Synthesis and characterization of bactericidal oligopeptides designed on the basis of an insect anti-bacterial peptide

Hisako SAIDO-SAKANAKA*, Jun ISHIBASHI*, Aki SAGISAKA*, Eiichi MOMOTANI† and Minoru YAMAKAWA*¹

* Laboratory of Biological Defense, National Institute of Sericultural and Entomological Science, Tsukuba, Ibaraki 305–8634, Japan, and † Laboratory of Molecular Pathology, National Institute of Animal Health, Tsukuba, Ibaraki 305–0856, Japan

Defensin from a beetle, *Allomyrina dichotoma*, is known to have anti-bacterial activity against Gram-positive bacteria. This peptide, which comprises 43 amino acid residues, was effective against methicillin-resistant *Staphylococcus aureus*. We identified the active site of beetle defensin by measuring anti-bacterial activity against *S. aureus* of 64 overlapping 12-mer peptides with either a free carboxylate or a free amide group at their C-termini. An LCAAHCLAIGRR-NH₂ (19L–30R-NH₂) fragment showed the greatest activity of the synthetic oligopeptides. The 19L–30R-NH₂ fragment was effective against both Gram-positive and Gram-negative bacteria. CD spectra showed that the 19L–30R-NH₂ fragment formed an α -helical structure in the lipidic environment. The anti-bacterial effect of the 19L–30R-NH₂ fragment was due to its interaction with bacterial membranes, judging from the leakage of liposome-entrapped glucose. Its anti-bacterial activity was increased when certain amino acid residues were replaced. Truncated peptides having had some amino acids

removed from the N-terminus of the 19L–30R-NH₂ fragment (8–10-mer peptides) still had strong anti-bacterial activity. Deleting some amino acids from the C-terminal region of the fragment dramatically reduced activity, indicating that the C-terminal region of the 19L–30R-NH₂ fragment, i.e. RR-NH₂, is important for exerting anti-bacterial activity. The AHCLAIGRR-NH₂ (22A–30R-NH₂) fragment and its analogues exhibited about 3-fold and 9–12-fold higher activity against *S. aureus* than did the 19L–30R-NH₂ fragment, and these analogues were effective against methicillin-resistant *S. aureus* and *Pseudomonas aeruginosa* isolated from patients. These oligopeptides showed no haemolytic activity and did not inhibit the growth of murine fibroblast cells.

Key words: *Allomyrina dichotoma*, cytotoxic activity, defensin analogues, membrane permeabilization, methicillin-resistant *Staphylococcus aureus*.

INTRODUCTION

Anti-bacterial peptides are one of the important factors in the innate immunity of vertebrates and invertebrates. They have been isolated from mammals [1], amphibians [2], insects [3] and chelicerates [4]. The search for novel anti-bacterial agents has become especially urgent because of the rapid spread of antibiotic-resistant pathogenic bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-antibiotic-resistant *Pseudomonas aeruginosa*, which cause serious problems in hospitals, especially in Japan. Most anti-bacterial peptides have broad-spectrum activities and disrupt bacterial membranes via a peptide–lipid interaction, unlike the mechanism of action of antibiotics used currently [5]. Some anti-bacterial peptides have been shown to be effective against antibiotic-resistant bacteria [6–8], suggesting their potential use as therapeutic agents. The cytotoxicity and antigenicity of these peptides, however, make them unsuitable for direct therapeutic use. Thus considerable attention has been focused on modifying their size and anti-bacterial activity. D-Enantiomers of cecropin A, magainin and melittin are as active as their parent peptides [9]. Hybrid peptides of cecropin A and melittin displayed higher anti-bacterial activity and a broader spectrum than the parent cecropin A, and show no haemolytic activity [10]. Furthermore, shortened cecropin A–melittin hybrid peptides (e.g. a 15-mer peptide) were found to have strong anti-bacterial activity [11]. An analogue of indolicidin, in which five tryptophan residues were replaced with

phenylalanines, showed strong anti-bacterial activity that was free of haemolytic activity [12]. Many other truncated anti-bacterial peptides have also been synthesized [13–15].

An understanding of the relationship between the structure and activity of anti-bacterial peptides is important in the development of new agents. In general, it is thought that the biological activity of amphipathic α -helical peptides results from their ability to form ion channels through membrane bilayers [16]. A study of the relationship between the secondary structure and biological activity of model short peptides (8–22-mer peptides) comprising leucine and lysine residues indicated that an amphipathic α -helical structure is an important condition for anti-bacterial activity [17]. The α -helical region of sapecin B, identified as the active site of this peptide, showed potent antimicrobial activity [18], and its modified analogue, an 11-mer peptide composed of leucine and lysine residues, showed greater activity against micro-organisms, including MRSA [19]. Synthetic histatin analogues with enhanced amphipathicity of the helical conformation were shown to have broad, improved antimicrobial activity [20].

