Rational Design of Engineered Cationic Antimicrobial Peptides Consisting Exclusively of Arginine and Tryptophan, and Their Activity against Multidrug-Resistant Pathogens

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The emergence of multidrug-resistant (MDR) pathogens underscores the need for new antimicrobial agents to overcome the resistance mechanisms of these organisms. Cationic antimicrobial peptides (CAPs) provide a potential source of new antimicrobial therapeutics. We previously characterized a lytic base unit (LBU) series of engineered CAPs (eCAPs) of 12 to 48 residues demonstrating maximum antibacterial selectivity at 24 residues. Further, Trp substitution in LBU sequences increased activity against both P. aeruginosa and S. aureus under challenging conditions (e.g., saline, divalent cations, and serum). Based on these findings, we hypothesized that the optimal length and, therefore, the cost for maximum eCAP activity under physiologically relevant conditions could be significantly reduced using only Arg and Trp arranged to form idealized amphipathic helices. Hence, we developed a novel peptide series, composed only of Arg and Trp, in a sequence predicted and verified by circular dichroism to fold into optimized amphipathic helices. The most effective antimicrobial activity was achieved at 12 residues in length (WR12) against a panel of both Gram-negative and Gram-positive clinical isolates, including extensively drug-resistant strains, in saline and broth culture and at various pH values. The results demonstrate that the rational design of CAPs can lead to a significant reduction in the length and the number of amino acids used in peptide design to achieve optimal potency and selectivity against specific pathogens.

Infectious diseases remain a worldwide health crisis despite the relatively remarkable progress achieved in the field of antimicrobial therapeutics during the last century (1–3). The most obvious concern is the increasing frequency of multidrug-resistant (MDR) and extensively drug-resistant (XDR) pathogens (4–10). Conventional classes of antimicrobial agents kill bacterial organisms or inhibit their growth by targeting specific metabolic pathways or microbial structures. A resulting undesired property of these antibiotics is the selection of resistance-conferring mutations and/or acquisition of other resistance determinants during antimicrobial therapy (11–13). This is a major obstacle to treatment efficacy in a number of infections, such as those associated with cystic fibrosis (CF) (14, 15), the use of medical implants (16, 17), intensive care (18), and other nosocomial infections (19). Hence, alternative sources of new antimicrobial therapeutics are urgently needed.

Antimicrobial peptides (AMPs) have been intensely investigated in the last 3 decades as a potential source of complementary antimicrobial agents with a lower propensity to select for drug resistance phenotypes than that of current antimicrobials (20–23). Most AMPs are cationic peptides that assume a specific amphipathic conformation, either in the presence of bacterial membranes (amphipathic α-helical peptides, e.g., cathelicidins and magainins [24, 25]) or constitutively (cyclic and β-sheet AMPs, e.g., the defensins, stabilized by disulfide bridges [26, 27]). It has been established that host-derived AMPs are an essential component of the defense system of most multicellular organisms and are found even in prokaryotes (28, 29). More importantly, AMPs have been engineered by evolution as anti-infective peptides that rapidly disrupt the membranes of specific pathogens in specific environments with no requirement for microbial metabolic activity (30–33).

It is clear that AMPs are a reliable prophylactic measure naturally used by the host defense system against the establishment of infections (22, 34–36). However, many pathogens are able to overcome the host AMPs and other components of the immune system. Of great concern are infections related to compromised immune systems and the selection of MDR phenotypes by conventional antimicrobial agents during unsuccessful attempts to eradicate these infections (14). One such example is demonstrated in infections related to CF. In CF, the mucociliary apparatus is highly compromised due to the dysregulation of sodium chloride levels resulting from a defective CF transmembrane conductance regulator (CFTR) (37–40). In addition, abnormally higher salt concentrations in the airway dampen the activity of AMPs associated with the respiratory epithelium (e.g., LL37 and defensins) partly contributing to uncontrolled colonization by Staphylococcus aureus and opportunistic organisms (e.g., Pseudomonas aeruginosa) (41). This important observation suggests that the working environment must also be considered when designing AMPs for clinical applications.

