Bioorganic & Medicinal Chemistry xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Structure–activity relationship of lipid core peptide-based Group A Streptococcus vaccine candidates

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ARTICLE INFO

Article history:
Received 11 February 2016
Revised 22 March 2016
Accepted 30 March 2016
Available online xxxx

Keywords:
Group A Streptococcus
Peptide vaccine
Lipopeptides
Adjuvant
Copper-catalysed azide–alkyne cycloaddition

ABSTRACT

Infection with Group A Streptococcus (GAS) can result in a range of different illnesses, some of which are fatal. Currently, our efforts to develop a vaccine against GAS focuses on the lipid core peptide (LCP) system, a subunit vaccine containing a lipoamino acid (LAA) moiety which allows the stimulation of systemic antibody activity. In the present study, a peptide (J14) representing the B-cell epitope from the GAS M protein was incorporated alongside a universal T-helper epitope (P25) in four LCP constructs of different spatial orientation or LAA lengths. Through structure–activity studies, it was discovered that while the alteration of the LCP orientation had a weaker effect on immunostimulation, increasing the LAA side chain length within the construct increased antibody responses in murine models. Furthermore, the mice immunised with the lead LCP construct were also able to maintain antibody activity throughout the course of five months. These findings highlight the importance of LAA moieties in the development of intranasal peptide vaccines and confirmed that its side chain length has an effect on the immunogenicity of the structure.

1. Introduction

Group A Streptococcus (*Streptococcus pyogenes* or GAS) is a Gram positive bacteria that can cause illnesses ranging from benign pharyngitis to invasive diseases like necrotising fasciitis and toxic shock syndrome. GAS-associated illnesses represent a global health problem, causing more than 600 million new cases of infection each year and resulting in a minimum of 500,000 deaths.[1,2] Delayed or inadequate treatment of GAS infections may also result in the development of post-infectious complications such as rheumatic fever (RF) and rheumatic heart disease (RHD), which are responsible for the majority of GAS-related mortality.[3] This highlights the need for the development of an effective prophylactic vaccine against GAS.

The development of a vaccine against GAS has primarily focused on the M-protein, a major virulence factor ubiquitous to all GAS strains.[4] Two areas of the M-protein present a particular research interest: the N-terminus and the C-repeat region.[5] The N-terminus is hypervariable and frequently undergoes genetic recombination, resulting in a rapid turnover of strains.[6–8] Due to this, antibodies induced against the N-terminus are serotype-specific and are only protective against particular strains of GAS, implicating the need to tailor vaccines for GAS endemic to different regions.[9] Conversely, the C-repeat region of the M-protein is highly conserved.[10] Epitopes derived from this region can overcome the restrictions regarding serotype specificity, allowing for the development of a vaccine that is protective against all GAS strains.

One such promising peptide epitope is p145 (LRRDLDASREAKKQVEKALE), which has been shown to generate protective antibodies capable of opsonising multiple serotypes of GAS.[10–14] However, immunisation with p145 poses a risk of generating T-cell autoimmune responses due to its sequence similarity to the human cardiac myosin.[15] Further research has identified J14 (KQAEDKVK***ASREAKKQVEKALE***QLEDKVK), a minimal peptide epitope that excludes the potentially deleterious T-cell sequences.[10] J14 is composed of 14 amino acids from p145 (sequence in bold) enclosed between two non-streptococcal sequences designed to maintain the α-helical conformation of a native epitope in the protein. It has been demonstrated that J14, when combined with the

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http://dx.doi.org/10.1016/j.bmc.2016.03.063
0968-0896/

appropriate delivery system, can confer protective B-cell antibodies in mice against heterologous GAS strains.[10,16,17]

As a short peptides, J14 is immunologically inert on its own. Therefore, it is coupled with a self-adjuvanting moiety in a delivery system called a lipid core peptide (LCP). LCPs contain immune stimulating lipoamino acids (LAAs) which are α-amino acids with a long alkyl side-chain. The number of LAAs incorporated into the LCP, and the length of their side-chains, can be easily modified.[18] LAAs have demonstrated potent adjuvanting activity by activating toll-like receptor 2 (TLR2) on antigen presenting cells (APCs), leading to the induction of an immune response.[19]

The LCP system is able to induce protective immunity in both the mucosal and systemic immune compartments.[19,20] This permits delivery of LCP vaccines via the intranasal route. As GAS typically resides on the mucosal epithelium of the upper respiratory tract, intranasal vaccination benefits by its ability to stimulate both IgA and IgG antibodies.[21,22] IgA, the primary antibody on mucosal surfaces, inhibits GAS adhesion and colonisation, thus preventing bacterial dissemination that could lead to systemic infection.[21,23] However, IgA is inadequate as an opsonin and as an activator of the complement system.[21] Under circumstances where bacteria does disseminate into the bloodstream, IgG, which is a powerful opsonin and activator of the complement system, can facilitate clearance of the bacteria.[21,24] The LCP system also features a lysine (K) moiety (or moieties) from which different vaccine components can be attached.[25] These components can be placed and rearranged at the α-amino, α-carboxyl, and ε-amino functional groups of the central lysine. This allows for the design of LCP systems with different molecular geometries but containing the same epitopes.

