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Review

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Antimicrobial Peptides (AMPs) with Dual Mechanisms: Membrane Disruption and Apoptosis

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Antimicrobial peptides (AMPs) are one of the critical components in host innate immune responses to imbalanced and invading microbial pathogens. Although the antimicrobial activity and mechanism of action have been thoroughly investigated for decades, the exact biological properties of AMPs are still elusive. Most AMPs generally exert the antimicrobial effect by targeting the microbial membrane, such as barrel stave, toroidal, and carpet mechanisms. Thus, the mode of action in model membranes and the discrimination of AMPs to discrepant lipid compositions between mammalian cells and microbial pathogens (cell selectivity) have been studied intensively. However, the latest reports suggest that not only AMPs recently isolated but also well-known membrane-disruptive AMPs play a role in intracellular killing, such as apoptosis induction. In this mini-review, we will review some representative AMPs and their antimicrobial mechanisms and provide new insights into the dual mechanism of AMPs.

Keywords: Antimicrobial peptide, apoptosis induction, membrane-active action

Introduction

Antimicrobial peptides (AMPs) are multifunctional molecules produced by not only specific cells but also many tissues of animals, plants, and invertebrates. They consist of diverse amino acids and are generally characterized by their size, sequence, net charge, structure, hydrophobicity, and amphipathicity [4]. Briefly, AMPs have approximately 12 to 50 amino acids and secondary structures like α-helix, β-sheet, or relaxed coils. Cationic antimicrobial peptides (CAPs) possess abundant positively charged amino acids, such as arginine (R) and lysine (K). The cationicity is specifically involved in the antibacterial activity, because the attraction between CAPs and the negatively charged head group of some phospholipids in the bacterial outer membrane, such as phosphatidylglycerol (PG) and cardiolipin, or lipopolysaccharide (LPS), and teichoic acid, is the first step for exerting antibacterial activity, followed by the interaction, insertion, and the membrane perturbation [46]. The hydrophobicity, relating to specific hydrophobic amino acids like tryptophan (W) or phenylalanine (F), is another significant factor, in terms of the affinity of water-soluble AMPs with target membrane lipid bilayer [4], which results in the antimicrobial effect. The hydrophobic region, such as hydrophobic terminus or hydrophobic amino acids, is also related to the self-association, forming α-helical bundles of AMPs [47]. It can additionally contribute to the toxicity of AMPs towards host cells. Therefore, designing cell-selective potent analogue peptides with reduced toxicity is a significant issue in peptide engineering study [23]. Owing to their unique properties, AMPs can be regarded as a novel pharmaceutical candidate for treating the diseases caused by pathogenic bacterial and fungal species, antibiotic-resistant microbial species, and even cancers.

AMPs Possessing Membrane-Active Mechanism

As is well known, AMPs exert their activity on microbial membrane or intracellular compartments. Specifically, membrane-disruptive peptides have been focused on thoroughly because of their direct potent activity against microbial plasma membranes. In the following subsections, we

Table 1. AMPs and their antimicrobial mechanisms.

Name		Origin	Mechanism	References
Membrane-active AMPs	Melittin	*Apis mellifera*	Toroidal pore/carpet	[14, 16, 28, 33, 49, 50]
	Cecropin	*Hyalophora cecropia*	Channel formation	[8]
	Magainin	*Xenopus laevis*	Toroidal pore	[34, 35]
Apoptosis-inducing AMPs	Coprisin	*Copris tripartitus*	1) No effects on *C. albicans* membranes 2) Hydroxyl radical generation and mitochondrial dysfunction in *C. albicans*	[26]
	Papiliocin	*Papilio xuthus*	ROS generation and mitochondrial dysfunction in *C. albicans*	[18]
	Melittin	*Apis mellifera*	ROS generation and mitochondrial dysfunction in *C. albicans*	[24, 42]
	Magainin 2	*Xenopus laevis*	RecA activation in *E. coli*	[29]

briefly review some key membrane-active AMPs (Table 1).