We have previously isolated and characterized defensin from a beetle, *Allomyrina dichotoma*. This peptide is composed of 43 amino acid residues, and has been shown to be effective against MRSA [7]. In the present study, we determined the active centre of defensin by measuring the anti-bacterial activity of 64 overlapping 12-mer peptides derived from it, and investigated the mode of action of this fragment. We also synthesized truncated

Abbreviations used: MRSA, methicillin-resistant *Staphylococcus aureus*; 19L–30R-NH₂ fragment, peptide fragment with the sequence LCAAHCLAIGRR-NH₂; 22A–30R-NH₂ fragment, peptide fragment with the sequence AHCLAIGRR-NH₂; Fmoc, 9-fluorenylmethoxycarbonyl; PEG-PS, poly(ethylene glycol)–polystyrene; CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; MIC, minimal inhibitory concentration.

¹ To whom correspondence should be addressed (e-mail yamakawa@nises.affrc.go.jp).

peptides and examined their anti-bacterial and cytotoxic activities. We found that some 9-mer analogues have greater anti-bacterial activity than the original peptide, but show no cytotoxic activity.

MATERIALS AND METHODS

Materials

9-Fluorenylmethoxycarbonyl (Fmoc) L-amino acids and Fmoc-tris(alkoxy)benzylamide-PEG-PS resins [where PEG-PS is poly(ethylene glycol)-polystyrene] were purchased from PerSeptive Biosystems. Phosphatidylglycerol (PG) from egg yolk lecithin, cardiolipin (CL) from bovine heart and phosphatidylethanolamine (PE) from *Escherichia coli* were obtained from Sigma. All other reagents were special-grade. Water was drawn from a Milli-Q system.

Peptide synthesis

The 64 overlapping 12-mer peptides, each offset by one residue, spanning the sequence of *A. dichotoma* defensin (VTCDLLSFE-AKGFAANHSLCAAHCLAIGRRGGSCERGVICRR) with either a free carboxylate or a free amide group at their C-termini were synthesized by the multi-pin technique (Chiron Mimotopes, Clayton, Victoria, Australia) [21]. Other peptides were synthesized by the solid-phase method in a 9050 Plus peptide synthesizer (Millipore). Each peptide was purified to homogeneity by the SMART system or an ÄKTA Explorer using a reverse-phase column of μ RPC C2/C18 PC 3.2/3 or of Resource RPC (1 ml) (Pharmacia, Uppsala, Sweden). The column of μ RPC C2/C18 PC 3.2/3 was eluted in 50 min at a flow rate of 0.1 ml/min, using a linear gradient from 0 to 30% (v/v) acetonitrile in water, both containing 0.05% (v/v) trifluoroacetic acid. The column of Resource RPC 1 ml was eluted in 20 min at flow rate of 2 ml/min, using a linear gradient from 0 to 25% (v/v) acetonitrile in water, both containing 0.05% (v/v) trifluoroacetic acid.

Anti-bacterial activity

Anti-bacterial activity assays were conducted in sterilized 96-well plates in a final volume of 100 μ l as follows. Samples of 90 μ l of suspensions of a mid-exponential-phase culture of bacteria in nutrient broth medium (Difco, Detroit, MI, U.S.A.) ($A_{600} = 0.001$) were added to 10 μ l of different concentrations of peptides dissolved in sterilized 10 mM sodium phosphate buffer, pH 7.0, containing 130 mM NaCl. The anti-bacterial activity of each peptide (50 μ g/ml) was examined by measuring absorbance at 600 nm after incubation for 15 h at 25 °C. The minimal inhibitory concentration (MIC) of peptides was observed 20 h after incubation at 37 °C. Cysteine-containing peptides were alkylated with 4-vinylpyridine [22,23]. The anti-bacterial activity of alkylated peptides was tested after purification by SMART using a reverse-phase column of μ RPC C2/C18 PC 3.2/3. The bacteria used were *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* JM109, MRSA and *Pseudomonas aeruginosa*. MRSA and *P. aeruginosa* were isolated from patients.

