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MINIREVIEW

Rediscovery of antimicrobial peptides as therapeutic agents

Minkyung Ryu, Jaeyeong Park, Ji-Hyun Yeom*, Minju Joo*, and Kangseok Lee*

Department of Life Science, Chung-Ang University, Seoul 06974, Republic of Korea

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In recent years, the occurrence of antibiotic-resistant pathogens is increasing rapidly. There is growing concern as the development of antibiotics is slower than the increase in the resistance of pathogenic bacteria. Antimicrobial peptides (AMPs) are promising alternatives to antibiotics. Despite their name, which implies their antimicrobial activity, AMPs have recently been rediscovered as compounds having antifungal, antiviral, anticancer, antioxidant, and insecticidal effects. Moreover, many AMPs are relatively safe from toxic side effects and the generation of resistant microorganisms due to their target specificity and complexity of the mechanisms underlying their action. In this review, we summarize the history, classification, and mechanisms of action of AMPs, and provide descriptions of AMPs undergoing clinical trials. We also discuss the obstacles associated with the development of AMPs as therapeutic agents and recent strategies formulated to circumvent these obstacles.

Keywords: antimicrobial peptide, drug candidate, clinical trial, SLAY, drug delivery system

History of AMPs

It is difficult for most animals to recover fully from injuries on their own. Further, plants and bacteria are confined to a limited space, and are unable to escape from fatal threats in the surrounding environment. Due to these reasons, all living species have evolved various host-defense mechanisms, including peptide-mediated defense systems. A large number of these peptides are found in frogs and toads. They have a soft skin that is exposed to harsh natural forces at all times; hence, they develop strategies for protection against microbes (Calhoun *et al.*, 2016). In 1922, Alexander Fleming demonstrated that a patient's nasal mucus had the ability to inhibit bacterial growth in a culture (Fleming, 1922), and the factor involved in the phenomenon was lysozyme, which is now used as a food preservative (Cunningham *et al.*, 1991). However, there was a delay in the substantiation of the non-enzymatic antibacterial mechanism of lysozyme; hence, it was designated as the first antimicrobial peptide (AMP) only in the early 1990s (Benkerroum, 2009). Following this finding, gramicidin was isolated in 1939 by Dubos from the soil bacterium *Bacillus brevis*; it has been shown to inhibit the growth of a broad-range of Gram-positive bacteria (Dubos, 1939a, 1939b). After several experimental studies using gramicidin against mice or guinea pig infected by bacteria, it was observed that this AMP exhibits potential for therapeutic use (Dubos, 1939b; Gause and Brazhnikova, 1944). Later, it was approved by the United States Food and Drug Administration (FDA) and commercially employed as the first natural antibiotic (Van Epps, 2006). In 1942, the first thionin that was widely dispersed in the plant kingdom was discovered. In the 1970s, it was named purothionin (Mak and Jones, 1976; Ohtani *et al.*, 1977). Melittin was isolated from a fraction of bee venom in 1967 and was found to have an antibacterial effect against penicillin-resistant *Staphylococcus aureus* and other Gram-positive and Gram-negative bacteria (Fennell *et al.*, 1967). These findings provide an answer about how plants or insects, which lack an adaptive immune system, can be safe from infection. The concept was evaluated by an experimental study with an AMP-deficient *Drosophila melanogaster*, which proved that this protein is important for protecting the insect from fungal infection (Lemaitre *et al.*, 1996). After the recognition of a cationic peptide that functions as the component of the innate immune system, which functions independent of the adaptive immune system (which only exists in animals), intensive studies have been conducted on defense mechanisms in plants and insects. Eukaryotic AMPs have been the focus of research with the discovery of cecropins and magainins in frogs (Phoenix *et al.*, 2013). During the 1970s and 1980s, α-defensin was discovered in rabbits (Selsted *et al.*, 1983, 1984) and humans (Ganz *et al.*, 1985). Later, β- and θ-defensins were identified in bovine granulocytes (Selsted *et al.*, 1993) and rhesus monkey leukocytes (Tang *et al.*, 1999), respectively. In 1981, *Hyalophora cecropia* pupae were infected with bacteria to induce the expression of the bacteriolytic proteins P9A and P9B (Hultmark *et al.*, 1980). The peptides were sequenced and renamed "cecropins," which are the first major α-helical AMPs (Steiner *et al.*, 1981). Since then, more than 3,236 AMPs have been found; most of these have been found in amphibians, followed by mammals, humans, and arthropods (https://dbaasp.org/home)

