Repurposing a peptide toxin from wasp venom into antiinfectives with dual antimicrobial and immunomodulatory properties

Osmar N. Silva\textsuperscript{a,b,1}, Marcelo D. T. Torres\textsuperscript{c,d,e,f,g,h,1}, Jicong Cao\textsuperscript{i,k,l}, Elaine S. F. Alves\textsuperscript{m}, Leticia V. Rodrigues\textsuperscript{m,n,o}, Jarbas M. Resende\textsuperscript{o}, Luciano M. Lião\textsuperscript{m}, William F. Portop,q,r, Isabel C. M. Fensterseifer\textsuperscript{p,r}, Timothy K. Lu\textsuperscript{j,k,l}, Octavio L. Franco\textsuperscript{b,p,r,2}, and Cesar de la Fuente-Nunez\textsuperscript{c,d,e,f,g,h,2}

\textsuperscript{1}Departamento de Biología, Instituto de Ciencias Biológicas, Programa de pós-graduação em Genética e Biotecnologia, Universidade Federal de Juiz de Fora, 36060-900 Juiz de Fora-MG, Brazil; \textsuperscript{2}Sinova, Pós-graduação em Biotecnologia, Universidade Católica Dom Bosco, 79117-010 Campo Grande-MS, Brazil; \textsuperscript{3}Machine Biology Group, Department of Psychiatry, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; \textsuperscript{4}Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; \textsuperscript{5}Institute for Biomedical Informatics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; \textsuperscript{6}Institute for Translational Medicine and Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; \textsuperscript{7}Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104; \textsuperscript{8}Synthetic Biology Group, Massachusetts Institute of Technology, Cambridge, MA 02139; \textsuperscript{9}Department of Electrical Engineering and Computer Science, Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge, MA 02139; \textsuperscript{10}Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; \textsuperscript{11}Bioengineering Department, The Broad Institute of MIT and Harvard, Cambridge, MA 02139; \textsuperscript{12}Laboratório de Resonância Magnética Nuclear, Instituto de Química, Universidade Federal de Goiás, 74001-870 Goiânia-GO, Brazil; \textsuperscript{13}Chemistry Department, Instituto Federal Goiano, 73900-000 Posse-GO, Brazil; \textsuperscript{14}Departamento de Química, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte-MG, Brazil; \textsuperscript{15}Centro de Análises Proteômicas e Bioquímicas, Universidade, Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, 71966-700 Brasília-DF, Brazil; \textsuperscript{16}Porto Reports, 72236-011 Brasília-DF, Brazil; and \textsuperscript{17}Programa de Pós-graduação em Patologia Molecular, Universidade de Brasília, 70910-900 Brasilia, Brazil

Edited by Frederick M. Ausubel, Massachusetts General Hospital, Boston, MA, and approved September 10, 2020 (received for review June 15, 2020)

Novel antibiotics are urgently needed to combat multidrug-resistant pathogens. Venoms represent previously untapped sources of novel drugs. Here we repurposed mastoparan-L, the toxic active principle derived from the venom of the wasp Vespula lewisi, into synthetic antimicrobials. We engineered within its N terminus a motif conserved among natural peptides with potent immunomodulatory and antimicrobial activities. The resulting peptide, mast-MO, adopted an α-helical structure as determined by NMR, exhibited increased antibacterial properties comparable to standard-of-care antibiotics both in vitro and in vivo, and potentiated the activity of different classes of antibiotics. Mechanism-of-action studies revealed that mast-MO targets bacteria by rapidly permeabilizing their outer membrane. In animal models, the peptide displayed direct antimicrobial activity, led to enhanced ability to attract leukocytes to the infection site, and was able to control inflammation. Permutation studies depleted the remaining toxicity of mast-MO toward human cells, yielding derivatives with antiflammatory activity in animals. We demonstrate a rational design strategy for repurposing venoms into promising antimicrobials.

venoms | antimicrobial peptides | immunomodulatory peptides | antiinfectives | structure–activity relationship

The excessive use and misuse of antibiotics has led to increasing rates of resistance in pathogenic bacteria (1). According to the Centers for Disease Control and Prevention in 2019, 2.8 million antibiotic-resistant infections occur in the United States each year, leading to ~35,000 deaths annually (2). In addition, 19.7% (~11 million) of all global deaths in 2017 were caused by sepsis, a syndrome most commonly linked to bacterial infections and for which antibiotics are the primary treatment (3). Drug-resistant infections are not only serious on their own; secondary infections are also highly relevant and represent a major cause of death in global pandemics, being responsible for 95% of deaths during the 1918 flu pandemic (4), and also playing a role in the current COVID-19 pandemic (5).

The emergence of multidrug-resistant bacterial pathogens has coincided with a severe decline in the development and approval of new antibacterial drugs (1). Thus, there is an urgent need for novel antibiotics effective against drug-resistant bacteria. Toxins represent a previously untapped source of potential antimicrobials.

Author contributions: O.N.S., M.D.T.T., T.K.L., O.L.F., and C.d.l.F.-N. designed research; O.N.S., M.D.T.T., J.C., E.S.F.A., L.V.R., J.M.R., L.M.L., W.F.P., I.C.M.F., and C.d.l.F.-N. performed research; T.K.L., O.L.F., and C.d.l.F.-N. contributed new reagents/analytic tools; O.N.S., M.D.T.T., J.M.R., L.M.L., and C.d.l.F.-N. analyzed data; and M.D.T.T. and C.d.l.F.-N. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

Published under the PNAS license.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2012379117/-/DCSupplemental.

First published October 12, 2020.