CD spectra

CD spectra were measured on a Jasco J-720 spectropolarimeter using a cell path length of 1 mm. Spectra were recorded at 25 °C in 10 mM sodium phosphate buffer, pH 7.0, containing 130 mM NaCl in the presence or absence of PG/CL (3:1; mol/mol) liposomes. Sixteen scans per sample were recorded over a

wavelength range of 190–270 nm. The baseline (liposomes without peptide) was subtracted. Unilamellar liposomes were prepared as described by Matsuzaki et al. [24]. α -Helical content was estimated as described by Greenfield and Fasman [25] and Wu et al. [26].

Liposomal membrane permeability assay

Liposomes containing trapped glucose were prepared as described by Yamada and Natori [18]. A sample of 2 μ mol of phospholipids mixed in chloroform was dried under nitrogen gas, then 0.2 ml of 0.3 M glucose was added and multilamellar liposomes were prepared using a vortex mixer. Liposomes were washed three times with 10 mM sodium phosphate buffer (pH 7.0)/130 mM NaCl to remove untrapped glucose and finally suspended in 0.6 ml of the same buffer. The liposome suspension (15 μ l) was added to different concentrations of peptides dissolved in 15 μ l of the same buffer. Mixtures were incubated for 1 h at 25 °C. Phospholipids in liposomes were calculated by measuring the amount of ester groups [27]. Glucose released from liposomes was assayed using a Glucose CII-Test kit (Wako Chemical, Osaka, Japan). α -Lactalbumin was used as a negative control.

Haemolytic activity

Erythrocytes were isolated from heparinized rabbit blood by centrifugation at 800 *g* for 10 min after washing three times with isotonic PBS (130 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄, pH 7.4) and resuspended in the same buffer. Erythrocyte solution [0.5% (v/v); final volume 100 μ l] was incubated with different concentrations of peptides for 30 min at 25 °C. Samples were centrifuged at 1000 *g* for 5 min and the absorbance of supernatants was measured at 540 nm. Lysis obtained with 1% (v/v) Triton X-100 was taken as 100%. Melittin was used as a positive control.

Cytotoxic activity

The cytotoxic activity of peptides was examined in a sterile 96-well plate using the murine macrophage cell line JA-4 [28] and the fibroblast cell line L929 [29]. Cells were cultured at 37 °C in RPM1 1640 medium (Nissui, Tokyo, Japan) containing 10% (v/v) fetal bovine serum, 100 μ g/ml streptomycin and 100 units/ml penicillin in a 5% CO₂ humidified atmosphere. Cultured cells were suspended in RPM1 medium (10⁵ cells/ml) and 100 μ l of cell suspension was transferred to culture plates. The mixture of PBS (10 μ l) containing different concentrations of peptides and Alamar Blue (10 μ l) (Iwaki, Chiba, Japan) was added to the cell suspension, which was then incubated at 37 °C for 24 h. Cell growth inhibition was determined by measuring fluorescence intensity on a Cytofluor 2300 (Millipore) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

RESULTS

Anti-bacterial activity of synthetic peptides

To determine the active site of defensin, we synthesized 64 overlapping 12-mer peptides spanning the sequence of defensin with either a free carboxylate or a free amide group at their C-termini, and studied the anti-bacterial activity of these peptides against *S. aureus*. Only one amidated peptide, corresponding to amino acid residues 19–30 of defensin, i.e. LCAAHCLAIGRR-NH₂ (19L–30R-NH₂), showed strong activity (100% growth inhibition). Other peptides had less or no anti-bacterial activity. For example, the amidated fragments comprising amino acid residues 18–29 and 20–31 of defensin did not inhibit growth. The

Table 1 Anti-bacterial activity of the synthetic 19L-30R-NH₂ fragment and its analogues

The MICs of defensin from a beetle are from [7]. ND, not determined.

Peptide	MIC ($\mu\text{g/ml}$)		
	<i>S. aureus</i>	MRSA	<i>E. coli</i>
Defensin	4	20	ND
LCAAHCLAIGRR-NH ₂ (19L-30R-NH ₂)	18	> 64	48
LCAAHCLAIRRR-NH ₂	7.5	> 64	24
LCAAHCLAILRR-NH ₂	10	64	24
LCAARCLAIGRR-NH ₂	7.5	> 64	32
LCAALCLAIGRR-NH ₂	6	48	24
LRAAHRLAIGRR-NH ₂	6	64	16
LLAAHLLAIGRR-NH ₂	10	64	48
AAHCLAIGRR-NH ₂	5	> 64	16
AHCLAIGRR-NH ₂ (22A-30R-NH ₂)	6	> 64	16
HCLAIGRR-NH ₂	9	> 64	24
CLAIGRR-NH ₂	40	> 64	> 48

Figure 1 CD spectra of the 19L-30R-NH₂ fragment

CD spectra were measured in sodium phosphate buffer in the presence (broken line) and absence (solid line) of PG/CL (3:1, mol/mol) liposomes. The concentration of the 19L-30R-NH₂ fragment was 5 μM , and that of liposomes was 1 mM.

