HEALTH AND MEDICINE

Enhanced therapeutic index of an antimicrobial peptide in mice by increasing safety and activity against multidrug-resistant bacteria

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The rising prevalence of antibiotic resistance underscores the urgent need for novel antimicrobial agents. Antimicrobial peptides (AMPs) are potentially effective therapeutics that disrupt bacterial membranes regardless of resistance to traditional antibiotics. We have developed engineered cationic AMPs (eCAPs) with broad activity against multidrug-resistant (MDR) bacteria, but stability remains an important concern. Therefore, we sought to enhance the clinical utility of eCAP WLBU2 in biological matrices relevant to respiratory infection. A designed substitution of D-Val for L-Val resulted in increased resistance to protease enzymatic degradation. We observed multiple gains of functions such as higher activity against bacteria in biofilm mode of growth, significantly lower toxicity to erythrocytes and white blood cells compared to WLBU2, with increased safety in mice. Direct airway delivery revealed a therapeutic index of >140 for the selected enantiomer compared to that of <35 for WLBU2. The data warrant clinical exploration by aerosolized delivery to mitigate MDR-related respiratory infection.

INTRODUCTION

The increasing prevalence of antibiotic resistance has threatened to reverse the unprecedented successes in the field medicine facilitated by the utilization of safe and efficacious antibiotics over most of the past century (1–3). Antimicrobial peptides (AMPs), which rapidly disrupt bacterial membranes regardless of resistance to traditional antibiotics, constitute a potentially effective therapeutic resource. However, for nearly four decades after their initial discoveries, there are no classical AMPs in clinical use yet, although the recent surge in preclinical and clinical development to overcome the urgent antibiotic resistance problems is a strong indication of the renewed interest in AMP development compared to the previous two decades (4–7). Notably, LL37 is in clinical trials for leg ulcer and melanoma, D2A21 for burn wound infections, LTX-109 for MRSA skin infections, NP213 for fungal nail infections, and AB103 for soft tissue infections as part of a list of AMPs in clinical development.

An important consideration is that the multifunctionality of natural AMPs and other shortcomings suggest that they have not evolved as dedicated antibiotics (8). One of the most critical limitations is their contextual activity (9, 10). Natural AMPs work only under specific test conditions, indicating that their evolution is largely influenced by tissue-dependent host-pathogen interactions over millions of years (8, 11). Hence, many natural AMPs are susceptible to serum, saline, and divalent cation concentrations (10, 12). Other inhibitory factors may include acidic pH and susceptibility to protease digestion (13).

The latter property is of particular importance, as it may automatically restrict the application of AMPs only to topical use (14, 15). To overcome this deficiency, several strategies have been used to enhance their pharmacological properties related to potency, stability, and bioavailability. An appealing approach to addressing the stability problem is the incorporation of D-amino acids into AMP structure. Early indications of potential success were demonstrated in the retention of antibacterial property by D-enantiomerization of a temporin A analog, which ruled out a protein receptor in the interaction of AMPs with bacteria (16). In addition, there has been a concerted effort to modify structures of natural AMPs (16) or to engineer AMPs de novo with the goal of enhancing antimicrobial potency and overcoming current limitations (17, 18). Using the latter approach, we have developed a series of engineered cationic AMPs (eCAPs) modeled as ideal amphipathic helices (12, 19–21).

The threat of antimicrobial resistance has incited a renewed enthusiasm for clinical investigations of AMPs, which has resulted in dozens of AMPs in preclinical and clinical trials in recent years. However, development for clinical applications tends to be limited to topical use (14, 22). The lead eCAP WLBU2, which is now in phase 1 clinical trial for knee arthroplasty–associated infections, demonstrates broad-spectrum activity against the most common multidrug-resistant (MDR) bacteria known as ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species) (23), both in planktonic and biofilm modes of growth (24, 25). Composed of Arg, Val, and Trp, WLBU2 displays in vivo activity against Pseudomonas aeruginosa when administered systemically in a murine model of septicemia (9, 21) or via intraperitoneal injection in a trauma-associated infection model (26). More recently, we have demonstrated its activity against bacteria in biofilm (abiotic and biotic) mode of growth and efficacy at a minimum therapeutic dose of 0.05 mg/kg, when delivered directly into the airway in a murine model of pneumonia (19, 27). Hence, WLBU2 has shown the property to overcome many of the limitations of AMPs, with significantly superior antimicrobial potency and efficacy than most known AMPs and standard of care antibiotics.