Significance

Novel antibiotics are urgently needed to treat the ever-increasing number of drug-resistant infections. Venoms constitute a treasure trove of novel potential medicines. Here, we converted a peptide derived from venom into potent antimicrobials capable of resolving otherwise lethal infections in mice. We demonstrate that the peptide acts directly on bacteria by targeting their membrane, while also modulating the host immune response and dampening unwanted inflammation. Venom-derived molecules such as the ones described here represent an exciting new source of antibiotics.
have been shown to develop resistance at a much lower rate than when exposed to conventional antibiotics (19).

Cationic peptides displaying an α-helical structure constitute a class of AMPs found in venoms. This class consists of small amphipathic peptides with net positive charge that tend to adopt an α-helical structure upon contact with hydrophilic/hydrophobic interfaces, such as the membrane of microorganisms (9). One such example is mast-L, an AMP from the mastoparan family (20–22), derived from the venom of the social wasp Vespa lewisi (23). However, the use of this peptide as a therapeutic has been hindered by its toxicity profile, as it displays high hemolytic activity (14) and promotes massive mast cell degranulation (24, 25). Here, using rational design, we increased the therapeutic properties of mast-L, while decreasing its inherent cytotoxicity. Through this approach, novel antiinfective synthetic peptides were generated that lacked toxicity and displayed potent antimicrobial and immunomodulatory activities in clinically relevant sepsis and skin infection animal models.

**Results and Discussion**

**Design and Structural Analyses of Wasp Venom-Derived Peptides.**

Despite the promise of mast-L as an antimicrobial, its activity must be improved to surpass that of standard-of-care antibiotics in order to enter clinical trials. In order to increase its antimicrobial and immunomodulatory properties, we performed a comprehensive search and analysis of the Antimicrobial Peptides Database (26) looking for conserved motifs associated with such biological functions. The pentapeptide motif (FLPII) was identified to be conserved in the N-terminal extremity of peptides with high antimicrobial and immunomodulatory (e.g., chemotactic) activities, including brevenins (27, 28), temporins (28), vespid chemotactic peptides (29) (Fig. 1A and SI Appendix, Table S1), and mediating interactions with membranes in plant lipocalins (30). In order to design a synthetic peptide with increased biological activity, we engineered the motif FLPII into the N-terminal extremity of mast-L (Fig. 1A), as changes in this part of the sequence are known to lead to loss of toxicity toward neutral vesicles mimicking human cell membranes (SI Appendix, Table S1).

Mastoparans, like other helical AMPs, are unstructured in aqueous solution and tend to structure into an α-helix upon contact with hydrophobic/hydrophilic interfaces, such as the one between bacterial membranes and the surrounding environment. The antimicrobial activity of such AMPs correlates with their ability to undergo the structural conformation change from unstructured to helical (8), and even small modifications to their sequence impact their structural tendency and biological activity.

In order to assess whether insertion of the FLPII motif affected peptide structure, we elucidated the 3D structure of both mast-L and mast-MO through circular dichroism (CD) (SI Appendix, Fig. S1A) and of mast-MO using NMR (Table 1 and SI Appendix, Fig. S1 B and C). The CD experiments indicated that both peptides (SI Appendix, Fig. S1) presented well-defined helical structures in the presence of trifluoroethanol (TFE)/water (1:1; v/v), SDS (100 mmol L⁻¹) micelles, and phospholipid vesicles. The positive band around 192 to 193 nm and two negative bands at 208 and 222 nm (SI Appendix, Fig. S1A) observed for both mast-L and mast-MO are characteristic profiles of α-helical structures (31). The highest α-helical content values, recorded for both peptides, was in SDS micelles (75.0% for mast-L and 79.5% for mast-MO), indicating that they undergo coil-helical transition upon contact with hydrophobic/hydrophilic interfaces, such as bacterial membranes. The same behavior was observed when peptides were added to other hydrophobic/hydrophilic interfaces, such as dodecylphosphocholine (DPC) vesicles, TFE/water mixture (3:2, v/v), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC): 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) (3:1, 10 mmol L⁻¹) vesicle.

The α-helical structure of mast-MO was confirmed by 2D-NMR spectroscopy in deuterated SDS-d25 micelles (100 mmol L⁻¹). The 1H resonance assignments were performed by simultaneous analyses of the TOCSY and NOESY contour maps (SI Appendix, Fig. S1 and S2), as previously described (32). The secondary structure of the peptides was predicted by chemical-shift index (CSI) using NMRView, and the experimental chemical shifts of Hα, Cα, HN, and N. CSI showed a well-defined α-helical structure throughout the whole sequence (between Leu³ and Leu¹⁴ residues) of mast-L and the maintenance of this helical behavior in mast-MO even with the addition of the pentapeptide motif (between Ile³ and Ile¹⁴ residues). We confirmed the α-helical structure using the TALOS+ software, in which we estimated the dihedral angles, ϕ (φ) and ψ (ψ).
Table 1. Cytotoxic activity profile of mast-MO and mast-L

| Cell line   | Mast-L | Mast-MO | LL-37 | Gentamicin | Imipenem |
|-------------|--------|---------|-------|------------|-----------|
| hRBCs       | 7      | 400     | 87    | >400       | >400      |
| HEK293      | 50     | 25      | NT    | NT         | NT        |
| L929        | 10     | >400    | 95    | >400       | >400      |
| RAW 264.7   | 10     | >400    | 100   | >400       | >400      |

The cytotoxicity of the peptides was evaluated against human red blood cells (hRBCs), murine fibroblasts (L929), murine macrophages monocytes (RAW 264.7), and human embryonic kidney (HEK293) cells. Results indicate the LC100, which is the concentration at which 100% of cells are killed. NT, not tested.

