ORIGINAL ARTICLE

# Chemical synthesis and biological evaluation of an antimicrobial peptide gonococcal growth inhibitor

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**Abstract** Gonococcal growth inhibitor 1 (GGI-1) is a 44-residue peptide with potent anti-*Legionella* activity. It has been isolated from *Staphylococcus haemolyticus* but, to date, its chemical synthesis has not been reported. Acquisition of this peptide via this means would enable a more detailed examination of its antimicrobial properties. However, its synthesis represents a significant challenge because of two predicted "difficult sequences" within the peptide. Its successful solid-phase assembly is reported in this paper, and was accomplished by use of simple palliative measures including the introduction of a single pseudo-proline isostere in order to counteract on-resin aggregation. The peptide had moderate antimicrobial activity against *Escherichia coli* but was inactive against another Gram-negative bacterium and two Gram-positive bacteria (*Bacillus* species). It had significant haemolytic activity, with a $H_{50}$ (concentration of peptide that causes 50 % haemolysis) of 20 and 125 μM for two blood samples from different donors. An alternative therapeutic index to that proposed for GGI-1 in a recent publication is proposed.

**Keywords** Antimicrobial peptide · "Difficult sequence" · Haemolytic activity · Gonococcal growth inhibitor 1 · Solid phase peptide synthesis

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## Introduction

Antimicrobial peptides (AMPs) are relatively short polypeptides (12–100 amino acids) that are positively charged (net charge of +2 to +9) and amphiphilic. They have been isolated from single-celled microorganisms, insects and other invertebrates, plants, amphibians, birds, fish and mammals, including humans, where they represent a defence system against invading pathogenic bacteria. This defence system includes direct inhibition of bacterial and viral pathogens combined with stimulation of the immune system, and the functions of the AMPs extend to innate and adaptive immunity, including immuno-modulation, chemotaxis, inflammatory response and wound repair. AMPs are important because of their potential to provide an alternative therapy against bacterial infections to conventional antibiotics in an age of increasing occurrence of antibiotic-resistant bacteria. A challenge in advancing AMPs to clinical use is the separation of potent antimicrobial activity from the unwanted cytotoxicity on normal mammalian cells, including erythrocytes (Dawson and Liu 2008).

The Australian Defence Science and Technology Organisation has an interest in medical countermeasures against bacteria that could be used in biological warfare or as bioterror agents, and has recently evaluated a number of AMPs in this context (Dawson and Liu 2008, 2010, 2011; Dawson et al. 2010, 2011). Recently, various peptides secreted by *Staphylococci* species were found to have potent anti-*Legionella* activity (Marchand et al. 2011), and it was decided to assess their antimicrobial activity against other bacteria. Most are easily chemically synthesised by conventional solid phase techniques. However, one, gonococcal growth inhibitor 1 (GGI-1), a 44-residue peptide that was isolated from *S. haemolyticus* (Watson et al.

1988), has not has its chemical synthesis reported to date, and presents a significant challenge due to long stretches of hydrophobic amino acids in the overall sequence, making it a recognised so-called "difficult sequence" (Fig. 1). We now report its efficient chemical synthesis, purification, antimicrobial properties and haemolytic activity.

## Materials and methods

### Materials

Human red blood cells were obtained free of charge under a contract with the Australian Red Cross Blood Service (Melbourne, Australia). All reagents for the antimicrobial and haemolytic assays were obtained from Sigma-Aldrich (Sydney, Australia). All amino acid derivatives for the synthesis of GGI-1 were purchased from GL Biochem (Shanghai, China).

