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Spectral and biological evaluation of a synthetic antimicrobial peptide derived from 1-aminocyclohexane carboxylic acid

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ABSTRACT

Ac-GF(A6c)G(A6c)K(A6c)G(A6c)F(A6c)G(A6c)GK(A6c)KKKK-amide (A6c = 1-aminocyclohexane carboxylic acid) is a synthetic antimicrobial peptide (AMP) that exhibits in vitro inhibitory activity against drug resistant strains of *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Enterococcus faecium* at concentrations ranging from 10.9 to 43 μM. Spectroscopic investigations were conducted to determine how this AMP interacts with simple membrane model systems in order to provide insight into possible mechanisms of action. CD and 2D-^{1}H NMR experiments indicated this AMP on binding to SDS and DPC micelles adopts conformations with varying percentages of helical and random coil conformers. CD investigations in the presence of three phospholipid SUVs consisting of POPC, 4:1 POPC/POPG, and 60% POPE/21%POPG/19%POPC revealed: (1) The interactions occurring with POPC SUVs have minimal effect on the conformational diversity of the AMP yielding conformations similar to those observed in buffer. (2) The interactions with 4:1 POPC/POPG, and 60% POPE/21%POPG/19%POPC SUVs exhibited a greater influence on the percentage of different conformers contributing to the CD spectra. (3) The presence of a high of percentage of helical conformers was not observed in the presence of SUVs as was the case with micelles. This data indicates that the diversity of surface bound conformations adopted by this AMP are very different from the diversity of conformations adopted by this AMP on insertion into the lipid bilayer. CD spectra of this AMP in the presence of SUVs consisting of LPS isolated from *P. aeruginosa*, *K. pneumoniae* and *Escherichia coli* exhibited characteristics associated with various helical conformations.

1. Introduction

Over the past two decades, the world has witnessed a dramatic increase in the emergence of multiple drug resistant pathogenic microorganisms (MDROs).[1–5] Clinical isolates such as those from *Staphylococcus* and *Pseudomonas* have evolved resistance to many of the traditional antibiotics.[6–8] The recent evolution of carbapenem-resistant *Enterobacteriaceae* (CRE), which includes the genera *Enterobacter aerogenes* and *Klebsiella pneumonia*, represents another critical health concern since the infections associated with these Gram-negative bacteria are very difficult to treat with traditional antibiotics and exhibit mortality rates as high as 50%.[5] This continued and wide spread evolution of drug resistant bacteria has resulted in an international health care crisis[9–11] that has stimulated a world-wide research effort to develop antibiotics that kill bacteria via novel mechanisms of actions.[10,12–17]

One potential source of new antibiotics that exhibit novel mechanisms of action are natural and synthetic antimicrobial peptides (AMPs).[18] AMPs have evolved as part of the innate defense mechanisms against invading micro-organisms such as bacteria, fungi, protozoa and parasites in a variety of organisms, including humans,[19] amphibians,[20] insects, mammals, birds, fish and plants.[21–23] They are also known to be involved in the innate immune response.[18,24–26] AMPs are generally small highly positively charged[27] amphipathic peptides with well-defined

Abbreviations: A6c, 1-aminocyclohexane carboxylic acid; AMP, antimicrobial peptide; CD, circular dichroism; DPC, dodecylphosphocholine; LPS, lipopolysaccharides; MIC, minimum inhibitory concentration; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser spectroscopy; Oic, octahydroindolecarboxylic acid; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt); SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicle; Tic, tetrahydroisoquinolinecarboxylic acid; TOCSY, total correlation spectroscopy.

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regions of hydrophobicity and hydrophilicity.[28–30] As of June 2012, no AMP based therapeutic has been approved by The Food and Drug Administration for human use.[31,32] This is the result of a combination of factors including low bioavailability, high cost of synthesis, moderate activity, low metabolic stability and relatively higher toxicity compared to conventional antibiotics.[31,32] Even in the face of the current lack of success in FDA approval of AMP based antibiotics the continued research into the development of AMPs into therapeutically useful agents is still fully justified, particularly in light of the continued failure of conventional antibiotic therapies to address the evolution of drug resistant bacterial.[31,33]

It is our hypothesis that it is possible to concurrently increase the selectivity and potency of AMPs for specific bacterial strains, as well as reduce mammalian cell toxicity by incorporating unnatural amino acids with well-defined physicochemical properties into the primary sequence of the AMP.[18,34] The incorporation of unnatural amino acids into AMPs offers two major advantages over those containing only the 20 naturally RNA encoded amino acids. (1) Unnatural amino acids provide greater control of the physicochemical properties and conformational flexibility of the AMP, thus increasing organism selectivity and potency.[9,34–37] (2) Unnatural amino acids exhibit increased metabolic stability.[28,38–41] Both factors should increase the therapeutic potential of AMPs containing unnatural amino acids.