Fig. 2. Antimicrobial activity and mechanism of action of wasp venom-derived peptide. (A) Antimicrobial activity of mast-L, mast-MO, and standard-of-care antibiotics gentamicin and imipenem against 15 clinically relevant pathogens. (B) Cytoplasmic membrane depolarization effects of mast-MO against eight different gram-negative bacterial strains (Fig. 2B), against which we had observed antimicrobial activity (SI Appendix, Fig. S2). On the other hand, mast-MO potently permeabilized the outer membrane of the same set of eight bacterial strains (Fig. 2C and SI Appendix, Fig. S4), as determined by increased fluorescence of the lipophilic dye NPN in the NPN assay, indicating that this is one of the mechanisms of action of the peptide. NPN generates weak fluorescence in aqueous environments and is unable to permeate bacterial membranes unless they are damaged, but its fluorescence increases upon contact with lipidic environments, such as the interior of the lipid bilayers of bacterial membranes, therefore indicating that the membrane has been permeabilized and is thus compromised.

Next, we assessed whether mast-MO was capable of synergistically interacting with different classes of commonly used antibiotics.
First, we screened 12 antibiotics with different mechanisms of action against three bacterial strains (colistin-resistant E. coli AIG222, Acinetobacter baumannii ATCC19606 and Pseudomonas aeruginosa PA01) (SI Appendix, Fig. S5). Then, we selected four antibiotics with moderate to high activity against each of the selected strains and screened, in checkerboard assays, serial dilutions of the antibiotics in the presence of increasing concentrations of mast-MO (SI Appendix, Fig. S6). Mast-MO presented its highest synergistic interactions (fractional inhibitory concentration index ∼0.1) when combined with antibiotics that block protein synthesis, such as kanamycin, chloramphenicol, and gentamicin, against colistin-resistant E. coli and A. baumannii cells (SI Appendix, Fig. S6). We speculate that mast-MO permeabilizes the outer membrane, thus allowing these antibiotics to penetrate into the cell reaching their target (i.e., blocking the protein synthesis machinery).

Once we elucidated the synergistic interactions of mast-MO with a range of antibiotics, in addition to the mechanisms by which the peptide targeted bacterial cells, we evaluated the effect of the motif insertion on peptide stability and cytotoxicity against mammalian cells. Mast-MO was as stable as mast-L (SI Appendix, Fig. S7) when exposed to proteolytic enzymes contained in fetal bovine serum. Mast-MO did not present hemolytic activity in human red blood cell assays at the concentration range tested (1 to 400 μmol L\(^{-1}\)) but it was toxic at 25 μmol L\(^{-1}\) against the human embryonic cell line HEK293, whereas mast-L was both hemolytic and toxic toward the different human and murine cell lines tested at 7 to 50 μmol L\(^{-1}\) (Table 1). Interestingly, mast-MO was not cytotoxic when incubated with mouse cell lines, such as murine fibroblasts (L929 cells) and murine macrophages (RAW 264.7 cells) (Table 1). Therefore, we proceeded to test the antinfective activity of mast-MO in preclinical mouse models. First, we analyzed the toxicity of a single dose of either mast-L or mast-MO administered peritoneally to mice in order to determine the range of concentrations to be used in subsequent animal experiments. We also tested the hemolytic activity of mast-MO (up to 200 μmol L\(^{-1}\)) against murine red blood cells (SI Appendix, Table S5) prior to conducting animal experiments.

In preliminary mouse experiments, mast-L was highly toxic at concentrations higher than 10 mg kg\(^{-1}\), whereas mast-MO lacked toxicity (SI Appendix, Table S4), in line with our results using mouse cell lines (Table 1). Based on these results, we used concentrations ranging from 1 to 10 mg kg\(^{-1}\) of mast-MO and 0.5 to 5 mg kg\(^{-1}\) of mast-L, which are not cytotoxic, in subsequent experiments.

To test whether the FLPII motif within the mast-MO sequence led to increased immunomodulatory and chemotactic properties with respect to mast-L, we performed ELISA experiments using the peritoneal fluid collected from mice (see for example, Fig. 4A). We infected C57BL/6 mice with both gram-negative and gram-positive pathogens and administered either mast-L or mast-MO intraperitoneally. For mice infected with E. coli ATCC8739, we monitored the peritoneal cavity to detect and quantify the release and presence of several immune mediators (Fig. 3E). Mast-MO–treated groups exhibited a pronounced ability to induce leukocyte migration to the site of infection, a key step into resolving infections, compared to the mast-L–treated group (Fig. 3B). This effect was similar to the positive control group of mice treated with tranalgulatinase (TGA) (Fig. 3B). Whereas mast-L did not significantly induce leukocyte recruitment over the time period tested, mast-MO stimulated recruitment at all time points with a peak of ∼10\(^{10}\) observed 3 h postinfection (Fig. 3B).

After 24 h, the leukocyte recruitment levels in mast-MO–treated mice dropped to ∼10\(^{7}\), likely attributed to the resolution of the infection by the peptide (Fig. 4B and C). These results are consistent with the intrinsic ability of the pentapeptide motif FLPII to act as a chemoattractant (Fig. 1A), in line with our initial design, and highlight the immune boosting capability of our designed peptide.

We also longitudinally tracked for 48 h the concentration of IL-12, a protein produced mainly by macrophages and neutrophils in response to antigenic stimulation, and the proinflammatory cytokine TNF-α. Mast-MO and mast-L repressed both proinflammatory cytokines, in addition to IL-6, thus showing their ability to repress inflammation in vivo (Fig. 3 C–F). We monitored, via ELISAs, the cytokine profiles present within the peritoneal cavity of mice infected with E. coli ATCC8739 and S. aureus ATCC29213 (Figs. 3E and 4F, respectively) and treated with a single dose of peptide. Treatment with peptides in both cases led to a significant decrease in the inflammatory response within the peritoneal fluid resulting from the bacterial infection, thus highlighting the potential of the peptides as antiinflammatory agents. These results confirmed that mast-MO appeared to control inflammation in addition to acting as a chemoattractant for leukocytes. Overall, our data suggest that mast-MO helps balance host responses by reducing the prolonged production of proinflammatory mediators while enhancing the activity and recruitment of leukocytes, which are important for baseline immune function.

