Antibiotic-resistant bacteria are a serious and growing threat to human health and national healthcare systems. These "superbugs" kill hundreds of thousands of people each year and are estimated to add $>20 billion in healthcare costs in the United States alone. Multidrug resistant Gram-negative (MDR G–ve) strains of Klebsiella pneumoniae, Escherichia coli, Acinetobacter baumannii, and Pseudomonas aeruginosa that possess extended spectrum β-lactamase (ESBL) and metallo-β-lactamases (MBL) are of grave concern. There are now extensive and widely dispersed pools of resistance elements that cover all of the classical pathways targeted by current antibiotics: cell wall, folic acid, protein, RNA, and DNA synthesis. Most antibiotics target specific receptors, enzymes, or proteins that are susceptible to single-point mutations that can lead to resistance. However, compounds that target the bacterial membrane (such as antimicrobial peptides, AMPs) or its biosynthesis (such as monomycin and the recently reported teixobactin), are much less likely to select for spontaneous, single-step resistant mutants. Today the only membrane-targeting antibiotics suitable for MDR G–ve infections are the closely related lipopeptides colistin (polymyxin E) and polymyxin B. Unfortunately, resistance to these key "last resort" antibiotics has appeared, with a recent study demonstrating a plasmid-mediated mcr-1 gene conferring colistin resistance found in China and Europe. Colistin and polymyxin B are both highly nephrotoxic with a low therapeutic index, often imparting severe adverse effects in humans at doses required for efficacy. Therefore, there is an urgent unmet medical need for safer antibiotics to treat highly drug-resistant G–ve infections, and this can potentially be achieved by developing drugs that target G–ve bacterial membranes with high selectivity over mammalian membranes.

AMPs are ubiquitous in nature. All multicellular organisms, microorganisms, plants, and animals have an innate immune defense system that secretes AMPs. Endogenous AMPs are produced when infections occur and can also be stored in exposed tissues of animals and plants, constituting a first line of defense against pathogens. However, AMPs are highly nephrotoxic in nature, and their indiscriminate use could lead to the development of resistance in pathogens. Therefore, there is a need for safer, less toxic AMPs that can target specific pathogens with minimal harm to the host.

In this study, we aimed to dissect and understand the correlation between antimicrobial activity and toxicity, membrane binding, and membrane permeability. The hydrophobicity, pI, net charge at physiological pH, and amphipathic moment for the β-hairpin antimicrobial peptides tachyplesin-1, polyphemusin-1, protegrin-1, gomisin, arenicin-3, and thanatin were determined and correlated with key antimicrobial activity and toxicity data. These included antimicrobial activity against five key bacterial pathogens and two fungi, cytotoxicity against human cell lines, and hemolytic activity in human erythrocytes. Observed antimicrobial activity trends correlated with compound amphipathicity and, to a lesser extent, with overall hydrophobicity. Antimicrobial activity increased with amphipathicity, but unfortunately so did toxicity. Of note, tachyplesin-1 was found to be 8-fold more amphipathic than gomisin. These analyses identify tachyplesin-1 as a promising scaffold for rational design and synthetic optimization toward an antibiotic candidate.
defense that is fast and efficient. These peptides have broad-spectrum antimicrobial activity and are also able to host repair and adaptive immune responses in a concerted response to multidrug-resistant bacteria.7 AMPs are generally small (<10 kDa), characterized by their charge and structural rigidity. They are grouped according to their secondary structure (α-helical, β-sheet, or extended peptides), 8,9 which confer distinct physicochemical properties and biological mechanisms of action. About 2000 AMPs have been reported (http://aps.unmc.edu/AP/main.php) from eukaryota having antibacterial activity. Approximately 90% of them have been determined to adopt α-helical structure. This category has been extensively explored and reviewed elsewhere.10