### Synthesis of GGI-1

The peptide was assembled on an automated PerSeptive Biosystems Pioneer peptide synthesiser using the continuous flow Fmoc solid phase synthesis methodology as previously described (Bathgate et al. 2006; Hossain et al. 2008, 2010). The solid support was appropriately C-terminal amino amide-linked Fmoc-PAL-PEG-PS for the preparation of C-terminal peptide amides (Applied Biosystems, Australia), and HBTU-activated Fmoc-amino acids were used throughout. Amino acid side chain protection was afforded by the following: Arg, Pbf; Asn and Gln, Trt; Asp and Glu, O-Bu$^t$; His, Trt; Lys, Boc; Ser and Thr, Bu$^t$. No repeat couplings were carried out. N$^\alpha$-Fmoc deprotection was with 20 % piperidine in DMF. Assembly of GGI-1 (1–44) commenced on a 0.1-mmol scale using a fourfold excess of activated amino acid and 60 min coupling times. The synthesis was programmed to pause at Ile$^{27}$ and Ala$^{14}$ at which stages one-half of the peptide resin was removed from the synthesiser and separately reacted with a twofold excess of activated Fmoc-Val-Ser(psiMe, MePro)-OH for 4 h and then returned to the automated instrument and the synthesis continued. After acylation and N$^\alpha$-deprotection of the final residue, Met$^1$, the peptide resin was treated with acetic anhydride in DMF for 30 min, washed and dried prior to cleavage from the solid support and simultaneous side chain deprotection by a 1.5-h treatment of the GGI-1(1–44) peptide resin with trifluoroacetic acid (TFA) in the presence of anisole and 2,2′-(ethylenedioxy)-diethanethiol (95/3/2, v/v) with a few drops of triisopropylsilane (TIS). The resulting crude peptide (183 mg) was subjected to preparative reversed-phase high performance liquid chromatography (RP-HPLC) on a Vydac C4 column (Hesperia, USA) using a 1 %/min gradient of CH$_3$CN in 0.1 % aqueous TFA. The major peak was collected, and subsequent freeze-drying gave 21.6 mg (11.8 %) of highly purified GGI-1(1–44).

### Chemical characterisation

The purity of the synthetic peptides was assessed by analytical RP-HPLC on a Phenomenex C18 column (pore size 300 Å, particle size 5 μm, 4.6 × 250 mm) using a gradient of acetonitrile in 0.1 % aqueous TFA. The product was confirmed by MALDI-TOF mass spectrometry using a Bruker Autoflex II instrument (Bremen, Germany) in the linear mode at 19.5 kV. The peptides were quantitated by amino acid analysis of a 24-h acid hydrolysate using Waters Accu-Tag chemistry together with a Shimadzu microbore RP-HPLC system.

### Antibacterial assays

The procedure has been described in a previous publication (Dawson et al. 2010, 2011). The bacteria used were *Escherichia coli* K91 BK, *Burkholderia thailandensis*, *Bacillus globigii* ATCC 9372 and *Bacillus anthracis* (Sterne strain 34F2) (Dawson et al. 2010, 2011). Briefly, a 5-mL aliquot of sterile NZCYM growth medium was inoculated with a colony of test bacteria and was incubated overnight at 37 °C with shaking at 200 rpm. A 1-mL aliquot was diluted 100-fold with fresh media and was incubated for a further 1 h with shaking at 37 °C. Aliquots (200 μL) of the bacterial suspension were added to 200 μL aliquots of peptide in the wells of a sterile 48-well cell culture plate that had been treated with 1 mL of 0.5 % bovine serum albumin in phosphate-buffered saline (PBS) to block peptide-binding sites (Zelezetsky et al. 2005) and washed twice with 1 mL of PBS. The plate was incubated at 30 °C with shaking at 150 rpm for 8 h. Readings of absorbance at 600 nm to monitor bacterial growth were taken at hourly intervals using a Synergy HT Plate Reader (BioTek Instruments, Winooski, Vermont, USA). Control wells contained no peptide (positive control) or no bacteria (blank). All experiments were repeated on a different day.

