Applications of Antimicrobial Peptides Derived from the Defensin of the Rhinoceros Beetle *Allomyrina dichotoma*

JUN ISHIBASHI*

*National Agriculture and Food Research Organization, Ibaraki, Japan*

Antimicrobial peptides (AMPs) are produced by multicellular organisms and play an important role in innate immunity. AMPs are considered to be promising candidates for novel antibiotics because the emergence of drug-resistant bacteria has become a serious global health problem. There are many advantages of AMPs over conventional antibiotics, including a broad antibacterial spectrum and unique antibacterial mechanisms. Importantly, microbial resistance to membrane-disruptive AMPs is very unlikely to occur rapidly because changes in the target cell membrane cannot occur within a short period. However, antigenicity, cytotoxicity, stability, and production cost are the main difficulties with the application of AMPs for therapeutic purposes. Therefore, modifications of the original AMPs have been performed to overcome these difficulties.

Here we describe our work on the potential therapeutic applications of AMPs as well as the development of beetle defensin-derived AMPs and their multiple functions and applications. The defensin-derived AMPs disrupt negatively charged phospholipids on the cell membrane, showing direct cytotoxic activity against bacteria, fungi, protozoa, and cancer cell lines as well as induce apoptosis by disrupting the mitochondrial membrane; however, it does not demonstrate cytotoxic activity against normal mammalian cells. AMPs also show telomerase inhibition activity. They showed therapeutic effects in MRSA and *Escherichia coli* (*E. coli*)-infected mice, with very weak antigenicity. The AMP-immobilized fibers exhibited potent antibiotic activity against *Staphylococcus aureus* (*S. aureus*), including MRSA, and the activity was maintained even after repetitive washing and sterilization by autoclaving. These results suggest that the AMP-immobilized fibers are promising for use as novel antimicrobial materials.

**Key words:** insect defensin, antimicrobial peptide, membrane disruption, multi-purpose application

### Introduction

Antimicrobial peptides (AMPs) are produced by multicellular organisms and play an important role in innate immunity. Pioneering studies of AMPs were first performed in insects, which do not have an adaptive immune system. The first reported AMP, cecropin, was isolated from the moth *Hyalophora cecropia*. Subsequently, thousands of AMPs have been found in a wide variety of organisms. The diversity of AMPs is so large that they are classified into several categories based on their structural features. AMPs are considered to be promising candidates for novel antibiotics because the emergence of drug-resistant bacteria has become a serious global health problem. In fact, the efforts for clinical application of AMPs have recently been accelerated. There are many
advantages of AMPs over conventional antibiotics, including a broad antibacterial spectrum and unique antibacterial mechanisms. The mechanisms of AMP action are generally categorized as follows: 1) membrane dysfunction, 2) inhibition of extracellular biopolymer synthesis, and 3) inhibition of intracellular functions. In particular, a number of AMPs are cationic peptides and disrupt negatively charged cell membranes through peptide–membrane interactions. On the other hand, the mechanisms of antimicrobial drug resistance are as follows: 1) degradation of antibiotics, 2) point mutations in target proteins, 3) acquisition of an efflux pump, and 4) changes in the membrane surface of the microbe. Importantly, microbial resistance to membrane-disruptive AMPs is very unlikely to occur rapidly because changes in the target cell membrane cannot occur within a short period.

Natural proteins, formed by evolutionary processes, have excellent designs created through evolutionary processes. However, modifications are often required to improve certain features of a natural protein prior to its use as an antibiotic. In particular, antigenicity, cytotoxicity, stability, and production cost are the main difficulties with the application of AMPs for therapeutic purposes. Therefore, modifications of the original AMPs have been performed to overcome these difficulties. For example, the truncation of peptide chains may decrease both antigenicity and production cost. The use of unnatural amino acids, such as D-amino acids, may also improve the stability of AMPs against protease degradation.

In this review, we describe our work on the potential therapeutic applications of AMPs as well as the development of beetle defensin–derived AMPs and their multiple functions and applications.