**Synthetic Peptide Mast-MO Confers Protection to Mice Against Lethal Septicemia Caused by Bacterial Infections.** To determine the antiinfective activity of mast-MO in a preclinical mouse model, we tested its ability to kill pathogenic and drug-resistant E. coli and S. aureus strains in a systemic sepsis infection mouse model (Fig. 4). We first determined the minimal bacterial load resulting in a lethal dose (LD\(_{100}\)) 24 h postinjection. The following LD\(_{100}\) were determined: ∼2 × 10\(^{5}\) for E. coli ATCC8739 and E. coli 1812446 and ∼2 × 10\(^{6}\) for S. aureus ATCC29213 and S. aureus (MRSA) ATCC33591. Mice were infected with the LD\(_{100}\) of bacteria and treated 3 h postinfection with either mast-L (1 and 5 mg kg\(^{-1}\)) or mast-MO (1, 5, and 10 mg kg\(^{-1}\)), which were also administered intraperitoneally. Mice infected with E. coli ATCC8739 and clinical isolate E. coli 1812446 (KPC\(^{+}\)) and subsequently treated with mast-L (5 mg kg\(^{-1}\)) displayed decreased survival compared to groups treated with mast-MO (5 and 10 mg kg\(^{-1}\)) (Figs. 4B and C and 5E and F, respectively). Overall, mice treated with mast-MO exhibited 80% survival, whereas those treated with mast-L displayed 40 to 60% survival. Mouse groups treated with mast-L at 10 mg kg\(^{-1}\) had to be killed due to observed toxic effects when the peptide was injected systemically. Conversely, mice treated with mast-MO (10 mg kg\(^{-1}\)) were completely protected from infection by S. aureus ATCC29213 (Fig. 4D and E), whereas those treated with mast-L (5 mg kg\(^{-1}\)) had 1 to 5 mg kg\(^{-1}\) survival. Finally, mice infected with S. aureus MRSA ATCC33591 and treated with mast-MO (10 mg kg\(^{-1}\)) exhibited 80% survival (Fig. 4H and J). Only 60% of mice infected with S. aureus MRSA ATCC33591 and treated with mastoparan-L (5 mg kg\(^{-1}\)) survived. Once again, mice treated with 10 mg kg\(^{-1}\) of mast-L had to be killed.

In summary, in all systemic infection experiments performed, 10 mg kg\(^{-1}\) of mast-MO led to significant reduction of bacterial counts and high survival rates, comparable to standard-of-care antibiotics, such as the aminoglycoside gentamicin and the β-lactam imipenem, which are usually administered at 1 to 30 mg kg\(^{-1}\) in mouse models (35). Our results further confirmed in vivo that mast-MO did not present toxicity in the mouse model tested, whereas the wasp venom peptide mast-L led to toxicity when injected systemically at 10 mg kg\(^{-1}\). Mast-MO, even at relatively low doses (10 mg kg\(^{-1}\)), proved to be an excellent antiinfective agent, leading to complete inhibition of otherwise lethal bacterial infections caused by drug-resistant and clinically relevant gram-negative and gram-positive pathogens. Our in vivo data also demonstrated the dual activity of our engineered synthetic peptide mast-MO to both modulate the immune response and directly kill bacteria (Fig. 5A).

**Rational Design Yields Mast-MO Derivatives Lacking Toxicity against Human Cells and with Increased Antimicrobial and Antiinfective Activity in a Murine Model.** In order to deplete the remaining toxicity observed in mast-MO toward certain human cells (e.g.,...
HEK293) (Table 1), we designed and synthesized a permutation library of 59 novel mast-MO derivatives (SI Appendix, Table S6) composed of single amino acid permutations of the FLPII motif (Fig. 5B). The FLPII motif was chosen for the permutations to preserve the natural antimicrobial activity of mast-L and also conserve the hydrophobic content inserted in the N-terminal extremity for the initial design of mast-MO. Next, we assessed the antimicrobial (SI Appendix, Fig. S8) and cytotoxic activities of the mast-MO analog library against HEK293 cells (Fig. 5C). This specific cell line was chosen as it derives from human embryonic kidney cells and potential antimicrobial (i.e., AMPs, antibiotics) toxicity affects the kidneys, which are responsible for clearing such molecules (36). We also measured whether the peptides aggregated at the highest concentration tested (128 μmol L⁻¹) (SI Appendix, Table S6), since the motif inserted is composed of residues with aliphatic or aromatic side chain groups that may be prone to aggregation when in proximity within the primary peptide sequence, thus altering biological activity. Minor changes to the first five residues inserted in the mast-MO primary sequence led to a pronounced decrease in cytotoxic activity (2.5- to 5-fold) compared to mast-MO (Fig. 5C).

