Understanding interactions of Citropin 1.1 analogues with model membranes and their influence on biological activity

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Abstract

The rapid emergence of resistant bacterial strains has made the search for new antibacterial agents an endeavor of paramount importance. Cationic antimicrobial peptides (AMPs) have the ability to kill resistant pathogens while diminishing the development of resistance. Citropin 1.1 (Cit 1.1) is an AMP effective against a broad range of pathogens. 20 analogues of Cit 1.1 were prepared to understand how sequence variations lead to changes in structure and biological activity. Various analogues exhibited an increased antimicrobial activity relative to Cit 1.1. The two most promising, AMP-016 (W3F) and AMP-017 (W3F, D4R, K7R) presented a 2- to 8-fold increase in activity against MRSA (both = 4 μg/mL). AMP-017 was active against E. coli (4 μg/mL), K. pneumoniae (8 μg/mL), and A. baumannii (2 μg/mL). NMR studies indicated that Cit 1.1 and its analogues form a head-to-tail helical dimer in a membrane environment, which differs from a prior study by Sikorska et al. Active peptides displayed a greater tendency to form α-helices and to dimerize when in contact with a negatively-charged membrane. Antimicrobial activity was observed to correlate to the overall stability of the α-helix and to a positively charged N-terminus. Biologically active AMPs were shown by SEM and flow cytometry to disrupt membranes in both Gram-positive and Gram-negative bacteria through a proposed carpet mechanism. Notably, active peptides exhibited typical serum stabilities and a good selectivity for bacterial cells over mammalian cells, which supports the potential use of Cit 1.1 analogues as a novel broad-spectrum antibiotic for drug-resistant bacterial infections.

1. Introduction

The emergence of drug-resistant bacteria has made the development of novel antibacterial agents a pressing issue [1]. In fact, most experts consider bacterial infections one of the greatest threats to human health, which may result in more than 10 million deaths worldwide by 2050 [2]. Bacterial resistance to common antibiotics occurs through a variety of mechanisms [3]. For example, the Gram-positive methicillin-resistant Staphylococcus aureus (MRSA) copes with antibiotic agents by increasing its membrane thickness or altering penicillin binding proteins (PBP) [4]. Gram-negative bacteria such as Enterobacter spp., Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa present resistance by virtue of a double membrane, by the development of efflux pumps, by the production of degrading enzymes, by gene transfer or by altering drug binding targets [5,6]. Antimicrobial peptides (AMPs) are an interesting option for combating resistant bacteria...
to conventional small molecule antibiotics [7]. AMPs are capable of killing a wide range of microorganisms by membrane pore formation, or membrane disruption. In addition, AMPs can present antibacterial activity by inhibiting protein synthesis, enzymatic activity, or cell wall synthesis [8–10]. The most attractive feature of AMPs is the observation that bacteria are less likely to develop resistance to AMPs when compared to non-peptide antibiotics [11]. This stems from the fact that the fundamental structure of the membrane is not easily altered by mutations. In order to develop AMPs as antimicrobial agents it is essential to determine its mechanism of action and the structural features that give rise to their activities. This understanding is key to improving and evolving their efficacy.

Citopin 1.1 (Cit 1.1/AMP-001) is an AMP derived from the skin of an Australian tree frog of the Lithorina genus. Cit 1.1 has shown intriguing activity against Gram-positive and Gram-negative species including: Staphylococcus aureus, Staphylococcus epidermis, Enterococcus faecalis, Escherichia coli, and Klebsiella pneumonia [12]. Conversely, studies evaluated the antifungal activity of Cit 1.1 against Candida species using direct bioautography [13] and against Candida albicans, Candida tropicalis and Candida krusei isolated from patients with oral cavity and respiratory tract infections [14]. They found that the peptide was weekly active and less potent than amphotericin B and nystatin against clinical strains [13,14]. To our acknowledge, Cit 1.1 has not been further tested against any other fungal strains besides Candida spp., suggesting the antifungal potential of this peptide and its analogues can be further explored.

Previous structure-activity relationship (SAR) reports indicated that truncation of Cit 1.1 sequence, such as deletion of Gly1, Leu2, or Phe3, completely ablated the peptide’s activity. On the other hand, deletion of Gly14, Gly15, or Leu16 had no impact on Cit 1.1 activity [15]. Tyler and co-workers suggested that the α-helical conformation of Cit 1.1 was critical to antimicrobial activity [16]. In fact, replacement of the L-amino acids with their D-isomers yielded an AMP with similar biological activity [16]. Later studies showed that if an isoAsp bond is formed between Leu5 and the carboxylic acid side chain of Asp4 biological activity is abolished while the serum stability is greatly enhanced [17]. This conformational change does not affect the total charge of Cit 1.1 and only marginally changes the isoelectric point and hydrophobicity; however, an isoAsp bond significantly alters its secondary structure hinting to the importance of the peptide’s conformation [17]. It has been additionally proposed that two α-helices are formed upon contact of Cit 1.1 with bacterial membranes [15]. Accordingly, an α-helical structure for Cit 1.1 appears to play an important role in the peptide’s antimicrobial activity. It is important to note that these prior studies relied on the introduction of relatively large structural changes to investigate a relationship between amino acid composition and peptide conformation on biological activity.

In this report, we sought to develop new Cit 1.1 analogues enhanced antimicrobial action against various pathogens. Further, we expanded upon the previous structural investigations by further characterizing the conformation and interactions of Cit 1.1 and its analogues in a membrane mimetic environment. The membrane-bound solution structures of the Cit 1.1 peptides were determined using nuclear magnetic resonance (NMR) spectroscopy. The mode of membrane disruption was visualized using transmission and scanning electron microscopy techniques (TEM and SEM), flow cytometry and confocal microscopy. The toxicity of the most potent AMP was studied in vitro against mammalian and human red blood cells. Poor serum stability of peptide-based antimicrobials is another factor limiting the use of AMPs and, therefore, we have also studied the serum stability of Cit 1.1 and its analogs. The experimental data has enabled us to define a mechanism of action for Cit 1.1, to describe the structural arrangement of the peptide when in contact with membranes, and to understand the impact of subtle molecular and conformational changes on peptide activity.