*For correspondence. (K. Lee) E-mail: kangseok@cau.ac.kr; Tel.: +82-2-820-5241; Fax: +82-2-825-5206 / (J.H. Yeom) E-mail: jihyun82@cau.ac.kr / (M. Joo) E-mail: jsl8316@hanmail.net

(Pirtskhalava *et al.*, 2016).

Action mechanisms of AMPs

AMPs are known to have bactericidal activity by targeting the bacterial membrane and lysing it mostly via a non-specific manner which is driven by electrostatic effect. Cationic antimicrobial peptides have more assess to the stronger negative charge of the bacterial outer membrane compared to that of the animal host membrane from nanomolar to micromolar concentration of the peptides (Kumar *et al.*, 2018). In the case of the bacteriocins, which are proteinaceous toxins produced by bacteria and some archaea to inhibit the growth of closely related bacterial strains, they usually use docking molecule to exert their membrane lysis activities (Héchard and Sahl, 2002). For instance, nisin, one of the class I bacteriocins, interacts with Lipid II in exterior side of the plasma membrane to form pores which lead to leakage of small biomolecules like ATP, amino acids (Breukink and de Kruijff, 2006). The class II bacteriocin such as lactococcin A and microcin E492 bind to mannose phosphotransferase system (Man-PTS), which is a pore-forming receptor in the membrane (Cotter *et al.*, 2013).

Most of vertebrate and invertebrate AMPs, as well as some bacteriocins, target membrane and lyse bacteria without interacting with docking-molecules outside of cells (Kumar *et al.*, 2018). One of the cathelicidin families, which is a conserved component of innate immunity in vertebrate, LL-37 is attracted by lipopolysaccharide (LPS) of Gram-negative bacterial outer membrane or lipoteichoic acid (LTA) in the Gram-positive bacterial cell wall; this interaction facilitates the assessment of AMPs to Gram-positive bacterial membrane (Xhindoli *et al.*, 2016). The human α-defensins, human neutrophil peptide-2 and -3 (HNP-2, -3) are reported to generate membrane pore by direct binding to membrane, oligomerization, and induce leakage of cytosol molecules (Hill *et al.*, 1991; Wimley *et al.*, 1994). Gramicidin A, another bacteriocin, β-helical structure of which incorporates into membrane and dynamic dimerization of two monomers forms a channel that leads to osmotic swelling and cell lysis (Wang *et al.*, 2012). Also, Gramicidin S binds to membrane followed by re-orientation. The monomers of Gramicidin S are self-assembled to form oligomeric pore that destabilizes and depolarizes target membrane (Babii *et al.*, 2018).

In addition to membrane lysing function of those peptides, AMPs also exert modulation of immune responses by direct interactions with Toll-like receptors (TLRs). They activate immune cells to control inflammation and to increase killing of pathogens. When AMPs bind with each molecule of dsDNA, dsRNA, ssRNA, or LPS, they form AMP-immune ligand complexes. The ligands directly bind to TLRs and activate NFκB signaling pathway which leads to cytokine production, chemotaxis, and cellular differentiation (Lee *et al.*, 2019). Due to their complex function in immunomodulatory mechanism, such AMPs are referred as host-defense peptides (HDPs) (Mansour *et al.*, 2014). Immunomodulative action of AMPs are intensively studied on cathelicidin and defensin families (Lee *et al.*, 2019).

Some AMPs penetrate membrane and target intracellular molecules such as DNA or RNA. For instance, HNP-1, which is already known to disturb membrane, is also shown to inhibit bacterial cell wall synthesis through direct binding to Lipid II in *Staphylococcus aureus* (de Leeuw *et al.*, 2010). It has been shown that HNP-5 can bind to DNA strongly, suggesting that it inhibits DNA replication and/or transcription processes (Mathew and Nagaraj, 2015). Buforin II from frog was also shown to bind to DNA and RNA after penetrating the cell membrane, resulting in rapid bactericidal activity (Park *et al.*, 1998).