The most active peptides obtained, which were not cytotoxic and did not aggregate in aqueous solution, were selected and tested in a skin scarification mouse model that mimics an epithelial abscess infection (8, 13, 37). Mice were infected topically with the gram-negative pathogen *P. aeruginosa* and subsequently treated with a single low dose (16 μmol L⁻¹) of the seven lead synthetic peptides (analogs 14, 15, 26, 33, 47, 49, and 54) (Fig. 5D–F and SI Appendix, Table S5). The peptides did not cause any side effects or adverse toxicity (i.e., hemolysis or cytotoxicity) toward mouse or human cell lines (Fig. 5E). Synthetic analogs 15 (IFLPINLKALALKL-NH₂) and 49 (FPILINLKALALKL-NH₂) displayed increased antiinfective activity compared to their parent peptide mast-MO (Fig. 5E). Analog 15 is very similar to mast-MO as the permutation used to generate this molecule involved insertion an Ile residue at the beginning of the...
Fig. 4. Antiinfective activity of mast-MO against gram-negative and gram-positive pathogens in a lethal sepsis infection model. (A) Mice were infected with bacteria, treated with each peptide or antibiotic, and monitored for 8 d. Survival and cell counts after 24 h, respectively, are shown for E. coli ATCC8739 (2 × 10⁴ CFU/mL inoculum) (B and C), S. aureus ATCC29213 (2 × 10⁹ CFU/mL inoculum) (D and E), E. coli 1812446 (KPC⁺) (2 × 10⁴ CFU inoculum) (F and G), and S. aureus (MRSA) ATCC33591 (2 × 10⁹ CFU inoculum) (H and I). Mice were treated intraperitoneally with 1, 5, and 10 mg kg⁻¹ of mast-L, mast-MO, or 10 mg kg⁻¹ of the antibiotics gentamicin and imipenem. PBS was used as a control for the untreated group of mice. Treatments were administered 3 h postinfection. Data are expressed as the mean ± SD. Statistical analysis was performed using Bonferroni test. *P < 0.1, **P < 0.01, ***P < 0.001 compared to the untreated control.
Fig. 5. Permutations of the conserved motif lead to mast-MO variants that lack toxicity against human cells and display antinfective activity in a mouse model. (A) Schematic showing the dual immunomodulatory and antimicrobial activities of mast-MO demonstrated in this work. The peptide killed bacterial cells by destabilizing their outer membrane and displayed immunomodulatory properties by attracting leukocytes to the site of infection and anti-inflammatory activity by repressing proinflammatory mediators (e.g., TNF-α and IL-12). Despite these therapeutic properties, mast-MO retained some cytotoxicity toward human cells at concentrations immediately above its antimicrobial and immunomodulatory activities, hindering its use as a potential therapy in humans. (B) Design of mast-MO derivatives through permutation of its FLPII motif. (C) Cytotoxic activity of mast-MO and its permutation variants against human embryonic kidney cells (i.e., HEK293). (D) Schematic of the experimental design. Briefly, the back of mice was shaved, and an abrasion was generated to damage the stratum corneum and the upper layer of the epidermis. Subsequently, an aliquot of 50 μL containing 5 × 10^7 CFU/20 μL of P. aeruginosa in PBS was inoculated over each defined area. One day postinfection, peptides (16 μmol L^{-1}) were administered to the infected area. (E) Mouse body weight measurements throughout the experiment were taken and normalized by the body weight of noninfected mice as a proxy for potential toxicity. (F) Antinfective activity of the permutation-based synthetic analogs compared to mast-MO-treated and negative control groups. Briefly, mice were anesthetized with isoflurane, had their backs shaved, and a superficial linear skin abrasion was made with a needle in order to damage the stratum corneum and upper layer of the epidermis. A bacterial load 1 × 10^7 CFU/20 μL of bacteria (in 20 μL) in PBS was inoculated over each defined area containing the scratch with a pipette tip. One day after the infection, a single dose of each peptide (16 μmol L^{-1}) was administered to the infected area. Six animals per group were killed and the area of scarified skin was excised 2 d postinfection, homogenized using a bead beater for 20 min (25 Hz), and serially diluted for CFU quantification (statistical significance was determined using two-way ANOVA followed by Dunnett’s test, ***p < 0.001).
sequence while maintaining the last four residues of mast-MO. On the other hand, analog 49 presented all its aliphatic residues next to each other in positions three to five of the added motif, contrary to mast-MO that has a leucine residue in position two. Overall, we designed several bactericidal mast-MO analogs by permuting the 5 amino acid residues that compose the FLPII and two of these analogs presented high antiinfective activity with a single dosage in a skin abscess formation mouse model at low micromolar concentration (16 μmol L⁻¹), comparable to the most active antiinfective peptides (8, 13).

Conclusions
Multidrug-resistant pathogens are becoming increasingly prevalent and no new classes of antibiotics have been discovered for decades. Therefore, strategies enabling the discovery of new strategies for treating infections are urgently needed. Toxins from the venom of insects and arthropods are rich in antimicrobials that represent an untapped source of template molecules for the design of novel drug candidates. Here, we leverage a sequential rational design strategy to convert a highly toxic peptide derived from wasp venom into nontoxic synthetic derivatives with drug-like properties and antiinfective activity in preclinical animal models. Initially, we engineer into mast-L a pentapeptide motif highly conserved in existing chemotactic and antimicrobial peptides. In relation to mast-L, the resulting synthetic peptide, mast-MO, presented enhanced antimicrobial activity by destabilizing the bacterial outer membrane, and exhibited immunomodulatory properties by increasing leukocyte migration to the infection site and repressing proinflammatory factors, crucial to allow infection clearance. Multifunctional antimicrobial and immunomodulatory actions of mast-MO enable it to directly kill bacterial pathogens and selectively enhance host immune responses to clear infections while maintaining control of the inflammatory response. Multifunctional antimicrobials, such as the ones presented here, represent a new paradigm for treating infections by targeting both bacteria and the host. Moreover, the peptide did not induce any apparent immunotoxicities in any of the mouse models tested. Our study is an example of how leveraging template-based design strategies coupled with sequential rational design approaches can be used to modulate multiple biological functions leading to promising antimicrobial leads. We envision that the principles and approaches exploited here can be applied to better understand the role of motifs and sequence determinants underlying the immunomodulatory and antimicrobial activities of these molecules, and to successfully repurpose venom peptides into potential therapeutics.

Materials and Methods
Peptide Synthesis. Mast-L, mast-MO, and LL-37 were purchased from Shanghai Hanhong Chemical and mast-MO analogs were purchased from Biopolymers (Massachusetts Institute of Technology). The peptides were synthesized using the solid-phase peptide synthesis and N-9-fluoromethyloxycarbonyl (Fmoc) strategy and purified by HPLC (HPLC). Peptide purity used in biologic assays was higher than 95%.