### Table 1

<table>
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<th>Peptide sequence</th>
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<th>Logd</th>
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2. Results

2.1. Minimum inhibitory concentration (MIC assay)

Cit 1.1 analogues were prepared using solid phase peptide synthesis (SPPS). Table 1 shows the amino acid sequence of the synthesized peptides and the observed MIC values against S. aureus JE2 (MRSA) and E. coli K12 (see Experimental SS for detailed procedures). Cit 1.1 (AMP-001) displayed activity against MRSA (32μg/mL) and E. coli (32μg/mL) or reduced antibacterial action (AMP-002, AMP-003, AMP-004, AMP-005, AMP-006, AMP-007 and AMP-008, respectively. These changes diminished the potency against Gram-positive pathogen when compared to Cit 1.1 (Table 1). The peptides displayed equal (AMP-006 and AMP-007, 32μg/mL) or reduced antibacterial action (AMP-008, >32μg/mL) against Gram-negative bacteria. On the AMP-009 analogues Lys7 was replaced with citrulline, which substitution resulted in a lack of antibacterial activity. AMP-010 was synthesized by methylating all of the available Cit 1.1 amine groups, whereas AMP-011 was generated by methylating the Lys amine moieties to produce analogues with a resident positive charge. Both AMP-010 and AMP-011 exhibited a complete lack of antibacterial activity. AMP-012 was constructed by replacing Lys7 and Lys8 with arginine residues, while the AMP-013 analogue was prepared by only substituting Lys7 for an arginine. The MICs for both AMP-012 and AMP-013 decreased 2-fold against the MRSA strains (16μg/mL) while the activity against Gram-
negative organisms was retained or diminished (MIC > 32 μg/mL) when compared to Cit 1.1 (Table 1 and 2). Interestingly, AMP-012 and AMP-013 exhibited antifungal activity with a MIC of 16 μg/mL against C. neoformans while AMP-012 displayed MIC of 16 μg/mL against Candida albicans (Table 2). A glycine residue was then inserted following Lys 8, in order to alter the relative position of potentially key α-helical residues. AMP-014 was observed to be inactive against the tested bacterial strains. The AMP-015 analogue was designed with a modest substitution of Asp4 for Glu. Surprisingly, AMP-015 was inactive against all bacterial strains tested (MIC > 32 μg/mL) but it inhibited the growth of C. neoformans with a MIC of 16 μg/mL.

The AMP-016 analogue was constructed by replacing Phe3 with Trp, which yielded an AMP with improved antibacterial activity against MRSA with MIC values of 8 μg/mL and 4 μg/mL for S aureus JE2 and S aureus ATCC 43300, respectively. AMP-016 also exhibited a better antibacterial activity against E. coli K12 with an MIC value of 16 μg/mL. AMP-016 did not demonstrate antibacterial activity against K. pneumoniae or P. aeruginosa, but it was active against A. baumannii (16 μg/mL), C. albicans (32 μg/mL) and C. neoformans (8 μg/mL). The Asp4 and Lys7 residues where changed to Arg in the AMP-017 analogue, producing the most potent analogue of the series which was effective against MRSA and E. coli (MIC 4 μg/mL), K. pneumoniae (MIC 8 μg/mL) A. baumannii (MIC 2 μg/mL). AMP-017 was also active against fungi with a MIC of 2 μg/mL against C. neoformans. Replacement of Gly1 with PhenylGly (AMP-018), Phe3 with a biphenylalanine residue (AMP-019), or 1-Naphyl (AMP-020) resulted in AMPs with improved antimicrobial activity against bacteria and fungi, when compared with the lead compound. For example, the MIC against MRSA was between 4–8 μg/mL. AMP-019 and AMP-020 were also active against the dangerous A. baumannii with MIC = 16 μg/mL. Gly1 was replaced with Sar to yield the AMP-021 analog, which presented promising MIC values against MRSA (8 μg/mL), E. coli (16 μg/mL), A. baumannii (4 μg/mL) and C. neoformans (16 μg/mL).

### Table 2

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<td>4</td>
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* Staphylococcus aureus ATCC 43300 MRSA.
* Escherichia coli ATCC 25922.
* Pseudomonas aeruginosa ATCC 27853.
* Klebsiella pneumoniae ATCC 700603.
* Acinetobacter baumannii ATCC 19606.
* Candida albicans ATCC 90028.
* Cryptococcus neoformans var. grubii H99 ATCC 20882.

**Fig. 1.** (A) Plot showing the Hα deviations (ppm) from statistical-coil chemical shifts. Amino acids are numbered according to their sequence in Cit 1.1 (red), AMP-003 (green), and AMP-016 (blue). (B) TOCSY Hα region for Cit 1.1 (red), AMP-003 (green), and AMP-016 (blue). Peaks are labeled according to their respective amino acid position.

2.2. NMR spectroscopy

Negatively charged sodium dodecyl sulfate (SDS) micelles, which mimics the charge of Gram-positive bacterial membranes, were therefore chosen as a model for the structural elucidation of Cit 1.1, AMP-003, and AMP-016 peptides by NMR [20]. Standard two-dimensional (2D) TOCSY and NOESY datasets were collected for backbone and side-chain resonance assignments [21]. NMR assignments for Cit 1.1, AMP-003, and AMP-016 were nearly complete with 94% HN, 100% Ha, 100% Hβ, 88% Hγ, 90–100% Hδ, 60–100% He, 0–33% Hε, and 100% Hζ of the assignments made (Table S7). Fig. 1A shows the Hα chemical shift deviations from statistical-coil values for the three peptides bound to SDS micelles [22,23]. The three peptides showed negative chemical shift differences across the entirety of the sequence, which is strongly suggestive of an α-helical conformation along the SDS micelle. Chemical shift differences between the Cit 1.1, AMP-003, and AMP-016 peptides were more apparent when HN chemical shifts were included. This is illustrated by an overlay of the Hα region of the 2D TOCSY
spectra for the three peptides as shown in (Fig. 1B). Major chemical shifts changes were observed for residues 3 to 7, which are consistent with the Hα chemical shift changes plotted in Fig. 1A. In order to reinforce this observation, we performed circular dichroism (CD) studies. It was found the AMPs, presented α-helical character (with some random coils) in aqueous media (Fig. S6). The secondary structure of AMP-016 was also studied after 1 h incubation with E. coli and the data showed the conformation was retained upon interaction with the bacteria membrane (Fig. S7).