Conventionally, a typical GAS LCP vaccine consists of C12 (2-amino-D,L-dodecanoic acid) LAAs attached on the α-carboxyl side-chain of the lysine core via glycine spacers, and peptide epitopes (usually B-cell epitopes) attached to the lysine amine moieties.[26] In recent designs of vaccine candidates against GAS, a T-helper (Th) epitope (P25) was included on the N-terminus to confer immunity in heterologous populations, two copies of serine (S) were included as spacer, and the C12 LAA was replaced with the more lipophilic C16 LAA (2-amino-D,L-hexadecanoic acid).[26,27] The newly designed LCP was able to stimulate IgG antibody production upon intranasal delivery.[17,28] These alterations suggest that structural changes to an LCP have the potential to affect its immunological properties.

Herein, LCP vaccine candidates (Fig. 1) were designed to incorporate the same J14 B-cell epitope and P25 Th-epitope, but to differ in lipopeptide structure (LAA side-chain length) or spatial orientation. Following LCP synthesis and self-assembly into nanoparticles, immunological evaluation was performed upon intranasal administration in mice. Both spatial orientation of epitopes and lipophilicity of LAAs were found to influence humoral immune responses generated by LCP **1**–**4**.

2. Materials

Protected L-amino acids were purchased from Mimotopes (Melbourne, Australia) or Novabiochem (Laufelfingen, Switzerland). pMBHA resin was obtained from Peptide International Inc. (Kentucky, USA). Trifluoroacetic acid (TFA) was purchased from Merck (Kilsyth, Australia). 1-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was obtained from Mimotopes. HPLC grade acetonitrile (MeCN) and *N,N*-dimethylformamide (DMF) were purchased from Ajax Finechem (Sydney, Australia). All other reagents were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia). Microwave-assisted Fmoc-SPPS was performed on a CEM Discovery reactor (CME Corporation, Matthews, NC, USA). Microwave-assisted Boc-SPPS was carried out using a CEM Discovery automated peptide synthesiser. HF cleavage was achieved with an AKel-F HF apparatus (Peptide Institute, Osaka, Japan). ESI-MS was performed on a Perkin-Elmer-Sciex API3000 with Analyst 1.4 software (Applied Biosystems/MDS Sciex, Toronto, Canada). Analytical RP-HPLC was performed on an Agilent instrument. Separation was achieved by running gradient mode of solvent B (MeCN/H_2O/TFA; 90:10:0.1) over solvent A (H_2O/TFA; 100:0.1) on a Vydac analytical C4-column (214TP54; 10 μm, 4.6 × 250 mm) or a Vydac analytical C18-column (218TP54; 10 μm, 4.6 × 250 mm). Purification was performed on a preparative RP-HPLC using a Waters Delta 600 system with a 10.0 ml/min flow rate. Compounds were detected at 230 nm and separation was achieved with solvent B and solvent A on a C4-column (214TP1022; 10 mm, 22 × 250 mm). DLS (dynamic light scattering) measurements were taken on a Nanosizer instrument (Zetasizer Nano Series ZS, Malvern Instruments, Worcestershire, UK) using the Zetasizer 6.2 software. Particle-imaging was achieved with a JEM-1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

3. Methods

3.1. Synthesis of LAAs with Dde-protective group (Dde-C16 and Dde-C20)

C16 (2-amino-D,L-hexadecanoic acid) and C20 (2-amino-D,L-eicosanoic acid) were synthesised following similar methods

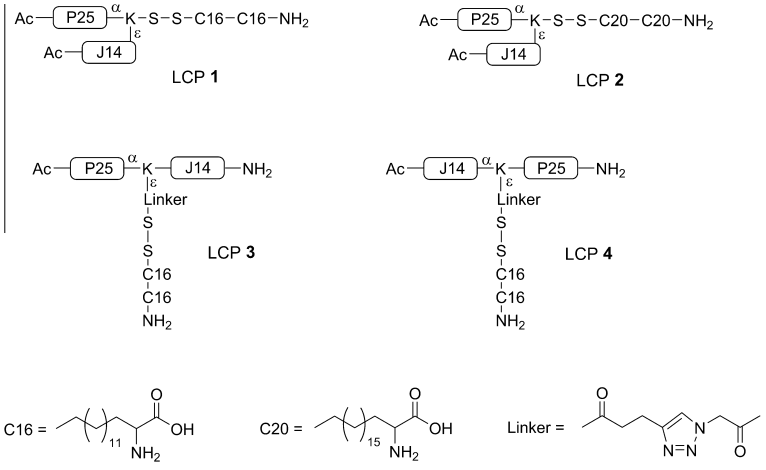


Figure 1. Structures of synthetic LCP **1**–**4**. The LCPs incorporated a GAS B-cell epitope (J14: KQAEDKVKASREAKKQVEKALEQLEDKVK), a universal Th-epitope (P25: KLIPNASLIENCTKAEL), and LAAs with differing side-chain length (C16 or C20). Ac = acetyl group; S = serine; K = lysine.

described before.[29] Sodium ethoxide solution was prepared by dissolving sodium in an ethanol solution, to which diethyl acetomidomalonate and 1-bromo-tetradecane or 1-bromo-octadecane was added to produce C16 or C20, respectively. The mixture was refluxed for 4 days and the resultant precipitate was filtered and washed with cold water. The solid was dried then dissolved in DMF and fuming 37% HCl and refluxed again for 4 days. Ammonia solution was added to yield precipitate, which was filtered and washed with cold water and acetone. The final product was dried until powdery.