Despite these advances in eCAP design, structural optimization to continuously improve safety and lability remains critical to enhance the therapeutic window and bioavailability for broader clinical applications of WLBU2. It is in this context that we aimed at addressing potential lability concerns with a systematic conversion of L-WLBU2 to D-WLBU2. Although D-enantiomerization could
potentially enhance the stability of AMPs, there is no universally adopted rules on how to incorporate $d$-amino acid modifications into any given AMP. Furthermore, $d$-enantiomers may lose part of their antimicrobial activities due to stereotypical changes in the original secondary structures. Our data illustrate an unexpected finding that $d$-enantiomerization of WLBU2 resulted in a novel form of the WLBU2 eCAP with reduced host toxicity while gaining enhanced stability when D8 is directly delivered into the airway. In addition, apparent were other gains of functions such as higher activity against bacteria in biofilm growth and a remarkable increase in therapeutic index. Therefore, rational $d$-enantiomerization of AMPs may provide an insightful approach to their structural optimization in overcoming major limitations to the clinical applications of AMPs.

RESULTS

$d$-enantiomerization of WLBU2

The $d$-enantiomerization of WLBU2 was tailored toward the treatment of respiratory infection induced by MDR bacterial pathogens, a potential application not yet considered for WLBU2 in its clinical development. Accordingly, we engineered three $d$-enantiomers of WLBU2 based on the predicted enzyme digestion sites of neutrophil elastase (Fig. 1, A and B), which is a common and abundant protease during respiratory infection (28). The three enantiomers are named D4, D6, and D8, as each number represents the sum of $l$-Val to $d$-Val substitutions in each structure. We designed three different $d$-structures instead of one because it was not clear and there were no obvious rules on how the shift from $l$ to $d$ would have affected antimicrobial activity. The $d$-structures were then extensively examined for their antibacterial activity, toxicity to mammalian cells, functional stability, and in vivo efficacy in a respiratory infection model.

Enantiomerization enhanced activity in biological matrices

We first compared WLBU2 with the $d$-enantiomers using a standard bacterial killing assay in broth medium [MHB2, cation-adjusted Mueller-Hinton broth (MHB)]. Activities were identical among all isomers, and complete killing was achieved at 4 $\mu$M (minimum bactericidal concentration or MBC, minimum concentration causing complete bacterial killing; Fig. 1C). Next, we examined antibacterial effects using an ex vivo bacteremia model by treating human blood inoculated with $P$. aeruginosa (PAO1) with each peptide. Two of the enantiomers

Fig. 1. Antibacterial activity of WLBU2 and its $d$-enantiomers. WLBU2 was designed so the sequences segregated into an ideal amphipathic alpha helix (A). To enhance peptide stability, $d$-enantiomers of WLBU2 (D4, D6, and D8) were engineered on the basis of the modification of valine, the digestion sites of neutrophil elastase. Each number indicates a site of $l$-Val to $d$-Val substitution (B). Using $P$. aeruginosa PAO1 [10$^6$ colony-forming units (CFU)/ml], WLBU2 was compared with the $d$-enantiomers for bactericidal activities (1-hour bacteria-peptide treatment) under various conditions: MHB2 (cation-adjusted Mueller-Hinton broth (MHB)) (C), human whole blood (D), and divalent cations (E) WLBU2 and (F) D8. Minimum growth inhibitory concentrations in varying serum concentrations were also examined (G). Arrows indicate the direction of the hydrophobic moment. The helical wheel was generated using the online software Heliquest: http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py.
(D4 and D8) completely eradicated *P. aeruginosa* from human blood at two- to four-fold lower concentrations than the parent peptide WLBU2, whereas the human AMP LL37 was completely inactive at the test concentrations (Fig. 1D). Notably, the shift to the right (Fig. 1D) in the blood environment was demonstrated by all peptides, although the complete bacterial killing at 6 μM (compared to 4 μM in MHB2) for D8 was within the margin of error for this assay. Therefore, the antibacterial activity of D8 was similar under the two test conditions for D8 (Fig. 1, C and D), in contrast to the activities of WLBU2 and its other analogs, which demonstrated a two- to six-fold reduction in activity in whole blood. On the basis of these initial results, we selected D8 as the most potent enantiomer for confirmatory experiments in subsequent studies. Divalent cations Ca\(^{2+}\) and Mg\(^{2+}\) exerted moderate inhibitory effects on both WLBU2 and D8, although the MBC remained at low micromolar concentrations (0.25 to 1 μM). (Fig. 1, E and F). Consistent with the activity in whole blood, serum had no inhibitory effects on D8, in sharp contrast to WLBU2. D8 retained the same minimum inhibitory concentration (MIC) at 4 μM in MHB regardless of the addition of serum (Fig. 1G). In addition, the activity of D8 against *P. aeruginosa* was enhanced (reduced MIC) by 16- and 32-fold compared to WLBU2, when phosphate-buffered saline (PBS) was combined with 25 and 50% serum, respectively. Thus, serum inhibited the activity of WLBU2 whether the background environment was MHB or PBS but did not affect the antimicrobial activity of D8. Next, we examined the killing kinetics of WLBU2 and D8 in PBS. At the concentration of 1 μM, both WLBU2 and D8 completely killed an inoculum of 10^6 bacterial cell/ml within the first 15 min of bacteria-peptide treatment (Fig. 2A). Colistin (also known as polymyxin E), a membrane-active antibiotic, achieved complete killing within 30 min, while the control antibiotic ceftazidime required up to 3 hours for 99% (two logs) bacterial killing (much slower killing activity compared to the cationic antimicrobials as expected).

