Low cationicity is important for systemic in vivo efficacy of database-derived peptides against drug-resistant Gram-positive pathogens

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As bacterial resistance to traditional antibiotics continues to emerge, new alternatives are urgently needed. Antimicrobial peptides (AMPs) are important candidates. However, how AMPs are designed with in vivo efficacy is poorly understood. Our study was designed to understand structural moieties of cationic peptides that would lead to their successful use as antibacterial agents. In contrast to the common perception, serum binding and peptide stability were not the major reasons for in vivo failure in our studies. Rather, our systematic study of a series of peptides with varying lysines revealed the significance of low cationicity for systemic in vivo efficacy against Gram-positive pathogens. We propose that peptides with unbiased amino acid compositions are not favored to associate with multiple host factors and are more likely to show in vivo efficacy. Thus, our results uncover a useful design strategy for developing potent peptides against multidrug-resistant pathogens.

antimicrobial peptides \mid basic amino acids \mid peptide design \mid in vivo efficacy \mid protease stability

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ccording to the 2013 report of the Centers for Disease Control and Prevention, antibiotic-resistant pathogens caused 23,488 deaths per year. Among them, methicillin-resistant \textit{Staphylococcus aureus} (MRSA) alone caused 11,000 deaths per year (1). Therefore, novel and potent antibiotics against MRSA are urgently needed. Antimicrobial peptides (AMPs) are important innate immune molecules that protect the host from the infection of invading pathogens. A majority of such peptides are cationic and amphipathic, allowing them to rapidly eliminate pathogens by targeting anionic membranes. Such a mechanism confers power to AMPs so that they can also kill persisters (dormant bacteria) and disrupt biofilms (bacterial community). In addition, cationic peptides regulate the host immune response to clear infection. The combination of these peptide actions renders it difficult for bacteria to develop resistance (2–5).

While a few bacterial peptides have found practical applications clinically or as food preservatives (4, 5), amphipathic AMPs from animal sources have not been approved by the US Food Drug Administration (FDA) for use in humans as new antibiotics. To facilitate the discovery of new peptide antibiotics, we constructed and made use of the Antimicrobial Peptide Database (APD) (6). Our systematic annotation of peptides not only facilitated information retrieval and analysis but also enabled us to arrive at a unified peptide classification method irrespective of peptide source (active and 3D structure) (7). The APD also allowed us to develop innovative methods for identifying useful candidates with desired antimicrobial activity. One natural method was to screen the representative candidates from the database against dangerous pathogens such as HIV type 1 and MRSA (8, 9). Using an ab initio approach, we also designed pathogen-specific peptides by developing a database-filtering technology (DFT) (10). In this approach, thousands of database peptide sequences can pass through multiple filters (Fig. 1A), each of which rejects one unwanted feature and retains the needed parameter. A major algorithm used in that design is the most probable principle that selects the maximum in each step for peptide parameters such as length, charge, hydrophobicity, and structure. A combination of these parameters defines the peptide DFTamP1, which effectively kills Gram-positive MRSA USA300 (10), a major community-associated resistant pathogen. In addition, other peptide discovery approaches have been developed based on the data from the APD (11–13).

However, there is a great gap from peptide discovery to successful implementation as an antimicrobial agent. Most of the current AMP developments focus on topical treatment (2–5, 14–19), and what determines peptide systemic in vivo efficacy is poorly understood. It is proposed that serum/plasma binding is responsible for the loss of activity in vivo for cationic AMPs (20, 21). Indeed, recent studies have attempted to identify cationic peptides with reduced plasma binding (15). However, the authors did not show the advantage of reduced plasma binding in vivo. This study attempted to identify peptides with efficacy in mice by overcoming both in vitro and in vivo barriers (Fig. 1A). The in vitro barriers include peptide cytotoxicity, stability, effects of physiological salts, pH, and serum binding, which could compromise peptide activity (22, 23). By using various sequence-modulating methods, we generated numerous peptides based on our database-designed peptide template (Fig. 1B). One of our designed peptides crossed multiple in vitro barriers, allowing us to test this peptide in vivo models. Our in vivo evaluation of a series of peptides demonstrated that peptides with low cationicity are active in vivo against Gram-positive pathogens, those with more charged lysines are unable to reduce bacterial burden in mice. It appears that biased amino acid compositions are necessary to avoid activity loss in vivo. Our discovery could form the basis for developing potent peptides against multidrug-resistant pathogens.