Lessons learned from structure-function studies of host defense α-helical peptides have led to the design of diverse engineered cationic antimicrobial peptides (eCAPs) with a range of in
vitro antimicrobial activities (23, 42, 43). Over the last decade, our laboratory has pioneered the design of eCAPs with potent antimicrobial activities based on the examination of structure-function relationships of host-derived synthetic AMPs (43–52). Thus, we previously characterized a series of eCAPs called lytic base unit (LBU) series composed of only Arg and Val arranged to fold into idealized amphipathic helical motifs in the presence of lipid membranes or membrane mimotope solvents. The design of the LBU series was intended to exploit the cost-effectiveness of condensation chemistry by serially linking LBUs to synthesize longer eCAPs as needed. Just as most natural AMPs are between 15 and 40 amino acids long, it was interesting to observe that maximal activity of the LBU series was achieved at 24 residues (47). Further, Trp substitution into the LBU sequence significantly enhanced activity against both Gram-positive and Gram-negative bacteria in environments that are considered challenging to AMPs, such as saline, serum, and whole blood (46, 47). In fact, systemic administration of the most potent of the LBU derivatives (WLBU2) resulted in complete protection of mice injected intravenously with a lethal dose of \textit{P. aeruginosa} with no obvious harmful side effects (53). Based on these and other studies of Trp-rich AMPs (e.g., indolicin and tritrpticin) (43, 49, 50, 54–56), we reasoned that the membrane perturbation properties of Arg and Trp, presented in the context of an optimized amphipathic helix, can be further exploited to engineer shorter and more highly potent eCAPs, thereby reducing the cost of production. Thus, we designed a new series of eCAPs (6 to 18 residues long) consisting exclusively of Arg in the hydrophilic face and Trp in the hydrophobic face. The current data demonstrate that it is indeed possible to engineer AMPs that display broad and potent in vitro activity against MDR pathogens at only 12 residues in length, thus achieving specific potencies that are comparable to those of the 24-residue eCAP WLBU2, but at one-half the length.

MATERIALS AND METHODS

**Peptide design and synthesis.** The WR peptide eCAPs were designed using helical wheel diagrams (Fig. 1A) by serial addition of one Arg residue and one Trp residue at a time. The order in which the amino acids were added in the primary sequences was dictated by the ability to obtain idealized amphipathic helices. The amphipathicity of each peptide was then calculated using the online program HydroMCalc on a combined consensus hydrophobic scale (MM(Da), molecular mass in daltons; \( \mu \)H, mean hydrophobic moment; \( \mu \)Hrel, relative hydrophobic moment).

![helical wheel diagrams](image-url)

**FIG 1** Engineered cationic antimicrobial peptide (eCAP) design. (A) As shown in helical wheel diagrams, cationic peptides were designed to form idealized amphipathic helices with the hydrophilic and hydrophobic domains consisting of Arg and Trp, respectively. (B) Primary sequences of the WR peptide series used in the present study and their molecular masses in daltons. The peptides were engineered by serial addition of one Arg and one Trp residues starting from the shortest peptide WR6. Relative hydrophobic moments, or hydrophobic moments relative to ideal amphipathicity, were examined using the online program HydroMCalc on a combined consensus hydrophobic scale. MM(Da), molecular mass in daltons; \( \mu \)H, mean hydrophobic moment; \( \mu \)Hrel, relative hydrophobic moment.
TABLE 1 MICs of the indicated peptides against multiple clinical isolates of Gram-positive and Gram-negative organisms in MHB

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Resistance</th>
<th>LL37</th>
<th>WR12</th>
<th>WLBU2</th>
<th>Colistin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii</td>
<td>AB1</td>
<td>XDR</td>
<td>6 ± 2 (1.3)</td>
<td>5 ± 3 (2.4)</td>
<td>11 ± 5 (3.2)</td>
</tr>
<tr>
<td></td>
<td>AB2</td>
<td>XDR</td>
<td>6 ± 2 (1.3)</td>
<td>5 ± 3 (2.4)</td>
<td>11 ± 5 (3.2)</td>
</tr>
<tr>
<td></td>
<td>AB3</td>
<td>XDR</td>
<td>19 ± 12 (4.2)</td>
<td>11 ± 5 (5.2)</td>
<td>6 ± 2 (1.8)</td>
</tr>
</tbody>
</table>

| Klebsiella pneumoniae | KP1 | XDR | 29 ± 7 (6.4) | 27 ± 9 (12.8) | 10 ± 4 (2.9) | 8 (7.3) |
| | KP2 | >32 (7.1) | >32 (15.2) | >32 (9.4) | >32 (9.1) |
| | KP3 | 19 ± 4 (4.2) | 32 (15.2) | 14 ± 2 (2.9) | 0.5 (0.5) |
| | KP4 | >32 (7.1) | 32 (15.2) | 16 (4.7) | >32 (9.1) |

| Pseudomonas aeruginosa | TRPA108 | MDR | 10 ± 6 (2.2) | 4 (1.9) | 4 (1.2) | 0.5 (0.5) |
| | TRPA111 | MDR | 10 ± 6 (2.2) | 3 ± 1 (1.4) | 3 ± 1 (0.9) | 1 (0.9) |
| | PAO1 | 19 ± 9 (4.2) | 11 ± 5 (5.2) | 6 ± 2 (1.8) | 0.5 (0.5) |