Less known are the antimicrobial peptides that adopt a β-sheet structure; these include β-hairpin and cyclic α, β, and θ-defensins.11 This paper focuses on the β-hairpin AMPs, which have undergone few studies and limited characterization. A recent compilation of β-hairpin compounds was reported by Panteleev in 2015.12 Here we focus on the physicochemical properties of this AMP class, and their contribution to the AMP antimicrobial activity and toxicity. β-Hairpin AMPs are usually small (<30 amino acid residues) and cationic and adopt amphipathic structures that confer excellent binding to the lipid bilayer of bacterial membranes. Fernandez-Vidal et al.13 demonstrated that amphipathicity is more important than hydrophobicity for binding to microbial membranes. Most of the β-sheet AMPs cause membrane disruption due to electrostatic interactions with the cell membrane.7,14,15 However, other modes of action have been suggested.16,17 Unfortunately, most AMPs are not directly suitable as antibiotic leads for clinical development as they often cause hemolysis and/or mammalian cell cytotoxicity. Modification is required to generate therapeutically valuable molecules.18 For example, a synthetic cyclic peptide derived from the β-hairpin AMP protegrin-1 has been developed by Polyphor Ltd. (Basel, Switzerland),19 demonstrating the potential for this class of compounds. POL7080 (RG7929) completed a phase 2 trial for non-cystic fibrosis bronchiectasis in November 2015 (clinical trial identifier NCT02096315) and is currently undergoing phase 2 testing in patients with P. aeruginosa ventilator-acquired pneumonia (VAP) co-administered with standard of care (NCT02096328). POL7080 is proposed to act against G−ve bacteria by targeting the β-barrel protein LptD (Imp/OstA), which is involved in the outer-membrane biogenesis of lipopolysaccharide (LPS).19 Another β-hairpin AMP is being developed by Adenium Biotech (Denmark), which has been working with variants of arenicin-3, leading to one analogue (NZ17074) undergoing preclinical studies.20,21

### RESULTS AND DISCUSSION

#### Structure Analysis

Some β-hairpin AMPs are intrinsically disordered and require contact with their lipophilic membrane target to adopt an ordered conformation. Others adopt a preferred structural organization, but may change conformation between being in solution and in contact with the surface of a cell membrane. AMPs, often cationic at physiological pH, form amphipathic structures that mirror that of the phospholipids, allowing the AMPs to interact with bacterial lipid membranes.7 Previous studies on β-hairpin AMPs have been conducted using different strains of bacteria and assay methods, which does not allow for robust comparison of antimicrobial activity and toxicity. We have now examined representatives of the β-hairpin class under a standard set of conditions to directly

| Compound     | Origin                          | Type of turn | PDB ID | Cartoon representation |
|--------------|---------------------------------|--------------|--------|------------------------|
| arenicin-3   | coelomocytes of marine polychaeta, lugworm Arenicola marina | I’ β-turn | - | ![Cartoon](cartoon.png) |
| tachyplesin-1| hemocyte debris of Tachyples tridentatus | II four-residue β-turn | IWO0 | ![Cartoon](cartoon.png) |
| polyphemusin-1| hemocyte debris of Limulus Polyphemus | I’ four-residue β-turn | 1RRK | ![Cartoon](cartoon.png) |
| gomesin      | hemocytes of the spider Acanthoscurria gomesiana | noncanonical four-residue β-turn | 1KFP | ![Cartoon](cartoon.png) |
| protegrin-1  | porcine neutrophils             | distorted four-residue β-turn | 1PG1 | ![Cartoon](cartoon.png) |
| thanatin     | spined soldier bug, Podissus maculiventris | standard β-turn | 8TFV | ![Cartoon](cartoon.png) |

*The cartoon representation was generated using PyMOL (Schrödinger). Disulfide bridges are shown as sticks.*

Table 1. β-Hairpin AMP Origin, Turn Type, PDB ID, and Cartoon Representation*
correlate the therapeutic parameters of the peptides in relation to their origin, length, number of disulfide bridges, and turn type.

The β-hairpins originate from diverse sources, including both invertebrates and mammals. They adopt the common characteristic of antiparallel β-sheets linked by a small turn of three to seven amino acids, forming a hairpin shape that is maintained by interstrand disulfide bridges in both aqueous and lipid-mimicking environments.22–26 Table 1 summarizes the six β-hairpin AMPs chosen for our analysis. The peptides vary from 17 to 21 residues in length, with most containing two disulfide bonds that adopt a common linkage pattern, Cys1–Cys4 and Cys2–Cys3. The exception is thanatin, which has only one disulfide bridge. NMR solution structures indicate that the length of the β-sheet differs between β-hairpins, with the arenicin-3 β-sheet pair rigidifying almost the whole structure, but a less dominant β-sheet conformation in polyphemusin-1 results in greater flexibility.