Defensins and Cathelicidins

In mammals, the epithelium of the intestine, respiratory tract, or skin is the first line of defense regarding barrier function and homeostasis because it directly adjoins an external environment [11]. Therefore, antimicrobial proteins derived from epithelial cells (ECs) are thoroughly investigated in the epithelial cell defense system. Defensins are the most well-established AMPs, consisting of 30–40 amino acids containing six cysteine residues [12, 31]. They consist of two major groups, α-defensins and β-defensins.

α-Defensins are highly expressed in the small intestine. HD5 (DEFA5) and HD6 (DEFA6) peptides in humans are representative α-defensins [39]. Cryptdins are murine α-defensins [39]. α-Defensins are small peptides containing conserved amphipathic structures with positively charged/hydrophobic residues [52]. This structural feature allows these peptides to bind with negatively charged cell surfaces of invading pathogens and to be inserted into the membrane [52].

β-Defensins are secreted from the epithelium of the skin (keratinocytes), respiratory tract (respiratory ECs), and large intestine (mainly enterocytes) [11]. This group of AMPs also exerts antimicrobial activity through selective microbial membrane permeabilization [45]. Cathelicidins (*e.g.*, LL37 in humans and CRAMP in mice) are abundant in resident mast cells of the skin and also exist in ECs of the lung, urinary tract, and large intestine [2]. They are generally cationic α-helical peptides and these properties contribute to the binding affinity between cathelicidins and negatively charged phospholipids of bacteria [2].

Paneth cells are specialized secretory cells residing at the base of small intestinal crypts [41]. For intestinal homeostasis, they produce the antimicrobial proteins against enteric pathogens, such as α-defensins (cryptdins in mice), cryptdin-related sequence (CRS) peptide, regenerating islet-derived protein (Reg) family of C-type lectins, and lysozymes [5, 7, 38]. HIP/PAP, hepatointestinal pancreatic/pancreatitis-associated protein (Reg3α) in humans and Reg 3β and Reg3γ in mice are representatives of the Reg family [38]. They have carbohydrate recognition domains selectively recognizing peptidoglycan of the gram-positive bacterial cell wall [30]. They are not membrane-disruptive AMPs. However, they play critical roles by interacting with the cell surface of bacteria.

Melittin

Melittin is the most distinguished lytic peptide, which is the main component of bee venom (40–50%) isolated from honey bee *Apis mellifera* [13]. This α-helical peptide is hydrophobic and possesses a high positive net charge of +6 [9]. It is generally used for membrane studies as a control peptide, as it exhibits definite disruption of the lipid membrane. Briefly, melittin binds to lipid membranes and forms a α-helical structure with both parallel and perpendicular positions. The perpendicular position is thought to be involved in pore formation [14, 16, 28, 33, 49, 50]. Characteristically, melittin as a monomer, over 1 μg/ml, can bind to membrane lipids of erythrocytes, resulting in hemoglobin release within a few seconds [15]. Therefore, the design of analogs with lower cytotoxicity is important in melittin studies. Many studies focused on the leucine zipper motif contributing to the toxicity towards mammalian cells and simultaneous nonselective activity [40, 53].

Cecropin

Cecropins were the first insect AMPs isolated from a giant silk moth, *Hyalophora cecropia* [17]. This peptide is cationic and adopts α-helical structures in the hydrophobic

condition. Cecropins display a broad spectrum of antibacterial activity against gram-negative and gram-positive bacterial strains, and originate from the amidated C-terminus conferring to the interaction between membranes and these peptides [32, 37]. Christensen *et al*. [8] demonstrated in detail that cecropins interacted with the lipid bilayer with electrostatic adsorption, followed by the insertion of the hydrophobic C-terminus in contrast with residual amphipathic helix in the interface. Moreover, cecropin showed channel formation in membranes in a voltage-dependent manner [8]. In mammals, cecropin P1 derived from the porcine small intestine has similarity in amino acids with insect cecropins [27]. Cecropins are also used as a reference peptide, like melittin, in membrane studies and antimicrobial mechanism studies of peptides and proteins. As is well-documented, cecropin A/melittin (CAME) hybrid peptides are established analog AMPs showing advanced antimicrobial effects [3, 36].

