cytelisa™ Human VEGF kit, CYTIMMUNE Sciences Inc., Maryland, USA).

Acknowledgments

The authors thank Dr. Ross McGeary, Dr. Vera Bender and Dr. Marina Ali for helpful discussions, and Dr. Aniko Schubert and Dr. Joanne Blanchfield for their excellent technical assistance. This project was supported by grants from the NHMRC Australia and The Wellcome Trust UK.

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