The amino acid sequence alignment, presented in Table 2, demonstrates the sequence homology and importance of particular amino acids shared between β-hairpin AMPs. Two areas are defined as conserved: (i) tyrosine placed adjacent to a disulfide bond and either included in the turn (tachyplesin-1, polyphemusin-1, and gomesin) or forming part of a β-sheet (arenicin-3, protegrin-1, and thanatin) and (ii) the conserved C-terminal cysteine forming the external disulfide bridges of all six compounds. The arrangement of the cationic Arg and Lys residues included in the turn and the distal C- and N-termini regions, flanked by hydrophobic, membrane-insertive Val, Leu, Ile, Tyr, or Trp residues, results in an amphipathic structure with one hydrophobic and one basic face, which creates a hydrophobic moment in the structure and allows the molecules to disrupt the bacterial lipid membrane.

The high representation of Tyr and Arg, as displayed by an amino acid frequency graph of all β-hairpin peptide sequences (Supporting Information Figure S1), consistently confers the elements of hydrophobicity and overall positive charge, respectively, to this class of antibiotics.

**Physicochemical Analysis.** Many AMPs, including all of the β-hairpin class, act on the membrane of microorganisms by utilizing physicochemical peptide–lipid interactions to bind to, and disrupt, the lipid bilayer of the bacterial membrane. The most important physicochemical property widely reported is amphipathicity, which allows the peptides to attack the membrane by interacting with the hydrophobic–hydrophilic character of the lipids. The physicochemical properties of the β-hairpin AMPs are reported in Table 3. The peptide isoelectric point (pI) was calculated using the PepCalc.com online tool. Hydrophobicity was calculated on the basis of the normalized consensus scale of Eisenberg.33

**Table 2. β-Hairpin AMPs Sequence Alignment**

| peptide     | sequence | sequence | sequence |
|-------------|----------|----------|----------|
| arenicin-3  | GFCWYVC  | RNRGVR   | RYRRCN   |
| tachyplesin-1| KWCFRVC  | YRGIC    | -CYRRCR  |
| polyphemusin-1| RRWCFC   | YRGFC    | -CYRKCRI |
| gomesin     | ZCRRLC   | YKQRR    | -CVTGYCR |
| protegrin-1 | RGRLC    | YCRRR    | -RFCVC   |
| thanatin    | GSKKPVYP  | IIYCNRRTG | -KRCQRM  |

*α = C-terminal amidation and Z = N-terminal pyroglutamic acid. The gray sections are the conserved region. The cystines are marked in bold. The cationic residues are marked in red and the hydrophobic in green. The boxes indicate the turn region.

**Table 3. Physicochemical Properties of β-Hairpin AMPs**

| peptide     | pI  | net charge | hydrophobicity | amphipathic moment |
|-------------|-----|------------|----------------|--------------------|
| arenicin-3  | 11.17 | +4        | −0.11          | 0.44               |
| tachyplesin-1| 12.58 | +6        | −0.43          | −0.11              |
| polyphemusin-1| 12.41 | +7        | −0.56          | 0.48               |
| gomesin     | 12.58 | +6        | −0.61          | 0.83               |
| protegrin-1 | 13.10 | +6        | −0.42          | 0.36               |
| thanatin    | 12.71 | +6        | −0.34          | −0.69              |

*See Figure 1.

The pI is directly correlated with solubility, and proteins are soluble, avoiding aggregation, at greater than 1 or 2 pH units on either side of the pI. Because AMPs need to access lipid membranes from an aqueous phase, they need to be soluble in both environments. If AMPs aggregate in solution, they will lose their ability to interact with the cell membrane. All of the molecules presented in Table 3 have a calculated pI between 11 and 13, which avoids self-association driven by a loss of side-chain charges at physiological pH.