The NMR structures for the Cit 1.1, AMP-003, and AMP-016 peptides in SDS micelles were determined to further elucidate the role that structure plays in antimicrobial activity. The structural statistics for the three peptides (Table S8) indicates that the experimental data agrees well with the calculated structures. Backbone RMSDs for each of the peptides was 0.3 Å, 0.7 Å, and 0.4 Å for Cit 1.1, AMP-003, and AMP-016, respectively. The structures did not contain any NOE violations > 0.5 Å or dihedral angle violations > 5°. The high quality of the NMR structures is also evident by the PROCHECK z-scores of -0.3, -0.95, and -0.47 for Cit 1.1, AMP-003, and AMP-016, respectively [24]. Further, 92–94% of all residues are located in the most favored region of the Ramachandran plot. An overlay of the water refined 20 lowest energy NMR structures for the Cit 1.1, AMP-003, and AMP-016 peptides are shown in Fig. 2A–C.

The resulting NMR structures indicate that the Cit 1.1, AMP-003, and AMP-016 peptides adopt a head to tail helical dimer (Fig. 2D–F). The head to tail orientation of the peptides was confirmed by the presence of NOEs from Leu2 of strand A to Ile13/Leu16 of strand B. These long-range structural restraints are only possible for a head to tail dimer. Of particular note, a significant number of NOEs exist at the N-terminus (Fig. 2G, H), which is consistent with the presence of both an α- and 31-α-helix for the biologically active peptides (Cit 1.1 and AMP-016). It is possible that the peptides adopt both conformations (or are in equilibrium) when bound to a SDS micelle. In this case, the NMR structure would simply represent an overall average of the conformational exchange. Importantly, the inactive AMP-003 lacks a similar NOE pattern (and number of NOEs) at the N-terminus and, thus, it exclusively adopts an α-helical structure (Fig. 2I). Since the only difference between Cit 1.1 and AMP-003 is the N-terminal acetylation, this clear difference in helix composition between the peptides is due to the acetylation.

Amide proton temperature titrations up to 70°C were performed on the three peptides to characterize helical stability (Figs. S9–11). The titration data was binned according to the relative magnitude of the HN chemical shift changes (ΔH/ΔN): minor (0 to −0.05, yellow), intermediate (−0.05 to −0.25, orange), and major (−0.25 to −0.45, red). The peptide ribbon diagrams in Fig. 2D–F are colored based on these HN chemical shift changes in order to qualitatively assess the thermal stability of the helices. Amino acid residues exhibiting major chemical shift changes upon heating are transitioning from a stable and structurally-ordered state to a disordered and unstructured state. Conversely, residues with relatively unchanged chemical shifts are likely already in a transiently disordered state at room temperature.

Next the peptides were titrated with gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) and monitored by NMR to identify the solvent exposed residues while the peptide was bound to SDS micelles (Figs. S12–14). The addition of a paramagnetic species will reduce the NMR signal intensity for residues in contact with the solvent proportionally to the amount of the added paramagnetic species. Upon the addition of Gd-DTPA, the majority of the amino acids exhibited an approximate linear decrease in peak intensity regardless of location in

![Fig. 2. The ensemble of the lowest energy, water refined structures of (A) Cit 1.1, (B) AMP-003, and (C) AMP-016. Peptide ribbon structures for (D) Cit 1.1, (E) AMP-003, and (F) AMP-016 are colored by the binned amide proton temperature titration data according to the ΔH ranges 0 to −0.05 (minor chemical shift, yellow), −0.05 to −0.25 (intermediate chemical shift, orange), and −0.25 to −0.45 (major chemical shift, red). NOE connectivity plots for (G) Cit 1.1, (H) AMP-003, and (I) AMP-016. Weights of the connectivities are normalized to the NOE intensities.](image-url)
the dimer structure. This is most likely due to multiple binding sites for Gd-DTPA on each peptide, an aggregation of the peptides along the surfaces of the micelles, and a dynamic association/dissociation of the dimer [25]. Leu2 in Cit 1.1 and AMP-016 was an exception to this general observation. The NMR peak intensity for Leu2 increased, at first, with the addition of Gd-DTPA. Of note, the peak intensity for Leu2 was relatively weak compared to the other residues (Fig. 1B), which is suggestive of a high exchange rate and/or a transiently disordered structure.

2.3. Scanning electron microscopy (SEM)

S. aureus JE2 and E. coli K12 cells were treated for 1 h with Cit 1.1 or AMP-016 at 2X MIC values. As clearly evident from the SEM images, AMP treatment led to membrane disruption accompanied by cytoplasmic leakage, deep holes and severe membrane deformations (Fig. 3). These morphological changes indicate that the AMPs kill bacteria by disrupting cell membranes, although the results do not exclude additional mechanisms of action. Please note the SEM micrographs present some differences between S. aureus and E. coli, which may suggest a different mechanism of action for AMP antimicrobial activity between Gram-positive and Gram-negative organisms. In the case of S. aureus, holes and cytoplasm release were observed. Conversely, membrane solubilization due to a mixed peptide/phospholipid vesicle formation (budding) with subsequent membrane thinning leading to only a small amount of cytoplasm release was observed for E. coli.

