Imcroporin, a New Cationic Antimicrobial Peptide from the Venom of the Scorpion Isometrus maculates

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The pace of resistance against antibiotics almost exceeds that of the development of new drugs. As many bacteria have become resistant to conventional antibiotics, new drugs or drug resources are badly needed to combat antibiotic-resistant pathogens, like methicillin-resistant Staphylococcus aureus (MRSA). Antimicrobial peptides, rich sources existing in nature, are able to effectively kill multidrug-resistant pathogens. Here, imcroporin, a new antimicrobial peptide, was screened and isolated from the cDNA library of the venomous gland of Isometrus maculates. The MIC of imcroporin against MRSA was 50 µg/ml, 8-fold lower than that of cefotaxime and 40-fold lower than that of penicillin. Imcroporin killed bacteria rapidly in vitro, inhibited bacterial growth, and cured infected mice. These results revealed that imcroporin could be considered a potential anti-infective drug or lead compound, especially for treating antibiotic-resistant pathogens.

The war between human beings and bacteria is still going on. On one hand, conventional antibiotics defend against bacterial infection. On the other hand, antibiotic resistance fights back. Among the pathogens of concern, methicillin-resistant Staphylococcus aureus (MRSA) takes priority. From 1999 to 2005, the estimated number of MRSA-related hospitalizations in the United States more than doubled, from 127,036 to 278,203, and MRSA-related deaths averaged about 5,500 per year (range, 3,809 to 7,372) (27). Vancomycin was most commonly used in the past 2 decades to treat MRSA infections. However, vancomycin-intermediate S. aureus has emerged (1, 3, 34). Therefore, new kinds of antimicrobial agents are badly needed.

Cationic host defense peptides play an important role in the innate immune response. These peptides have potent antimicrobial activity against gram-positive and gram-negative bacteria, fungi, parasites, and some viruses (19). Such peptides can be constitutively expressed or induced by bacteria in many organisms (20, 23, 29, 38). They are widely distributed in nature, from insects and plants to highly evolved animal species with more complex immune systems (6, 7). More than 2 decades ago, these defense molecules were initially isolated from insect lymph, the skin of frogs, and mammalian neutrophil granules and were demonstrated to have antibacterial properties. Since then, interest in the distribution and application of these peptides has been escalating, leading to the discovery of more than 1,300 cationic peptides from numerous species (16, 31, 37).

Cationic antimicrobial peptides are defined as peptides of 12 to 50 amino acids with a net positive charge of 2 to 9 (5, 7, 20). Despite their small size and common physicochemical features, cationic antimicrobial peptides exhibit a range of structures. The peptides can be divided into four groups according to their secondary structures: amphipathic α-helices, amphipathic peptides with two to four β-strands, loop structures, and extended structures (7, 17, 26). Moreover, many cationic antimicrobial peptides have activity against antibiotic-resistant bacteria. As antibiotic-resistant pathogens have become a threat to human health (2, 4, 25, 36), the cationic antimicrobial peptides are one of the new strategies against infective diseases (21, 22, 31, 33).

Several cationic antimicrobial peptides have been found in scorpion hemolymph and venom (9, 15), including hadrurin (35), scorpion (10), opistoporins (30), parabutoporin (30), IsCTs (13, 28), pandinins (11), and mucroporin (12). In the present study, we isolated a cationic antimicrobial peptide, termed imcroporin, from the cDNA library of the venomous gland of the scorpion Isometrus maculates. Imcroporin showed potent growth-inhibitory activity against antibiotic-resistant gram-positive pathogens, but not gram-negative bacteria, and relatively low hemolytic activity against human erythrocytes. What is more, imcroporin killed the bacteria rapidly and cured infected mice. These results indicate that imcroporin could be considered a potential anti-infective drug or lead compound, especially for treating antibiotic-resistant pathogens.

MATERIALS AND METHODS

cDNA library construction. I. maculates scorpions were collected in Hainan Province, China. Their glands were collected 2 days after electrical extraction of their venom. Total RNA was prepared from the glands, using Trizol reagent (Invitrogen). Poly(A) mRNAs was purified with a Poly(A) Tract mRNA isolation system (Promega). A cDNA library was constructed with the Superscript plasmid system cDNA library construction kit (Gibco/BRL). cDNAs were cloned into the pSPORT1 plasmid (Gibco/BRL) and transformed into Escherichia coli DH5α. Randomly chosen cDNA clones were sequenced to obtain a reliable representation of the toxin content in the venom gland.