CD Spectroscopy. The CD experiments were performed to determine the ideal conditions for carrying out the NMR experiments, including conformational preferences and the stability ratio at different pH and media concentrations. Mast-L at 70 μmol L⁻¹ and mast-MO at 50 μmol L⁻¹ were analyzed in SDS

NMR Spectroscopy. The samples were prepared by dissolving the peptide stock solution (1 mmol L⁻¹) in 500 μL H₂O/D₂O (9/1 vol/vol) in a micellar solution containing 100 mmol L⁻¹ of SDS (SDS-d₂₀), pH 4.0 and 25 °C. All spectra were recorded on a Bruker Avance III 500 spectrometer equipped with a 5-mm broadband inverse probehead. Proton chemical shifts were referenced to trimethylsilyl-2,2,3,3-d₄-propionate sodium (TSP-d₄). The conditions for the ¹H-¹H TOCSY and ¹H-¹H NOESY experiments are detailed in SI Appendix.

Bacterial Strains and Media. Strains used included clinical isolates E. coli KPC ID 1812446, E. coli multdrug-resistant ID 2101122, and carbapenemase-producing K. pneumoniae KPC ID 1825971, as well as reference strains Bacillus subtilis ATCC6633, Enterococcus faecalis ATCC12953, S. aureus ATCC29213, MRSA ATCC35951, Streptococcus pyogenes ATCC19615, E. coli ATCC8737, K. pneumoniae ATCC13885, Proteus mirabilis ATCC25933, P. aeruginosa ATCC 15492, Salmonella enterica ATCC14028, A. baumannii ATCC19606, E. coli ATCC11775, E. coli AIG221, E. coli AIG222, E. coli BL21, K. pneumoniae ATCC133883, P. aeruginosa PA01, P. aeruginosa PA14, and S. enterica RFP. Bacteria were plated on brain heart infusion broth (BHI, HiMedia) from a frozen stock. Following 24 h of incubation of the agar plate, three isolated colonies were transferred to 1 mL of BHI. The broth culture was incubated overnight (12 to 16 h) at 37 °C with shaking.

Antibacterial Assays. MIC of peptides and antibiotics were evaluated using the broth microdilution technique in Mueller-Hinton Broth medium (MHB) with an initial inoculum of 5 × 10⁶ cells in nontreated Polystyrene microtiter plates (Corning) in accordance with Wiegand et al. (41). The MIC was interpreted as the lowest concentration of peptide or antibiotic that completely inhibited the visible growth of bacteria after 12 h of incubation of the plates at 37 °C. Each agent was tested in triplicate in at least three independent experiments. The MIC assays were also performed using the broth microdilution method in sterile 96-well polypropylene microtiter plates. Peptides were added to the plate as solutions in 8M2 minimal medium in concentrations ranging from 0 to 128 μg mL⁻¹, and the bacteria (E. coli BL21, S. aureus ATCC12600, P. aeruginosa PA01 and PA14) were grown overnight at 37 °C and inoculated at a final concentration of 5 × 10⁵ colony forming units (CFU) mL⁻¹ per well. The plates were incubated at 37 °C for 24 h and read in a plate reader at 610 nm. All assays were done in triplicate.

Bacterial Killing Assays. The bacterial killing experiments involved performing 1:100 dilutions of overnight cultures of E. coli BL21, S. aureus ATCC12600, P. aeruginosa PA01 and PA14 in the absence or presence of increasing concentrations of the samples (0 to 200 μg mL⁻¹). After 24 h of treatment, 10-fold serial dilutions were performed, bacteria were plated on LB agar plates (E. coli BL21 and S. aureus ATCC12600), and Pseudomonas Isolation Agar (P. aeruginosa PA01 and PA14) and allowed to grow overnight at 37 °C after which CFU counts were recorded. All experiments were done in triplicates.

Antibiofilm Assays. Experiments were performed as described previously. Biofilms were grown in BM2 glucose medium for 72 h at 37 °C in flow cell chambers with channel dimensions of 1 × 4 × 40 mm, as previously described by de la Fuente-Nunez et al. (42). For the treatment of preformed biofilms, bacteria were allowed to develop structured 2-d-old biofilms prior to treatment with peptides for the following 24 h. Biofilm cells were then stained using the Live/Dead BacLight bacterial viability kit (Molecular Probes) and subsequently examined using a confocal laser scanning microscope (Olympus, Fluoview FV1000). 3D reconstructions were generated using the Imaris software package (Bitplane).

Homolysis Assays. Hemolytic activity of peptides was determined by using fresh mouse red blood cells; by measuring the peptide-induced changes of the optical density (OD) at 540 nm (Víctor X, Perkin-Elmer), 100% lysis was
tetrazolium bromide (MTT) protocol was performed. Briefly, 60% of the
Bacterial Toxicity Mouse Model.

Membrane Permeabilization Assays. The membrane permeability of the mast-
MO (20 μmol L−1) was determined by using the NPN uptake assay. All of the
bacterial strains were grown to OD600 of 0.4, centrifuged (10,000 rpm at 4 °C for
3 min) and resuspended in buffer (5 mmol L−1; 1 glucose, pH 7.4). Next, 4 μL of NPN solution (0.5 mmol L−1; working concentration of 10 μmol L−1 after dilutions) was added to 100 μL of the bacterial solution in a white 96-wells plate. The background fluorescence was recorded at λex = 350 nm and λem = 420 nm. Mast-MO samples in water (100 μL solution at 1.2 μmol L−1) were added to the 96-wells plate, and fluorescence was recorded as a function of time until no further increase in fluorescence was observed (20 min).