2.4. Propidium iodide (PI) uptake and flow cytometry

A PI uptake assay was performed to confirm the ability of AMPs to disrupt bacterial cell membranes. S. aureus JE2 and E. coli K12 cells were treated with Cit 1.1 or AMP-016 at 2X MIC values. The PI fluorescence intensity was measured relative to Polymyxin B (PMB) as a reference. Cit 1.1 and AMP-016 showed high PI uptake relative to controls (Fig. 4), further suggesting that bacterial cell death is associated with membrane damage, since PI can only cross damaged cell membranes. The cell membrane disruption of S. aureus JE2 and E. coli K12 was further evaluated with flow cytometry. Untreated (no AMPs) and treatment with 70% EtOH were used as negative and positive controls, respectively (Experimental S15 and Fig. S16). After 1 h treatment with either Cit 1.1 or AMP-016 at 2X MIC values, S. aureus cells showed an increased uptake, when compared to control, of 9.4% or 12.5%, respectively (Fig. S16). Similarly, E. coli cells treated with either Cit 1.1 or AMP-016 exhibited cell viability of 13.9% or 27.5%, respectively.

2.5. In vitro serum stability

Serum stability was assessed by incubating the peptides in human serum for over two hours followed by quantitation using LC–MS/MS. AMP-002 and -006 displayed the highest serum stability. Approximately 60%–80% of the peptides remained in the serum after 2h. Cit 1.1, -012, -013, and -016 displayed similar stabilities with only 40–50% of the peptide remaining in the serum after 2 h (Fig. 5).

2.6. Cytotoxicity

The cytotoxicity of the active AMPs against human embryonic kidney cells (ATCC CRL-1573) was performed using a resazurin test and the IC50 values were determined from dose response curves. The hemolytic activity was determined against human red blood cells and the HC50 values (concentration at 50% hemolysis) were calculated by curve fitting of the inhibition values versus log(concentration). The IC50 and HC50 values are summarized in Table 3. In general, the AMPs showed...
The data is plotted as mean ± SD from triplicate measurements.
replaced Lys7 with an arginine. The MICs for both AMP-012 and AMP-013 decreased 2-fold against *S. aureus* to 16 μg/mL. The increase in antimicrobial activity may be explained by an enhanced translocation of peptides within the *S. aureus* cells [38,39]. However, the AMP-012 and AMP-013 displayed diminished activity against most of the Gram-negative organisms tested. Interestingly, AMP-012 exhibited antifungal activity against both *C. albicans* and *C. neoformans* (16 μg/mL). To further explore the importance of cationic residues on activity, citrulline a structural mimic of arginine containing a urea functional group instead of the charged guanidium group was constructed (AMP-009). This substitution resulted in a complete lack of antibacterial activity, further establishing the importance of a charged H-bonding donor at position 7 (the citrulline urea group is capable of forming a hydrogen bond but its side chain is uncharged). The citrulline substitution reduces the net charge of AMP-009, which suggests the net charge of the peptide also contributes to the antimicrobial activity of AMPs.

Previous studies have shown that *Cit 1.1* adopts an α-helical conformation, but there has also been some conflicting evidence about the exact details of the *Cit 1.1* structure [15]. Instead of a single α-helical chain, two helices have been suggested for the *Cit 1.1* structure [15]. To address this possibility, a glycine residue was inserted into the *Cit 1.1* sequence following Lys8 to create the AMP-014 analog. The insertion of Gly9 was expected to provide added flexibility to the peptide [40,41] and potentially enhance its activity if the two-helix model was correct. AMP-014 was observed to be inactive against all the bacterial strains tested and suggests the two-helix model for *Cit 1.1* may be incorrect. If the *Cit 1.1* structure is comprised of two α-helices separated by a flexible link, then the inserted Gly may allow enough mobility for the correct surface of the α-helices to interact with the membrane. Nevertheless, it is important to note the Gly insertion lengthens the peptide and shifts the sequence beyond Lys8 affecting the charge distribution at the *Cit 1.1* surface. Thus, the loss in activity may also be explained by a change in the position of the hydrophobic and charged residues.

The AMP-015 analog, which contains a Glu for Asp4 substitution, was also inactive. Others have suggested a potential H-bond between the N-terminus and residue 4 [16]. This change may affect such interaction but no change in activity was observed. AMP-016 was constructed by replacing Phe3 with Trp. Tryptophan-rich peptides have been shown to enhance the activity of AMPs presumable due to a larger hydrophobic area [34,42] and the insertion of Trp residues at the membrane-water interface [41]. The AMP-016 analogue exhibited a 4-fold and 2-fold increase in activity against *S. aureus* and *E. coli*, respectively. In addition, AMP-016 was active against *C. neoformans* (8 μg/mL). Thus, we think the indole ring in tryptophan is likely acting as a better anchor to the bacterial membranes than the smaller phenyl ring [42].

AMP-017, AMP-018 and AMP-21 were designed to maintain the increased antimicrobial activity resulting from the inclusion of Trp at position 3, while investigating the impact of other amino acid substitutions at different positions. AMP-017 (Asp4 and Lys7 were replaced by Arg) showed an increase in activity against *E. coli, K. pneumoniae, A. baumannii* and *C. neoformans* when compared with Cit 1.1 or AMP-016. However, replacing Gly1 with PhenylGly and Lys7 with Arg (AMP-018) dramatically decreased the activity of AMP-017 against bacteria (still, this compound was better than *Cit 1.1*). AMP-021,

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**Fig. 6. Summary of the Cit 1.1 SAR.**
where Gly1 was replaced by Sar and Lys7 by Arg, showed a slight decrease in activity against Gram-negative strains that AMP-017. However, the molecule had better activity than Cit 1.1 or AMP-018. This result indicates the key role of Trp3 or Arg4 on activity.