After all these observations, a logical question is whether the modes of actions for both WLBU2 and D8 in killing bacteria are by membrane permeabilization, as do many cationic AMPs. Thus, large amount of *P. aeruginosa* [10^8 colony-forming units (CFU)/ml] was treated with WLBU2 or D8 for 1 hour and then interrogated for membrane permeabilization by propidium iodide (PI) incorporation using flow cytometry (Fig. 2B). Both WLBU2 and D8 displayed PI incorporation in a dose-dependent manner, with >90% membrane permeabilization at the highest test concentration of 4 μM. At 1 μM, however, WLBU2 and D8 demonstrated a differential PI incorporation of 42 and 68%, respectively. PI incorporation due to colistin was 80% at 4 μM but negligible (10%) at 1 μM, which is comparable to that of ceftazidime, a cell-wall synthesis inhibitor, at both test concentrations. As the d-enantiomers demonstrated similar in vitro antimicrobial activities to that of WLBU2, except for the observed superior antimicrobial activity of D8 in the presence of serum and blood, we proceeded to compare the peptides for toxicity to mammalian cells.

**Enantiomerization resulted in reduced toxicity to mammalian cells**

One of the important factors determining the effectiveness of a drug is its toxicity to mammalian cells in vitro or to the host in vivo. Because...
AMPs may exert toxic effects against tumor cells and sometimes have anticancer properties (29), toxicity against mammalian cell lines may not be indicative of toxicity to noncancerous (normal) primary cells or to the host. Thus, we compared WLBU2 and the $\alpha$-enantiomers for toxicity against freshly isolated human erythrocytes and blood leucocytes to include both nonnucleated and nucleated cells in our examination of toxicity to mammalian cells. Unexpectedly, all $\alpha$-peptides demonstrated negligible red blood cell (RBC) lysis ($\leq 1\%$ lysis; Fig. 2C) and minor effects ($\leq 15\%$ toxicity; Fig. 2D) on the white blood cells (WBCs) at a broad range of concentrations of up to $100 \ \mu M$, measured by flow cytometry using live/dead stain. WLBU2 displayed moderate effects ($20\%$ hemolysis and $30\%$ WBC toxicity at $100 \ \mu M$). The $\text{TC}_{50}$ (toxic concentration $50\%$, corresponding to $50\%$ toxicity) was unattainable at the maximum test concentration for all peptides. Thus, we defined a selectivity index at $20\%$ hemolysis ($\text{SI}_{20} = \text{TC}_{20}/\text{MIC}$ in MHB2 = 100/4 or 25) for WLBU2, which is undetermined for D8 (but significantly higher than 25) due to the minimal hemolysis ($\leq 1\%$) even at $100 \ \mu M$.

**Enantiomerization enhanced eCAP stability**

Consistent with the application to respiratory infections, we examined the influence of biological secretions on the peptides. We used *P. aeruginosa* for growth inhibition assays after the peptides were preincubated for 1, 2, and 4 hours at $37^\circ C$ in apical wash (representing epithelial lining fluid) of primary human airway epithelial cells (AECs). The $\alpha$-peptides remained unaffected by apical secretions of polarized AEC regardless of the length of exposure to the biological secretions (Fig. 3). In contrast, WLBU2 lost its bactericidal activity after the 4-hour incubation in the presence of the epithelial secretions (Fig. 3A), suggesting that the most effective enantiomer D8 is stable in the airway environment and may be a more appropriate treatment option than WLBU2 for respiratory infections. Thus, we selected D8 for further examination. Because the enantiomers were designed on the basis of the abrogation of the recognition sites of neutrophil elastase, we assessed whether D8 could be digested by human neutrophil elastase by exposure of the peptide to the protease for 5 and 24 hours. As shown in Fig. 3 (E and F), the activity of D8 was not affected by neutrophil elastase even after 24-hour treatment with the enzyme. In contrast, WLBU2 lost its activity at the test concentration of $16 \ \mu M$ after just 5-hour incubation with neutrophil elastase.