<table>
<thead>
<tr>
<th>Bacterial strain</th>
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<th>LL37</th>
<th>WR12</th>
<th>WLBU2</th>
<th>Colistin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA USA100</td>
<td>&gt;32 (7.1)</td>
<td>7 ± 2 (3.3)</td>
<td>6 ± 2 (1.8)</td>
<td>&gt;32 (9.1)</td>
<td></td>
</tr>
<tr>
<td>USA300</td>
<td>&gt;32 (7.1)</td>
<td>5 ± 3 (2.4)</td>
<td>6 ± 2 (1.8)</td>
<td>&gt;32 (9.1)</td>
<td></td>
</tr>
<tr>
<td>4003</td>
<td>&gt;32 (7.1)</td>
<td>3 ± 1 (1.4)</td>
<td>5 ± 2 (1.5)</td>
<td>&gt;32 (9.1)</td>
<td></td>
</tr>
</tbody>
</table>

| Acinetobacter baumannii | USA300 | XDR | 6 ± 2 (1.3) | 5 ± 3 (2.4) | 11 ± 5 (3.2) | 11 ± 5 (3.2) |
| | 4003 | XDR | 19 ± 12 (4.2) | 11 ± 5 (5.2) | 6 ± 2 (1.8) | 2 (1.8) |

| Klebsiella pneumoniae | KP1 | XDR | 29 ± 7 (6.4) | 27 ± 9 (12.8) | 10 ± 4 (2.9) | 8 (7.3) |
| | KP2 | >32 (7.1) | >32 (15.2) | >32 (9.4) | >32 (9.1) |
| | KP3 | 19 ± 4 (4.2) | 32 (15.2) | 14 ± 2 (2.9) | 0.5 (0.5) |
| | KP4 | >32 (7.1) | 32 (15.2) | 16 (4.7) | >32 (9.1) |

| Pseudomonas aeruginosa | TRPA108 | MDR | 10 ± 6 (2.2) | 4 (1.9) | 4 (1.2) | 0.5 (0.5) |
| | TRPA111 | MDR | 10 ± 6 (2.2) | 3 ± 1 (1.4) | 3 ± 1 (0.9) | 1 (0.9) |
| | PAO1 | 19 ± 9 (4.2) | 11 ± 5 (5.2) | 6 ± 2 (1.8) | 0.5 (0.5) |

a P. aeruginosa PAO1 was used as a reference organism. WR12 and WLBU2 displayed broad activity against both types of organisms. However, the peptide WR12 was less active against K. pneumoniae strains, but cross-resistance between colistin and the engineered peptides was generally not observed.

b XDR, extensively drug resistant; PDR, pandrug resistant; MDR, multidrug resistant.
c Mean values (± standard deviation) are presented. Concentration conversions: colistin, 1 µM = 1.1 µg/ml; WLBU2, 1 µM = 3.4 µg/ml; WR12, 1 µM = 2.1 µg/ml; and LL37, 1 µM = 4.5 µg/ml.

CD analysis. Circular dichroism (CD) was performed as previously described (47). Briefly, CD measurements were taken with a Jasco spectrometer (Jasco Instruments, Lakewood, NJ) at room temperature, over the range of 185 to 260 nm, and in deionized water, various concentrations of sodium dodecyl sulfate (SDS), or 30% trifluoroethanol (TFE) in water. The mean residue ellipticities ([degree/1,000 × (degree × square centimeters)/decimeter]) for five scans per sample were plotted against the wavelength (in nanometers).

Bacterial killing and growth inhibition assays. Some features of the standard assay endorsed by the Clinical and Laboratory Standards Institute (CLSI) were modified to satisfy the objective of the present study, which was to compare a repeated sequence folded into amphipathic helical structures for the effects of length, NaCl, and pH on bactericidal activity. First, we adopted a modified CLSI bacterial killing assay identical to that previously described (47) and used two prototypical organisms, a laboratory strain of *P. aeruginosa* (PAO1) and a clinical strain of methicillin-resistant *S. aureus* (MRSA 4003) kindly provided by Jane Marsh (University of the Pittsburgh School of Medicine). Also used for the present study was a panel of 11 other clinical bacterial strains. This panel included two National Institutes of Health (NIH) reference strains, MRSA USA100 and MRSA USA300, and nine strains isolated from patients at UPMC Presbyterian Hospital consisting of MDR, XDR, and pandrug-resistant (PDR) strains of the Gram-negative pathogens *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *P. aeruginosa* (Table 1).