Dde-OH (*N*-(4,4-dimethyl-2,6-dioxycyclohexylidene)ethyl alcohol) was synthesised following previously described methods.[30] Then, Dde-OH (1.1 equiv) was dissolved in ethanol, to which triethylamine (3 equiv) and C16 (1 equiv), or C20 (1 equiv), was added. The mixture was refluxed for 2 days and ethanol was evaporated. Ethyl acetate was added, and 5% HCl and brine solution were used to wash the organic phase. The organic layers were collected and filtered by manual short flash chromatography using a silica column. The solution was evaporated and stored at −20 °C to afford yellow crystals. The crystals were washed with cold MeCN, filtered, and dried until powdery. The resulting Dde-C16 and Dde-C20 were analysed by ^{1}H NMR (300 MHz, $CDCl_3$) to produce spectra identical with those reported previously.[30]

3.2. Synthesis of LCP 1 and LCP 2

LCP **1** was synthesised by coupling Dde-C16 onto *p*MBHA resin (0.45 mmol/g; 0.2 mmol scale) by using HBTU (4.0 equiv) and DIPEA (6.2 equiv) in DMF (2 × 1 h). Removal of the Dde-protecting group was performed by treatments with 2% hydrazine in DMF (2 × 30 min). The remaining LCP sequence was synthesised by microwave-assisted Boc-SPPS. Amino acid activation was achieved by dissolving Boc-amino acids (4.2 equiv) in 0.5 M HBTU (4.0 equiv) in DMF, followed by the addition of DIPEA (6.2 equiv). The amino acid coupling cycle consisted of Boc-deprotection with neat TFA at room temperature (2 × 1 min), 2 min DMF flow wash, followed by 10 min coupling with the pre-activated amino acid. The temperature was set at 70 °C (35 W, 10 min) for all amino acids, except for Cys and His, which were coupled at 50 °C (35 W, 10 min). N-terminal acetylation and final cleavage from resin was performed as reported previously.[29] The lyophilised products were purified by RP-HPLC. The collected fractions were analysed by ESI-MS and RP-HPLC. Where appropriate, fractions were combined and lyophilised to yield pure product.

For the synthesis of LCP **2**, Dde-C20 was coupled onto *p*MBHA resin (0.45 mmol/g; 0.2 mmol scale). The remaining procedures followed that of the synthesis of LCP **1**, as mentioned above.

LCP **1** yield: 12.5%. Purity: 90%. Molecular weight: 6086.4. ESI-MS: $[M+4H]^{4+}$ *m/z* 1523.4 (calcd: 1522.6), $[M+5H]^{5+}$ *m/z* 1218.4 (calcd: 1218.3), $[M+6H]^{6+}$ *m/z* 1015.7 (calcd: 1015.3), $[M+7H]^{7+}$ *m/z* 870.6 (calcd: 870.4), $[M+9H]^{9+}$ *m/z* 677.5 (calcd: 677.3), $[M+10H]^{10+}$ *m/z* 608.8 (calcd: 609.5). R_t = 25.0 min (0–100% solvent B, 40 min, C4-column).

LCP **2** Yield: 18.5%. Purity:>99%. Molecular weight: 6198.5. ESI-MS: $[M+5H]^{5+}$ *m/z* 1241.1 (calcd: 1240.7), $[M+6H]^{6+}$ *m/z* 1034.7 (calcd: 1034.1), $[M+7H]^{7+}$ *m/z* 886.6 (calcd: 886.5), $[M+9H]^{9+}$ *m/z* 690.4 (calcd: 689.7). R_t = 29.0 min (0–100% solvent B, 40 min, C4-column).