Being highly cationic is a key feature of β-hairpin AMPs with net charge varying only slightly, ranging from +4 to +7. It has been demonstrated that the AMP net charge has a direct correlation to their attraction/interaction to negatively charged bacterial membranes, with a lower net charge reducing attraction.34,35

The hydrophobicity is relatively similar for all six β-hairpin AMPs with an average value of −0.41. Gomesin has the lowest hydrophobicity at −0.61. Hydrophobic residues facilitate interactions with the fatty acyl chains. The relatively low hydrophobicity prevents binding to the zwitterionic membranes found in mammalian cells, resulting in low toxicity.36,37 Hydrophobicity is also required for bacterial membrane permeabilization, but some studies have demonstrated that above an optimum level or threshold (as determined by Glukhov et al.38) of hydrophobicity, further increase leads to a loss in antimicrobial activity and increase in toxicity.39

Figure 1 summarizes the lipophilicity, or amphipathic moment, of the β-hairpin AMPs based on analysis of the NMR solution structures, taking the direction of the Cα-atom of each residue (the rest of the side chain being too flexible for accurate calculation). The amphipathic moment (Table 3) was calculated using our own model, specific for β-hairpin compounds. Our model considers the most probable orientation of the protein–membrane complex, where the AMPs make contact with the membrane with a perpendicular interaction between the surface of the bacterial or mammalian membrane and the β-sheets, taking into account the twisting of the molecule. This approach agrees with the reported findings of Matsuzaki et al.40 that is, that the mean axis of the β-sheet of
tachyplesin-1 is parallel to the surface of the lipid film when binding to the bacteria membrane. For each molecule the amphipathic moment was calculated as the difference between the average value of hydrophobic phase residues and the average value of polar phase residues using the Eisenberg_M scale. The resulting amphipathic moment allowed the classification of the selected hairpin peptides into three groups. The first group constitutes the more amphipathic compounds, which have an amphipathic moment $<$abs$0.4$], including tachyplesin-1 and protegrin-1. The second group consists of those with a medium amphipathic moment between abs$0.4$ and abs$0.5$ (arenicin-3 and polyphemusin-1), with the third group comprising those that are least amphipathic, including gomesin and thanatin with values $>$abs$0.5$.

Figure 1. Lipophilicity/amphipathic moment calculation of $\beta$-hairpin AMPs. All residues pointing down, toward the membrane, are classified as hydrophobic phase (orange) and those pointing up, away from the membrane, as polar phase (green).

Figure 2. Two-sided view of electrostatic and molecular hydrophobicity: (a) electrostatic views based on the continuum model of the Poisson–Boltzmann equation; (b) molecular hydrophobicity views based on the normalized consensus scale value of Eisenberg.41
NMR conformational analysis showed that the two-stranded β-sheet in all six β-hairpin AMP structures studied here possesses a significant twist. This distortion allows shielding of the hydrophobic side of the β-sheet from contact with the polar solvent. Surface representations that display the electrostatic and molecular hydrophobicity (Figure 2) of the six β-hairpin AMPs illustrate the absence or presence of amphipathicity in aqueous solution. Although amphipathic in nature, the charged and hydrophobic residues are scattered throughout the peptide sequences, rather than grouped. Therefore, the structures possess a more distributed amphipathicity compared to the two clear and distinct faces of opposing properties often seen by other amphipathic molecules, such as the helical AMPs.

**Antimicrobial Activity.** To determine correlations between the physicochemical properties of the β-hairpin AMPs and their antimicrobial activity and/or toxicity, the selected peptides were tested against a panel of bacteria and fungi, with toxicity determined on the basis of hemolytic activity and cytotoxicity assays against mammalian cells. Potent and broad-spectrum antimicrobial activity was seen for tachyplesin-1, polyphemusin-1, protegrin-1, and arenicin-3 (Table 4). Gomesin showed less potency yet retained broad-spectrum antimicrobial activity, with the exception of *K. pneumoniae* (ATCC 700603, MDR) and *S. aureus* (ATCC 43300, MRSA), for which the activity was reduced 4–8-fold. Thanatin showed a much narrower spectrum of antimicrobial activity, with the exception of *K. pneumoniae* ATCC 13883, with activity against other species or resistant strains 8–32-fold less active.