### Haemolysis

The procedure has been described in a previous publication (Dawson et al. 2010, 2011). Briefly, serial (1 + 1) dilutions

**Fig. 1** Primary structure of GGI-I

Ac-MQKLAEAIAAAVSAGQDKDWGKMGTSIVGIVENGITVLGKIFGF-CONH$_2$

1      5      10      15      20      25      30      35      40

of the peptide in PBS (100 μL) were prepared in the wells of a Nunc Immuno 96-microwell flat-bottom plate (Nunc A/S, Roskilde, Denmark). A 100-μL aliquot of 2 % washed human red blood cells in PBS was added to each well and the plate was incubated for 30 min at 37 °C at 100 rpm. The plate was then centrifuged at $1,000 \times g$ for 20 min in a Heraeus Multifuge 3S-R centrifuge (DJB Labcare Ltd., Newport Pagnell, UK). Aliquots (100 μL) of the well supernatants were transferred to a fresh 96-well plate and the absorbance at 414 nm was read in the plate reader as a measure of the release of haemoglobin. Controls (in triplicate) were 0.1 % Triton X-100 in PBS (positive control) and PBS only (blank). Replicate experiments were performed with each of two separate samples of blood cells (i.e., different donors). The concentration of peptide that causes 50 % haemolysis ($H_{50}$) was determined by fitting a second-order polynomial curve to the linear-log version of the (percent haemolysis)–concentration plot.

## Results and discussion

### Synthesis of GGI-1

GGI-1 is a 44-residue, highly hydrophobic peptide that was reported to contain an N-terminal formyl-Met and free C-terminal carboxylate (Watson et al. 1988). In order to further characterise its antimicrobial properties we undertook its chemical synthesis as an N-terminal acetylated and C-terminal peptide amide as the latter modification generally enhances structural stability and antimicrobial activity or decreases cytotoxicity (Kim et al. 2011). A prior, default solid-phase synthesis of GGI-1 using well-established in-house protocols that consistently produces good quality crude peptides of varying lengths and sequences in our laboratory was initially employed but was found to yield no identifiable target product (data not shown). Re-examination of the peptide sequence highlighted the presence of numerous hydrophobic and bulky residues with many in long stretches within GGI-1 indicating it was a so-called "difficult sequence" (Milton et al. 1990). Such sequences are often refractory to chemical synthesis for any one of a number of reasons including on-resin aggregation. The

phenomenon remains a subject of intense investigation with many palliative or, even, remedial measures proposed and demonstrated to be at least moderately effective (Tickler and Wade 2007; Abel Rahman et al. 2007). Indeed, GGI-1 sequence analysis by a software prediction program (Peptide Companion; Lebl et al. 1995) suggested that two broad regions of significant synthetic difficulty could be expected, one within the C-terminal 20 residues and the other comprising the N-terminal 10 residues (Fig. 2). For this reason, a repeat assembly was undertaken in which one-third of the way through the assembly, one-half of the peptide resin was removed to result in higher molar excesses of activated amino acid being used for the remainder of the synthesis. Then, later in the assembly and prior to the commencement of the N-terminal "difficult sequence" region, additional peptide resin was removed and a single pseudo-Pro isostere was introduced in order to reduce or eliminate on-resin aggregation which is often associated with failed assemblies (Tickler and Wade 2007). At the end of the synthesis, not only was GGI-1 obtained but it was also in good quality, making its subsequent purification in overall yield of 12 % straightforward (Fig. 3). Such a result confirms earlier reports that seemingly modest alterations to synthetic schedules or protocols can have dramatic effects on the success of synthesis of "difficult sequences" (Tickler et al. 2004). The use of pseudo-Pro isosteres is especially effective and their increased cost is far outweighed by the consistently positive outcomes that these confer on the acquisition of complex peptides (Mutter et al. 1995; White et al. 2004).

### Antimicrobial activity

Gonococcal growth inhibitor 1 failed to significantly inhibit growth of any of *B. thailandensis*, *B. globigii* and *B. anthracis* at concentrations up to 100 μM (the highest concentration tested). Some (but not complete) inhibition of growth of *E. coli* was observed, and the $I_{50}$ (the concentration that reduced net absorbance at 600 nm by 50 %) was found to be $71 \pm 10.6$ μM (SEM; $n = 2$). In simultaneous experiments, tetracycline had a minimum inhibitory concentration (MIC) of $65 \pm 0$ μM and an $I_{50}$ of $13.6 \pm 1.5$ μM.