3.3. Synthesis of alkyne derivative 9

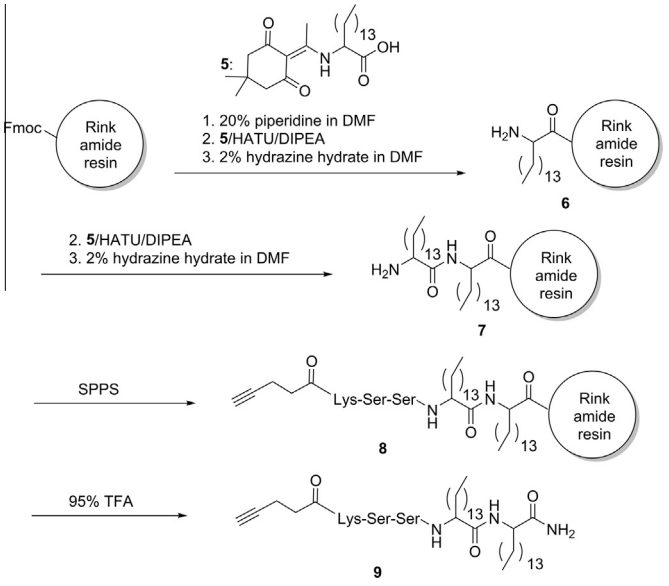
The synthesis of alkyne derivative **9** was performed with manual Fmoc-SPPS (Scheme 1). Dde-C16 was coupled onto rink amide MBHA resin (0.2 mmol scale) by using HATU (4.0 equiv) and DIPEA (4.2 equiv) in DMF (2 × 1 h). The Dde-protecting group was removed by treatment with 2% hydrazine in DMF (8 × 30 min). Two serine moieties and a lysine residue were incorporated using standard Fmoc-SPPS. Amino acid activation was achieved by dissolving Fmoc-amino acids (4.2 equiv) in 0.5 M HATU (4.0 equiv) in DMF, followed by the addition of DIPEA (4.2 equiv) The coupling cycle consisted of Fmoc deprotection with 20% piperidine in DMF (twice, 10 min and 20 min), a 1 min DMF flow-wash, followed by couplings with preactivated Fmoc-amino acids (2 × 1 h). Pentynoic acid (4.2 equiv) was coupled to the N-terminus of the sequence by using HATU (4 equiv) and DIPEA (4.2 equiv) at room temperature (2 × 1 h), followed by washing with DMF, DCM, MeOH, and drying under vacuum. The peptide was cleaved from the resin in a solution of neat TFA triisopropylsilane/water (95:2.5:2.5) for 4 h. The cleaved peptide was precipitated, filtered, and washed with ice-cold Et_2O, dissolved in acetonitrile/water 90:10 and lyophilised. The resulting crude product (**9**) was purified by RP-HPLC.

Peptide **9** yield: 24%. Purity: >95%. Molecular weight: 906.3. ESI-MS: $[M+H]^+$ *m/z* 906.9 (calcd: 907.3). R_t = 33 min (35–75% solvent B, 60 min, C4-column).

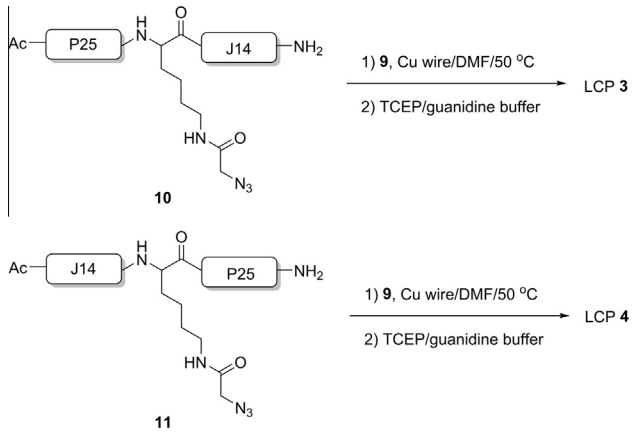
3.4. Synthesis of peptides 10 and 11

The two azido derivative peptides **10** and **11** (Scheme 2) were synthesised according to the general procedure by microwave-assisted Fmoc-SPPS with 2 × 5 min couplings for each Fmoc-amino acid (4.2 equiv) using HATU (4.0 equiv) and DIPEA (4.2 equiv). The temperature was set at 70 °C (20 W, 10 min) for all amino acids, except for Cys and His, which were coupled at 50 °C (20 W, 10 min). For His, Cys, and Arg, initial coupling was performed at rt for 3 min before microwave-assisted couplings.[31] Each amino acid coupling cycle consisted of Fmoc-deprotection with 20% piperidine in DMF at 70 °C (twice, 2.5 min and 5 min), 2 min DMF flow wash, followed by 10 min coupling with the pre-activated amino acid. In the case of Fmoc-deprotection for Asp, 0.1 M HOBT in 20% piperidine/DMF was used. Upon completion of synthesis, the resin was washed with DMF, DCM, MeOH, and vacuum dried. The peptide was cleaved from the resin by stirring in a solution of TFA (99%)/triisopropylsilane/water (95:2.5:2.5) for 4 h. The crude products (**10** and **11**) were purified by RP-HPLC.

Peptide **10** yield: 6%. Purity: >95%. Molecular weight: 5446.2. ESI-MS: $[M+4H]^{4+}$ *m/z* 1362.1 (calcd: 1362.6), $[M+5H]^{5+}$ *m/z* 1090.1 (calc: 1090.3), $[M+6H]^{6+}$ *m/z* 908.8 (calcd: 908.7),



Scheme 1. Synthesis of alkyne derivative **9**.



Scheme 2. Synthesis of LCP **3** and **4** (J14: KQAEDKVKASREAKKQVEKALEQLEDKVK), P25: KLIPNASLIENCTKAEL)).

$[M+7H]^{7+}$ m/z 779.0 (calcd: 779.0), $[M+8H]^{8+}$ m/z 691.9 (calcd: 681.8). R_t = 23.8 min (25–45% solvent B, 20 min, C18-column).