Screening the cDNA library with a PCR strategy. A specific primer was designed and synthesized to screen for the gene for imcroporin, which is homologous with BmKb1, an antimicrobial peptide from Buthus martensii Karsch, from the cDNA library of the venomous gland of I. maculates by a PCR method, as described previously (39, 40). The specific forward primer and the reverse primer were 5’-TCGACCCAGCGTCCG-3’ and 5’-TCTTCTCAAATCGGCAT-3’.
corresponding to the Sall adapter region of the recombinant vector and the conserved processing region of BmkB1 propeptide, respectively.

cDNA sequencing and computer analysis. The plasmids characterized as positive clones were determined by using an ABI Prism 377XL DNA se-
quencer with a universal T7 promoter primer. Sequence analysis was carried out with BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences of imcroporin homologues were retrieved from GenBank by the BlastP method (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple-sequence alignments of imcroporin proteins were carried out using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw/).

Chemical synthesis. Imcroporin was synthesized by GL Biochem Ltd. (China), with a purity of >95%.

Bacterial strains. S. aureus (AB94004), Pseudomonas aeruginosa (AB93066), E. coli (AB94012), Bacillus thuringiensis (AB92037), and Micrococcus luteus (AB93133) were purchased from the China Center of Type Culture Collection.

Antibiotic-resistant strains were obtained from the 302nd Military Hospital of Beijing, Beijing, China, and included penicillin-resistant Staphylococcus epidermidis (PSSE) P1389, MRSA P1386 and P1374, and methicillin-resistant coagulasenegative Staphylococcus (MR CNS) P1369. Penicillin-sensitive S. epidermidis (PSSE) P1111 was also obtained from the 302nd Military Hospital of Beijing, Beijing, China.

Antimicrobial assay. Antimicrobial activities and MICs were assayed by liquid inhibition growth assay in 96-well microtiter plates in a final volume of 100 μl containing microbes at a concentration of 10^5 to 10^8 CFU per ml in LB culture medium and synthetic imcroporin at final concentrations of 100, 75, 50, 20, 10, 5, and 0 μg/ml. Inhibitory growth was examined by monitoring the absorbance (optical density) at 630 nm (OD630) with a microplate reader after incubation of 37°C for 12 h with continuous shaking.

Hemolysis. To measure hemolytic activity, freshly obtained human red blood cells (2% [vol/vol, final]) were washed several times in HEPES buffer (pH 7.2) by centrifugation for 10 min at 1,200 g. The red blood cells were then incubated for 1 h in 0.9% saline with a series of concentrations of imcroporin (100, 75, 50, 40, 20, 10, 5, and 2.5 μg/ml). Hemoglobin released in the supernatant was measured at 570 nm (OD570) with a microplate reader.

Cytotoxicity. Cytotoxicity was measured by the method of 3-(4,5-dimethylthia-
zol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Sterile filtered MTT solution (5 mg/ml in phosphate-buffered saline) was stored protected from light at −20°C until it was used. HEK293T and SMMC-7721 cells, 10^4 cells/well, were seeded in 96-well plates and grown in Dulbecco’s modified Eagle’s medium-10% fetal calf serum to confluence. Imcroporin peptide was added to the wells at different concentrations (0, 1, 2.5, 5, 10, 20, 50, and 100 μg/ml). After 24 h of incubation, 10 μl of MTT solution was added to each well, and the plates were incubated for 2 to 4 h in 5% CO2 at 37°C. The plates were gently swirled for 10 min at room temperature to dissolve the precipitate. The absorbance was monitored at a wavelength of 550 nm.

Killing kinetics. In vitro time-kill curves for imcroporin, ampicillin, penicillin G, vancomycin and cefazolin were determined against S. aureus (AB94004) that was resistant to antibiotics. The bactericidal effects of imcroporin were tested at one, two, and five times the MIC; ampicillin at five times the MIC; vancomycin at five times the MIC; and penicillin G at five times the MIC. Overnight-cultured S. aureus was transferred to LB and was cultured to exponential phase (OD600 approximately 0.6). Two hundred microliters of treated bacterial suspension was transferred to a sterilized 1.5-ml tube. After the tube was centrifuged at 1,000 × g for 5 min, the supernatant was removed and the pellet was resus-
pended in 400 μl of broth. After being centrifuged at 5,000 rpm for 5 min, the supernatant was transferred to a sterilized 1.5-ml tube, and the catalase activity was measured with a catalase assay kit (Boyctone, Jiangsu, China) based on measuring peroxidase activity.