Structures of AMPs

The diverse targeting ability of AMPs is due to their physiological characteristics related to their structure. Thus far, thousands of AMPs have been discovered, and they do not appear to have conserved amino acid sequences. However, AMPs can be classified into three groups based on the properties of amino acids that determine the secondary structure of AMPs.

The first group of AMPs are the α-helical AMPs, which are composed of a continuous hydrophobic surface (Takahashi *et al.*, 2010). Several mechanisms underlying the action of α-helical AMPs on cellular membranes have been suggested, including the "toroidal pore model," "interfacial activity model," "carpet model," and "charged lipid clustering model." According to the "toroidal pore model," α-helical AMPs usually trigger antimicrobial activity by embedding into the cellular membrane and via the alignment of the hydrophilic side of the AMP towards the phospholipid head and the hydrophobic side of the AMP in the acyl tail core (Nguyen *et al.*, 2011). Then, the AMP disrupts the membrane by associating itself with the toroidal pores in the membrane, leading to the leakage of cellular components and death of the cell (Brogden, 2005). According to the "interfacial activity model," α-helical AMPs possess only a hydrophobic face. The AMPs are embedded into the cellular membrane in a parallel orientation; they disrupt the membrane without self-assembly (Wimley, 2010). Some AMPs act like detergents to break down membrane lipids into micelle-like structures. The "carpet model" is related to the toroidal pore model, but proposes that a higher concentration of AMPs is needed to exert an antimicrobial effect (Brogden, 2005). Further, as per the "charged lipid clustering model," differently charged membrane lipids can be clustered around AMPs, leading to membrane destabilization and depolarization (Epand and Epand, 2011).

The second group of AMPs are the β-sheet AMPs, which possess conserved cysteine residues and are stabilized via disulfide bridges between these residues. These covalent bonds do not appear to be necessary for membrane positioning, and may or may not affect the activity of the peptide (Wu *et al.*, 2003; Klüver *et al.*, 2005; Doherty *et al.*, 2008; Schroeder *et al.*, 2011). β-Sheet AMPs have more diverse targets than α-helical AMPs. Usually, β-sheet AMPs build a β-barrel in the anionic membrane. However, in cholesterol-enriched membranes, β-sheet AMP aggregates are formed (Tang and Hong, 2009). β-Sheet AMPs have a non-lytic mechanism of action. However, the cyclic β-sheet AMP tachyplesin I directly binds

to the minor groove of DNA and disturbs the DNA-protein interaction (Brogden, 2005). The membrane-targeting peptide human defensin can also bind to the precursor of peptidoglycans and inhibit cell wall biosynthesis in staphylococci (Sass *et al.*, 2010).

The last group of AMPs are the extended AMPs, which do not have a regular secondary structure and often contain a high proportion of Arg, Trp, or Pro residues (Chan *et al.*, 2006). Most of the extended peptides do not target the cell membrane, but usually accumulate in the cytoplasm and interact with proteins to inhibit their functions. For example, the Pro-rich peptides pyrrhocoricin, drosocin, and apidaecin penetrate the cell membrane and interact with intracellular proteins to inhibit DnaK activity and chaperone-associated protein folding (Brogden, 2005).

AMPs approved by FDA or undergoing clinical trials

The emergence of antibiotic-resistant bacteria is a growing concern as the development of antibiotics is falling behind the increase in resistance of pathogens (Lee, 2019; Lei *et al.*, 2019). For the alternative, numerous AMPs have been examined as therapeutic agents, and patented (Kang *et al.*, 2017). Among them, only a limited number of AMPs have been approved by the FDA as therapeutics. The list of AMPs in clinical trials is provided on the websites "Data Repository of Antimicrobial Peptides (DRAMP, http://dramp.cpu- bioinfor.org/) with 76 clinical antimicrobials (Kang *et al.*, 2019), and "Adis Insight" (https://adisinsight.springer.com/). AMPs approved by the FDA are listed in the "Therapeutic Protein Database (THPdb, http://crdd.osdd.net/raghava/thpdb/) (Usmani *et al.*, 2017). This section will cover the AMPs that are currently undergoing clinical trials and the limitations of AMPs due to which they failed to get FDA approval. The AMPs approved by the FDA or undergoing clinical trials are listed in Tables 1 and 2.