Peptide **11** yield: 12%. Purity: >95%. Molecular weight: 5446.2. ESI-MS: $[M+3H]^{3+}$ m/z 1816.3 (calcd: 1816.4), $[M+4H]^{4+}$ m/z 1362.9 (calcd: 1362.6), $[M+5H]^{5+}$ m/z 1090.2 (calcd: 1090.3). R_t = 24.3 min (25–45% solvent B, 20 min, C18-column).

3.5. Synthesis of LCP 3 and LCP 4

For the synthesis of LCP **3**, a mixture of azide derivative (**10**) (0.4 μmol, 1 equiv) and lipoalkyne (**9**) (2.2 μmol, 5 equiv) was dissolved in DMF/DMSO (1:0.6), and a piece of copper wire was added (Scheme 2). Most of air in the reaction mixture was removed by quick nitrogen bubbling. The reaction mixture was covered and protected from light with aluminium foil and stirred at 50 °C under nitrogen. The progress of the reaction was monitored by analytical HPLC (C4-column) and ESI-MS until the peptide **10** was completely consumed after 5 h. The reaction mixture was added to a buffer (6 M guanidine, 50 mM sodium phosphate, 20% acetonitrile, 5 mM EDTA, ~pH 7.3) containing TCEP (tris(2-carboxyethyl)phosphine) and stirred for 1 h. The reaction mixture was purified using a semi-preparative HPLC. After lyophilisation, the pure LCP **3** was obtained as an amorphous white powder.

For the synthesis of LCP **4**, a mixture of azide derivative (**11**) (0.3 μmol, 1 equiv) and lipoalkyne (**9**) (2.2 μmol, 7 equiv) was dissolved in DMF/DMSO (1:0.6), and a piece of copper wire was added (Scheme 2). The remaining procedures follow those of the synthesis of LCP **3**, as mentioned above.

LCP **3** yield: 43%. Purity: >97%. Molecular weight: 6352.5. ESI-MS: $[M+6H]^{6+}$ m/z 1059.7 (calcd: 1059.8), $[M+7H]^{7+}$ m/z 908.5 (calcd: 908.5). R_t = 27.8 min (30–70% solvent B, 60 min, C4-column).

LCP **4** yield: 72%. Purity: >97%. Molecular weight: 6352.5. ESI-MS: $[M+6H]^{6+}$ m/z 1059.7 (calcd: 1059.8), $[M+7H]^{7+}$ m/z 908.5 (calcd: 908.5), $[M+8H]^{8+}$ m/z 795.0 (calcd: 795.1), $[M+9H]^{9+}$ m/z 706.7 (calcd: 706.8). R_t = 23.0 min (30–70% solvent B, 60 min, C4-column).

3.6. Measurement of LCP particle size

LCP compounds were dissolved in PBS (phosphate buffered saline) (0.2 mg/mL). The particle size of each LCP compound at 25 °C was measured in capillary cuvettes using a Nanosizer (Zetasizer Nano Series ZS, Malvern Instruments, UK). Measurements were taken with a scattering angle of 173°. Correlation times were set at 10 s per run, and a total of 5 runs were made for each measurement. The average size of the particles was calculated by the Zetasizer 6.2 software (Malvern Instruments, UK).

3.7. Transmission electron microscopy

Samples of the LCP **1**–**4** in PBS used for the Nanosizer experiments were placed on carbon-coated 200 mesh grids. Excess liquid was removed with filter paper after 3 min. A JEM-1010 transmission electron microscope (JEOL Ltd, Japan) operating at 80 kV was used to take photographs of the compounds.

3.8. Intranasal immunisation of mice

Eight week-old female outbred Swiss mice were used in this study (Griffith University, Gold Coast, Australia). All animal protocols used were approved by the Animal Ethics Committee (University of Queensland) in accordance with National Health and Medical Research Council (NHMRC) of Australia guidelines. Access to food and clean water was provided ad libitum.

Cohorts of 5 mice were administered 60 μg of LCP dissolved in 30 μL (15 μL/nare) of sterile PBS following anaesthetisation with 1:1:10 mixture of ketamine/xylazine/water (Griffith University, Gold Coast, Australia). Similarly, a negative control group was administered 30 μL of PBS and a positive control received 30 μg of J14-K-P25 with 10 μg of CTB mixed with PBS (total volume of 30 μL). Additionally, one group was administered 30 μg of unconjugated P25+J14+CTB. Boosters of the same dosage were given on days 21 and 42 following primary immunisation.

3.9. Serum collection

Serum was collected on days 20, 41 and 60, and at five months post-primary immunisation (day 155). 10 μL of blood was collected from the tail artery of each mouse, and serum was extracted by centrifugation.

3.10. Determination of antibody titres

An ELISA was used to measured J14-specific murine serum IgG as described elsewhere.[10] Serial two-fold dilutions of samples were produced in 0.5 % skim milk/PBS-Tween 20 buffer, starting at the concentration of 1:100 for sera samples. A titre was defined as the highest dilution that gave an optical density >5 × SDs above the mean optical density of control wells containing mouse sera collected before immunisation. Statistical significance (p <0.05) was determined using a one-way ANOVA with Tukey post-hoc test. Horseradish peroxidase-conjugated antimouse IgG1 and IgG2a were used as secondary antibodies.