To test for the bactericidal activity of the various antimicrobial peptides against these index bacteria, bacterial suspensions (−10⁶ CFU/ml in 10 mM potassium phosphate buffer or phosphate buffer containing 150 mM NaCl (PBS; pH 7.2) were incubated with serial 2-fold dilutions of peptides for 1 h at 37°C. Serial broth dilutions were subsequently performed, and suspensions were plated on Luria-Bertani (LB) agar (Sigma, St. Louis, MO). Surviving colonies were enumerated the next day to determine viable bacterial counts (CFU/ml). Using GraphPad Prism 6.00 for Windows (GraphPad Software, San Diego, CA), the CFU/ml data (on a log scale) were plotted against peptide concentrations, and the minimum bactericidal concentration (MBC; the molar concentration of peptide reducing viable bacteria within a suspension by 3 orders of magnitude) was derived from the nonlinear regression of the survival curves using GraphPad Prism 6.00. MBC values were expressed on a molar basis as averages from three independent experiments, with a lower MBC corresponding to a higher peptide potency. To determine MICs, the same assay was modified by replacing the testing condition with Mueller-Hinton broth (MHB) and adopting an incubation time of 16 to 18 h. The values of *A*₅₀₀ were then measured using a BioTek microplate reader (BioTek Instruments, Winooski, VT). The MIC was determined as peptide concentration reducing bacterial growth by at least 90%. To determine the influence of pH on activity, this assay was further modified by varying the pH (6.5, 6.0, and 7.4) in MHB. Unpaired *t* tests were performed by comparing activities at acidic and neutral pH using GraphPad Prism 6.00.

Kineti cs of bacterial killing. The procedure used for bacterial killing assays was modified as previously described (46) to determine the rate of bacterial killing by WLBU2, WR12, and LL37. The strain *P. aeruginosa* PAO1 or MRSA 4003 (−10⁶ CFU/ml) was treated with the indicated concentrations of peptide in PBS or MHB. Aliquots of 20 µl of the peptide-treated suspension were withdrawn at different times from 0 to 45 min at room temperature, serially diluted, and plated on LB agar to determine bacterial counts. Values expressed as the log CFU/ml plotted against time were representative of three to four independent experimental trials.

Mammalian cytotoxicity assays. Both hemolytic assays and MTT assays were performed. (i) Hemolytic assays were performed using red blood cells (RBCs) isolated from heparinized human blood from healthy donors (with the approval of the University of Pittsburgh Institutional Review Board) by Histopaque gradient centrifugation and then resuspended to 2% (vol/vol) in PBS, as previously described (46). To determine RBC lysis, a volume of 50 µl (1:4) of the RBC suspension was mixed with peptides at variable concentrations ranging from 0 to 10 µM to a total volume of 200 µl in a round-bottom 96-well plate. The reaction mixture was incubated at 37°C for 60 min with gentle shaking. To analyze the RBC lysis, the RBC-peptide mixture was spun at 600 × *g* for 5 min, and 80 µl of the supernatant transferred to 120 µl (1:2.5) of RBC lysis buffer (final
dilution 1:10) in a flat-bottom 96-well plate for spectrophotometric analysis. Similarly, 0 to 50 μl of untreated RBCs was diluted in RBC lysis buffer to a final volume of 500 μl (up to 1:10 dilution), and the hemoglobin suspensions were used to produce a standard RBC lysis curve. The average absorbance values of the supernatants of all samples (200 μl) in triplicates were measured by using a microplate reader at 550 nm as an indicator of hemoglobin released from lysed cells. These experiments were verified by three independent trials. (ii) Cytotoxicity against peripheral blood mononuclear cells (PBMCs) was examined by a tetrazolium-based (MTT) assay using PBMCs also isolated from human blood by Histopaque gradient centrifugation. PBMCs were treated with various peptide concentrations in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum in 96-well plates. After 6 h, the cells were washed with PBS and treated with MTT formazan (Sigma, Lakewood, NJ) at a concentration of 0.5 mg/ml and 37°C. After 4 h, the crystals were dissolved in 0.1 N HCl-isopropanol, and absorbances were determined at 570 nm using a Dynatech MR5000 (Germantown, MD) to determine the percent cytotoxicity.

RESULTS

Peptide design. Studies of our LBU peptide series established that antimicrobial peptides of 24 residues containing only two different amino acids (Arg and Val) can be designed to achieve substantial bactericidal potency (MBC/H11021/H9262 M) (47). In addition, Trp substitutions can enhance antimicrobial activity under challenging conditions (e.g., saline, divalent cations, and blood) without significant enhancement of host toxicity (46, 47). Based on these and other investigations (43, 50, 56), we hypothesized that the minimum length required for antimicrobial selectivity under physiologically relevant conditions and, therefore, the cost of production can be substantially reduced. Further, eCAPs with idealized amphipathicity can be designed using exclusively two amino acids, Arg and Trp. Hence, using computational helical wheel analyses, we designed a new peptide series (WR) of 6 to 18 residues (Fig. 1) by serial additions of one Arg in the hydrophilic face and one Trp in the hydrophobic face. As previously described, Arg and Trp were chosen because of their membrane perturbation tendencies (43, 50, 55, 56, 58–61). Amphipathic analysis revealed that the relative hydrophobic moments (Hrel), or the hydrophobic moments relative to a perfect amphipathic peptide, ranged from 0.93 (WR6) to 1.02 (WR16). Hence, because of the relative conservation of the amphipathic properties of the peptides with various lengths, it was possible to discern specifically the influence of peptide length on antibacterial activity.