Enzyme release assay. Overnight-cultured S. aureus (AB94004) was trans-
ferred to LB medium and was allowed to grow to the exponential phase. The bacterial suspension and serially diluted peptides were mixed at a ratio of 1:1 in different tubes in a final volume of 2 ml and were then incubated at 37°C with continuous shaking. At each time point, 200 μl treated bacterial suspension was transferred to sterilized tubes. After being centrifuged at 5,000 rpm for 5 min, the supernatant was transferred to a sterilized 1.5-ml tube, and the catalase activity was measured with a catalase assay kit (Boyctone, Jiangsu, China) based on measuring peroxidase activity.

In vivo activity. The antimicrobial efficacy of imcroporin was tested in the mouse model. Specific-pathogen-free KM mice weighing 20 to 25 g (half of them female) were purchased from the Hubei Research Center of Laboratory Ani-
mals. Exponential-phase S. aureus (AB94004) was diluted in 3 to 5% gastric mucin (Sigma-Aldrich) at a concentration of 10^7 times the determined 50% lethal dose (LD50), and the mice were injected intraperitoneally with 0.5 ml of this suspension. One hour after infection, mice were injected intraperitoneally with 0.9% saline alone (n = 7), with 60 mg of imcroporin/kg of body weight (n = 7), or with 60 mg/kg vancomycin (n = 7). The mice were observed twice or three times each day, and the survival rate was recorded on the seventh day of infec-
tion. Alternatively, 24 h after treatment, the mice were sacrificed and 2 ml sterile 0.9% saline was injected intraperitoneally into each mouse. The abdominal cavity was then opened, and the fluid was sampled with a pipette. Colony counts from the peritoneal fluid were determined. All animal studies were approved by the Institutional Animal Care and Use Committee at Wuhan University.

Nucleotide sequence accession number. The sequence of imcroporin has been deposited in the GenBank NCBI database (accession number FJ780499).

RESULTS

Sequence analysis. After 450 systemic clones from the cDNA library of the venomous gland of I. maculatus were screened, there were 9 clones with a positive result. Sequencing of all these positive clones revealed a novel precursor (Fig. 1), termed imcroporin, with high similarities to BmkB1, which is a disulfide bridge-free venom peptide from B. martinensis Karchs that has antimicrobial activity (39).

Imcroporin has a putative 22-residue signal peptide, identified with the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/), followed by a presumed 17-residue mature peptide and an uncommon acidic propeptide at the C terminus. The 35-residue propeptide starts with a conserved posttranslational processing signal, Gly-Arg-Arg, at positions 40 to 42. Conventionally, removal of a propeptide with such a processing signal would result in a mature peptide with C-terminal amidation, as described previously (14).

The secondary structure of the mature peptide was pre-
dicted by the SOPM method (18; http://npsa-pbil.ibcp.fr/cgi-
-bin/npsa_automat.pl?page=/NPSA/npsa_server.html). The secondary structure of the mature peptide of imcroporin is predicted to be 100% α-helix. The helical wheel is divided into two parts by a line. One part is the hydrophobic face, and the other is the hydrophilic face. This result suggests that imcroporin is a good dipolar molecule (Fig. 2).
In vitro antibacterial activity of imcroporin. The MIC of imcroporin was studied by the microdilution method. Table 1 shows that imcroporin has a special antimicrobial activity against gram-positive organisms and is potent against antibiotic-resistant pathogens. The MICs of imcroporin against various bacteria are as follows: *M. luteus* AB93113, 20 µg/ml; *B. thuringiensis* AB93066, 50 µg/ml; *S. aureus* AB94004, 20 µg/ml; and *B. subtilis* AB91021, 50 µg/ml. In contrast, *E. coli* AB94012 and *P. aeruginosa* AB92037 were insensitive to 100 µg/ml imcroporin. In addition, we tested the MIC of imcroporin against antibiotic-resistant bacteria, including MRSA, MRCNS, and PRSE. For comparison with the resistant bacteria, a sensitive bacterium, PSSE, was used as the control.