4. Results and discussion

In our attempts to optimise delivery systems for peptide-based GAS vaccines, three novel analogues of lead vaccine candidate LCP **1** were designed (Fig. 1). LCP **2** incorporated C20 LAAs (2-amino-D, L-eicosanoic acid), a longer version of C16 LAAs presented in LCP **1**. LCP **3** and **4** differ in special orientation from **1** and **2**; both have C16 LAAs on the ε-amino group of the central lysine, attached via a triazole linkage. In LCP **3**, P25 was attached on the α-amino side-chain and J14 on the α-carboxyl side-chain, while in LCP **4**, these epitopes replaced their positions. In all compounds, the self-adjuvanting lipopeptide moiety was attached to the branching lysine via the lipopeptide N-terminal serine, and therefore, all LCPs possessed C-terminal LAAs in amide form.

LCP **1** and **2** were synthesised using standard SPPS while LCP **3** and **4** were produced with the help of a copper-catalysed

Table 1
Average diameter of the LCP particles (d nm) dissolved in PBS (0.2 mg/mL)

Compound	Size by DLS (d nm)	Size by TEM (d nm)
LCP **1**	9.2 ± 0.3	7–11
LCP **2**	11.1 ± 0.6	8–12
LCP **3**	10.0 ± 1.0	8–15
LCP **4**	13.9 ± 3.0	8–25

azide-alkyne cycloaddition (CuAAC) reaction. At first, the alkyne derivative **9** was synthesised by Fmoc-SPPS (Scheme 1). Couplings of Dde-C16 (**5**) to the Rink amide resin was achieved with the help of HATU and DIPEA in DMF (2 × 1 h). The Dde-protecting group of **5** was removed by using 2% hydrazine in DMF (8 × 30 min) to give **6**, then the process was repeated to afford lipopeptide-resin **7**. The serines were also coupled using the HATU/DIPEA method. To increase the solubility of **9**, a lysine residue was introduced into the lipopeptide sequence. Finally, pentynoic acid was coupled to the N-terminus end of the peptide to give the alkyne lipopeptide (**8**) that was cleaved with 95% of TFA to give **9** in 24% yield (Scheme 1). The CuAAC was applied to conjugate the azide derivatives **10** and **11** with the lipopeptide **9** in the presence of Cu wire[32] at 50 °C (Scheme 2). The reaction mixture was added to a guanidine buffer solution containing TCEP to reduce the disulphide bond formed during the click reaction. The final constructs LCP **3** and **4** were purified and obtained in 43% and 16% yields, respectively.

As LCPs are amphiphilic compounds, they can self-assemble into particles of distinct sizes in aqueous solutions.[33–36] The size of the particles they form is important in determining whether they can be absorbed through mucosal surfaces and thus, whether they can be delivered effectively as intranasal vaccines.[20] Their size can also affect how they are recognised and processed by APCs, as well as their trafficking in the lymphatic system, and in turn, affecting the immune response that follows.[37,38] The size distribution of the particles formed by LCP **1**–**4** was measured by DLS (Table 1). Particles were also visualised by transmission electron microscopy (TEM) (Fig. 2). With the exception of LCP **4**, all the compounds formed particles with a homogenous distribution. LCP **4** formed the largest nanoparticles with the highest variability in size (8–25 d nm). However, this size difference is negligible and is unlikely to cause differences in immunogenicity of the compounds.

At sizes smaller than 100 d nm, LCP **1**–**4** are all optimal for absorption through the mucosal epithelium and for entering lymph vessels via gaps between epithelial cells.[39] This permits easy access for the particles to the systemic immune system. As all the compounds in this study formed particles of similar sizes, it was therefore assumed that any immunological difference between the compounds was caused by factors other than their size.

Outbred Swiss mice were used for the immunological study of LCP **1**–**4**. Following the initial intranasal immunisation and two boosters of LCP compounds, blood serum was collected to determine J14-specific IgG antibody titres by enzyme-linked immunosorbent assay (ELISA). All LCP groups elicited significant IgG titres (Fig. 3), with LCP **2** inducing the highest titres ($p < 0.0001$ vs the negative control group PBS). LCP **2** titres were also significantly higher than those of LCP **3** and **4**, and the positive control (J14-K-P25 + CTB). LCP **2**, having LAAs with longer side-chains than LCP **1**, also induced titres higher than those of LCP **1**.