We also replaced Phe3 with other aromatic amino acids; biphenylalanine or naphthylalanine (AMP-019 and AMP-020). These replacements maintained the activity observed with AMP-016 against S. aureus, while reducing activity against Gram-negative bacteria and fungi. The analogue was slightly more potent than Cit 1.1. These finding may also be correlated to the increasing hydrophobicity (LogP -1.85, -2.61, and -3.60 for AMP-019, AMP-020 and Cit 1.1, respectively) which may facilitate the peptide insertion into the bacteria membrane. Fig. 6 summarizes the structure-activity relationship for the series of AMP analogs.

3.2. NMR spectroscopy

Cit 1.1, AMP-003, and -016 were analyzed by NMR to elucidate the relationship between structure and antimicrobial activity. Interestingly, Fig. 1A indicates that the largest Hα chemical shift difference between the three peptides occurs at the N-terminus between residues Asp3 and Lys7. Although the dissimilarity between AMP-016 and Cit 1.1/AMP-003 can be attributed to the tryptophan substitution at position 3, the minor deviation of Asp4 is likely a result of the acetylation of the N-terminus. In the case of AMP-016, the steric and electronic effects from the indole side chain at position 3 may lead to a significant decrease in helicity. N-terminal acetylation does not induce any significant effect on the overall helicity of AMP-003 except for a slight decrease at Asp4. The removal of the charged N-terminus changes the polarity or the hydrogen-bond network around Asp4, which contributes to a decrease in helicity. Fig. 1B shows the overlay of the Hα region of the 2D TOCSY for the three peptides. The significant chemical shift differences of the N-terminal residues highlight a changing local environment for residues 3 to 7 that is likely a result of minor alterations in the structure of the three peptides. Accordingly, these subtle changes in the peptide’s structure may be directly related to differences in antimicrobial activity.

Our NMR derived structures for Cit 1.1, AMP-003, and -016 differ from the previous results of Sikorska et al. that describe the presence of two helices within Cit 1.1 structure [15]. Experimentally determined NOEs were found along the full length of each of the peptides (Fig. 2G–I) and between the helices in a dimer orientation. Although the Cit 1.1, AMP-003, and AMP-016 dimers are not completely superimposable, there are no major structural differences between the three peptide structures that appear to explain the difference in activity. In all cases, the dimers pack together along a hydrophobic face, which then exposes charged residues to the solvent. Consequently, the peptides’ NMR structures are consistent with the prior observation that the number and distribution of hydrophobic residues is crucial for antimicrobial activity [43]. Increasing peptide hydrophobicity increases antimicrobial activity, but only if the overall hydrophobicity remains below an optimal upper threshold. Peptide aggregation likely occurs if too many hydrophobic residues are inserted into the peptide sequence, which leads to a loss in activity. Therefore, the Cit 1.1 peptide must maintain a hydrophobic surface to allow for a stable dimer in order to carpet a bacterial cell membrane, but must also avoid the formation of inactive aggregates. The tryptophan substitution in AMP-016 and the corresponding increase in antimicrobial activity supports the above hypothesis. The hydrophobic and electrostatic properties of tryptophan [44] allows AMP-016 to maintain a similar overall hydrophobicity to Cit 1.1 while also promoting peptide aggregation at the membrane surface, and a positive electrostatic interaction with the negatively-charged membrane. This, however, does not explain the differences in activity between the active (Cit 1.1/AMP-016) and inactive (AMP-003) peptides since all of the peptides form a similar head-to-tail dimer. Instead, the primary difference between the active and inactive peptide dimers is the N-terminal acetylation.

It has been previously observed that N-terminal acetylation increases the propensity of α-helix formation in peptides regardless of the amino acid sequence [45]. N-terminal acetylation also increases the overall stability of an α-helix. In the case of Cit 1.1, the apparent increase in helical stability due to acetylation resulted in a loss of activity. Consequently, antimicrobial activity may be correlated with a certain amount of structural disorder or mobility within the peptide. Of course, this result can also be linked to the loss of basicity as explained earlier. The NMR also leads to the theory that antimicrobial activity may depend on an equilibrium between a disordered monomer in solution and helical dimers coating the membrane surface. In the presence of bacteria, the equilibrium shifts from disordered monomer to the helical dimer. The dimer is then able to associate with other dimers on the membrane surface and promote antimicrobial activity through a proposed carpet mechanism. However, if the peptide is stabilized in solution then the equilibrium is shifted away from interacting with the bacterial membrane and the antimicrobial activity is abolished. We hypothesize that Cit 1.1 exists as a disordered monomer in solution and dimerizes and aggregates along the anionic surface of the bacterial membrane. In the inactive form, the N-terminal acetylation stabilizes the transiently formed α-helix in solution. Consequently, the aggregation on the membrane surface, which promotes antimicrobial activity, is greatly diminished. Again, the observation that the inactive AMP-003 dimer is exclusively α-helical and the Cit 1.1/AMP-016 dimers are a mixture of α- and 3_10-helix is supportive of the proposal that helix stability affects antimicrobial activity. To further investigate the role of α-helical stability in regard to antimicrobial activity, NMR was used to measure the thermal stability and solvent accessibility of the Cit 1.1, AMP-003, and AMP-016 peptides.

The temperature-dependent chemical shift changes clearly define the dimer interface as the residues with the largest Δampaign values (Figs. 2D–F, S1–S3). The residues in the dimer interface correspond to: Ile6, Val9, Ala10, Ile13, and Gly14. Importantly, the inactive AMP-003 peptide exhibited the smallest Hα chemical shift changes for these residues. This suggests the inactive AMP-003 is relatively less stable as a dimer in the SDS micelle compared to the active peptides. Interestingly, there are additional AMP-003 residues outside of the dimer interface that also exhibit a temperature-dependent chemical shift change. This was not observed for the Cit 1.1 or AMP-016 peptides. One possible explanation for this difference is that the AMP-003 peptide still maintains an α-helical structure as a monomer, which undergoes further temperature-dependent denaturation. This does not appear to occur for Cit 1.1 or AMP-016. In fact, more residues in the Cit 1.1 peptide compared to AMP-003 are disordered based on the lack of a temperature-dependent chemical shift change. These results suggest that a certain amount of structural disorder or dynamics is necessary for antimicrobial activity.