**Both WLBU2 and D8 overcome bacterial resistance to traditional antibiotics**

One of the hypotheses driving the development of AMPs is that they will remain effective against MDR bacterial pathogens when traditional antibiotic treatment fails. One way to test this hypothesis is to examine both antibiotic-susceptible (S) and antibiotic-resistant (R) clinical strains. Thus, we selected two panels of clinical strains of *P. aeruginosa* and *Acinetobacter baumannii* that are both on the critical category (priority 1) of the World Health Organization priority pathogens list of bacteria for which new antibiotics are urgently needed. Both WLBU2 and D8 were active (MIC, $\leq 8 \ \mu M$; Table 1) against most of the *P. aeruginosa* strains that have obtained antibiotic resistance through different mechanisms. In addition, the significance of the *A. baumannii* panel is that it comprises multiple pairs of colistin-S/R strains, with the resistant strains isolated from the same patients only after colistin treatment as last resort because of the recalcitrance to treatment with traditional antibiotics. Thus, all *A. baumannii* strains are carbenpenem resistant, and half are colistin-S or colistin-R (Table S1). All these clinical strains remain susceptible to both WLBU2 and D8, with WLBU2 displaying a slightly lower MIC than D8. The natural AMP LL37 was generally not effective.

**Enantiomeric optimization enhanced activity against bacteria in biofilm mode of growth**

Biofilm-related infections are frequently tolerant to treatment by traditional antibiotics, and we have recently demonstrated the remarkable antibacterial activities in biofilm mode of WLBU2 against...
ESKAPE pathogens (27). It is, therefore, important to compare WLBU2 with the lead d-enantiomer (D8) for the ability to either prevent or disrupt biofilm formation, particularly in using a biotic biofilm assay system in the context of air-liquid interface (ALI) culture model of polarized AEC. This is a physiologically important model for biotic biofilm assays consisting of bacterial growth over the apical side of well-differentiated and polarized human AEC. Toxicity of WLBU2 and D8 to primary AECs was negligible when assessed by lactate dehydrogenase (LDH) release at 32 and 64 μM (24). We used 16 μM concentration to measure activity against bacteria in biofilm mode of growth and 32 μM for assessment of the integrity of gap junctions by transepithelial electrical resistance (TEER; Fig. 4A). We compared the peptides for the ability to prevent biofilm formation after allowing the bacteria to attach to the surfaces of the AECs. Both WLBU2 and D8, similar to the natural AMP LL37, showed no permanent damage to the integrity of gap junctions formed by AECs, as TEER returned to baseline within 24 hours of peptide treatment. While WLBU2 was effective at preventing biofilm formation (1.5-log reduction in CFU; Fig. 4B), D8 enantiomer was more effective than WLBU2 with ~3-log (1000-fold) reduction in biofilm CFU. These results are consistent with differential activity of the two eCAPs after exposure to the apical secretions from polarized AEC (Fig. 3).

Enantiomeric optimization enhanced eCAP therapeutic index
In vivo data are usually lacking in most AMP studies, which is consistent with scant preclinical and clinical trials data. Therefore, it is essential to determine whether the in vitro activities of antimicrobials are translatable to in vivo efficacy. However, in vivo toxicity is critical to the assessment of a therapeutic window. Thus, we initially examined the toxic effects of the peptides on C57BL/6 mice by intratracheal (i.t.) instillation at up to 140 μg using mice at 20-g weight. While cellularity among the different doses was modestly increased compared to mock-treated mice (Fig. 5A) for D8 (70 to 140 μg or up to 7 mg/kg i.t.), the most notable difference was in the number of neutrophils at the 35-μg dosage for WLBU2, which was three times as many for WLBU2-treated mice compared to PBS (control). The WLBU2 dosage escalation did not exceed 35 μg (1.75 mg/kg of body weight) because that dose was not well tolerated and resulted in ~17% death (one of six mice died; Fig. 5B), while the remaining mice appeared initially lethargic. The highest test dose of D8 (140 μg or 7 mg/kg i.t.) resulted in no fatalities and appeared well tolerated by observation of apparent effects (e.g., mobility). Concerning in vivo efficacy, we previously showed that WLBU2 could attenuate a P. aeruginosa infection of the respiratory system effectively at a dose as low as 1 μg (0.05 mg/kg) delivered by intratracheal instillation (19). On the basis