MIC of the 19L-30R-NH₂ fragment is shown in Table 1. The 19L-30R-NH₂ fragment showed anti-bacterial activity against Gram-positive bacteria (MIC of 18 $\mu\text{g/ml}$ for *S. aureus*) and against Gram-negative bacteria (MIC of 48 $\mu\text{g/ml}$ for *E. coli*). A 19L-30R fragment containing a free carboxylate group had no great activity against *S. aureus* (MIC of > 50 $\mu\text{g/ml}$). The activity of these peptides was not affected by alkylation (results not shown). Anti-bacterial activity is not affected by either the free thiol groups in these peptides or by a disulphide bond.

CD spectra

The first nine amino acids of the 19L-30R-NH₂ fragment are identical with the C-terminus of the presumed α -helical structure region, by sequence identity with some other insect defensins [7], suggesting that this fragment has the potential to form an α -helical structure. CD spectra of the 19L-30R-NH₂ fragment were measured using a spectropolarimeter in the absence or presence of PG/CL (3:1, mol/mol) liposomes. This fragment formed an α -helical structure in the lipidic environment, whereas it showed a random-coil structure in the absence of liposomes (Figure 1). The α -helical content of the fragment was approx. 60%.

Figure 2 Effect of the 19L-30R-NH₂ fragment on glucose release from liposomes

S. aureus (PG/CL, 3:1, mol/mol) (a) and *E. coli* (PE/PG/CL, 7:2:1, by mol) (b) types of liposomes with entrapped glucose were prepared. The amount of glucose released by 0.3% Triton X-100 was defined as 100%. The amount of glucose entrapped in liposomes was 1.3 mol of glucose/mol of *S. aureus*-membrane-type phospholipids and 1.2 mol of glucose/mol of *E. coli*-membrane-type phospholipids. The phospholipid concentration of the reaction mixture for the *S. aureus*-membrane-type was 236 μM and that for the *E. coli*-membrane-type was 214 μM . ●, 19L-30R-NH₂ fragment; ■, α -lactalbumin.

Membrane permeability

Most anti-bacterial peptides are known to interact with bacterial membranes [5]. We investigated the interaction of the 19L-30R-NH₂ fragment and its analogues with phospholipids. To do this, we prepared both *S. aureus*- and *E. coli*-membrane-type liposomes, i.e. the phospholipid composition for *S. aureus* was PG/CL (3:1, mol/mol) and that for *E. coli* was PE/PG/CL (7:2:1, by mol) [30]. The 19L-30R-NH₂ fragment displayed activity against liposomes of both phospholipid compositions (Figure 2). The amount of glucose leakage from liposomes depended on the peptide concentration. Glucose leakage from the *S. aureus*-membrane-type liposomes was greater than that from the *E. coli*-membrane-type liposomes. These results are consistent with anti-bacterial activity of the peptide.

Anti-bacterial activity of analogues of the 19L-30R-NH₂ fragment

The anti-bacterial activities of the 19L-30R-NH₂ fragment and of its analogues are summarized in Table 1. The MICs of analogues against *S. aureus* were 1.8–3-fold higher than that of the 19L-30R-NH₂ fragment. The anti-bacterial activity of the analogues against *E. coli* was greater than or comparable with that of the 19L-30R-NH₂ fragment. Some indicated weak activity against MRSA.

The truncated peptides AAHCLAIGRR-NH₂, AHCLAIGRR-NH₂ and HCLAIGRR-NH₂, which show deletions of

Table 2 Anti-bacterial activity of the synthetic 22A–30R-NH₂ fragment and its analogues

MRSA and *P. aeruginosa* were isolated from infected patients. ND, not determined.