Significance

New strategies are required to develop the next generation of peptide antibiotics. This study has advanced our understanding by discovering an important factor that determines in vivo success of database-derived cationic peptides. While designed peptides with low cationicity are active in vivo against Gram-positive pathogens, those with more charged lysines are unable to reduce bacterial burden in mice. It appears that biased amino acid compositions are necessary to avoid activity loss in vivo. Our discovery could form the basis for developing potent peptides against multidrug-resistant pathogens.

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Data deposition: The atomic coordinates, NMR restraints, and chemical shifts reported in this paper have been deposited in the Protein Data Bank, http://www.robj.org (PDB ID: 6MKK) and in the Biological Magnetic Resonance Bank, http://www.bmrb.wisc.edu/ (BMRB ID: 30524).

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with a varying number of lysines (Fig. 1B) led to an important discovery that the more lysines in the peptide, the more likely it will fail to reduce bacterial burden in our murine models. Here we report these results and discuss the importance of biased amino acid compositions for designing potent peptides with in vivo efficacy. The knowledge gleaned here may inspire the design of new antibiotic activity against multiple types of infections, including those involving persisters and biofilms.

**Results**

**Peptide Optimization and In Vitro Testing.** Our main goal is to identify peptides that display in vivo efficacy in a systemic murine model (outlined in Fig. 1A). There are multiple hurdles one must overcome toward the development of novel peptide antibiotics. One of them is peptide cytotoxicity. To minimize hemolysis of the peptide template DFTamP1, we altered its amino acid sequence (Fig. 1C) in three ways (sequences and properties of peptides in SI Appendix, Table S1). First, a single alanine was used to substitute each of the eight leucines. The resulting analogs DFT506–DFT513 retained high antimicrobial activity against S. aureus USA300 with a minimal in vitro inhibitory concentration (MIC) ranging from 0.78 to 1.56 μM (SI Appendix, Table S2). Two variants, DFT507 (L3A) and DFT510 (L5A), were found to have the same antibacterial activity spectrum against S. aureus as the original peptide DFTamP1. Other alanine-substituted analogs showed good activity against Gram-negative Escherichia coli and Klebsiella pneumoniae. In addition, DFT506 and DFT511 were also active against Pseudomonas aeruginosa PAO1. Interestingly, the double-alanine substitution analogs DFT514–DFT516 (L5A and L8A) were active only against S. aureus, E. coli, and K. pneumoniae (SI Appendix, Table S2). They were also less hemolytic to human red blood cells. Triple-substituted analogs (DFT521, DFT528, and DFT529) of DFTamP1 retained only moderate activity (MIC 12.5–25 μM) against S. aureus. Finally, quadruple-alanine substitution led to inactive peptides DFT522 and DFT530 at 25 μM (SI Appendix, Table S2). These results indicate the limitation of the alanine-shuffling method for obtaining highly selective peptides.

As a second approach, we also applied single-amino-acid deletions to DFTamP1. When L6 was removed, the peptide DFT523 retained moderate activity (12.5–25 μM) against S. aureus and E. coli. Further removal of one more leucine from the sequence led to inactive peptides (DFT524–DFT526 in SI Appendix, Table S3). Third, interesting peptides emerged when the sequence of DFTamP1 was shuffled, i.e., altering amino acid order without a change of peptide composition. To avoid a loss of antimicrobial activity from random shuffling, we conducted a local sequence shuffling to generate sequences unexplored in our previous peptide design. For example, we introduced a less common triple leucine (LLL) motif into the peptide DFT505 (double leucine [LL] was identified in 704 AMPs in the APD whereas LLL was found only in 21 AMPs) (6, 24). Remarkably, DFT505 became much less hemolytic than DFTamP1 (SI Appendix, Table S4). Meanwhile, all of the peptides in this category retained the same activity spectrum as DFTamP1, which is primarily active against S. aureus (MIC 0.78–6.25 μM), but not P. aeruginosa, E. coli, and K. pneumoniae. Of note, DFT504 (MIC 6.2 μM) was four times less active than the DFTamP1 (SI Appendix, Table S4).