### Table 1. Activity of WLBU2 and D8 against a panel of MDR P. aeruginosa.

| P. aeruginosa isolates | MIC (µM) | Resistance profiles |
|------------------------|----------|---------------------|
|                        | WLBU2    | D8                  |
|                        |          | 1 2 3 4 5 6 7 8 9 10 11 12 13 | |
| **P. aeruginosa isolates** | **MIC (µM)** | **Resistance profiles** |
| 230 aac(3)-IId, aadA2, cmlA1, dfrB5, OXA-4, OXA-50, PAO, tet(G), VIM-2 | 8 | 4 | R | S | R | R | R | S | R | R | R | R | R |
| 233 catB7, OXA-50, PAO | 8 | 8 | S | R | S | S | S | S | R | S | R | S | S |
| 237 catB7, OXA-50, PAO | 8 | 8 | S | S | S | S | S | S | S | S | S | S | S |
| 239 aac(6′)-Iia, aadB, aph(3′)-lc, cmlA1, dfrB5, GES-1, OXA-10, OXA-50, strA, strB, tet(G), VIM-11 | >32 | 16 | R | R | R | R | R | R | R | R | R | R | R |
| 241 aac(6′)-Iic, cmlA7, catB7, IMP-1, OXA-101, OXA-50, OXA-9, PAO, sulI | 8 | 16 | I | R | R | R | S | R | R | R | R | R | R |
| 242 aac(3)-IId, aadA2, cmlA1, dfrB5, OXA-4, OXA-50, PAO, VIM-2 | 4 | 8 | I | S | R | R | R | S | I | R | R | I | R |
| 243 aac(3)-IId, aadA2, cmlA1, dfrB5, OXA-4, OXA-50, PAO, tet(G), VIM-2 | 4 | 4 | I | S | R | I | R | S | R | R | R | R | R |
| 763 GES-19, GES-20 | 8 | 8 | R | R | R | R | R | S | R | R | R | R | R |
| 510 PDC-112 | 4 | 8 | S | S | S | R | S | S | S | S | S | S | S |
| 513 PDC-35 | 4 | 4 | I | R | R | R | S | R | R | R | R | R | R |
| 609 VEB | 8 | 8 | R | R | R | R | S | S | S | R | S | S | R |
| PA-14 | 4 | 4 | n/a | n/a | n/a | S | S | S | n/a | n/a | S | n/a | n/a |
| PAO1 | 4 | 4 | n/a | n/a | n/a | S | S | S | n/a | n/a | S | n/a | n/a |
of these previous results, we compared WLBU2 with D8 in the same respiratory model of infection. When peptides were administered intratracheally at 1 hour after bacterial inoculation (3 × 10⁶ CFU in 50 μl of PBS intratracheally), both peptides demonstrated a significant reduction in the lung burden at the 1-μg (0.05 mg/kg) treatment (Fig. 5C). While the statistically significant differences among the peptides were comparable (P < 0.0001) with respect to mock-treated mice, D8 demonstrated a noticeably lower bacterial burden in lung homogenates. Both WLBU2 and D8 reduced lung burden from a lethal to a nonlethal infection, allowing the mice to recover from the infection (19). Histopathologic examination revealed markedly reduced infiltrates of inflammatory cells for mice treated with both WLBU2 and D8 (Fig. 5D), compared to mock-treated mice. As WLBU2 is in the early phase of clinical trials for the treatment of infection related to knee arthroplasty, the results indicate a novel application of D8 via direct airway delivery, with a therapeutic index
greater than 140. Assessment of aerosolization of D8 for the treatment of respiratory bacterial infection is strongly supported by these results.

DISCUSSION
Antibiotic resistance represents an enormous public health challenge, and many of the most important MDR pathogens classified as ESKAPE organisms are often responsible for respiratory infections that are recalcitrant to traditional antibiotic treatment (1, 23). As a result, there is an urgent need for novel antimicrobials that can overcome the current mechanisms of resistance displayed by MDR pathogens. AMPs constitute an appealing alternative because they tend to display broad-spectrum activity and usually remain active against MDR pathogens, even in biofilm form (26, 27). However, the activity of AMPs is often inhibited in certain biological matrices (e.g., salt and serum) (9), which may explain the lack of activity of LL37 activity in our biotic biofilm assay and largely accounts for the lack of in vivo efficacy of most AMPs. Although newly designed AMPs are beginning to show more promising results for clinical development, in vivo studies typically indicate a potential for topical applications. Such is the case of another promising AMP, SAAP-148, which displays topical efficacy in animal models of skin infection (30). We have previously demonstrated that it is possible to overcome most of the shortcomings of natural AMPs by engineering AMPs de novo based exclusively on the principle of cationic amphipathicity, which reduces the diversity in amino acid composition and, therefore, partially (at least in theory) decreases the susceptibility to protease digestion compared to natural AMPs. Hence, the peptide WLBU2 has emerged as a lead compound with in vivo efficacy in mice using multiple models of infection (e.g., sepsis, trauma, and respiratory) (9, 19, 21, 26). Enantiomerization of WLBU2 revealed a higher potential for systemic application based on negligible effect of human blood and serum on antibacterial activity of D8, although the exploration of this gain of function in sepsis models was beyond the scope of the current study. While divalent cations had a slightly negative effect on the activity of WLBU2 and D8, complete killing in antibacterial assays in PBS combined with CaCl₂ and MgCl₂ or with serum remained at ≤1 μM. Therefore, while WLBU2 is now in advanced preclinical development and currently in phase 1 trial, our data indicate the need to effectively produce the next generation of long-lasting antimicrobials and broaden the clinical applications of current and novel antimicrobial agents.