Magainin

In 1987, Zasloff [51] designated the magainin peptides (magainin 1 and magainin 2), which originated from the skin of *Xenopus laevis*, an African clawed frog [51]. Interestingly, he suggested these AMPs could be expressed in not only eosinophilic and granule-laden intestinal cells, like mammalian Paneth cells, of *Xenopus* small intestine, but also the skin [43]. This site specificity suggested that magainins play a conserved role in the host defense system in both mammalians and non-mammalian vertebrates [43, 51]. These two 23 mer peptides have an α-helical structure and a net positive charge of +4 [51]. They also showed remarkable antibiotic activity against a broad spectrum of bacteria, fungi, and protozoa [51]. Magainins have been thoroughly investigated regarding their biological properties and their notable features. They show high cell selectivity between pathogens and mammalian cells at the concentrations exhibiting antimicrobial activities, which allowed them to be employed as a template for the design of novel analog peptides [6, 35, 51]. In terms of the mechanism of action, magainins bind to acidic lipid compositions through electrostatic interactions and permeabilize the cell plasma membrane by forming pores [34, 35]. The analog of cecropin A/magainin 2 (CAMA) hybrid peptide, an antibacterial peptide [48], is still being investigated for its clinical potential in microbial diseases in humans [44].

AMPs Possessing Apoptosis-Inducing Mechanism

In this part, we introduce some AMPs containing the apoptosis-inducing mechanism. Additionally, the established membrane-active AMPs showing a dual mechanism are reviewed (Table 1).

Coprisin

Coprisin (VTCDVLSFEAKGIAVNHSACALHCIALRKK GGSCQNGVCVCRN-NH_2) is a defensin-like 43 mer peptide containing three disulfide bonds (positions: 3-34, 20-39, and 24-41), which was isolated from the dung beetle, *Copris tripartitus*, in 2009 [19]. Coprisin exhibited broad-spectrum antifungal activities against various fungal pathogens, such as *Aspergillus* and *Candida* species, without any cytotoxicity towards human erythrocytes [26]. Interestingly, several membrane studies, such as 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence analysis, calcein leakage measurement from large unilamellar vesicles (LUVs), and rhodamine-conjugated single giant unilamellar vesicle (GUV) analysis, suggested that coprisin did not disrupt both the cell plasma membrane of *Candida albicans* and fungal model membranes [26]. Notably, in a rhodamine-conjugated single GUV, which is consisted of phosphatidylchoine (PC)/phosphatidylethanolamine (PE)/phosphatidylinositol (PI)/ergosterol (5:4:1:2 (w/w/w/w)), the absence of membrane-active action was well visualized [26]. Therefore, it was hypothesized that coprisin exerted its activity after the cell penetration. Based on the hypothesis, some apoptosis markers, such as phosphatidylserine (PS) exposure for early apoptosis, and DNA fragmentation for late apoptosis, were examined. The results showed that corpisin significantly induced apoptosis in *C. albicans* [26]. Furthermore, reactive oxygen species (ROS), specifically hydroxyl radicals (•OH), are suggested as key players in coprisin-induced apoptosis [26]. Coprisin additionally caused mitochondrial dysfunction and cytochrome *c* release/caspase activation as downstream events [26]. In addition, in terms of antibacterial activity, coprisin, which possesses an amphipathic α-helix (A^{19} to R^{28}) and a electropositive surface formed by R^{28}, K^{29}, K^{30}, and R^{42}, showed potent activity by targeting bacterial LPS [22]. However, the antifungal study of coprisin provided new insight regarding the mechanism of AMPs.

Papiliocin

In 2010, a novel cecropin-like AMP was isolated by Kim *et al*. [21] and named papiliocin. Papiliocin (RWKIFKKIE KVGRNVRDGIIKAGPAVAVVGQAATVVK-NH_2) is a 37 mer peptide isolated from the swallowtail butterfly, *Papilio xuthus* [21]. It exhibited potent antimicrobial activities against both gram-positive and negative bacteria, and fungi, without cytotoxicity against human erythrocytes

[21]. The first mechanism study of paliliocin showed that papiliocin effectively disrupted the fungal plasma membrane of *C. albicans* [25]. In model membranes mimicking the outer leaflets of the *C. albicans* plasma membrane, papiliocin formed pores on the membrane within minutes [25]. The secondary structure, and antibacterial and anti-inflammatory properties of papiliocin were further investigated [20]. Kim *et al*. [20] suggested that papiliocin contained two α-helices (K^3 to K^{21} and A^{25} to V^{36}) with the hinge region [20].