In addition, there are other factors (pH, salts, and serum) that compromise peptide activity (15, 16, 21). The activity of defensins is usually salt-sensitive (5, 22), while human cathelicidin LL-37 becomes less active at pH 6.8 or after binding to human serum (22, 23). Encouragingly, antibacterial activity of DFT503 and its D-form (DFT503d) was not significantly affected by salts (1–2 mM CaCl₂ or 50–200 mM NaCl) (Table 1 and SI Appendix, Table S5). However, their MIC values increased over eightfold in a medium containing a physiological amount (5%) of human serum or 5% mouse plasma (Table 1). To search for peptides that can tolerate serum/plasma, we substituted a single serine or glycine in DFT503 with alanine (SI Appendix, Table S1). The substitution of S3 of DFT503 by K3 increased peptide solubility. Antimicrobial assays revealed that DFT561 maintained the same activity spectrum as DFTamP1 (10). Importantly, the peptide did not become more hemolytic. We also compared the positional effect of one additional lysine on peptide properties. While DFT560, DFT562, and DFT563 all showed high antimicrobial potency, they were more hemolytic as well. When additional basic amino acids were incorporated, peptides DFT564 and DFT565 (with three and four lysines) became even more hemolytic than DFT503. Thus, additional positively charged lysines did not offer an advantage to the peptide in terms of cytotoxicity, despite increased membrane depolarization of S. aureus (SI Appendix, Fig. S1).

We then compared antimicrobial activity of these peptides in the presence of serum. Remarkably, DFT561 inhibited S. aureus USA300 in the presence of 5% human serum or mouse plasma (Table 1). Thus, DFT561 emerged as a more robust antimicrobial candidate with an excellent therapeutic potential (high potency, low hemolysis, and robust activity). Next, we intended to improve peptide stability to proteases. Since a peptide made of D-amino acid is usually more stable (e.g., SI Appendix, Fig. S2) (3, 10, 16, 17), we also made the D-form of DFT561 (DFT561d). Interestingly, both forms of the DFT561 peptides retained bacterial killing in the presence of mouse peritoneal cavity fluid or homogenized tissue supernatants from uninfected mice (SI Appendix, Fig. S4).

To further evaluate peptide potency, we tested resistant bacterial strains, biofilms, and persisters. DFT503, DFT561, and their
d-forms showed potent activity against Gram-positive MRSA and vancomycin-resistant enterococci (VRE) strains, but not multi-resistant Gram-negative E. coli, P. aeruginosa, and K. pneumoniae (SI Appendix, Table S6). DFT561d appeared more potent than DFT561 in disrupting static biofilms of S. aureus USA300 formed in 24 h. At 25 μM, DFT561d essentially eliminated bacterial burden in the biofilms, while DFT561 killed 60% of the S. aureus (SI Appendix, Fig. S3B). Because of the antibiofilm potency of DFT561d, we further tested its effects on other forms of S. aureus. It was able to rapidly kill S. aureus in exponential (SI Appendix, Fig. S4) and stationary phases (SI Appendix, Fig. S3C), as well as persisters that were not killed by traditional antibiotics (SI Appendix, Fig. S3D). These results further validate the potency of DFT561d.