In this study, we sought to enhance the relative stability of this well-characterized engineered AMP (WLBU2) by methodically modifying the enzymatic cutting sites of human neutrophil elastase using a rational approach to d-enantiomerization. As predicted, the D-WLBU2 peptides are functionally more stable in physiologically relevant mammalian cell secretions compared to WLBU2 (Fig. 3). The peptide with the highest content of d-Val (D8) was the most active against bacterial biofilm and displayed notably higher intratracheal maximum tolerated dose (MTD) in mice. In addition, the principle of cationic amphipathicity still applies to both D8 and WLBU2, with the same charge and hydrophobic content and similar bacterial killing kinetic property in PBS. While it is not clear how d-enantiomerization affects the secondary structure, the bacterial killing by both peptides displays a similar mode of action. However, this study reveals functional differences that are not obvious from the structural changes (L-Val to D-Val) such as differential levels of activity against bacteria under biofilm condition, better activity in serum and whole blood, and significantly lower toxicity to mammalian cells and in the mouse airway. Although a recent study demonstrated that D8 enhanced the stiffness of erythrocyte membrane (31), the data underscore the need for more in-depth biophysical studies for a greater understanding of the structural impact of l-to-d enantiomerization.

There have been previous attempts to reduce the lability of AMPs. However, these studies are rare and do not typically result in proof of concept using in vivo models (16, 32, 33). Our approach includes a rational framework for l-to-d enantiomerization using the cutting sites of a common enzyme (protease) relevant to the intended clinical application (e.g., respiratory infection treatment). The structure-function examination includes a comprehensive series of in vitro and in vivo experiments that are consistently relevant to the treatment of respiratory infection. Because WLBU2 was initially designed to form an ideal amphipathic helix, it was thought that the d-enantiomerization could adversely affect its antimicrobial function. This potential outcome is weakly supported, only when considering broad-spectrum activity (slightly lower activity of D8; Table 1 and table S1). However, both peptides still display activity at low micromolar concentrations against a distinct panel of MDR P. aeruginosa [from Centers for Disease Control and Prevention (CDC)] and A. baumannii strains (all are meropenem resistant with 50% of the strains resistant to colistin). The modification greatly favors D8 for both reduced host toxicity and increased antimicrobial activity under physiologically relevant conditions such as whole blood or exposure to serum and apical secretions of AECs. Thus, the stability of the d-enantiomers resulted in significant gains of functions that may be clinically consequence, compared to the parent eCAP WLBU2. Notably, activity in the presence of epithelial secretions or neutrophil elastase is evident because of enhanced resistance to protease digestion, consistent with a slightly differential impact on bacterial burden and inflammation in the mouse airway. In addition, d-enantiomerization enhanced activity in whole blood and serum, probably by minimizing the interactions of the d-peptide with serum proteins (34), whereas the effects of divalent cations remain similar between the two peptides.