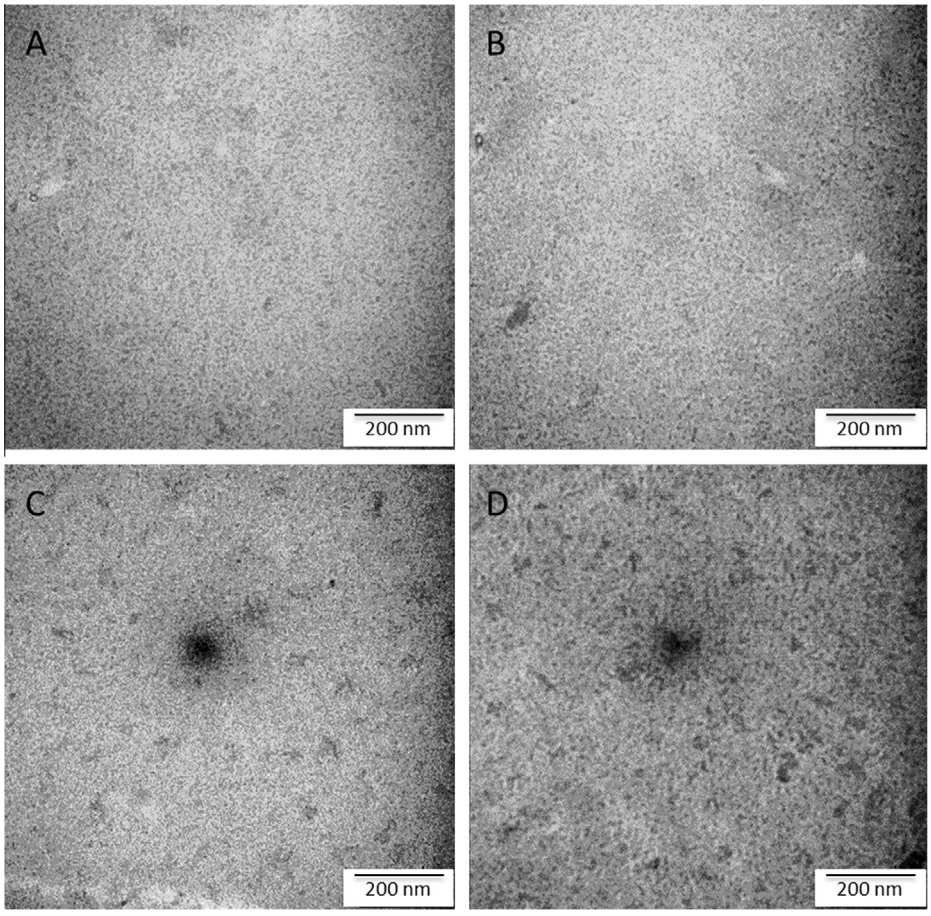


Figure 2. Transmission electron microscopy photographs of the investigated compounds in 2 mg/mL concentration dissolved in PBS: (A) LCP **1**. (B) LCP **2**. (C) LCP **3**. (D) LCP **4**. Bar = 200 nm.

Please cite this article in press as: Chan, A.; et al. *Bioorg. Med. Chem.* (2016), http://dx.doi.org/10.1016/j.bmc.2016.03.063

However, this difference was not statistically significant as the induced immune response differed greatly between individual mice.

As no human-approved mucosal adjuvant exists for the delivery of peptides, CTB (which is restricted to animal use) represents a standard adjuvant to compare against the investigated compounds.[40–42] Thus, LCP **2** is not only more efficient at inducing IgG production, it is also expected to be a safer alternative for vaccines adjuvanted with CTB. Furthermore, it should be noted that there was no significant difference in induced titres between mice immunised with Th-epitope and B-cell epitope that were conjugated (J14-K-P25 + CTB), and mice immunised with Th-epitope and B-cell epitope that were not conjugated (P25 + J14 + CTB). This contrasts with some reports suggesting that such conjugation is crucial for the induction of immune responses.[43]

LCP **2** was compared to LCP **1** to determine the effect of varying the LAA length on compound immunogenicity. Despite the lack of significance in the current data, it does appear that LCP **2** produced higher IgG titres than LCP **1**. This suggests that an increase in LAA length may also increase the immunogenicity of the compound. LCP **4** and **5** were compared to LCP **1** to determine the effects of spatial orientation on compound immunogenicity. Neither LCP **3** nor **4** induced immune responses that were statistically different from LCP **1**. This suggests that, with the same LAA length, the spatial orientation of the LCP has a rather limited effect on its immunogenicity.

Overall, LCP **2** stimulated the highest short-term systemic immune response. In order to further characterise the J14-specific immune response elicited by LCP **2**, IgG antibody isotypes were evaluated by ELISA and compared to LCP **1** (Fig. 4). In the murine system, IgG1 is predominantly induced by Th2 activity and IgG2a induced by Th1 activity.[44] Both IgG1 and IgG2a activity were observed after immunisation with LCP **1**, as well as with LCP **2**, suggesting a mixed Th1/Th2 response. Notably, LCP **2** was able to generate a significantly greater Th1 response than LCP **1**. This may be more desirable as Th1-type cytokines are responsible for stimulating phagocytes and promoting pro-inflammatory responses against intracellular pathogens, which may facilitate faster bacterial clearance.[45] Furthermore, the high Th2 activity prevents a Th1-dominated response that may cause host tissue damage.[46] In comparison, the J14-K-P25 + CTB positive control mounted a Th2 response only.