In Vivo Toxicity and Efficacy of Cationic Peptides in a Neutropenic Mouse Model. Because DFT561 had crossed multiple in vitro barriers (Table 1), we tested its in vivo toxicity and efficacy. We used a well-established neutropenic mouse model, which is often utilized to evaluate the efficacy of antibiotics and peptide mimics (25, 26). In this model, the effect of immune cells on bacterial killing is minimized via two injections of cyclophosphamide before infection (Fig. 2). To evaluate the safety of the peptide in vivo, we compared the toxicity of DFT561 injected intraperitoneally (i.p.) at 10, 20, and 40 mg/kg per mouse. Mice injected at 10 mg/kg behaved normally, but those treated at 20 or 40 mg/kg showed reduced activity for 30 min (SI Appendix, Table S7). We observed small weight losses for all of the treatment groups, but the changes were statistically insignificant (SI Appendix, Fig. S5). In addition, alanine transaminase normally resides in serum and tissues but can be released after liver injury. We did not see differences in a quantitative alanine transaminase assay of liver homogenates from mice treated at 0, 10, and 20 mg/kg (SI Appendix, Fig. S6). All these results indicate that DFT561 is unlikely to be toxic when treated at 5 mg/kg.

In our in vivo efficacy study, each mouse was i.p. infected with 2 × 10^6 colony forming units (CFU) of S. aureus USA300 LAC (Fig. 2A). As a positive control, we observed 1–4 logs decrease in bacterial burden in different tissues after daptomycin treatment at 10 μg/kg via a single i.p. injection. In contrast, we did not see a decrease in bacterial burden in tissues when the infected mice were treated with an LL37-derived analog (SI Appendix, Fig. S7). We then treated the infected mice with DFT561 at a single dose of 5 mg/kg since it is less affected by serum/plasma binding (Table 1). The 5-mg/kg dose was chosen based on a dose-dependent study (SI Appendix, Fig. S8). We initiated the peptide treatment at 2 h post infection when bacteria had spread to various tissues, indicating a systemic infection (SI Appendix, Fig. S9). At 24 h after treatment, bacterial burdens in spleen, lung, liver, and kidney with and without peptide treatment were enumerated. On average, we observed a bacterial load decrease by 1.8 logs in liver and 1.4 logs in kidney (up to 90% killing). There were also decreased bacterial loads in spleen and lung, but the differences were statistically insignificant compared with the untreated controls (Fig. 2C). We also evaluated DFT561d since it is resistant to proteinases. Interestingly, DFT561 and DFT561d displayed a similar in vivo effect (SI Appendix, Fig. S10), although DFT561d showed a much reduced antibacterial activity in vitro in the presence of human serum or murine plasma (Table 1). Perhaps, serum/plasma binding is not a limiting factor for in vivo efficacy of these peptides in this mouse model when intraperitoneally administered. To test this possibility, we extended our in vivo study to DFT503, which is inactive in the presence of serum/plasma (Table 1). Compared with DFT561, we observed a similar in vivo efficacy for DFT503 in kidney and liver, but a slightly better efficacy in spleen and lung (Fig. 2B). Thus, our data suggest that serum binding plays a less important role for in vivo efficacy of these peptides. Likewise, peptide stability may not be critical here based on the similar in vivo effects of the L- and D-forms of D561 when injected intraperitoneally (SI Appendix, Fig. S10). We then asked whether a further increase in basic lysine could influence peptide in vivo efficacy. For this purpose, we evaluated DFT564 and DFT565 (Fig. 2A). Surprisingly, while a DFT503 analog with one lysine worked, DFT564 and DFT565 with three to four lysines were entirely inactive (Fig. 2D and E), underscoring that a high number of positively charged lysines is not favorable for in vivo efficacy. While a single dose injection facilitated our comparison of different peptides, we also investigated the effect of multiple peptide doses. Remarkably, the bacterial burdens were decreased by 4–5 logs in spleen, lung, and kidney after two injections of DFT503 at 5 mg/kg per mouse 2 and 24 h post infection (SI Appendix, Fig. S11). These results emphasize the advantage of multiple dose treatment with DFT503.