While both peptides (WLBU2 and D8) were effective in vivo at the same dosage of 0.05 mg/kg, as shown by the lower bacterial burden and inflammation (compared to mock treatment), D8 displayed a much higher TI (≥140) than WLBU2 (<35) in the context of the intratracheal MTD. Toxicity to the airway cells is also addressed in Fig. 4 as a measure of intercellular gap junction. The tested AMPs are not toxic to AECs, as they (including LL37) only temporary changed the intercellular tight junction without actually causing epithelial cells death at high concentration. It is possible that the transitory effect of the peptide on the TEER similarly contributes to bacterial biofilm disruption. However, this action would not explain the significant (log) differences in the impact on biofilm disruption by D8 and WLBU2, because the effects on the tight junction are similar among the three peptides. Toxicity of WLBU2 to AECs was also previously assessed in another publication by other methods (e.g., LDH release), which was negligible (24). We adopted the TEER assay in this study because the extent of recovery from an initial drop in TEER appears to be more sensitive than direct toxicity assay using these polarized AECs, which showed no measurable cytotoxicity at the tested concentrations. Nonetheless, WBC toxicity assay, which (Fig. 2) already includes the use of human neutrophils and monocytes or macrophages, demonstrated minimal toxicity for D8 compared to the parent peptide. In addition, in vivo toxicity is directly reflective of the minimal toxicity of D8 to the host airways/lungs. At up to
140-fold (140 µg) of the therapeutic dose, there was negligible effect on cellularity in bronchoalveolar lavage (BAL) fluid for D8 compared to WLBU2 at only 35 µg. Together, these results demonstrate the extremely low toxicity of D8 in vitro (cells) and in vivo (mice), which addresses the concern regarding D8 toxicity to airway cells at the therapeutic dose. Considering host toxicity has been a major concern in AMP development, the results reveal a potentially novel application for D8 in treating respiratory infection in patients with pneumonia and cystic fibrosis (CF) sufferers. Comparative studies of WLBU2 and D8 alone or in combination with other antibiotics using aerosolized formulation for the treatment of respiratory infection could further delineate the therapeutic potential of these two promising eCAPs in overcoming MDR bacterial infections.

In summary, we report herein that D-enantiomerization of WLBU2 resulted in increased functional stability coupled with lower toxicity to mammalian cells. One of the D-enantiomers (D8) further displayed superior activity against bacteria under biofilm condition and a significantly increased therapeutic index in a murine model of P. aeruginosa pneumonia. This report underscores the therapeutic potential of D8 in the context of respiratory infections such as those related to CF and ventilator-associated pneumonia, which are often complicated by drug resistance. The current approach to structural optimization may provide a new hope and viable option for developing AMPs with enhanced pharmacological properties.

**MATERIALS AND METHODS**

**Reagents**

Bacterial media MHB and MHB2 were purchased from Millipore Sigma (St. Louis, MO, USA). PI and fixable live/dead stain were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Peptide stock solution was prepared autoclaved PBS (pH 7.4) and filter-sterilized unless stated otherwise.

**Bacteria**

To examine basic antimicrobial function of the D-enantiomers, P. aeruginosa PAO1 (American Type Culture Collection, BAA-47) was used as a standard strain that is well known and widely used in many microbiological laboratories. For activity against resistant strains, two clinical panels of MDR isolates were used. The P. aeruginosa panel was from CDC, and the other panel consists of A. baumannii strains (table S1) as a unique collection of colistin-S/R pairs in which each colistin-S strain became resistant (colistin-R) only after colistin treatment (29, 35). All A. baumannii clinical isolates were anonymously provided by the clinical microbiology laboratory of the University of Pittsburgh Medical Center. Bacteria were recovered from −80°C freezer stock and single colonies cultured in agar plates. Overnight, bacterial cultures were diluted at 1:50 with fresh MHB or MHB2 and cultured for additional 3 to 4 hours for exponential growth. Bacteria were centrifuged at 3000g for 10 min. The pellet was resuspended under test condition media (Sigma-Aldrich) to determine bacterial turbidity by optical density at 500 nm (OD_{500}). Bacterial OD was always adjusted to 0.5 ± 0.01 (approximately 10^{8} CFU/ml) using a spectrophotometer.

**Human AECs**

Primary human AECs were isolated from airway tissues obtained anonymously from donors who died of nonrespiratory disease, through the Center for Organ Recovery and Education (Pittsburgh, PA, USA) as previously described (19). AECs were then cultured and maintained at ALI culture until fully differentiated as polarized AECs for experiments.

**Peptide synthesis**

Synthetic WLBU2, D4, D6, and D8 were synthesized using standard Fmoc (9-fluorenylmethoxy carbonyl) synthesis protocols as previously described (10), and purification was achieved by reversed-phase high-pressure liquid chromatography on Vydac C18 or C4 columns (The Separations Group). The identity of each peptide was established by mass spectrometry (Electrospray Quatro II triple quadrupole mass spectrometer) (9).