The mechanism of action of β-hairpin AMPs has been suggested to involve disruption of the integrity of the bacterial lipid bilayer.25,26,42–44 We carried out a fluorescent probe study (SYTOX green dye; data included in the Supporting Information) to assess the inner (cytoplasmic) bacteria membrane permeability. The results show that all compounds except thanatin permeabilized the inner bacteria membrane, suggesting that they act via disruption of the membranes of G−ve and G+ve bacteria. The ability of each β-hairpin peptide to permeabilize the *E. coli* cytoplasmic membrane was in agreement with previous studies.31,33,49–51 To further investigate this mechanism, we compared the antimicrobial activity of the peptides against three strains of *E. coli*: (i) a control *E. coli* strain (ATCC 25922) that possessed a “smooth” lipopolysaccharide (LPS) outer layer; (ii) a membrane mutant, *E. coli* K12 (ATCC 700926), a “rough” strain deficient in the O-antigen chain of the LPS; and (iii) a membrane mutant, *E. coli* MB 4902, ΔlpxC mutant, where LpxC is a key enzyme involved in the biosynthesis of lipid A, a phosphorylated glycolipid that anchors the LPS to the outer membrane of the cell. All peptides were slightly more active (2–16-fold) against the “rough” K12 mutant than against the “smooth” strain, except for thanatin, for which activity remained the same. With the ΔlpxC mutant, there was a significant increase in potency for all compounds (4–128-fold decrease in minimum inhibitory concentrations (MICs)), with protegrin-1 and polyphemusin-1 showing the greatest potencies at 0.015 and 0.03 μg/mL, respectively. Taken together, these data imply the peptides are able to more readily penetrate the lipid bilayer, resulting in cell death with decreased lipid A in the outer membrane (OM). The potencies of protegrin-1 and polyphemusin-1 against the ΔlpxC *E. coli* mutant suggest that their mode of action may differ subtly from that of the other peptides.

### Table 4. Antimicrobial Activity of β-Hairpin AMPs

| Compound Name | ATCC 25922 (control strain) | ATCC 700926 (K12) | ATCC 700603 (MDR) | ATCC 43300 (MRSA) |
|---------------|-----------------------------|-------------------|-------------------|-------------------|
| vancomycin    | 0.03                        | 0.03              | 0.03              | 0.03              |
| amphotericin  | 0.5                         | 0.25              | 0.25              | 0.25              |
| arenicin-3    | 0.06                        | 0.25              | 0.25              | 0.25              |
| polyphemusin-1| 0.05                        | 0.25              | 0.25              | 0.25              |
| protegrin-1   | 2.0                         | 0.25              | 0.25              | 0.25              |
| tachyplesin-1 | 0.06                        | 0.25              | 0.25              | 0.25              |
| thanatin      | 8.0                         | 0.25              | 0.25              | 0.25              |

Colistin, vancomycin, and amphotericin B were used as positive inhibitor controls for G−ve, G+ve, and fungi, respectively. Data: 4 n.

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Interestingly, of the six β-hairpin AMPs tested, one group (arenicin-3, tachyplesin-1, and thanatin) was most active against G−ve bacteria and, in particular, *E. coli*, whereas the second group (polyphemusin-1, protegrin-1, and gomesin) was more potent (2−10-fold) against *B. subtilis* (G+ve bacteria) and *C. neoformans* (fungi) than any other strains of bacteria and fungi. It is likely that this second group of compounds has a different mode of action to the other AMPs studied, an observation supported by the inner bacteria membrane permeabilization study (data in the Supporting Information), where all three peptides (polyphemusin-1, protegrin-1, and gomesin) showed permeabilization at a lower concentration than their MIC for *E. coli* permeabilization at a lower concentration than their MIC for peptides (polyphemusin-1, protegrin-1, and gomesin) showed an observation supported by the inner bacteria membrane permeabilization study (data in the Supporting Information), where all three compounds (polyphemusin-1, protegrin-1, and gomesin) showed permeabilization at a lower concentration than their MIC for the same *E. coli* ATCC 25922 strain.