**Mechanism of Action and Structural Basis of Cell Selectivity of the DFT Peptides.** Our previous study established membrane targeting of DFTamP1 (10). It is logical to hypothesize that the improved DFT peptides (Table 1) work in the same manner. To test this hypothesis, we used the nonmembrane permeable probe propidium iodide. A fluorescence increase was observed in the presence of DFT503, DFT503d, DFT561, and DFT561d (SI Appendix, Fig. S12A), or membrane-active daptomycin, but not membrane-inactive vancomycin and rifamycin (SI Appendix, Fig. S13). Thus, peptide-mediated membrane permeation may have caused the loss of viability of S. aureus USA300 since no bacteria could grow (SI Appendix, Fig. S12B). Because we obtained even better in vivo effects for DFT peptides with reduced positive charge, we conducted additional experiments using DFT503. First, the L- and D-forms of DFT503 showed essentially the same MIC (Table 1). Second, DFT503 and DFT503d at the MIC showed similar rapid killing kinetics and were able to eliminate S. aureus USA300 at 120 min (SI Appendix, Fig. S12C). Third, we observed fluorescein isothiocyanate entry into S. aureus cells (green cells) by confocal microscopy after treatment with either DFT503 or DFT503d (SI Appendix, Fig. S12D).
experiments further support the membrane targeting nature of DFT503. However, DFT503d appeared to be more effective in depolarizing bacterial membranes than DFT503. A similar difference existed between DFT561 and DFT561d (SI Appendix, Fig. S14). DFT503d was more potent than DFT503 in killing *S. aureus* USA300 in established biofilms (SI Appendix, Fig. S15).

To shed additional light on the peptide action, we then determined the 3D structure of DFT503 in a membrane-mimetic micelle. For a higher quality structure, we used the improved 2D NMR method (27), which is essential for structural determination of peptides rich in certain amino acids (28, 29). Compared with the classic homonuclear 2D 1H NMR method (30), the improved method includes additional heteronuclear correlated 2D NMR spectra to validate 1H assignments and refine structure. DFT503, a leucine-rich peptide, adopted a helical structure based on the chemical shift analysis (SI Appendix, Fig. S16). In the same figure, our NMR analysis revealed identical secondary shift plots for peptides DFT561 and DFT564, indicating no structural change under similar sample conditions. The 3D structure of DFT503 was determined based on 87 distance and 22 angle restraints (Fig. 1). All of the backbone dihedral angles are located in the allowed region of the Ramachandran plot. Similar to DFTamP1 (Fig. 1C and D), DFT503 also adopted a helical structure (Fig. 1E and F), indicating that our local sequence shuffling is structurally tolerated. However, leucine side chains position differently. While DFTamP1 has a regular amphipathic helix with nearly all of the leucines located on the hydrophobic surface (Fig. 1C), some leucines deviated from the hydrophobic surface of DFT503 (Fig. 1E).

In particular, a glycine (G9) is located on the hydrophobic surface. Because G9 does not have a side chain, it generates a structural defect locally (Fig. 1F). We propose that this structural defect (Fig. 1F) is responsible for reduced hemolysis of DFT503 compared with DFTamP1 (Fig. 1D). Thus, local sequence shuffling provides an alternative approach to generating hydrophobic defects, different from the case of D-amino-acid incorporation (31).

The membrane targeting of DFT peptides offers an advantage. We found no spontaneous resistance development for DFT561d in a multiple passage experiment (SI Appendix, Fig. S3E) (16, 32). Nevertheless, *S. aureus* is known to respond to the action of cationic peptides via the major two-component system GraRS that regulates the modification of bacterial phosphatidylglycerols with lysine by MprF (33, 34). To view the extent of resistance, we then compared the MIC values of these DFT peptides using the *S. aureus* JE2 (wild type) and ΔmprF mutant strains in the Nebraska Transposon Mutant Library (35). Indeed, we observed only a twofold MIC increase for DFT561, DFT564, and DFT565 in 50% tryptic soy Fig. 2. In vivo efficacy of database-designed peptides against *S. aureus* infection using neutropenic mice. (A) Scheme for in vivo efficacy experiment. Mouse neutropenia was induced by two injections of cyclophosphamide (150 mg/kg and 100 mg/kg) −4 and −1 d before infection (25, 26). Animals infected with *S. aureus* USA300 (2 × 10^6 CFU/mouse) were treated intraperitoneally 2 h post infection with a single peptide dose (n = 5 mice per group) of (B) DFT503; (C) DFT561; (D) DFT564; and (E) DFT565 at 5 mg/kg. Animals were euthanized 24 h post treatment; organs were harvested and weighed; and CFU were enumerated in spleen, lung, kidney, and liver. The bacterial loads from each mouse were plotted as individual dots, and error bars represent the deviation from the average within the experimental group. *P < 0.05, **P < 0.01, and n.s. represents “no significance” (determined by Mann–Whitney test).
broth (TSB) (SI Appendix, Table S8). Remarkably, DFT503 did not show any change in MIC against these two strains at different concentrations of TSB (25, 50, and 100%), revealing another important merit of DFT503 as a potent peptide against MRSA (10).