**Fig. 2** Aggregation and corresponding synthetic difficulty of GGI-1 as predicted by "Peptide Companion". Aggregation potential greater than 1.2 is indicative of amino acid acylation difficulty
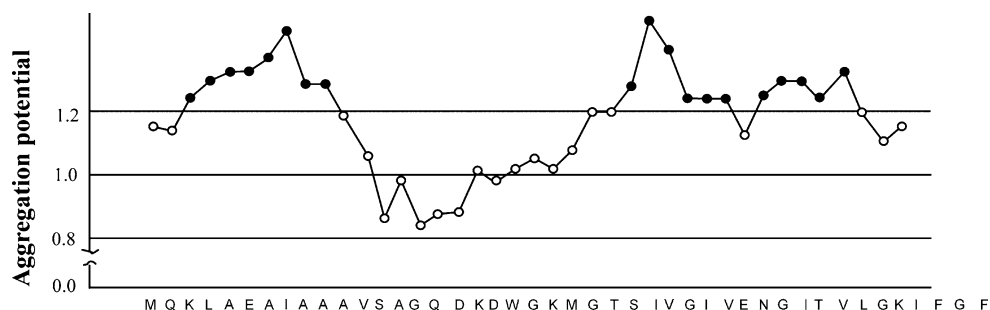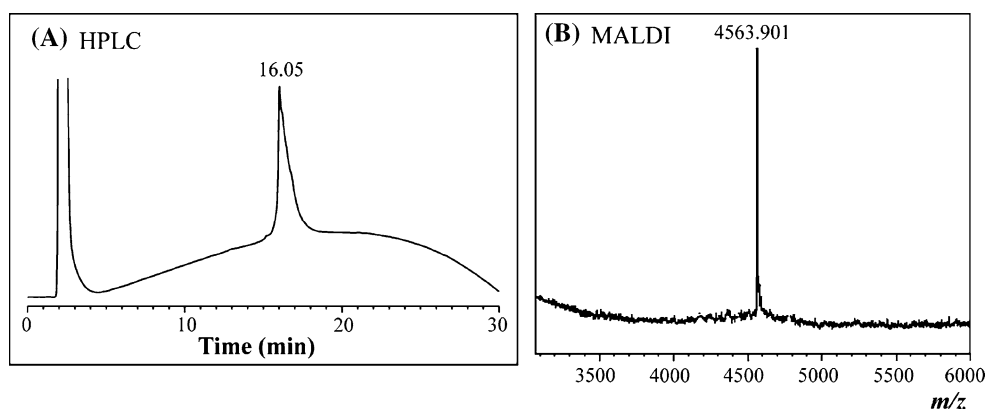
Fig. 3 **a** Analytical RP-HPLC of purified synthetic GGI-I on a Vydac C4 column (4.6 × 250 mm). Buffer A: 0.1 % aqueous TFA; Buffer B: 0.1 % TFA in $CH_3CN$. Gradient: 20–100 % B in 30 min. Wavelength 214 nm; flow rate 1.5 ml/min. Column temperature: 50 °C. **b** MALDI-TOF mass spectrum of purified synthetic GGI-I (calcd. [MH+] 4,564.39, found [MH+] 4,563.90)

Gonococcal growth inhibitor 1 has been reported to have a MIC against *Legionella pneumophila* of 4.2 μM (Marchand et al. 2011). This appears to be the only quantitative report of the antibacterial activity of GGI-1, as descriptions of its inhibitory properties against growth of *Neisseria gonorrhoeae* (Marchand et al. 2011; Beaudet et al. 1982) are qualitative only.