**Antibacterial assays**

Antibacterial activity was examined by a standard growth inhibition assay endorsed by the Clinical and Laboratory Standards Institute with minor modifications (36). Bacteria were incubated with each of the indicated peptides in MHB or MHB2 for 18 hours, at which time A570 (absorbance at 570 nm) values were measured to examine growth inhibition using a BioTek microplate reader (BioTek Instruments) (37). To test the influence of serum on antimicrobial activity, fetal bovine serum was combined with either MHB or PBS. MICs were defined as the peptide concentrations completely preventing detectable growth. Peptide concentrations of up to 32 µM were evaluated for antibacterial activity. The growth inhibition assay was also modified to include whole blood instead of MHB2 or the incubation of the peptide in the presence of apical secretion of AECs (dilated in MHB2 at 1:1, v/v). For bactericidal assays, bacteria-peptide mixtures were serially diluted after 1-hour incubation and plated on LB agar at 37°C overnight. MICs or MBCs of multiple bacterial strains were analyzed using GraphPad Prism software. Bactericidal assays were modified to assess bacterial killing as a function of time and concentration of the antimicrobial agent in PBS (peptides) or broth medium (MHB, ceftazidime, which requires bacterial growth for killing).

**WBC toxicity assay**

We used flow cytometry as a direct measure of mammalian cytotoxicity to complement the hemolysis assays. Freshly isolated human peripheral blood mononuclear cells were treated with each peptide for 1 hour at 37°C and immediately washed with PBS using a round-bottom 96-well plate. Fixable blue live/dead stain from Life Technologies was added to each sample according to the manufacturer’s instructions. The cells were washed with PBS and then fixed with 4% formaldehyde (Thermo Fisher Scientific). After washing twice with PBS, the samples were stored at 4°C overnight before analysis by flow cytometry using the Guava flow cytometer. (Millipore, MA, USA). Peptide-treated samples were compared with untreated control for incorporation of the dye, and data were analyzed using FlowJo software. Percent toxicity (dye incorporation) was plotted in GraphPad (Prism software) against concentrations.

**Membrane permeabilization assay**

To examine the ability of the peptides to permeabilize bacterial membrane, PAO1 (10^{8} CFU/ml) was treated with peptide in PBS for 1 hour at 37°C. The bacteria-peptide mixture was washed with PBS and exposed to PI for 15 min at room temperature in the dark. To assess whether PI incorporation was due specifically to membrane permeabilization and not necessarily by other cell killing mechanisms, ceftazidime was used as a control for cell death not occurring by
direct membrane disruption. Broth medium (MHB) was used for ceftazidime treatment for 3 hours to allow this antibiotic to kill bacteria. The bacterial cells were also washed after PI staining and resuspended in PBS before measuring PI incorporation by flow cytometry.

**Biotic biofilm and TEER assays**

Our biotic biofilm assay was performed using transwell-based air-liquid cocultures of polarized human AECs and *P. aeruginosa* PAO1, with a starting multiplicity of infection of 30 as previously described (19, 38, 39). Polarized human AECs were inoculated with PAO1 in 50 μl of minimum essential medium at the apical side for 1 hour to allow attachment of the bacteria to the AECs in ALI culture. Each AMP (final concentration, 16 μM), diluted in 50 μl of PBS, was added for 5 hours. After a total of 6 hours, the biofilms were disrupted by sonication for 30 s (PRO DPS-20 sonicator), with subsequent serial dilution and enumeration on tryptic soy agar plates to determine CFU. For TEER measurements, the biotic biofilm assay was modified by adding the peptide without addition of bacteria. TEER was measured over time to assess the effects of each peptide, compared with PBS, on the tight junction integrity of the in vitro epithelium.

**Mouse toxicity**

All animal experiments were carried out based on a protocol (#17081148) approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh according to the National Institutes of Health (NIH) guide for the care and use of laboratory animals. Seven-week-old female wild-type C57BL/6J mice were anesthetized by isoflurane inhalation and instilled intratracheally with ~3 × 10^6 CFU of *P. aeruginosa* PAO1 in 50 μl of PBS before measuring PI incorporation by flow cytometry.

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**Murine infection model**

Seven-week-old female wild-type C57BL/6J mice were anesthetized by isoflurane inhalation and instilled intratracheally with ~3 × 10^6 CFU of PAO1 in 50 μl of PBS. One hour after exposure, each peptide was intratracheally administered at 0.05 mg/kg (predetermined in a pilot experiment as the minimum dose of peptide required to rescue mice from a lethal infection) in 50 μl of PBS. Control mice were instilled with 50 μl of PBS without peptide. Mice were monitored for signs of morbidity and euthanized at 18 hours after bacterial exposure for bacterial burden in total lung homogenates and histopathology of the lungs.

**Statistical analysis**

When applicable, data are typically expressed as means ± SD with error bars and differences between test samples compared by multiple *t* tests for statistical significance. For in vivo studies, we performed statistical comparisons between groups of mice by analysis of variance (ANOVA), which was followed by one-way ANOVA using Dunnett’s multiple comparison test or two-way ANOVA using the Bonferroni multiple comparison test. A *P* < 0.05 was considered to be statistically significant.
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