The presence of a helical dimer is also suggested by the Gd-DTPA titration experiments. At low concentrations of Gd-DTPA, the amide peak intensity for Leu2 was found to increase, which indicates a reduction in the chemical exchange with the solvent. It is plausible that the coordination of Gd-DTPA by the active peptides stabilizes the dimer structure and reduces the amide chemical exchange of Leu2 causing an initial sharpening of the peak. Only at higher Gd-DTPA concentrations does the Leu2 NMR resonance broaden like the other amino acid residues. This effect is not seen in the inactive peptide (AMP-003), suggestive of a more stable α-helix along the entirety of the peptide monomer (Fig. S4–6).

3.3. Mechanism of action in bacteria membrane

Bacterial membrane disruption can be described by the worm-hole, the barrel-stave, or the carpet models [46,47]. Previously reported data on the Cit 1.1 interaction with negatively charged lipid vesicles hinted towards involvement of the carpet mechanism [12]. In the carpet-
model of membrane disruption, the AMPs bind parallel to the bacterial membrane covering its entire surface. This causes cell membrane permeation and eventually release of small vesicles from the membrane [46,47]. A separate study involving giant unilamellar vesicles (GUVs) further supported the carpet mechanism proposed earlier [40]. Conversely, an atomic force microscopy study by Henderson and colleagues indicates that Cit 1.1 can promote the formation of worm-like micelles, which could be an outcome of many toroidal pore formations [48]. However, those prior studies relied on negatively charged phospholipid model membranes, which are structurally simple compared to bacterial membranes. These model membranes do not accurately represent the complexity of single or double-membraned Gram-negative organisms. Furthermore, those studies relied on indirect fluorometric observations instead of direct microscopic imaging [12,46]. Treatment of *S. aureus* and *E. coli* with AMP-016 lead to membrane disruption accompanied by the formation of protruding blebs and holes as visualized by SEM (Figs. 3) and transmission electron microscopy (not shown). The carpet mechanism of membrane disruption is commonly associated with detergent-like antibacterials displaying indiscriminate cell lytic properties and AMPs acting by this mechanism may possess some basic structural requirements [8]. Herein, we have shown that AMPs that disrupt membranes in a detergent like fashion are sensitive to subtle structural modifications. Additionally, the membrane damage in MRSA JE2 and *E. coli* K12 was also confirmed by a PI uptake assay and by flow cytometry experiments, showing an increase in the fluorescent intensity of PI after treatment with AMP-016.

3.4. In vitro serum stability studies

AMPS have found limited use due to their susceptibility to metabolic enzymes in serum and the liver. Accordingly, some of the Cit 1.1 analogues were tested for serum stability. AMP-002 was the most stable peptide of the tested AMPs with about 80% of the peptide remaining after 2 h. of incubation. This was expected because peptides can be degraded starting from the N-terminus by exopeptidases [49]. Thus, an alkylated N-terminus may be a successful strategy for enhancing AMP stability [30], which may explain the two-fold increase in serum half-life for AMP-002 compared to Cit 1.1. AMP-006 was also more stable in serum compared to Cit 1.1, with approximately 60% of the peptide remaining after a 2-hr. incubation in serum. AMP-006 had Lys7 and Lys8 substituted by Orn residues. The presence of this unnatural amino acid may prevent recognition by LysC and LysN endopeptidases [50]. N-terminus modification and substitution with unnatural amino acids may enhance AMP plasma stability and bioavailability [51], but retaining bioactivity is still paramount. The data suggests alkylation of the N-terminus is an interesting strategy to enhance the stability of Cit 1.1 analogues.

3.5. Cytotoxicity

For therapeutic use, AMPs should display good selectivity for bacterial cells over mammalian cells. The active peptides, AMP-016 and AMP-017 showed both IC50 and HC50 values higher than their MICs and displayed minimal hemolytic activity against human red blood cells. We theorized that AMPs should possess a greater affinity for negatively charged bacterial membranes compared to zwitterionic mammalian cell membranes. In fact, prior studies have suggested that AMPs exhibit a higher affinity to bacteria compared to mammalian cells because of their distinct membrane characteristics [7,47,52]. For example, the transmembrane potential is more negative in bacteria cells, which suggest an increase affinity or sensitivity of bacteria cells for cationic antimicrobial peptides [7].

4. Conclusion

We have shown that AMP-membrane interactions impact antimicrobial activity. Rationally designed Cit 1.1 analogues were used to develop SAR and to elucidate previously unknown interactions responsible for membrane disruption and antibacterial activity. Our insights regarding Cit 1.1 structural features essential for activity are summarized in Fig. 6. Hydrogen bond donors with a basic pKa at the N-terminus were found to be indispensable for antibacterial activity. Ablation of either the hydrogen bond donors or the positive charge resulted in inactivity; suggesting a complementary effect between hydrogen-bonding and electrostatic interactions in disrupting membranes. Also, sufficient projections of the side chain amine groups were shown to be essential for membrane disruption. AMP-016 and AMP-017 are highly potent Cit 1.1 analogues with a 2- to 8-fold increase in antimicrobial activity against MRSA. Both analogues have a Trp residue that replaces the native Phe. Thus, the improved activity may be due to an increase in the bulk of the hydrophobic anchor and an improved electrostatic interaction with the negatively-charged membrane. The W3F modification also made Cit 1.1 analogues active against fungi. NMR studies revealed the propensity of AMPs to form head-to-tail helical-dimers on the surface of anionic membranes and bacterial surfaces. NMR spectroscopy exposed a positive correlation between AMP helicity and antibacterial activity, where N-terminal acetylation increased the α-helical structure of the peptides in the absence of a membrane, and, correspondingly, lead to a decrease in antibacterial activity. These experiments provide further support for the carpet model of membrane disruption, which was subsequently confirmed by electron microscopy in both Gram-positive and Gram-negative bacteria. The results presented herein provide the critical foundation for further evolving Cit 1.1 analogues into novel broad-spectrum antibiotics for the treatment of drug-resistant bacterial infections.