The novel antimicrobial mechanism of papiliocin was proposed in succession [18]. The results showed that papiliocin caused apoptotic events, such as PS flip-flop, chromatin condensation, and DNA fragmentation in *C. albicans* [18]. It was also suggested that ROS accumulation and mitochondrial membrane damage could be the key in papiliocin-induced fungal apoptosis [18]. Unlike coprisin, papiliocin peptide showed a dual mechanism, membrane-active action, and apoptosis induction, specifically in fungal pathogens [18, 25]. The exact demonstration of the coexistence between two discrepant mechanisms is still largely unknown. However, it will enable more effective clinical approaches in treating human fungal disease.

Melittin

As noted previously, melittin is widely known as a membrane-active AMP. However, a novel antimicrobial mechanism of melittin has been suggested [24]. In 2010, the potential of melittin in *C. albicans* was suggested for the first time by using some hallmarks of apoptosis, such as Annexin V, DAPI, and TUNEL staining [42]. However, the in-depth mechanism was still elusive. In 2014, the intracellular mechanism of melittin-induced apoptosis in *C. albicans* was further characterized [24]. Melittin caused ROS generation to play a pivotal role in the apoptosis induction, and specifically $^{\bullet}OH$ is significantly involved [24]. The results also suggested the mitochondrial dysfunction and the caspase activation induced by melittin and further indicated the role of mitochondria by investigating Ca^{2+} homeostasis between the ER and mitochondria [24]. In the study, mitochondrial Ca^{2+} levels were highly increased, suggesting the mitochondrial perturbation or rupture by the decreased mitochondrial membrane potential ($\Delta\Psi_m$) [24]. In summary, it was suggested that melittin also possessed a dual antifungal mechanism.

Magainin 2

As discussed previously, magainin 2 is a pore-forming AMP [34, 35]. It was recently proposed that magainin 2 caused bacterial cell death in *Escherichia coli*, like eukaryotic apoptosis [29]. Magainin 2 showed the apoptotic phenotype in a caspase-dependent manner, after membrane disruption [29]. Furthermore, RecA protein, which is essential for DNA repair in bacterial SOS responses [10], was suggested as a key player in magainin 2-induced bacterial cell death [29]. The result suggested that RecA was involved in the cleavage of LexA protein, which regulates SOS response in the damaged bacteria [1, 29], and that RecA also acted as a caspase substrate in this apoptosis-like death [29]. It suggests that membrane-active peptides can successively exert the antimicrobial activity.

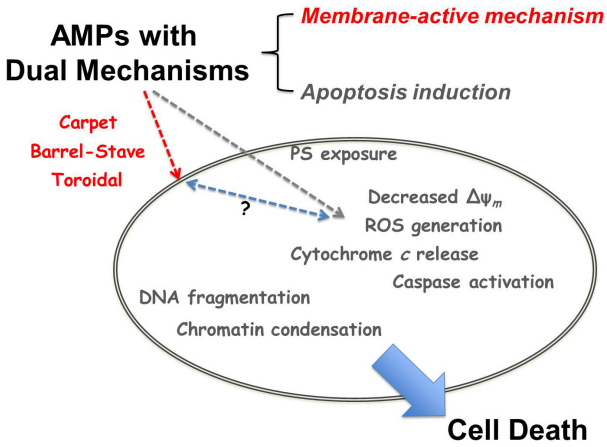

Fig. 1. Dual mechanisms of AMPs.

In conclusion, we have reviewed several membrane-active AMPs and comparatively novel AMPs showing apoptosis-inducing ability (Fig. 1). As noted, AMPs are still the most potent candidates as alternatives of conventional antibiotics. Ongoing studies, in terms of understanding the diverse mechanism of AMP, will contribute to the development of more potent AMPs without unexpected side effects.

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