CD spectroscopy. To confirm the helicity of the WR peptides and establish the relationship between length and helicity, we compared CD spectra of the WR eCAPs in aqueous and membrane mimotope solvents, TFE and SDS. In general, the peptides demonstrated random coil or disordered conformations in aqueous solvent as indicated by the broad minimum peak at 200 nm (Fig. 2A to F). Interestingly, as peptide length reaches 18 residues, this minimum at 200 nm is shifted to a weaker but broad peak between 200 nm and 220 nm, indicating the formation of an ordered structure (Fig. 2G). Strong ordered conformations become evident in TFE (molar ellipticities < −5,000 at 204 and 222 nm).
and increasing concentrations of SDS (6 and 30 mM) (molar ellipticities < -5,000 at 208 and 222 nm). The maximum at 190 nm and strong minima at 208 and 222 nm displayed by WR eCAPs of 10 residues in length or longer are highly indicative of helicity (62). More importantly, all of the peptides demonstrate greater levels of helical structure in SDS compared to 30% TFE. However, the minima are often broad (208 to 228 nm) for the longer eCAPs WR16 and WR18, perhaps indicating some aggregation of these longer peptides. Although the molar ellipticity at 228 nm is likely due to a high Trp content (63, 64), these bands also overlap with the characteristic broad minimum at 210 to 220 nm normally displayed by β-stranded peptides (62). Hence, the shorter peptides WR6 and WR8 demonstrated modest helicity in TFE and SDS, whereas the longer WR10, WR12, WR14, WR16, and WR18 eCAPs adopted strong helical conformations.

Relationship between length and in vitro bactericidal potency of the WR peptides. To define the minimum length for achieving optimal antibacterial activity, we first determined the bactericidal potency of the WR peptides against MRSA 4003 in phosphate buffer, and bacterial survival was determined by broth dilution assays. Bactericidal activity was optimal at 12 and 14 residues in length (with statistical significance using two-way analysis of variance [P < 0.05]) but reduced for the longer peptides WR16 and WR18.

and optimal bactericidal effects (lowest MBC) were achieved at 12 residues in length, with complete bacterial killing occurring below 1 μM. No significant gain in activity was observed beyond 12 residues, and the shorter eCAPs WR8 and WR10 were similar in potency to the longer WR18 and WR16 peptides, respectively. The next logical step was to determine how salinity at physiological concentration (150 mM NaCl in 10 mM phosphate buffer) would influence antibacterial activity. Using the same standard bacterial killing assay, we tested the WR eCAPs against both MRSA 4003 and P. aeruginosa PAO1 and examined the correlation between length and MBC (the peptide concentration required for a 3-log reduction in activity). As shown in Fig. 4, no appreciable change in activity was observed for WR12 and WR14 in the presence or absence of saline against both bacteria. However, a dual length-dependent effect of salinity was noted for the other eCAPs. Salinity dampened the activity of WR8 (MBC, 8 μM) against P. aeruginosa, but the greatest effect was observed on antistaphylococcal potency (MBC, >10 μM for WR8 and 3.0 μM for WR10). In contrast, salinity enhanced activity of WR18 against MRSA by 4-fold (from an MBC of 2 μM in PB to 0.5 μM in PBS).

Cytotoxic and antibacterial selectivity of select eCAPs in vitro. As a primary characterization of the cytotoxic property and selective potential of the WR series, we compared the hemolytic and bactericidal activities of select WR peptides in PBS, using LL37 as a reference natural AMP and WLBU2 as a reference eCAP (47, 48). Only the eCAPs WR10, WR12, and WR16 were selected for this assay because the shorter eCAPs (<10 residues) did not display substantial bacterial inactivation in PBS, whereas WR14 and WR18 demonstrated bactericidal activities similar to those of the shorter WR12 and WR16, respectively. As shown in Fig. 5, the hemolytic activity of the WR peptides increased with length, reaching up to 25% hemolysis at a concentration of 10 μM (WR16). Importantly, however, the WR peptides (WR10 (Fig. 5A) and WR12 (Fig. 5B) displayed no considerable hemolytic activity below 10 μM (<10% hemolysis), a concentration that is at least 10-fold greater than the MBC of the active peptides WR12 and WLBU2 (Fig. 5D). WR10 demonstrated the lowest hemolytic activity, but its activity against MRSA was significantly lower than that of WR12 and WLBU2. Similarly, the hemolytic effect of the host peptide LL37 was negligible, and its activity against MRSA was inhibited in PBS with complete bacterial killing achieved only at 10 μM (Fig. 5E). Hence, the eCAP WR12 displayed similar antibacterial selectivity to WLBU2, while significant activity...
against *P. aeruginosa* and negligible hemolytic effects were also demonstrated by WR10 and LL37. Further examination of cytotoxicity against nucleated cells using human PBMCs and a tetrazolium-based assay (Fig. 5F) revealed no substantial toxic effects of any of the eCAPs (WR10, WR12, WR16, and WLBU2) at up to a 20 μM peptide exposure to PBMCs for 6 h. Based on the combined antibacterial activity and cytotoxicity data, we focused most of the remaining experiments on WR12 as the shortest WR eCAP with the highest antibacterial selectivity.