Modification of beetle antimicrobial proteins

Insect defensins show potent antimicrobial activity, particularly against Gram-positive bacteria. Structure of defensins is stabilized with three pairs of intramolecular disulfide bonds, which is referred to as a cysteine-stabilized α-helix/β-sheet motif (CSαβ). A defensin from the rhinoceros beetle *Allomyrina dichotoma* (*A. dichotoma*) that consisted of 43 amino acid residues (VTCDDLSFEAKGFAANHSLCAAHCLAIGRRGG-SCERGVCICRR-OH), which was likely to be an antigen. We searched for the smallest fragment of the defensin with antibacterial activity. We synthesized 12-mer fragment peptides, covering the whole molecules (1V-12G, 2T-13F, and ~31G-43R), using both a C-terminal free form (−OH) and amide form (−NH₂). The 19-30-NH₂ (LCAAHCLAIGRR-NH₂) fragment showed potent antimicrobial activity against *S. aureus*. We further truncated the peptide and found a 9-mer fragment, 22-30-NH₂ (AHCLAIGRR-NH₂), which was the smallest peptide, showing potent antibacterial activity. This fragment was located in the α-helix of the characteristic CSαβ structure of the defensin. Amino acid substitutions were then performed to enhance the antimicrobial activity of the peptide without increasing its cytotoxicity in mammalian cell lines. Finally, we obtained four defensin–derived peptides (peptide A: RLYLRIGRR-NH₂, peptide B: RLRLRIGRR-NH₂, peptide C: ALYLAIRRR-NH₂, and peptide D: RLLLRIGRR-NH₂) with potent antibacterial activity and low cytotoxicity against mammalian cell lines and low hemolytic activity (Figure-1). The original *A. dichotoma* defensin showed potent antibacterial activity against Gram-positive bacteria but weak activity against Gram-negative bacteria. However, the defensin–derived AMPs killed both Gram-negative and –positive bacteria. The defensin–derived AMPs also showed killing activity against several fungi and protozoa. The defensin–derived AMPs did not inhibit the growth of murine fibroblasts or macrophages, and we found no hemolytic activity against rabbit erythrocytes *in vitro*. The secondary structures of these 9-mer peptides were analyzed by CD spectra and showed α-helical properties in a hydrophobic environment. The defensin–derived AMPs induced the leakage of entrapped glucose from liposomes composed of acidic phospholipids, suggesting that the target of AMPs may be acidic phospholipids in the cell membrane. In addition, D-enantiomers of these peptides showed higher activity against MRSA and *Pseudomonas aeruginosa* (*P. aeruginosa*) than their original L-enantiomers due to protease resistance.

We analyzed the effects of the defensin–derived AMPs in combination with different antibiotics on the growth of MRSA and *P. aeruginosa* using the
checkerboard titration method. The fractional inhibitory concentration (FIC) index was then calculated. AMPs showed synergistic or additive effects when used in combination with different antibiotics.

**Bacterial resistance to defensin-derived AMPs**

We also examined bacterial resistance to defensin-derived AMPs. MRSA and *P. aeruginosa* were treated with a combination of defensin-derived AMPs and antibiotics, and changes to the minimal inhibitory concentration (MIC) under extended culture periods were observed. The bacteria were cultured in the presence of AMPs and antibiotics for 30 days. The MIC value of the antibiotics increased during the culture; however, the MIC value of AMPs did not change. These results suggest that the bacteria could not develop resistance to AMPs in a short period.

**Antigenicity**

To test the antigenicity of the defensin-derived AMPs, they were conjugated with keyhole limpet hemocyanin (KLH) and immunized to mice. After immunization, little or negligible anti-AMP antibody production was detected, although antibodies against KLH were significantly produced. These results suggest that the antigenicity of the defensin-derived AMPs in mice is very weak.