Beyond direct antimicrobial killing, cationic AMPs also regulate the immune response of host cells (21, 36). At a bactericidal concentration (1.6–3.1 μM) of DFT561d, there was essentially no change in viable THP-1 macrophages (SI Appendix, Fig. S3F). Upon S. aureus infection, there was an increased release of chemokine TNF-α from THP-1 cells. Notably, DFT561d could suppress this bacteria-induced TNF-α (SI Appendix, Fig. S3G). In contrast, the level of MCP-1 was up-regulated due to the effect of DFT561d (SI Appendix, Fig. S3H). Hence, the designed peptide regulated the chemokine levels of THP-1 cells in vitro. To further verify this observation, we also evaluated these chemokine changes in vivo. This requires efficacy testing of the peptide in nonneutropenic mice. We observed a similar in vivo efficacy when treated at a single dose of 10 mg/kg DFT503 due to a 10-fold higher inoculum of 2.0 × 10^6 CFU S. aureus USA300 per mouse (SI Appendix, Fig. S17A). We then measured the TNF-α and MCP-1 levels in mouse plasma. Interestingly, we also observed suppression of TNF-α and induction of the level of MCP-1 (SI Appendix, Fig. S3I), consistent with our observation in vitro (SI Appendix, Fig. S3 G and H). In addition, the levels of IL-17A and IL-10 were also increased at 24 h (SI Appendix, Fig. S17B). While IL-17 can recruit neutrophils, IL-10 may neutralize TNF-α, attenuate tissue pathology, and reduce mortality (37). In addition, the balance between the IL-10 and IL-17 cytokines is also crucial for bacterial elimination and protecting the host from a pathological condition at the site of infection (38, 39). These results depict a picture that DFT peptides play a critical role in eliminating S. aureus USA300 via direct killing as well as immune boosting both in vitro and in vivo.

**Discussion**

Antimicrobial peptides are widely recognized candidates for developing novel antibiotics. However, it remains challenging to design peptides with systemic in vivo efficacy. Indeed, no AMPs designed from vertebrate hosts have been approved by the FDA for use in humans as new antibiotics (2, 5, 21, 26). Part of the reason may be that we do not yet fully understand the principle of peptide design. Our results indicate that peptide amino acid composition plays a major role in determining its activity against bacteria. Some variation in amino acid sequence is allowed since numerous peptides remained active against MRSA after an alanine scan or local sequence shuffling (SI Appendix, Tables S2 and S4). However, amino acid sequences are also important for peptide activity and toxicity and could become critical for nonmembrane-targeting AMPs. Of note, the DFT peptides displayed systemic in vivo efficacy at 5 mg/kg. Although they are not as potent as daptomycin (10 mg/kg) after single-dose treatment, an improved outcome was obtained via two-dose treatment, revealing the therapeutic potential of the peptide (SI Appendix, Fig. S1I). Our results also indicate the requirement of new ideas and concepts for developing cationic AMPs into novel antibiotics. First, in contrast to the common perception that serum/plasma binding is responsible for activity loss of cationic AMPs in vivo, the fact that we observed in vivo efficacy for serum-binding DFT503 indicates that this requirement is not general. Second, despite higher stability to proteases of the D-form than the L-form of DFT561, we found similar in vivo efficacy in mice when the peptides were i.p. administered. This may result from fast bacterial killing of the membrane-targeting peptide. Thus, increased peptide stability may not be generally required for in vivo efficacy. From a different point of view, peptide degradation after bacterial killing can avoid unwanted effects due to compound accumulation. In addition, peptide degradation removes selection pressure, minimizing the likelihood of bacterial resistance development. Third, it appears that high hydrophobicity and low cationicity provides one useful avenue to the design of peptides with systemic in vivo efficacy against Gram-positive MRSA and VRE. This is an important observation because a further increase in positively charged lysine demonstrated unwanted outcomes both in vitro and in vivo. It is likely that high cationicity is also responsible, at least partially, for the failure of some typical AMPs (four to nine lysines/arginines) in vivo. These include human cathelicidin LL-37–derived HC10 (this study) and amphibian-derived peptides MSI-78 and DS4 (1–16) (SI Appendix, Table S9) that were assessed in the same murine model by others (26).