**Engineered peptides promote rapid bacterial killing in PBS and MHB.** Because resistance-conferring mutations can occur during bacterial division under antimicrobial selective pressure, we first compared the rates of bacterial killing in PBS for the peptide WR12 (1.5 μM), the well-characterized eCAP WLBU2 (1.2 to 1.5 μM), and the natural host peptide LL37 (1.5 to 3 μM). As shown in Fig. 6, 99.9% of the bacterial killing occurred in the first 2 min of peptide exposure, a finding consistent with published data (46). Complete inactivation of *P. aeruginosa* was achieved by all three peptides within the first 5 min of peptide treatment in PBS (Fig. 6A), and similar killing kinetics were observed in the treatment of MRSA with WR12 and WLBU2 (Fig. 6B). In marked contrast, LL37 failed to inactivate MRSA in physiological saline. Similarly, when bacteria (*P. aeruginosa* and MRSA) were treated with peptides at their respective MICs under growth condition (in MHB), 90 to 99% killing was achieved in the first 2 min, with complete bacterial killing occurring by 30 min (Fig. 6C and D). This finding demonstrates the rapid killing kinetics of the WR series, even under robust bacterial growth conditions and salt concentrations that may inhibit natural AMP activities. This rapid kinetics of bacterial killing can reduce the potential for the development of resistance to eCAPs compared to the slower kinetics observed with standard antimicrobial agents.

**Antibacterial activities of engineered peptides under acidic conditions depend on length.** Bacterial infections associated with CF tend to be highly drug-resistant and yet are predicted to be largely susceptible to eCAPs. A recent report demonstrated that natural AMP-mediated inhibition of bacterial growth was reduced by the acidification of the airway surface liquid (ASL) in a CF pig model (65). Thus, we tested the influence of pH on eCAPs activity by determining the MIC values in MHB at various pH

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**FIG 5** Selective toxicity. Human RBCs and bacteria were independently treated in PBS with the selected peptides WR10 (A), WR12 (B), WR16 (C), WLBU2 (D), and LL37 (E) at 37°C for 1 h. The percent hemolysis was determined by spectrophotometric analysis of hemoglobin release by using a standard curve of RBC lysis, and bacteria were enumerated as described in Materials and Methods. Bacterial survival (CFU/ml) and the percent hemolysis are plotted against peptide concentrations. (F) To determine the cytotoxicity against nucleated cells, human PBMCs were treated with peptides for 6 h, and the cytotoxicity was determined by MTT assay. The data plotted are average values from three independent experimental trials.
conditions. For these assays, the eCAPs WR8, WR10, WR12, WR16, and WLBU2 and the natural AMP LL37 were compared. In general, WR12 and WLBU2 retained their activities at an acidic pH. As shown in Fig. 7, WR12 (MIC, 1.25 to 2.5 μM) and WLBU2 (MIC, 2.5 to 5 μM) displayed no statistically significant differences in activity against *P. aeruginosa* and MRSA at an acidic pH compared to a neutral pH (*P* > 0.05). The lung AMP LL37 displayed a modest (2-fold), but statistically significant, reduction in antipseudomonal activity (MIC 10 μM) at pH 6.0 and 6.5 compared to activity at the more physiologically neutral pH (MIC, 5 μM), with a *P* value of 0.002 (Fig. 7A). Similarly, the activities of the shorter peptides WR8 (MIC, 20 to 40 μM) and WR10 (MIC, 5 to 10 μM) were reduced (*P* = 0.002), whereas WR16 (MIC, 10 μM) retained its activity against both *P. aeruginosa* (Fig. 7A) and MRSA (Fig. 7B) at an acidic pH compared to a neutral pH (*P* > 0.05). Thus, these data indicate that the longer eCAPs (WR12, WR16, and WLBU2), in contrast to the natural AMP LL37 and the shorter eCAPs, are not significantly affected by changes in environmental pH.