**Therapeutic effects of the defensin-derived AMPs in vivo**

The defensin-derived peptides showed a therapeutic effect in vivo. The survival rates of mice that had been previously infected with MRSA or *E. coli* were significantly higher in mice that were administered the defensin-derived AMPs than in the non-treated control mice. Histopathological observations of the mice that received AMPs revealed that there were few bacterial colonies and no damage to the liver or kidney. The defensin-derived AMPs bind to lipopolysaccharide (LPS) at a comparable affinity to polymyxin B, and they inhibited the expression of TNF-α. Additionally, treatment with AMPs blocked lethal shock in mice that were challenged with LPS and D-galactosamine (GalN). The survival rates of wild-type mice challenged with LPS and GalN was significantly higher in the AMP-injected group than in the non-injected group. In contrast, all of the TNF-α deficient mice that were injected with LPS and GalN survived with or without the injection of AMPs. These results suggest that the defensin-
derived AMPs inhibit the expression of TNF-α and block endotoxin shock in mice. Thus, these results highlight the therapeutic potential of defensin-derived AMPs. However, all of the mice that were given defensin-derived AMPs at a dose of 50 mg/kg died. This dose was exceeded by several-fold the effective therapeutic dose for MRSA-infected mice. This suggests that the defensin-derived AMPs have a narrow safety margin, limiting their therapeutic use in infectious disease.

Multiple targets of the defensin-derived AMPs

1. Effects against cancer cell lines

The defensin-derived AMPs disrupt phospholipid membranes that have a negative charge but do not disrupt electrically neutral phospholipid membranes. Therefore, these peptides show selective killing activity against bacteria without harming normal mammalian cells that have acidic phospholipids only on the inside leaflet of the lipid bilayer. However, several cancer cell lines expose the negatively charged lipid phosphatidylserine (PS) on the outer leaflet of the cell membrane, making the outer membrane of these cell lines negatively charged. Thus, these cancer cell lines could be susceptible to AMPs. We screened various cancer cell lines for susceptibility to the defensin-derived AMPs. We found several susceptible cancer cell lines; in particular, d-peptide B showed potent cytotoxicity against the human leukemia cell line Jurkat and the mouse myeloma cell line P3-X63-Ag8.653. AMPs induced the leakage of the cytosolic enzyme lactic dehydrogenase, and the loss of membrane potential suggested that AMPs also disrupted the cell membrane. Microscopic observations confirmed rapid disruption of the cell membrane. The IC₅₀ values correlated with the relative levels of surface PS in these cells. Taken together, these results showed that the defensin-derived AMPs could be useful to treat cancer.

2. Intracellular targets

The defensin-derived AMPs were submitted to the Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Innovative Areas, Scientific Support Programs for Cancer Research, from The Ministry of Education, Culture, Sports, Science and Technology, Japan to test other potential activities of AMPs as novel anticancer drugs. The committee conducted several assay and found that AMPs may have telomerase inhibition activity. Telomerase is a cytosolic enzyme, and therefore, AMPs must be delivered into the cell to inhibit telomerase activity. To transport AMPs across the cell membrane, we attached cell-penetrating sequence octa-arginine to d-peptide C (d-peptide C2: ALYLAIRRRRRR-NH₂). Actually the defensin-derived AMPs showed telomerase inhibition activity against telomerase extracted from Jurkat cells, the d-peptide C2 showed much higher activity than the other four peptides (d-peptide A, B, C, and D). The d-peptide C2 was able to cross the cell membrane and induce rapid cell death. This was surprising because typically telomerase inhibitors have a relatively slow effect. Additionally, the d-peptide C2 showed cytotoxicity against a cell line that has no activated telomerase enzymes, suggesting that the major target of the d-peptide C2 in Jurkat is not telomerase. We then hypothesized that the mitochondria may be a target of the defensin-derived AMPs because the mitochondria is considered to have originated from a symbiotic bacteria and its membrane also contains acidic phospholipids. Furthermore, the disruption of the mitochondrial membrane induces loss of energy and leads to apoptosis. The d-peptide C2 was found to affect the mitochondrial membrane potential and morphology. Furthermore, in a mitochondrial swelling assay using isolated mitochondria showed not only cell-penetrating peptide, d-peptide C2 the other four defensin-derived AMPs also disrupted mitochondrial membrane in vitro. These results indicated that the defensin-derived AMPs induce cellular apoptosis if they are delivered to cells using a drug delivery system (DDS). Because the cell-penetrating peptide octa-arginine is not cell specific, the d-peptide C2 showed cytotoxic activity against all of the cell lines that were examined. Therefore, the use of a cell-specific DDS may allow for selective cytotoxicity.