Haemolytic activity

The only report of haemolytic activity of GGI-1 quotes the factor $H_{10}$, which is the concentration that causes 10 % haemolysis of human erythrocytes. This was found to be 2.1 μM (Marchand et al. 2011). We have determined the full spectrum of haemolytic activity as a function of peptide concentration, and this is shown in Fig. 4. Results for blood from each of two donors are shown, rather than overall mean values, because of the substantial variation of values between the two samples. Values of $H_{50}$ (concentration of peptide that causes 50 % haemolysis) were found to be 19.6 μM and approximately 125 μM for the two blood samples, respectively. A value of $H_{10}$ could not be determined for one of the blood samples, since haemolysis was over 20 % at the lowest concentration tested (0.39 μM; Fig. 4). For the other blood sample, $H_{10}$ is approximately 0.5 μM. This value is consistent with the literature value of $H_{10}$ (2.1 μM, above), given the variability in results with blood from different donors (Fig. 4). These results suggest that the value of $H_{10}$ is not a reliable indicator of haemolytic activity. Thus, although one blood sample had $H_{10} \sim 0.5$ μM for GGI-1, a concentration 200 times higher than this (100 μM) failed to achieve even 50 % haemolysis, let alone 100 %. Marchand et al. (2011) calculated a therapeutic index for various peptides, which they defined as $H_{10}$/MIC. This index of selectivity was found to be 0.5 for GGI-1. We propose that $H_{50}/I_{50}$ is a better definition of the therapeutic index, since both haemolytic and antibacterial activity are expressed in terms of 50 % efficacy (Dawson et al. 2011). Our determination of $H_{50}$ for GGI-1 (20–125 μM) suggests that the refined

therapeutic index for GGI-1 in the systems of Marchand et al. (2011) would be of the order of 20–125 (since the $I_{50}$ of GGI-1 vs. *L. pneumophila* appears to be approx. 1 μM), which puts its potential clinical application in an entirely different light from that with a therapeutic index of 0.5 based on use of the value of $H_{10}$ (Fig. 4).

Conclusion

Increasing bacterial resistance is one of the world's most serious health concerns. It has been forecast that within 10 years, today's generation of antibiotics may be largely ineffective. The development of antimicrobial resistance is multi-faceted, but unanimous agreement amongst healthcare professionals and the pharmaceutical industry is the urgent need for new, potent and selective antimicrobial agents. AMPs have rapidly emerged as an important alternative to small molecule antibiotics due to their potency and varying modes of action. Numerous such peptides have been identified and subjected to detailed investigation but, thus far, few have been reported to possess potent, selective activity without cytotoxic effects.
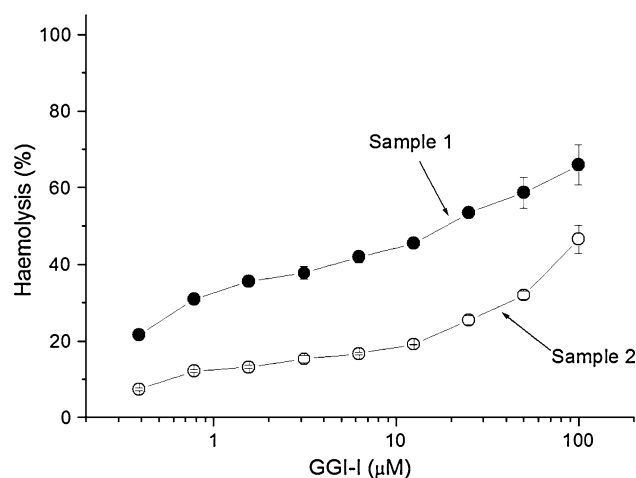


Fig. 4 Haemolytic activity of GGI-1

The identification of an antimicrobial gonococcal growth inhibitor-derived peptide, GGI-1, represents another source of potential antimicrobial therapy that necessitates detailed examination. Its acquisition was only achieved using modified solid phase synthesis protocols to overcome its "difficult sequence" resistance which highlights the continued importance this technology makes to such studies. The data showed that although the peptide lacks significant activity against common bacteria and possesses haemolytic activity that restricts its utility, chemical synthesis may provide a means of obtaining designed analogues with improved activity spectrum.

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