FDA-approved AMPs

AMPs are synthesized in two ways: by non-ribosomal synthesis and ribosomal translation of mRNA. The non-ribosomally synthesized peptides, such as glycopeptides and bacitracin, are produced by bacteria and have been developed as therapeutic agents owing to their similarities with natural peptides. They present interesting biological properties ranging from antibiotic to biosurfactant activities. Almost all FDA-approved AMPs are non-ribosomally synthesized peptides that require peptide synthetases (Mankelow and Neilan, 2000). However, ribosomally synthesized AMPs with diverse translational modifications are produced by all life forms and considered as new clinical agents with antimicrobial potential.

Oritavancin, dalbavancin, and telavancin are derivatives of vancomycin (Chen *et al.*, 2007; Zhanel *et al.*, 2010). They are semisynthetic lipoglycopeptides that treat infections caused by multidrug-resistant Gram-positive pathogens. The lipoglycopeptides have lipophilic side chains that prolong their alf-life. The half-lives of oritavancin, dalbavancin, and telavancin are 195.4 h, 14 days, and 8 h, respectively. The modified peptides are more effective than the compound of origin, vancomycin, and can even inhibit the growth of vancomycin-resistant bacteria by disrupting bacterial cell wall formation. AMPs are used for treating complicated skin and skin structure infections (cSSSIs) caused by *S. aureus* and are also effective against other Gram-positive bacteria (Saravolatz *et al.*, 2009). These AMPs are likely to serve as an alternative to vancomycin in the treatment of cSSSIs.

Daptomycin is a 13-amino acid peptide that targets the bacterial membrane and lyses it (Lee *et al.*, 2018). It binds to the cell membrane and causes a fast depolarization of the membrane potential; it is active only against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). It has a long half-life (approximately 8 to 9 h) and a prolonged post-antibiotic effect, which lasts up to 7 h. Daptomycin and its derivative Cubicin were approved in 2003 to treat *S. aureus* infection and cSSSIs (Carpenter and Chambers, 2004).

AMPs at the preclinical trial stage

Information about AMPs at the preclinical stage is insufficient, which may be due to it being company-classified information.

HB-1345 is a hexameric lipopeptide developed for the treatment of fungal skin infections. It is administered topically for the treatment of skin infections, such as chronic wounds and burn wounds, through immunomodulation. The MIC value of HB-1345 renders it effective against *Propionibacte-*

Table 1. Antimicrobial peptides approved by the FDA or undergoing clinical trials

Clinical stage	Name	Characteristic	Source	Medical use	Mode of action	Company
FDA approved (2014)	Dalbavancin	Antimicrobial	Semisynthetic lipoglycopeptide, derivate of teicoplanin	Bacterial skin infection	Disruption of cell wall biosynthesis	Dalvance / Allergan, Inc.
FDA approved (2014)	Oritavancin	Antimicrobial	Semisynthetic lipoglycopeptide, derivate of chloroeremomycin	Bacterial skin infection	Disruption of Gram-positive bacterial cell membrane and inhibition of trans-glycosylation and transpeptidation	Orbactiv / The Medicines Company
FDA approved (2009)	Telavancin	Antimicrobial	Semisynthetic lipoglycopeptide, derivate of vancomycin	Bacterial skin infection	Interference in cell wall and peptidoglycan synthesis	Vibativ / Theravance Biopharma, Inc.
FDA approved (2003)	Daptomycin	Antimicrobial	*Streptomyces roseosporus*	Bacterial infections of skin and underlying tissues	Membrane interruption, and inhibition of DNA, RNA, and protein synthesis	Cubicin / Cubist pharmaceuticals