Membrane Depolarization Assays. The cytoplasmic membrane depolarization activity of mast-MO (20 μmol L−1) was determined by the membrane potential-sensitive dye, DiSC3(5). Briefly, the bacterial strains were grown at 37 °C with agitation to the midlog phase (OD600 = 0.5). The cells were centrifuged and washed twice with washing buffer (20 mmol L−1 glucose, 5 mmol L−1 KCl, 0.5% yeast extract) and resuspended in an OD600 of 0.03-fold MIC. Plates were incubated at 37 °C for 24 h. All assays were done
ATCC19606 were chosen for the synergy assays because of their relevance as
Stability Assays.

Skin Abscess Invasion Mouse Model. P. aeruginosa strain PAO1 was used for
inducing a skin infection in the mouse model as described by Pane et al. (37). Briefly, bacteria were grown in tryptic soy broth medium. Subsequently, cells were washed twice with sterile PBS (pH 7.4, 13,000 rpm for 1 min), and resuspended to a final concentration of 1 × 107 CFU/20 μL. Female CD-1 mice (6-wk-old) were anesthetized with isoflurane and had their backs shaved and a superficial linear skin abrasion was made with a needle in order to damage the stratum corneum and upper-layer of the epidermis. An aliquot of 20 μL containing 1 × 102 CFU of bacteria in PBS was inoculated over each defined area containing the scratch with a pipette tip. One day after the infection, peptides (16 μmol L−1) were administered to the infected area. Animals were killed and the area of scarified skin was excised 2 and 4 days postinfection (100 μL per area). Animals were killed and the area of scarified skin was excised 2 and 4 days postinfection (100 μL per area) and serially diluted for CFU quantification. Two independent experiments were performed with three mice per group in each condition (n = 6 per group).

Data Availability. All study data are included in the article and supporting information.

ACKNOWLEDGMENTS. We thank Robin M. Kramer for help establishing the mouse scarification model protocol. C.d.I.F.-N. holds a Presidential Profes-
sorship at the University of Pennsylvania, is a recipient of the Langer Prize by the AIChE Foundation, and acknowledges funding from the Institute for Diabetes, Obesity, and Metabolism and the Penn Mental Health AIDS Research Center of the University of Pennsylvania. This work was also supported by Fundação de Amparo à Pesquisa do Estado de Goiás (2017/0062; Conselho de Aperfeiçoamento de Pessoal de Nível Superior, Conselho Nacional de Pesquisas (Process: 407812/2013-0); and Finan-
ciadora de Estudos e Projetos. All figures were prepared using the Biorender drawing toolkit.