Engineered peptides displayed broad-spectrum activity against multiple clinical isolates of MRSA, *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*. The activity of WR12 against both the index organism PAO1 and a clinical isolate of MRSA 4003 does not predict its breadth of efficacy against clinical isolates or how comparable it would be to colistin, a bacterium-derived AMP modified for clinical use against MDR and XDR pathogens.

**FIG 6** Comparative killing kinetics of WR12, WLBU2, and LL37 peptides. Bacteria, as indicated, were treated with peptides, and bacterial survival was determined by broth dilution assays at different times in PBS at the indicated concentrations (A and B) and in MHB at the corresponding MICs (C and D), as described in Materials and Methods. Rapid killing was observed for *P. aeruginosa* for all peptides, but LL37 activity was inhibited in PBS and MHB against MRSA.

**FIG 7** Influence of pH on the activity of the indicated peptides. Bacteria (*P. aeruginosa* [B] or MRSA [B]) were treated with serially diluted peptides (40 to 1.25 μM) in MHB with pH ranging from 6.0 to 7.4 at 37°C for 16 to 18 h and bacterial growth inhibition determined by spectrophotometric analysis at a wavelength of 600 nm. MICs are peptide concentrations demonstrating at least 90% of bacterial growth inhibition. The data plotted are averaged values from three independent experimental trials. *P* values were obtained by comparison with activities for the reference pH (7.4) using multiple unpaired *t* tests. An asterisk (*) adjacent to a peptide name indicates statistical significance (*P* < 0.05) for activities lower at any acidic pH. LL37 is not shown in panel B since it did not reach an MIC against MRSA.
WLBU2 with the intent to reduce the minimum length required for optimal antimicrobial activity (47, 59). Liu et al. previously examined a series of Arg- and Trp-based eCAPs for antimicrobial activity, but the peptide sequences were not optimized as idealized amphipathic motifs, and the peptide length was limited to 10 residues (75). In the present study, a sequence of 12 residues (WR12) with optimized amphipathicity and helicity (i) demonstrates broad activity against Gram-positive and Gram-negative organisms that have a high propensity to develop multidrug resistance to conventional antimicrobial agents, (ii) exhibits rapid killing of MRSA and P. aeruginosa in saline and in growth conditions (MHB), (iii) retains activity under acidic conditions (pH 6.0 to 6.5), and (iv) displays limited cytotoxic tendencies.

Most AMPs are relatively short (15 to 40 residues) by nature (69) and, within the range of 15 to 40 residues, it is intuitive that longer peptides, by virtue of greater membrane-spanning capacity, would presumably be more active. Studies of LL37 and other AMPs suggest that the consequence of reducing the length of natural peptides without affecting activity varies widely (76–78). In addition to a common amphipathic motif, the proportion of a specific amino acid in a peptide (e.g., Trp-rich AMPs) is likely to influence its antimicrobial or immunomodulatory properties (23, 79). Nevertheless, an important lesson learned from the engineering of the LBU series was that longer did not necessarily mean higher activity. In the present study, substantial in vitro efficacy was reached even at 10 residues in length (Fig. 4) in PBS (WR10). Interestingly, despite their greater length, the peptides WR14, WR16, and WR18 failed to surpass the antibacterial properties of WR12, indicating a minimum critical peptide length of 12 residues. Although the CD spectra of the longer eCAPs WR16 and WR18 show some evidence for peptide aggregation, which could account for their lower antibacterial activities, WR14 demonstrates strong helical conformations but did not display higher bactericidal effects than WR12. In addition, the results of the hemolytic assays (Fig. 5) suggest that the longer peptides (e.g., WR16) are potentially more hemolytic, although this modest hemolytic tendency was not consistent with the low cytotoxic effect on nucleated cells (human PBMCs). Hence, the eCAP WR12 appears to provide a fine balance between amphipathicity, length, and helicity to remain highly selective in saline conditions against both Gram-positive and -negative bacteria; it is the logical choice as a lead candidate peptide for further investigations.

Also important is the understanding that, compared to saline, complex biological environments may markedly reduce the potential for eCAP toxicity to mammalian cells. Previous studies of the eCAP WLBU2 indicate that mammalian cytotoxicity at high micromolar concentrations (up to 100 μM) is considerably depressed in human serum and whole blood, whereas bacterial killing was still observed at low micromolar concentrations (46, 53). These observations, although requiring further studies for generalization, suggest that it is essential to look at cytotoxicity or antimicrobial selectivity in appropriate physiologically relevant environments. Hence, further studies of the cytotoxic potential of the eCAPs WR12 and WLBU2 are warranted both in vitro and in vivo.

An important feature of AMPs is the rapid killing of microbial targets. As reported here, the WR12 killing kinetics were comparable to those of WLBU2; the peptides kill Gram-positive and Gram-negative bacteria within the first 5 min of exposure in PBS or within 30 min in MHB, with 90 to 99% reduction in viability occurring in the first 1 to 2 min under both conditions. This finding, which is consistent with previous reports (46), is an important feature because it decreases the cost of production.