5. Experimental section

5.1. Peptide synthesis and purification

AMPS were synthesized using solid phase peptide synthesis (SPPS) on a Focus XC automated peptide synthesizer (AAPPTec, Louisville, KY) on a 0.25 mmol scale. All peptides were synthesized using Fmoc-Rink Amide AM resin support (AAPPTec, Louisville, KY). The resin was wetted in dichloromethane (DCM) for 30 min. to promote swelling. The Fmoc-group was then deprotected twice using 20% 4-methyl piperidine in dimethyl formamide (DMF) (10 mL). The resin was thoroughly washed twice for 10 min. using a 15 mL 1:1 mixture of DCM and DMF. The desired amino acids were then added to obtain a predetermined sequence in the peptide synthesizer. Molecular weight of the AMPs was confirmed by MALDI using a 4800 MALDI TOF/TOF analyzer (SCIEX, Ontario, Canada). The AMPs were purified using a preparative high-performance liquid chromatography (HPLC) using a water (0.1% trifluoroacetic acid (TFA)) and acetonitrile (0.1% TFA) gradient on an HPLC system (Agilent, Santa Clara, CA) with a reverse phase column (Kinetix5 u XB-C18 100 A, 150 × 30.0 mm column). Each AMP was confirmed to be > 95% pure. The relative hydrophobicity of the AMPs was determined by simultaneously measuring retention times with the analytical HPLC system described above.

Special peptide modifications were done on resin and described as follows:

- **AMP-002** was synthesized by coupling a Sar instead of glycine following the procedure described above.
- **AMP-003** was acetylated by shaking the complete peptide loaded resin in 1 mL acetic anhydride dissolved in 5 mL (DCM:DMF = 1:1) for 6 h at ambient temperature.
- **AMP-004** was synthesized by methylating the Fmoc-deprotected N-terminus by adding 4 mL methyl iodide and 200 µL triethylamine to the resin in 10 mL DCM and then gently heating at 45 °C for 5 days.
- **AMP-005** was synthesized by substituting the glycine residue with bromoacetic acid using the same coupling procedure as described above. The bromine was then displaced with 10 mL hydrazine hydrate.
by reacting it on the resin for 24 h.

AMP-010 was synthesized by first making GLFDVIK(Alloc)K (Alloc)VASVIGGL on the resin. The N-terminus Fmoc was deprotected following the procedure described above. The side chain Alloc was deprotected on resin by treatment with 0.35 eq of Pd(PPh₃)₄ and 20 eq of Phenyl silane in DCM for 45 min. Methylation of the free primary amines was achieved by adding 4 mL methyl iodide and 200 μL triethylamine to the resin in 10 mL DCM and then gently heating at 45 °C for 5 days.

AMP-011 was synthesized by first making Fmoc-GLFDVIK(Alloc)K (Alloc)VASVIGGL on the resin. The side chain Alloc was deprotected on the resin by treatment with 0.35 eq Pd(PPh₃)₄ and 20 eq Phenyl silane in 10 mL DCM for 45 min. Methylation of the free primary amines were achieved by adding 4 mL methyl iodide and 200 μL triethylamine to the resin in 10 mL DCM and then gently heating at 45 °C for 5 days. Finally, the Fmoc- group was deprotected following the procedure described above.

5.2. Minimum inhibitory concentration assay (MIC)

MICs for Cit 1.1 and the Cit 1.1 analogues were measured using the following bacterial strains: S. aureus JE2 and E. coli K12. The MICs was determined using the broth microdilution method as previously described [52]. A stock solution of each peptide was prepared in milliQ water and then serial 2-fold dilutions were made in Difco Muller Hinton Broth (MHB) (BD Diagnostics, Becton Drive, NJ) in Cellstar 96-well microtiter plates (Greiner Bio-One, Kremsmünster, Austria). Bacterial cultures were prepared using the direct colony suspension method to 1.5 × 10⁸ colony forming unit (CFU)/mL (0.5 McFarland) and dilution to 10⁷ cfu/ml using PBS buffer at pH 7 (uncorrected). NMR experiments were collected at 25 °C on a 700 MHz Avance III spectrometer (Bruker, Billerica, MA) equipped with a 5 mm QCI-P probe with cryogenically cooled carbon and proton channels. 1H chemical shift and NOE assignments were accomplished with TOCSY, DQF-COSY, and NOESY experiments with chemical shifts referenced to TMS. NMR spectra were collected with 32 transients and 512 points in the indirect dimension. NOESY spectra were collected with mixing times of 100 ms and 200 ms.

5.3. NMR structure determination

Unlabeled Cit 1.1 and the Cit 1.1 analogues samples were shifted similarly to previous studies [15]. Briefly, 2 mM of Cit 1.1, AMP-003, or AMP-016 were dissolved in 90% H₂O and 10% D₂O with 120 mM of SDS-d₂₅ (Cambridge Isotope, Ewksbury, MA), 50 mM trimethylsilylpropanoic acid (TMSp), and PBS buffer at pH 7 (uncorrected). NMR experiments were collected at 25 °C on a 700 MHz Avance III spectrometer (Bruker, Billerica, MA) equipped with a 5 mm QCI-P probe with cryogenically cooled carbon and proton channels. 1H chemical shift and NOE assignments were accomplished with TOCSY, DQF-COSY, and NOESY experiments with chemical shifts referenced to TMS. NMR spectra were collected with 32 transients and 512 points in the indirect dimension. NOESY spectra were collected with mixing times of 100 ms and 200 ms. Inter-strand NOEs were identified using a 3D X-filtered experiment with a mixing time of 120 ms and a 50/50 mixture of unlabeled peptide and a peptide uniformly ¹³C,²⁵N labeled at Leu2 [53]. Initial models of the AMPs were generated from Hx chemical shifts using the CS-Rosetta webserver at the BMRB [54]. Structural refinement was carried out with XPLOR-NIH with dimer symmetry enforced [55]. The three AMP dimer structures were determined with 536 to 622 intramolecular NOE restraints, and 16 intermolecular (peptide-peptide) NOE restraints. A total of 400 models were calculated per AMP and the lowest 20 energy structures were subsequently subjected to water refinement in XPLOR-NIH. Validation of the AMP ensembles was done using the Protein Structure Validation Software (PSVS, http://psvs-1.5-dev.nesg.org/) suite from the Northeast Structural Genomics Consortium (NESG) [56].