Peptide	MIC ($\mu\text{g/ml}$)			
	<i>S. aureus</i>	MRSA	<i>E. coli</i>	<i>P. aeruginosa</i>
AHCLAIGRR-NH ₂ (22A–30R-NH ₂)	6	> 64	16	ND
ALRLAIRRR-NH ₂	2	> 48	1.5	3
ALLLAIRRR-NH ₂	2	32	2	3
AWLLAIRRR-NH ₂	2	20	3	4
ALYLAIRRR-NH ₂	1.5	24	3	3
ALWLAIRRR-NH ₂	2	32	3	4

Figure 3 Haemolytic activity of 22A–30R-NH₂ fragment analogues

Rabbit erythrocytes were incubated with different concentrations of the following synthetic peptides: ○, ALRLAIRRR-NH₂; ●, ALLLAIRRR-NH₂; □, AWLLAIRRR-NH₂; ■, ALYLAIRRR-NH₂; △, ALWLAIRRR-NH₂; ▲, melittin. Details of experimental conditions are given in the Materials and methods section.

two, three and four amino acids respectively from the N-terminal region of the 19L–30R-NH₂ fragment, still had greater activity against *S. aureus* than did the 19L–30R-NH₂ fragment (MICs: AAHCLAIGRR-NH₂, 5 $\mu\text{g/ml}$; AHCLAIGRR-NH₂, 6 $\mu\text{g/ml}$; HCLAIGRR-NH₂, 9 $\mu\text{g/ml}$). Peptides lacking C-terminal amino acids from the 19L–30R-NH₂ fragment (between one and four amino acids deleted) had no effect against *S. aureus* (results not shown), suggesting that the C-terminal structure of the 19L–30R-NH₂ fragment, RR-NH₂, is important for anti-bacterial activity.

Thus the truncated peptides had greater activity than the 19L–30R-NH₂ fragment. Because smaller peptides have an advantage for the design of therapeutic agents because of decreasing antigenicity, we synthesized five analogues of the 9-mer peptide AHCLAIGRR-NH₂ (22A–30R-NH₂ fragment) in order to obtain more effective anti-bacterial peptides. The anti-bacterial activities of these analogues are summarized in Table 2. The activity of ALYLAIRRR-NH₂ against *S. aureus* (MIC 1.5 $\mu\text{g/ml}$) was 4-fold greater than that of the 22A–30R-NH₂ fragment, and 12-fold greater than that of the 19L–30R-NH₂ fragment. The other 9-mer analogues had 3-fold and 9-fold greater anti-bacterial activities (MIC 2 $\mu\text{g/ml}$) than 22A–30R-NH₂ and 19L–30R-NH₂ respectively. The anti-bacterial activity of the analogues against *E. coli* was about 5–10-fold greater than that of the original fragment. Four analogues showed anti-bacterial activity against MRSA. AWLLAIRRR-NH₂ showed the highest activity against MRSA (MIC 20 $\mu\text{g/ml}$). The MIC of

Figure 4 Cytotoxic activity of 22A–30R-NH₂ fragment analogues on macrophages

The murine macrophage cell line JA-4 was used to observe growth inhibition by the following 22A–30R-NH₂ analogues: ○, ALRLAIRRR-NH₂; ●, ALLLAIRRR-NH₂; □, AWLLAIRRR-NH₂; ■, ALYLAIRRR-NH₂; △, ALWLAIRRR-NH₂. For other experimental conditions, see the Materials and methods section.

A. dichotoma defensin against MRSA was 20 $\mu\text{g/ml}$ when examined 10 h after incubation of the peptide [7]. These analogues were also active against *P. aeruginosa* isolated from a patient.

Haemolytic and cytotoxic activities of defensin analogues

All 9-mer analogues were tested for haemolytic activity against rabbit erythrocytes. No haemolytic activity was observed (Figure 3). Similar results were obtained with 12-mer analogues (results not shown). The cytotoxic activity of 9-mer analogues against mouse macrophages and fibroblasts was examined, and results indicated that these analogues had no cytotoxic activity against fibroblasts (< 2000 $\mu\text{g/ml}$ peptide). Although AWLLAIRRR-NH₂ inhibited the growth of macrophages (20% inhibition at 400 $\mu\text{g/ml}$ and 80% inhibition at 1 mg/ml), other 9-mer analogues did not show such inhibition (Figure 4).

DISCUSSION

In order to apply anti-bacterial peptides to the development of therapeutic agents, we looked for the active region of *A. dichotoma* defensin by measuring anti-bacterial activity against *S. aureus*. For this purpose, we synthesized 64 overlapping 12-mer peptides and examined their activity. Intact *A. dichotoma* defensin is known to be active against Gram-positive bacteria, but not against Gram-negative bacteria [7]. The 19L–30R-NH₂ fragment identified as an active region of defensin was effective against both Gram-positive and Gram-negative bacteria. The lipopolysaccharide of Gram-negative bacteria is reported to be a barrier for anti-bacterial peptides [31], so deletion of the other amino acid sequences of the peptide may enable the 19L–30R-NH₂ fragment to bind efficiently to the membranes of Gram-negative bacteria.