1. C. de la Fuente-Núñez, M. D. Torres, F. J. Mijocía, T. K. Lu, Next-generation precision antimicrobials: Towards personalized treatment of infectious diseases. Curr. Opin. Microbiol. 37, 95–102 (2017).
2. Centers for Disease Control and Prevention, “2019 Antimicrobial Resistant Threats Report” (2019), https://www.cdc.gov/drugresistance/biggest-threats.html. Accessed 1 June 2020.
3. K. E. Rudd et al., Global, regional, and national sepsis incidence and mortality, 1990–2017: Analysis for the Global Burden of Disease Study. Lancet 395, 200–211 (2020).
4. D. M. Morens, J. K. Taubenberger, A. S. Fauci, Predominant role of bacterial pneu-
omonia as a cause of death in pandemic influenza: Implications for pandemic influenza preparedness. J. Infect. Dis. 198, 962–970 (2008).
5. F. Zhou et al., Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. Lancet 395, 1054–1062 (2020).
6. R. J. Lewis, M. L. Garcia, Therapeutic potential of venom peptides. Nat. Rev. Drug Discov. 2, 790–802 (2003).
7. N. Mookherjee, M. A. Anderson, H. P. Haagsman, D. J. Davidson, Antimicrobial host defense peptides: Functions and clinical potential. Nat. Rev. Drug Discov. 19, 311–332 (2020).
8. M. D. T. Torres et al., Structure-function-guided exploration of the antimicrobial peptide polybia-CP identifies activity determinants and generates synthetic therapeutic candidates. J. Immunol. Biol. 1, 221 (2018).
9. M. D. T. Torres, S. Sothiselvan, T. K. Lu, C. de la Fuente-Nunez, Peptide design principles for antimicrobial applications. J. Mol. Biol. 431, 3547–3567 (2019).
10. M. H. Cardoso et al., A computationally designed peptide derived from Escherichia coli as a potential drug template for antibacterial and antibiofilm therapies. ACS Infect. Dis. 4, 1727–1736 (2018).
11. E. S. Cândido et al., Short cationic peptide derived from Archaea with dual antibacterial properties and anti-infective potential. ACS Infect. Dis. 5, 1081–1086 (2019).
12. O. N. Silva et al., An anti-infective synthetic peptide with dual antimicrobial and immunomodulatory activities. Sci. Rep. 6, 35465 (2016).
13. W. F. Porto et al., In silico optimization of a guava antimicrobial peptide enables combinatorial exploration for peptide design. Nat. Commun. 9, 1480 (2018).
14. K. G. N. Oshiro et al., Computer-aided design of mastoparan-like peptides enables the generation of nontoxic variants with extended antibacterial properties. J. Med. Chem. 62, 8140–8151 (2019).
15. M. D. T. Torres et al., Decorin analogs with increased resistance to degradation and lower hemolytic activity. ChemistrySelect 2, 18–23 (2017).
16. A. Nijink, R. Hancock, Host defence peptides: Antimicrobial and immunomodulatory activity and potential applications for tackling antibiotic-resistant infections. Emerg. Health Threats J. 2, e1 (2009).
17. S. M. F. Lima et al., Antimicrobial and immunomodulatory activity of host defense peptides, clavanins and LL-37, in vitro: An endodotropic perspective. Peptides 95, 16–24 (2017).
18. W. H. Kim, H. S. Lilheiro, W. Min, Evaluation of the immunomodulatory activity of the chicken NK-Lys-Derived peptide cNK-2. Sci. Rep. 7, 45099 (2017).
19. V. Lázár et al., Antibiotic-resistant bacteria show widespread collateral sensitivity to antimicrobial peptides. Nat. Microbiol. 3, 718–731 (2018).
20. M. B. Choi, Y. Lee, The structure and antimicrobial potential of wasp and hornet (Vespidae) mastoparans: A review. Entomol. Res. 50, 369–376 (2020).
21. X. Chen et al., Evaluation of the bioactivity of a mastoparan peptide from wasp venom and of its analogues designed through targeted engineering. Int. J. Biol. Sci. 14, 599–607 (2018).
22. A. L. Hijchlie et al., Mastoparan is a membrane-lytic anti-cancer peptide that works synergistically with gemcitabine in a mouse model of mammary carcinoma. Biochim. Biophys. Acta 1858, 3195–3204 (2016).
23. T. Higashijima, S. Uzu, T. Nakajima, E. M. Ross, Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). J. Biol. Chem. 233, 6491–6494 (1988).
24. M. Moussi et al., Activation of rat peritoneal mast cells by substance P and mastoparan. J. Pharmacol. Exp. Ther. 250, 329–335 (1989).
25. T. Higashijima, J. Burnier, E. M. Ross, Regulation of cAMP and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines. Metabolic and structural determinants of activity. J. Biol. Chem. 265, 14716–14816 (1990).
26. G. Wang, X. Li, Z. Wang, APoD: The antimicrobial peptide database as a tool for research and education. Nucleic Acids Res. 44, D1087–D1093 (2016).
27. J. M. Conlon et al., Peptides with potent cytolytic activity from the skin secretions of the North American leopard frogs, Lithobates blairi and Lithobates yavapaiensis. Toxicon 53, 699–705 (2009).
28. J. Goraya et al., Peptides with antimicrobial activity from four different families isolated from the skins of the North American frogs Rana luteoventris, Rana berlandieri and Rana pipiens. Eur. J. Biochem. 267, 894–900 (2000).
29. X. Yang, Y. Wang, W.-H. Lee, Y. Zhang, Antimicrobial peptides from the venom gland of the social wasp Vespa tropica. Toxicon 74, 151–157 (2013).
30. F. Hernández-Gras, A. Boronat, A hydrophobic proline-rich motif is involved in the intracellular targeting of temperature-induced lipocalin. Plant Mol. Biol. 88, 301–311 (2015).
31. N. J. Greenfield, Using circular dichroism spectra to estimate protein secondary structure. Nat. Protoc. 1, 2876–2890 (2006).
32. K. Wütrich, NMR of Proteins and Nucleic Acids, (John Wiley and Sons, New York, 1986).
33. L. N. Irazabal et al., Selective amino acid substitution reduces cytotoxicity of the antimicrobial peptide mastoparan. Biochim. Biophys. Acta 1858, 2699–2708 (2016).
34. B. M. Souza et al., Structure-activity relationship of mastoparan analogues: Effects of the number and positioning of Lys residues on secondary structure, interaction with membrane-mimetic systems and biological activity. Peptides 72, 164–174 (2015).
35. D. Pleiter, S. C. Mansour, K. Wuerth, N. Rahanjam, R. E. W. Hancock, New mouse model for chronic infections by gram-negative bacteria enabling the study of anti-infective efficacy and host-microbe interactions. mBio 8, e00140-e17 (2017).
36. C. P. J. M. Brouwer, M. Wulfertink, M. M. Welling, The pharmacology of radiolabeled cationic antimicrobial peptides. J. Pharm. Sci. 97, 1633–1651 (2008).
37. K. Pane et al., Identification of novel cryptic multifunctional antimicrobial peptides from the human stomach enabled by a computational-experimental platform. ACS Synth. Biol. 7, 2105–2115 (2018).
38. N. Sreerama, R. W. Woody, Estimation of protein secondary structure from circular dichroism spectra: Comparison of CONTIN, SELCON, and CDSSSTR methods with an expanded reference set. Anal. Biochem. 287, 252–260 (2000).
39. N. Sreerama, R. W. Woody, On the analysis of membrane protein circular dichroism spectra. Protein Sci. 13, 100–112 (2004).
40. Y. H. Chen, J. T. Yang, K. H. Chau, Determination of the helix and beta form of proteins in aqueous solution by circular dichroism. Biochemistry 13, 3350–3359 (1974).
41. I. Wiegand, K. Hilpert, R. E. W. Hancock, Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. 3, 163–175, 10.1038/nprot.2007.521 (2008).
42. C. de la Fuente-Nüeze et al., D-Enantiomeric Peptides that Eradicate Wild-Type and Multidrug-Resistant Biofilms and Protect against Lethal Pseudomonas aeruginosa Infections. Cell Chem. Biol. 22, 196–205 (2015).
43. M. F. Powell et al., Peptide stability in drug development. II. Effect of single amino acid substitution and glycosylation on peptide reactivity in human serum. Pharm. Res. 16, 1268–1273 (1999).
44. National Research Council, Guide for the Care and Use of Laboratory Animals, (National Academies Press, Washington, DC, 8th Ed., 2011).
45. S. Navon-Venezia, R. Feder, L. Gaidukov, Y. Carmeli, A. Mor, Antibacterial properties of dermaseptin S4 derivatives with in vivo activity. Antimicrob. Agents Chemother. 46, 689–694 (2002).
46. A. Ray, B. N. Dittel, Isolation of mouse peritoneal cavity cells. J. Vis. Exp., e1488 (2010).
47. S. E. Moreno et al., IL-12, but not IL-18, is critical to neutrophil activation and resistance to polymicrobial sepsis induced by colic ligation and puncture. J. Immunol. 177, 3218–3224 (2006).