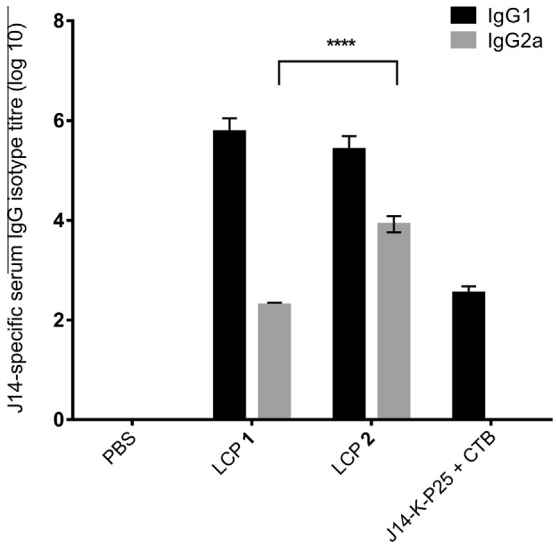


Figure 4. J14-specific serum IgG isotype titre (log 10) at day 60 post-primary intranasal immunisation of Swiss mice (n = 5/group). Error bars represent standard deviation.

Serum samples were obtained from the LCP **2** group at 155 days post-primary immunisation (with the last booster given on day 42) to determine whether this compound was capable of generating long-term J14-specific antibody response (Fig. 5). It was found that vaccination with LCP **2** maintained high IgG titres after five months (p <0.05 vs the negative control PBS), which was similar to those induced by the adjuvanted control (J14-K-P25 + CTB).

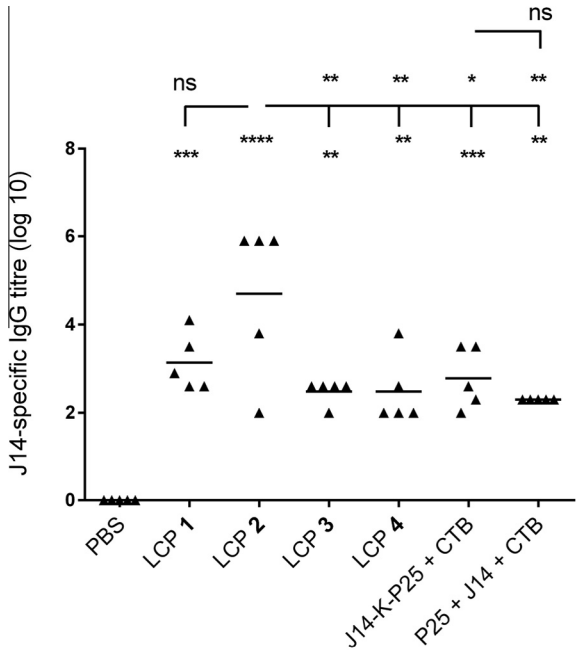


Figure 3. J14-specific IgG titres (log 10) elicited in response to vaccination of Swiss mice (n = 5/group) at day 60. Mice received a primary immunisation on day 0 followed by two boosters on days 21 and 42. Statistical analysis was performed by one-way ANOVA followed by the Tukey post-hoc test (ns, p <0.05; *, p <0.01; **, p < 0.001; ***, p <0.0001; ****).

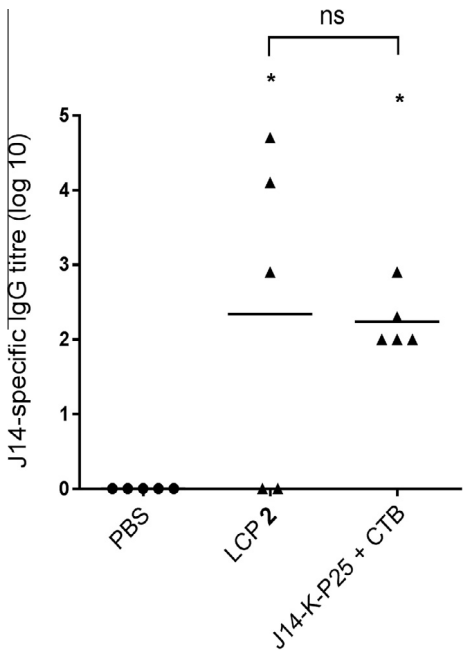


Figure 5. J14-specific IgG titres (log 10) elicited in response to vaccination of Swiss mice (n = 5/group) after five months (155 days). Mice received a primary immunisation on day 0 followed by two boosters on days 21 and 42. Statistical analysis was performed by one-way ANOVA followed by the Tukey post-hoc test (ns, p <0.05; *, p <0.01; **, p <0.001; ***, p <0.0001; ****).

5. Conclusion

Recent trends in GAS vaccine development have focused on the application of peptide-based antigens, mainly from M-protein. For this purpose, we have studied a multicomponent LCP construct incorporating minimal peptide epitopes, Th-epitopes, and LAA. The adjustable design of the LCP allowed the alteration of its spatial orientation and the length of incorporated LAAs. This study confirmed that the lipid length and position of vaccine components has an effect on the immune response in vivo in terms of IgG antibody production. The leading vaccine candidate (LCP **2**) in this study featured a C-terminal C20 LAA moiety attached via a lysine residue to P25 on the N-terminus, and J14 on the lysine ε-amino side-chain. LCP **2** was able to induce a strong mixed IgG1/IgG2a immune response, even after five months post-primary immunisation. This compound outperformed the other constructs involved in this study and its induced IgG response was even higher than those induced by the classical adjuvant CTB.

Acknowledgements

The authors acknowledge the facilities and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy and Microanalysis, The University of Queensland. This work was supported by the National Health and Medical Research Council of Australia (NHMRC 496600).

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