**Toxicity.** For a peptide to be considered for therapeutic development, it needs to not only possess potent antimicrobial activity but also have low toxicity to human cells. Here we evaluated the hemolytic activity of the profiled peptides, reported in Table 5 as a percentage value of hemolysis (compared to 100% hemolysis induced by 1% Triton X-100) when tested at 300 μg/mL (see Supporting Information, Figure S2). Protegrin-1 showed the highest hemolytic activity with 65% disruption at the concentration tested. Tachyplesin-1 and polyphemusin-1 both possessed hemolytic activity of approximately 30%, whereas arenicin-3 and gomesin were much less hemolytic (4%). Thanatin was the only compound deemed to be nonhemolytic. Cytotoxicity was assessed using two different human cell lines (kidney and liver) in combination with two serum concentrations, 1 and 10%. With reduced serum in the assay, the cells are more susceptible to damage as their growth is not as robust. The compounds were far less toxic when 10% FBS was present (Table 5), but these conditions can potentially lead to false negatives as there is likely to be less unbound active compound available (due to plasma protein binding) to interfere with the cells. Both cell lines were affected to a similar degree and in the same order of magnitude for all compounds except gomesin, which showed approximately 2-fold more toxicity toward the kidney cells (HEK293) than the liver cells (HepG2). In terms of cytotoxicity, only thanatin remained nontoxic at the highest concentration (300 μM) tested. Plasma protein binding (PPB), as measured by ultrafiltration in 100% human plasma, ranged from 65% for polyphemusin-1 to 92% for thanatin (Table 5). The lack of toxicity observed with thanatin may be partly due to its high protein binding, but protegrin-1 (89%) was similarly highly bound, yet showed the greatest toxicity. The therapeutic potential of β-hairpin AMPs is valued on the basis of their selectivity toward microbial cells as compared to normal mammalian cells. This cell selectivity is expressed as the therapeutic index (TI) of the peptides, which in this study is calculated as the ratio of the average toxicity (hemolytic and cytotoxicity using media with minimal serum; 1% FBS) to the median of the MIC determined toward all tested bacterium and fungus strains.

**Figure 3.** (A) Antimicrobial activity and (B) toxicity ranking, from left (most) to right (least) comparing the antimicrobial activity across the whole panel of bacterial and fungal strains and the toxicity as a combination of hemolytic activity and cytotoxicity.
hydrophobic are those belonging to the MAMT group, such as tachyplesin-1, polyphemusin-1, and protegrin-1. Arenicin-3 is moderately amphipathic and quite hydrophobic, which explains its belonging to the LALT group. Gomesin, however, has poor amphipathicity but the lowest overall hydrophobicity, disallowing interaction with either mammalian or bacterial cells, which explains its belonging to the LALT group. The structure–function relationship study from Mattei et al.45 concluded that more hydrophobic analogues of gomesin have higher antimicrobial activity, whereas peptides more hydrophilic abolish antimicrobial activity. It was concluded that the interaction of gomesin with bacterial membranes depends on interplay between surface electrostatic interactions, which drives anchoring to the membrane surface and vesicle aggregation, and insertion of the hydrophobic portion into the membrane core, responsible for causing membrane rupture/permeabilization. Thanatin, unlike gomesin, has good hydrophobicity but is not overly amphipathic, leading to the same behavior and explaining the classification of this compound in the LALT group.

The correlation between antimicrobial activity and toxicity resides in cell selectivity. The cationic property of AMPs contributes to cell selectivity because the surface of bacterial membranes is more negatively charged than that of mammalian cells. However, the hydrophobicity and the amphipathicity of the peptide are key components of the interaction of AMPs with mammalian cell membranes. Many studies have demonstrated that hemolytic peptides exhibit strong interaction with the zwitterionic phospholipid, phosphatidylcholine, whereas nonhemolytic peptides do not.46,47 Our data correlate with these rules, with the most amphipathic compounds being the most hemolytic. For all AMPs, the maintenance of the peptide hydrophobic–hydrophilic balance may be the critical parameter for producing a highly bacterial selective peptide therapeutic.48