The antibiotic-resistant pathogens were clinical isolates, all of which were tested with traditional antibiotics to determine their resistance before experiments were performed. Imcroporin was effective against both the antibiotic-resistant pathogens (MRSA, MRCNS, and PRSE) and the antibiotic-sensitive pathogen (PSSE). The MIC of imcroporin for the antibiotic-sensitive pathogen P1111 was 20 µg/ml, and for the penicillin-resistant strain P1389, it was 50 µg/ml. Penicillin G was also tested as the control. The MIC of penicillin G for the penicillin-resistant strain P1389 was 1,000 µg/ml, which was about 1 order of magnitude higher than for the penicillin-sensitive strain. The MIC of imcroporin against the penicillin-resistant strain P1389 was 20-fold lower than that of penicillin G. The MIC of imcroporin against the methicillin-resistant strains P1386 and P1374 (MRSA) and P1369 (MRCNS) was 50 µg/ml. The MICs of cefotaxime for the methicillin-resistant strains were 100 to 400 µg/ml, which was about 1 order of magnitude higher than the MICs of cefotaxime for the methicillin-sensitive strains P1111 and P1389 (8 to 12.5 µg/ml), which indicated that the methicillin-resistant strains were indeed resistant to cefotaxime. The MICs of imcroporin for the methicillin-resistant strains were 20- to eightfold lower than that of cefotaxime. These data suggest that imcroporin was more effective in inhibiting the antibiotic-resistant pathogens than the traditional antibiotic drugs.

How the growth of bacteria was impacted by imcroporin can be seen in the time curve (Fig. 3A and B). The OD 600 was measured 3 to 8 h later with a microplate reader. Ampicillin, penicillin G, cefotaxime, and vancomycin were tested as controls. At the same concentration of 20 µg/ml, imcroporin inhibited *S. aureus* AB94004 growth completely, as did vancomycin, ampicillin, and penicillin G. When the concentration of imcroporin was decreased to 15 µg/ml, *S. aureus* growth was inhibited for the first 5 h. When the concentration of imcroporin was decreased to 10 µg/ml, *S. aureus* growth was barely inhibited. The inhibitory effects of imcroporin against *S. aureus* were concentration dependent. Penicillin (500 µg/ml) or cefotaxime (200 µg/ml) failed to inhibit the growth of MRSA P1386. In comparison, 50 µg/ml imcroporin could inhibit the growth of MRSA P1386. The ability of imcroporin to inhibit the growth of MRSA P1386 at different concentrations showed that the inhibitory effects of imcroporin on bacterial growth are concentration dependent.

The cytotoxicity of imcroporin against mammalian cells was tested by an MTT assay and a hemolysis assay (Fig. 4A and B). When the concentration of imcroporin reached 50 µg/ml, the hemolytic activity against human erythrocytes was lower than 50%. At this concentration, imcroporin effectively inhibited the growth of bacteria, including MRSA (P1386 and P1374). The inhibitory rates of imcroporin against SMMC-7721 and HEK293T cell lines were different. Imcroporin (100 µg/ml) could kill almost all of the SMMC-7721 cells compared to about 25% of HEK293T cells.

To study how the inhibitory effects of imcroporin on bacteria were exerted, the killing assay for imcroporin was used to test the model strain *S. aureus* AB94004. One times, 2×, and 5× the MIC of imcroporin for *S. aureus* was directly added to bacteria in the exponential phase, and the survival rates were determined by counting the viable colonies on the plate. The results showed that the number of viable colonies decreased dramatically in a very short time, and the bactericidal process started as soon as the bacteria were mixed with the peptide. An increased peptide concentration corresponded with an increased killing rate, as can be seen in Fig. 5A. This result suggested that imcroporin exerts rapid killing effects on microorganisms, which is probably the reason that it can effectively inhibit the growth of microorganisms, especially antibiotic-resistant bacteria. To further confirm the killing effects, the catalase activity of the supernatant was measured by enzyme release assay immediately after

![FIG. 2. Representation of imcroporin as a helical wheel showing the hydrophilic face and hydrophobic face.](http://aac.asm.org/content/early/2017/10/30/aac.4677/F2.large.jpg)