DISCUSSION

We report here the design of a series of naturally inspired and rationally engineered peptides based on a cationic amphipathic helical motif, exclusively with Arg in the hydrophilic face and Trp in the hydrophobic face. Based on lessons learned from the studies of the LBU derivative WLBU2 and other structure-function correlations of host-derived AMPs (e.g., Trp- and Arg-rich peptides (43, 45–47, 54, 56, 57, 68), we demonstrated that it is possible to optimize a short series (6 to 18 residues) of cationic amphipathic peptides consisting of a succession of only two different amino acids (Arg and Trp) to reach optimal antibacterial potencies. We further explored the activity-enhancing properties of Arg and Trp and the concept of lytic base unit previously used in the design of WLBU2 to shorten the minimum length required for optimal and broad spectrum of antimicrobial activity. Reducing the length of a peptide and restricting the number of different amino acids used in a peptide sequence without affecting activity is a highly desired feature because it decreases the cost of production.

The discovery of a large number of natural cationic peptides in the last three decades has resulted in numerous structure-function studies underlining the importance of a cationic amphipathic helix as a consensus motif required for antimicrobial activity, rather than a consensus peptide sequence (69). It has become increasingly clear that, in the context of this cationic amphipathic motif, there are unique, evolution-tested (70), primary amino acid sequences that determine the specificity of microbial targets and environments (23, 46, 47, 53, 71–73). The idea of using Arg as the component of the hydrophilic face of a peptide stems from our studies of conservation of Arg in lentiviral lytic peptides of HIV-1 combined with the fact that Arg retains its positive charge in the most basic physiological environment. Further studies have shown that polyarginine provides a better membrane-transducing domain than polylysine (58, 74). Taken together with earlier studies of the role of Trp as an enhancer of membrane perturbation in the context of a peptide motif (e.g., Trp-rich AMPs indolicidin and tritrypticin) (43, 50, 54, 56), the design of the WR series was a logical next step from the engineering of the LBU series and WLBU2 with the intent to reduce the minimum length required for optimal antimicrobial activity (47, 59).
property that may reduce the potential for the development of resistant phenotypes during antimicrobial peptide treatment. The selection of resistance entails the ability of the microbial target to grow, which is precisely prevented by rapid killing. This feature, coupled with a lipid membrane that does not readily change in the microbial life cycle, renders AMPs less likely to select for resistance phenotypes when exposed to their microbial targets. However, resistance to colistin in clinical settings suggests that the development of effective antimicrobial agents requires multiple approaches to the challenges presented by MDR and XDR pathogens.

The specificity of the targets and working environments of AMPs indicates the need to design peptides for specific applications, rather than trying to adapt a naturally occurring peptide to an unnatural target or condition. One potential application for eCAPs is CF-related infections. The CF respiratory tract is a complex environment in which AMPs of the airway are inhibited, partly contributing to the establishment of chronic bacterial infections. A recent study suggests that acidification of the ASL in CF may contribute to AMP inactivation (e.g., lactoferrin) (65). AMPs interact with bacterial targets by electrostatic interactions with negatively charged phosphate groups on endotoxins and other membrane-associated phospholipids. As pH decreases, these targets are increasingly protonated resulting in a reduction in negative charges. Nonetheless, our investigation demonstrates that acidic pH had no significant effects on WR12 and longer eCAPs. Although a moderate effect on the host synthetic peptide LL37 was observed at pH 6.5, it is not clear how a pH between 6.5 and 7.0 (as seen in the swine CF ASL) would substantially contribute to AMP inactivation in the CF airway. Further, whether most AMPs of the respiratory system are sensitive to acidic conditions needs to be further investigated. Based on these results, it is not expected that the acidic environment reported in the swine CF model could significantly dampen the activity of WR12 or WLB2. This finding underscores an important difference between computationally structure-optimized eCAPs (WR12 and WLB2) and host-derived peptides (e.g., cathelicidins and defensins) in their affinity for their microbial targets.

In summary, we have successfully designed a 12-residue amphipathic peptide, WR12, with optimal antibacterial selectivity against a panel of MDR clinical isolates. Similar to WLB2, the peptide retained its activity in saline and acidic conditions. Further, WR12 and WLB2 demonstrated a broader spectrum of activity than colistin. This observation suggests that these eCAPs could potentially enhance treatment options in the face of increasing resistance to the current antibiotic regimens. We are currently investigating the mechanisms of in vivo efficacy, selective toxicity, and potential immune modulatory properties of these two lead compounds in acute and chronic respiratory infections to explore their potential for the treatment of bacterial infections.

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