We then considered the use of cyclic RGD (cRGD), a peptide that binds to integrin αVβ3 and is subsequently internalized by endocytosis. Using this DDS, peptides are internalized only in integrin αVβ3- high-expressing cells, such as the tumor vasculature cells HUVEC. A cysteine residue was
added to the N-terminal of the defensin-derived AMPs, and cRGD was linked via a disulfide bond. The cRGD-AMPs were internalized and showed specific cytotoxicity against HUVEC cells. Anti-integrin αVβ3 antibody inhibited the cytotoxicity of cRGD-AMPs, suggesting that AMPs were successfully delivered into integrin αVβ3-expressing cells and were able to induce apoptosis.

3. Applications for use in antibacterial materials
Antimicrobial materials are often used in daily life, and the market for antimicrobial material is very large. Therefore, we examined the use of defensin-derived AMPs in antimicrobial materials, particularly fibers.

We first tested silk sutures and fibroin film, both of which are used as wound dressings; these materials were coated with the defensin-derived AMPs. Mice were stitched with MRSA-contaminated silk sutures with or without the defensin-derived AMPs. Observations of the biopsy specimens showed that MRSA was found on control sutures but not on the silk sutures containing the defensin-derived AMPs. MRSA was then seeded onto agar plates and covered with AMP-containing fibroin films and incubated for 24 h. There was no colony formation under the AMP-containing fibroin film. Therefore, the AMP-containing silk sutures and fibroin film suppressed the proliferation of MRSA both in vitro and in vivo. These results demonstrated that the defensin-derived AMPs could be useful in external applications.

The AMP-containing polymers with extended-release characteristics may be effective as a clinical tool. Moreover, these peptides disrupt bacterial cell membranes from outside of the cells, suggesting that the peptides do not require release from the polymer to exhibit antibacterial activity. The immobilization of antibacterial peptides to different materials would be effective, and this efficacy may be retained for extended time periods.

Solid materials, such as polymers immobilized with antibacterial peptides attached by covalent bonds, may prevent bacterial proliferation and biofilm formation and decrease bacterial adhesion onto solid surfaces with long-term stability. The defensin-derived AMPs also show cytotoxicity against cancer cell lines, such as mouse myeloma, by disrupting the cell membrane. Thus, we believe that peptide-immobilized materials are promising for the medical treatment of blood cancers.

4. Design and synthesis of defensin-derived AMPs-immobilized fibers
The D-peptides A and D were immobilized onto cotton fibers via flexible molecular spacer chains. Techniques to immobilize peptides onto a solid support have been developed in the biotechnology and material chemistry fields to construct peptide array chips that can replace many of the bioanalytical methods that are currently being utilized. Peptides generally contain a large number of functional groups that are available as binding sites for specific molecules. Such characteristics of peptides allow for chemical or physical binding on surface-modified solid supports. Uncontrolled immobilizations often lose their biological activity by inhibiting peptide-target interactions or by causing incorrect folding of the peptide. To introduce a molecular spacer chain, we first examined bromination of the cotton fiber. The introduction of the amino-terminal flexible spacer chains was accomplished by a reaction between the bromo group of the bromo-functionalized fiber and the amino group of the diamine compounds, producing amino-functionalized fibers.

Solid-phase peptide synthesis was then applied to bind AMP to the amino-functionalized fiber using Fmoc chemistry (Figure-2). After an amino acid coupling reaction and deprotection of the protection groups, peptide-immobilized cotton fibers were obtained. SEM observations confirmed that the characteristic cotton fiber structure was maintained after the chemical modifications.