5.4. One-dimensional NMR temperature titration

The unlabeled NMR Cit 1.1, AMP-003, or AMP-016 samples used for structure determination were also subjected to a temperature titration to monitor structure stability. 1D ¹H NMR spectra were recorded at temperatures of 25, 30, 40, 50, 60, and 70 °C with 64 transients, 131k data points, and a spectral width of 12.87 ppm. Between each experiment, the samples were allowed to rest at the target temperature for 5 min before data collection. Following the titration, the 25 °C spectrum was recollected to ensure that the sample did not undergo irreversible degradation upon heating.

5.5. Paramagnetic relaxation enhancement titration

The unlabeled NMR Cit 1.1, AMP-003, or AMP-016 samples used for structure determination were also used to observe the effects of a dihydroxynitraminepentaacetic acid gadolinium (III) dihydrogen salt hydrate (Gd-DTPA) titration. Gd-DTPA was titrated into the samples to concentrations of 0, 2, 4, 8, 16, and 24 mM. 1D ¹H NMR spectra were recorded with 128 transients, 64k data points, and a spectral width of 12.87 ppm. Effects resulting from sample dilution were accounted for by normalizing peak intensities to TMS.

5.6. Scanning electron microscopy (SEM)

E. coli K12 and S. aureus JE2 were cultured in MHB and incubated at 37 °C. The resultant mid-log phase cultures were diluted to a final concentration of 1.5 × 10⁶ CFU/ml (0.5 McFarland). The bacteria cells were treated with Cit 1.1 or a Cit 1.1 analogue at 2X the MIC value and incubated for 1 h at 37 °C. A control was prepared by adding only media. After AMP treatment, the cells were immediately washed thrice with HyClone™ Dulbecco’s PBS solution (GE Healthcare Life Science, Marlborough, MA) and fixed with 2.0% (v/v) glutaraldehyde [57]. The bacterial suspension was then placed on 0.1% Poly-L-Lysine coated glass slide and allowed to adhere for 30 min. The slides were washed thrice with PBS to remove excess fixative. Samples were post-fixed in a 1% solution of OsO₄ for 30 min. To facilitate conductivity. The samples were then dehydrated in a graded ethanol series (50, 70, 90, 95, and 100%). Ethanol was removed by washing the slides twice with hexamethyldisilazane (HMDS). HMDS was allowed to evaporate overnight to dryness. Glass slides were attached to aluminum SEM stubs with double-sided carbon tape. Silver paste was applied to enhance conductivity, which was allowed to dry overnight. Samples were then coated with approximately 50 nm gold-palladium alloy in a Hummer VI Sputter Coater (Anatech, Battle Creek, MI) and imaged at 30 kV in a Quanta 200 SEM (FEI, Hillsboro, OR) operating in high vacuum mode.

5.7. Propidium iodide (PI) uptake

The PI uptake assay was performed according to Shireen et al [58]. S. aureus JE2 and E. coli K12 cells were grown in BHI broth to the mid logarithmic-phase and the bacterial cells were harvested by centrifugation at 5000 rpm for 5 min and washed once with PBS buffer. Cells were diluted to 10⁷ cfu/ml using PBS buffer. AMPs were added to each well at twice the MIC. Similarly, 10 μg/mL of PI was also added to each well. Polymyxin B was used as a reference drug and cells with no AMP treatment were used as a control. PI fluorescence was measured at 37 °C and at 20 min intervals for a total duration of 120 min. The PI fluorescence was measured using a spectrofluorometer with excitation and emission wavelengths set at 544 nm and 620 nm, respectively.
5.8. In vitro human serum stability studies

Cit 1.1 and Cit 1.1 analogues were incubated in human serum for 2 h in a shaking water bath (90 rpm) at 37 °C. Peptide content in the reaction mixture was below 0.5% (v/v). At select time intervals of 0, 30, 45, 60 and 120 min, 100 µL of the serum-AMP mixture was extracted and quenched with 400 µL of methanol containing oxibendazole as an internal standard. Samples were mixed and centrifuged at 17,950 × g for 15 min. at 4 °C. 100 µL of the supernatant was removed for analysis. Stability results were expressed as a percentage of AMP remaining in the serum as a function of time. AMP stability was represented as the percentage of the AMP remaining relative to the first time-point (0 min; 100% AMP). All experiments were performed in triplicate.

A LabSolutions LCMS Ver.5.6 controlled UPLC and MS system consisting of a Shimadzu LC-MS/MS 8060 equipped with a DUIS source, two pumps (LC-30 AD), a column oven (CTO-30AS) and an auto-sampler (SIL-30AC) were used to quantify AMPS in human serum. The MS/MS system was operated at unit resolution in the auto-sampler (SIL-30AC) were used to quantitate AMPs in human serum. Two pumps (LC-30 AD), a column oven (CTO-30AS) and an auto-sampler (SIL-30AC) were used to quantify AMPS in human serum.

A search was performed in facilities renovated with support from the Nebraska Research Initiative (NRI), the University of Nebraska Foundation, and the UNMC-Office of the Vice Chancellor for Research. FCRF is also supported by The Fred and Pamela Buffett Cancer Center’s through the NCI.

Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version, at doi:https://doi.org/10.1016/j.peptides.2019.170119.

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