The results of glucose leakage tests from liposomes clearly indicated that the target of the 19L–30R-NH₂ fragment is the bacterial membrane. Many basic anti-bacterial peptides have been reported to interact with liposomes containing acidic phospholipids and to change bacterial membrane permeability [5]. Liposomes with the phospholipid composition of Gram-positive bacteria were more sensitive to the 19L–30R-NH₂ fragment than those mimicking Gram-negative bacteria (Figure 2). The content of acidic phospholipids is higher in Gram-positive compared with Gram-negative bacterial membranes

[30]. Thus we surmise that the 19L–30R-NH₂ fragment has a higher affinity for a bacterial membrane with a high content of acidic phospholipids. This was also supported by the MIC data for this peptide (Table 1).

CD spectra showed that the 19L–30R-NH₂ fragment formed an α -helical structure in the presence of liposomes, and its α -helical content was estimated to be 60%. The α -helical wheel projection of amino acid residues of the 19L–30R-NH₂ fragment indicated that the peptide forms a weak amphipathic structure (results not shown) [32].

It has been suggested that amphipathic α -helical anti-bacterial peptides permeate the bacterial membrane by forming an ion channel [16]. However, recent studies on the mode of action of several anti-bacterial peptides showed that the peptides kill bacteria by a 'carpet-like' mechanism [33–35]. The mechanism of anti-bacterial activity of the 19L–30R-NH₂ fragment remains to be elucidated.

We synthesized various analogues of the 19L–30R-NH₂ fragment by replacing amino acids in order to increase anti-bacterial activity. The results indicated that the enhanced anti-bacterial activity of peptides such as LRAAHLAIGRR-NH₂, LCAARCLAIGRR-NH₂, LCAAHCLAIRRR-NH₂ and LCAALCLAIGRR-NH₂ is due to their increased helical amphipathicity. Enhanced segmental amphipathicity seen in further modified peptides, such as LLAHLAIGRR-NH₂ and LCAAHCAIRRR-NH₂, induced strong activity. These two analogues may have detergent-like properties, due to enhanced hydrophobicity in their N-terminal region or enhanced hydrophilicity in their C-terminal region.

The results of peptide truncation indicated that the C-terminal region of 19L–30R-NH₂, i.e. RR-NH₂, is important for anti-bacterial activity. This region may be necessary for interaction with acidic phospholipids. All analogues derived from the 22A–30R-NH₂ fragment had cationic C-terminal regions and were rich in hydrophobic amino acids in their N-terminal portions. The 9-mer analogues may also act as a detergent to kill bacteria.

No analogue tested damaged murine erythrocytes (Figure 3) or fibroblasts significantly, suggesting an inability to lyse eukaryotic cells, whose outer leaflet is composed of zwitterionic and sphingomyelin phospholipids [36]. In addition, cholesterol in the membranes of eukaryotic cells may prevent peptides from disrupting the membrane [37]. In one case, an analogue strongly inhibited the growth of macrophages (Figure 4). The second tryptophan residue in AWLLAIRRR-NH₂ may be the cause of its cytotoxicity towards macrophages, since ALLLAIRRR-NH₂ and ALWLAIRRR-NH₂ have no cytotoxic activity.

In summary, in this study the 19L–30R-NH₂ fragment derived from *A. dichotoma* defensin was shown to exert anti-bacterial activity by permeabilizing the bacterial membrane. Some modifications of the 19L–30R-NH₂ fragment that enhanced helical or segmental amphipathicity resulted in increased anti-bacterial activity without haemolytic activity. These peptides were effective against pathogenic bacteria such as MRSA and *P. aeruginosa* isolated from patients, and could be used as lead peptides in the design of therapeutic agents. At present, the effect of a magainin analogue has been evaluated in clinical tests for the treatment of patients with diabetic ulcers [38]. Furthermore, analogues of sapecin B showed chemotherapeutic activity against MRSA in mice [39]. Finally, it is worth noting that the mechanisms of

action of these peptides are totally different from those of any antibiotics used thus far. This difference enables insect anti-bacterial peptides to kill antibiotic-resistant pathogenic bacteria. We believe that a strategy to modify insect anti-bacterial peptides is an important approach to developing novel antibiotics.

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