### Table 1. MICs of imcroporin compared to those of vancomycin, cefotaxime, and penicillin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vancomycin (µg/ml)</th>
<th>Cefotaxime (µg/ml)</th>
<th>Penicillin (µg/ml)</th>
<th>Imcroporin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. luteus</em> AB93113</td>
<td>3</td>
<td>3</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td><em>B. thuringiensis</em> AB93066</td>
<td>3</td>
<td>12.5</td>
<td>12.5</td>
<td>50</td>
</tr>
<tr>
<td><em>S. aureus</em> AB94004</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td><em>B. subtilis</em> AB91021</td>
<td>3</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td><em>E. coli</em> AB94012</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> AB92037</td>
<td>ND</td>
<td>12.5</td>
<td>ND</td>
<td>&gt;100</td>
</tr>
<tr>
<td>MRSA P1386</td>
<td>4</td>
<td>400</td>
<td>1000</td>
<td>50</td>
</tr>
<tr>
<td>MRSA P1374</td>
<td>4</td>
<td>400</td>
<td>1000</td>
<td>50</td>
</tr>
<tr>
<td>MRCNS P1369</td>
<td>4</td>
<td>100</td>
<td>2000</td>
<td>50</td>
</tr>
<tr>
<td>PRSE P1389</td>
<td>4</td>
<td>12.5</td>
<td>1000</td>
<td>50</td>
</tr>
<tr>
<td>PSSE P1111</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>20</td>
</tr>
</tbody>
</table>

*ND, not determined.*
treatment with 5× the MIC of imcroporin. In this experiment, ampicillin was used as the control. As shown in Fig. 5B, the supernatant of ampicillin-treated bacteria showed the increasing activity of catalase with time and reached a peak at 20 min. Conversely, the supernatant of imcroporin-treated bacteria harvested at each time point showed almost the same enzyme activity. These results suggested that the intracellular enzyme of S. aureus was released immediately and that the cell membrane of S. aureus degraded rapidly after the bacteria were exposed to the peptide.

**In vivo activity of imcroporin.** The in vivo activity of imcroporin was evaluated by determining the LD₅₀ of S. aureus in a mouse model. The LD₅₀ of S. aureus for mice was 6.8 × 10⁵ CFU/ml, calculated by the method of Dai et al. (12). The infective dose of bacteria was about 10 times the LD₅₀, so we chose the optimal infective dose for mice of about 5 × 10⁶ CFU/ml. The success of treatment was evaluated by the decrease of peritoneal bacterial counts in peptide-treated mice compared with untreated control mice and vancomycin-treated mice (Fig. 6A). Imcroporin decreased the bacterial counts in the mouse peritoneum by 10-fold after 24 h of treatment compared with the untreated mice.

One hour after infection, the mice were injected with a 60-mg/kg dose of imcroporin or vancomycin. The mice were inspected for 7 days. As shown in Fig. 6B, 100% survival after 7 days was found in the groups that had been treated with either 60 mg/kg of imcroporin or 60 mg/kg of vancomycin. The surviving mice were unaffected and behaved normally. These results suggested that imcroporin had biological activity in vivo.

![FIG. 3. Growth-inhibitory effects of imcroporin against S. aureus and MRSA.](image-url)

![FIG. 4. Cytotoxicity of imcroporin to mammalian cells.](image-url)
Imcroporin is an α-helix polypeptide composed of 17 amino acids with a net positive charge of 1. It is the first cationic antimicrobial peptide isolated and characterized from the scorpion *I. maculates*. In the past, few cationic antimicrobial peptides were found in scorpions, and fewer of them had potency against antibiotic-resistant bacteria. Imcroporin shows potency against antibiotic-resistant bacteria, as well as antibiotic-sensitive bacteria. The activities of the cationic antimicrobial peptides found in venom are difficult to test in mammals because of their hemolysis, so few of them have been studied for their antibacterial activities in vivo. The reduction of the number of microorganisms in a mouse model and the survival of infected mice suggest that imcroporin effectively protected the mice against microbial infection. However, imcroporin still leads to some hemolysis at a concentration of 50 μg/ml. These results suggest that imcroporin has the potential to become a new anti-infection drug.

Although the mechanism of the cationic antimicrobial peptides against bacteria is not well understood, the relationships between structure and function provide the clue to how to design the native peptide for better bioactivity (8). Increasing cationicity and α-helicity by progressively substituting neutral and acidic amino acid residues on the hydrophilic face of the α-helix, such as with IsCT (28), mucroporin (12), and Pseudin-2 (32), could increase antimicrobial activity while decreasing cytotoxicity. Compared with its toxicity to the HEK293T cell line, the cytotoxicity of imcroporin to the SMMC-7721 cell line (a type of human liver cancer cell line) is high. The selectivity of imcroporin may be attributable to the different cell membrane components of...
cancer cells and normal cells. Cancer cell membranes typically carry a net negative charge due to higher expression of anionic molecules, such as polysaccharide (>9% of the total phospholipids of membranes) and O-glycosylated mucins (24).

In summary, imcroporin is a native cationic antimicrobial peptide from the scorpion I. maculates. Cancer cell membranes typically have high potential for clinical use.

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