The key features required for efficient bactericidal or hemolytic activities arise from understanding the secondary and tertiary structures of AMPs. Knowledge of key structure–function relationships may assist in the rational design toward improved analogues for clinical use. As a common supportive structural feature of proteins and peptides, it is highly likely that the disulfide bridges play an important role in \( \beta \)-hairpin AMP antimicrobial activity. The role of disulfide bridges in AMPs has been studied for numerous cases, with their importance appearing to be compound dependent. There are two main categories of compounds: (i) AMPs having antimicrobial activity that is independent of the presence or absence of disulfide bridges, for example, human \( \beta \)-defensin-322 and bovine \( \beta \)-defensin-2; and (ii) AMPs that display decreased antimicrobial activity in the absence of disulfide bonds, for example, human \( \beta \)-defensin-2 and \( \alpha \)-defensin HNP-122 or bactenecin.22

The \( \beta \)-hairpin AMPs fall into both categories, but their activity appears dependent on the ability of the peptides to retain their amphipathic structure upon membrane contact.3,49–52 Therefore, to design new analogues, the secondary structure and amphipathic modifications have to be the key parameters to take into account for developing good structure–activity relationships. Cys could potentially be replaced by aromatic residues such as Tyr or Phe, which would allow the secondary structure of the peptide to be maintained and increase the hydrophobicity of the peptide, which should allow better cell selectivity.48

**Conclusion.** Despite the small number of \( \beta \)-hairpin AMPs found in nature, we can start to discern trends to determine key features required for optimal compound design. The AMPs have diverse structural arrangements and physicochemical properties, allowing them to act selectively against bacteria compared to mammalian cell types. However, some key elements are required to remain constant to maximize antimicrobial activity and minimize toxicity. The strategy for a good \( \beta \)-hairpin AMP does not involve only one factor but rather requires a good balance between charge, hydrophobicity, amphipathicity, secondary and tertiary structure, and mode of action. \( \beta \)-Hairpin AMPs offer a wide spectrum of antimicrobial activity with very limited bacterial resistance observed to date, likely due to the significant change in membrane properties that this would require. \( \beta \)-Hairpin AMPs therefore present many advantages over antibiotics already available in the clinic and are worthy of further investigation to develop as replacements for antibiotics that have become ineffective.

### METHODS

**Compounds Tested.** Tachyplesin-1, polyphemusin-1, gomesin, protegrin-1, and thanatin were synthesized by Mimotopes Pty Ltd. (Clayton, Australia) using Fmoc-based solid phase peptide synthesis and orthogonal Cys protection (Fmoc Cys(Acm)–OH for C3, and C16 and Fmoc Cys(Trt)–OH for C7 and C12); the first disulfide bond was formed by air oxidation following cleavage from the resin, with iodine treatment generating the fully cyclized peptide that was purified by RP-HPLC to >95% purity. Identity and purity were confirmed by LC-MS and HRMS. Arenicin-3 was supplied by Adenium Biotech.

**Antimicrobial Assay (MIC).** Both antibacterial and antifungal MIC assays were performed by a broth microdilution plate based method as per CLSI guidelines for antimicrobial susceptibility testing.53,54 The assay was performed in Mueller Hinton broth (MHB; Bacto Laboratories, 211443) for bacteria and yeast extract–peptone dextrose (YPD; Sigma-Aldrich, Y1500) for fungi, and the MIC was determined as the lowest concentration of compound that prevented microorganism growth after 18–24 h. Full assay details are included in the Supporting Information with a full list of strains tested in Table S1.

**Cytotoxicity Assay.** Cytotoxicity to HEK293 and HepG2 cells was determined using the Alamar Blue (resazurin) assay,55,56 with 24 h of incubation and either 1 or 10% serum concentration. Full assay details are included in the Supporting Information.

**Hemolysis Assay.** Hemolytic activity was performed as previously described in the literature with slight modifications.37 Full assay details are included in the Supporting Information.

**Plasma Protein Binding Assay.** Plasma protein binding was performed using an ultrafiltration method.58

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.6b00045.

Experimental procedures, amino acid frequency graph, hemolytic activity results, HRMS, and LC-MS (PDF)
The authors declare the following competing financial interest(s): M.A.C. is on the scientific advisory board of Adenium Biotech (Denmark), and Adenium Biotech has sponsored research on arenicin-3 analogues.

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