To gain additional insight, we generated the RK-Pho diagram based on the 3,014 AMPs currently registered in the APD (Fig. 3) (6). The RK-Pho diagram depicts a correlation between peptide hydrophobic contents (Pho) and the percentage of basic amino acids (arginines + lysines). When we mapped the RK and Pho for peptides that had been tested in vivo for systemic efficacy, we found that those peptides that worked in vivo are closer to the RK-Pho dots from the database (dotted green circle), whereas those that failed in vivo are more deviated (dotted red circle). However, we also found an outlier, A3-APO, a proline-rich peptide also rich in basic charges. Although the parameters of this peptide deviated from the RK-Pho plot, A3-APO showed in vivo efficacy (18). In contrast to the membrane-active peptides that we studied here, this outlier only kills Gram-negative pathogens by targeting intracellular molecules such as heat-shock proteins (18). In addition, proline-rich peptides have a low hydrophobic content (on average, 25.4% for the 67 such peptides in the APD) (6), while the peptides studied here have a high hydrophobic content (61.5%).

**Conclusions**

Although over 3,000 AMPs are registered in the antimicrobial peptide database, only a few have found practical applications (2, 4, 21). On average, AMPs have +3.3 positively charged amino acids with 41.5% hydrophobic amino acids (6). How to design AMPs with systemic in vivo efficacy is not clear. We found here...
that the requirement for decreased binding to plasma and increased peptide stability may not be general characteristics of cationic AMPs with in vivo efficacy. However, this study reveals the importance of low cationicity and high hydrophobicity for systemic in vivo efficacy of membrane-active peptides against Gram-positive pathogens because an increase in lysine led to loss of efficacy in murine models. Additionally, positive charges might have facilitated peptide binding to host factors in vivo, rendering them unavailable for bacterial killing. In contrast, proline-rich peptides with high positive charges but low hydrophobic amino acids may also be less favorable to bind to multiple host factors, yielding an alternative design for in vivo efficacy against Gram-negative pathogens (40–42). Collectively, these results define two biased amino acid compositions for designing narrow-spectrum peptides with systemic in vivo efficacy: low cationic database-devised peptides against Gram-positive pathogens and low hydrophobic proline-rich peptides against Gram-negative pathogens.

Materials and Methods

Peptide Properties and Activity in Vitro. Peptide properties were analyzed using the online AMP database website http://aps.unmc.edu/AMP (6, 24). Peptide antibacterial activity, hemolytic activity, killing kinetics, peptide stability, membrane permeation, and antibiofilm assays were conducted as described in SI Appendix.


NMR and Structural Calculations. NMR samples, data collection, analysis, and structural determination were conducted as described in SI Appendix.

Peptide Efficacy and Immune Regulation in Mice. All animal studies were performed with local ethical committee clearance following protocols approved by the institutional animal care and use committee (IACUC 816-076-08-FC). On the study termination day, animals were euthanized humanely using CO2 followed by harvesting vital organs for laboratory analysis. All efforts were made to minimize animal pain and suffering. Further details can be found in SI Appendix.

Statistical Analysis. Experimental results are represented as mean ± SD. However, MIC values were reported as a range. For in vivo studies, the bacterial loads from mouse tissues were plotted as individual points, and error bars represent the deviations from the mean within the experimental group. *(P < 0.05, **P < 0.01, ***P < 0.001, and n.s. represents “no significance” (determined by the Mann–Whitney U test).

Additional material and methods can be found in SI Appendix.

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