The AMP reported herein was designed by applying the knowledge obtained from investigating the biological activity and the binding interactions with various phospholipids of another series of synthetic AMPs developed in our laboratory.[9,34–37,42,43] The structure and physicochemical properties of these AMPs were defined by incorporating several Tic–Oic dipeptide units and other unnatural amino acids into the primary amino acid sequence. The AMPs designed using this protocol exhibited below 25 μM to above 400 nM in vitro inhibitory activity against a variety of bacterial strains including drug resistant strains as well as select agents.[9,37]

The new synthetic AMP **1**, [Ac-GF(A6c)G(A6c)K(A6c)G(A6c)-F(A6c)G(A6c) -GK(A6c)KKKK-amide where A6c = 1-aminocyclohexane carboxylic acid] reported in this investigation incorporates the tetra substituted C^{α} amino acid, 1-aminocyclohexane carboxylic acid, into the peptide's primary sequence to induce a stable secondary structure onto the peptide backbone in the presence of amphipathic surfaces such as micelles and phospholipids, as well as, control the physicochemical properties of the AMP. This AMP has exhibited in vitro inhibitory activity against drug resistant strains of *S. aureus*, *Acinetobacter baumannii*, *K. pneumoniae*, *P. aeruginosa*, *Enterobacter aerogenes*, and *Enterococcus faecium* at concentration range of 10.9 to 43 μM, as point of reference in the in vitro inhibitory activity of an analogues containing three Tic–Oic dipeptide units AMP **23**: Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-KKKK-$CONH_2$[44] is also given in Table 1. As seen in Table 1, AMP **1**, based on the incorporation of multiple 1-aminocyclohexane carboxylic acids residues has shown equally or greater in vitro inhibitory activity against these five bacterial strains than the corresponding Tic–Oic AMP **23**.

2. Methods

2.1. Peptide synthesis

AMP **1** was synthesized by the contract laboratory, New England Peptides, (Gardner, MA) using solid phase technology and used without further purification.

2.2. Preparation of mixed POPC/POPE/POPG SUVs

The phospholipids POPC, POPG and POPE were purchase from Avanti Polar Lipids and used without further purification. The appropriate amount of dry phospholipid: POPC(53.2 mg), 4:1 POPC(42.56 mg)/POPG(10.78 mg), and 60% POPE(30.11 mg)/21%POPG(11.32 mg)/19%POPC(10.11 mg) was weighed out to yield a final lipid concentration of 35 mM with the desired percentage of each phospholipid. The lipid was hydrated with 2 mL of buffer (40 mM sodium phosphate, pH = 6.8) and vortexed extensively. SUVs were prepared by sonication of the milky lipid suspension using a titanium tip ultra-sonicator (Qsonica Sonicators model Q55) for approximately 40 min in an ice bath until the solution became transparent. The titanium debris was removed by centrifugation at 8800 rpm for 10 min using an Eppendorf table top centrifuge. Final lipid concentration used for CD studies was 3.5 mM.[42,45]

2.3. Preparation of LPS SUVs

Commercially available (Sigma Aldrich) lipopolysaccharides isolated from *P. aeruginosa*, *Escherichia coli* and *K. pneumoniae* were used in this investigation. A 4 mg sample of the appropriate lipopolysaccharide was hydrated with 4 mL of buffer (40 mM sodium phosphate, pH = 6.8) and vortexed extensively. SUVs were prepared by sonication of the milky lipid suspension using a titanium tip ultra-sonicator for approximately 10 min at a temperature of 40 °C until the solution became transparent. The titanium debris was removed by centrifugation at 8800 rpm for 10 min using a table-top centrifuge.[46]

2.4. Circular dichroism

CD spectroscopy is very sensitive to conformational changes in peptides and proteins and its use to monitor such changes is well documented.[47–49] Traditionally SUVs have been employed almost exclusively to investigate the binding of peptides and proteins with lipids in CD studies in order to minimize the contribution of light scattering.[47,50] In addition, in this investigation anionic (SDS) and zwitterionic (DPC) micelles were used to isolate and investigate the surface binding interaction of these peptides from the aggregation and pore forming interactions that occur upon binding to phospholipids.

Peptide solutions of 75 μM dissolved in 40 mM phosphate buffer (pH = 6.8) were used in these investigations. Binding studies were conducted using SUV preparations consisting of 3.4 mM of the appropriate phospholipids in 40 mM phosphate buffer

Table 1
Minimum inhibitory concentrations (MIC) values to inhibit growth of the ESKAPE pathogens in vitro

Bacteria	**1**	**23**
Staphylococcus aureus (UAMS-1 (MSSA)) (methicillin-sensitive *S. aureus*)	10.9 μM	>40 μM
Acinetobacter baumannii (WRAIR #13) (multidrug resistance to ampicillin, gentamicin, kanamycin, streptomycin, and tetracycline)	10.9 μM	2.5 μM
Klebsiella pneumoniae (BAMC 07-18) (resistant to ampicillin, azithromycin, chloramphenicol, gentamicin, and tetracycline)	43 μM	>40 μM
Pseudomonas aeruginosa (PAO1)	21.9 μM	>40 μM
Enterobacter aerogenes	21.9 μM	>40 μM
Enterococcus faecium (MMC4)	10.9 μM	10 μM