5. Antibacterial activities of AMP-immobilized fiber
Antibacterial activities of the AMP-immobilized fibers against MRSA were investigated by quantitative measurements of bacteria in wash solutions. The number of bacteria on the AMP-immobilized fibers decreased to approximately 0.1% after 1 h and 0.05% after 18 h, while the number of bacteria on control fibers increased after 18 h. We also measured the viability of the bacteria on the fibers after washing out the inoculated bacteria with saline, and this revealed that no living bacteria were attached to the AMP-immobilized fibers.
The colony numbers in the AMP-immobilized fibers considerably decreased soon after the bacterial suspension was inoculated. As mentioned above, the 9-mer peptides interact with the hydrophobic region of a cell membrane, thereby disrupting the microbial membrane. The antibacterial activities of these fibers against MRSA support the conclusion that immobilized peptides with a molecular spacer maintain the same function on the fiber surface as the peptides in aqueous solution. To test the stability of this antibacterial activity, we conducted the same antibacterial assay of these fibers after washing with distilled water and sterilizing with an autoclave. The antibacterial activities of the fiber against MRSA were maintained even after the third trial. These repetitive measurements indicate that the fiber binds to the antibacterial agent by covalent bonding, not physical adhesive bonding, and is resistant to typical sterilization procedures. Furthermore, these results indicate that AMPs remained attached to the supported fiber. These features, particularly the durability, are very important for application to clinical instruments as an antibacterial material.

6. Cytotoxicity against cancer cell lines

The defensin-derived AMPs disrupt the membrane in a mouse myeloma cell line with no effect on normal fibroblasts or leukocyte cells. We examined the cytotoxicity of the peptide-immobilized fibers in anticipation of the development of a novel therapeutic apparatus, particularly for the treatment of blood cancers. Significant decreases in the viability of the human leukemia cell line Jurkat and mouse myeloma cell line P3-X63-Ag8.653 were observed in media containing the AMP-immobilized fiber. In addition, repetitive measurements indicated that the immobilized peptide has an effective and stable anticancer activity. Optical microscopic observations of the mouse myeloma cell line revealed that the mouse myeloma cells were destroyed in the presence of the AMP-immobilized fiber, whereas there was no effect on the cells in the presence of control fiber. Therefore, the results of the anticancer assay and optical images indicate that AMP-immobilized fiber is effective against multiple cell lines and functions by disrupting their cell membranes; this function is maintained even after repetitive sterilization procedures by autoclaving.

Conclusion

In this review, we summarized the development of beetle defensin-derived AMPs and their potential use for multiple purposes (Figure-3). The defensin-derived AMPs disrupt negatively charged phospholipids on the cell membrane. AMPs also show direct cytotoxic activity against bacteria, fungi, protozoa, and cancer cell lines as well as induce apoptosis by disrupting the mitochondrial membrane; however, it does not demonstrate cytotoxic activity against normal mammalian cells. AMPs also show telomerase inhibition activity. They showed therapeutic effects in MRSA- and E. coli-infected mice, with very weak antigenicity. Importantly, however, the safety margin was quite narrow for use in the treatment of infectious diseases.

The AMP-immobilized fibers exhibited potent antibacterial activity against S. aureus, including MRSA, and the activity was maintained even after repetitive washing and sterilization by autoclaving.
Furthermore, the AMP-immobilized fiber showed cytotoxicity against cancer cell lines. These results suggest that the AMP-immobilized fibers are promising for use as novel antimicrobial materials as well as in therapeutic apparatuses, particularly for the treatment of blood cancers.

However, there are many difficulties to overcome for a practical use of the defensin-derived AMPs. The use of appropriate DDS would enhance the efficacy of AMPs and decrease toxicity. In addition, the production cost to make the AMP-immobilized fiber is quite high. Therefore, the cost of the AMPs would need to be decreased and better techniques for immobilization are required before widespread use of AMPs for clinical purposes.

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References