Table 2. Antimicrobial peptides undergoing clinical trials

Clinical stage	Name	Characteristic	Source	Medical use	Mode of action	Company
Preclinical studies	HB-1345	Antimicrobial	Synthetically designed peptide	Acne	Interruption of cell wall synthesis by binding to lipoteichoic acid	Helix Biomedix, Inc.
Phase I trials	Histatin	Antimicrobial	Human saliva	Oral candidiasis (as a mouth wash)	Disruption of fungal plasma membrane	Demgen and Dow Pharmaceutical Sciences, Pacgen Life Science
	IDR-1	Anti-inflammatory	Bovine neutrophils	Infections in immunocompromised patient	Immunomodulation, protection of }monocytes	Unknown
Phase II trials	PAC-113	Antifungal	Human saliva	Oral candidiasis	Destabilization of microbial cell membrane and fungal cell wall	Pacgen Life Science
	CZEN-002	Antifungal	α-melanocyte-stimulating hormone-derived synthetic peptide	Candidiasis	Modulation of inflammatory and immune responses	Zengen, Inc.
	Novexatin (NP213)	Antifungal	Synthetic peptide	Onychomycosis (fungal nail infection)	Perturbation and lysis of fungal outer cell membrane	NovaBiotics Ltd.
	hLF1-11	Antimicrobial	Human lactoferrin	Mycoses	Modulation of human monocyte by inhibiting myeloperoxidase activity	AM-Pharma Holding
	EA-230	Anti-inflammatory	hCG-derived synthetic peptide	Systemic inflammatory response and renal failure	Immunomodulation and prevention of organ failure	Exponential Biotherapies, Inc.
	C16G2	Antimicrobial	Synthetically designed peptide	Dental caries	Bacterial cell membrane disruption	Unknown
	Glutoxim (NOV-002)	Anticancer	Glutathione-derived synthetic peptide	Breast cancer, ovarian cancer	Immunomodulation and stimulation of myeloproliferation	Pharma BAM/Novelos Therapeutics, Inc.
	LTX-109 (Lytixar)	Antimicrobial	Synthetically designed peptide	Impetigo and nasal decolonization of MRSA	Interaction with the bacterial cell membrane, resulting in pore formation	Lytix Biopharma
	P113	Antifungal	Human saliva	Candidiasis in Human immuno-deficiency virus patients	Reduction of cell wall of fungi	Demegen Inc.
	Omiganan (CLS001)	Antimicrobial	Synthetic analog of indolicidin	Acne and rosacea	Enhancement of bacterial membrane permeability	Migenix Inc.
Phase III trials	D2A21 (Demegal)	Antimicrobial	Synthetically designed peptide	Burn infection, skin infection with multidrug-resistant pathogens	Formation of multimeric pores in bacterial cell wall, stimulation of apoptosis	Par Advance Technologies, Inc.
	XOMA-629	Antimicrobial	Synthetic peptide derived from bactericidal/permeability-increasing protein	Skin flora by *Cutibacterium acnes*	Inhibition of endotoxins	Xoma Ltd.
Phase III trials (Discontinued)	Talactoferrin-α	Anti-inflammatory	Synthetic peptide	Non-small cell lung cancer, diabetic neuropathic ulcers	Immunostimulation	Agennix, Inc.
	Pexiganan (MSI-78)	Antimicrobial	Analog of magainin peptide	Foot ulcers in diabetic patients	Disruption of bacterial cell membrane and stimulation of defensin	Genaera Corporation
	Iseganan (IB-367)	Antimicrobial	Synthetic analog of protegrin I	Nosocomial pneumonia	Modulation of bacterial cell membrane	Ardea Biosciences, Inc.
	Opebacan ($rBPI_{21}$, Neuprex)	Antimicrobial	Synthetic peptide	Graft-versus-host disease, post-traumatic infections	Enhancement of bacterial cell membrane permeability, inhibition of angiogenesis	Xoma Ltd.

hCG, Human chorionic gonadotropin.
MRSA, Methicillin-resistant *Staphylococcus aureus.*

rium acnes (Pirri *et al.*, 2009).

Phase I

Histatins are small histidine-rich cationic peptides that exhibit antifungal activity by inducing AMP release, and generation of reactive oxygen species, which leads to cell death. It is active against pathogenic yeast and fungi that are resistant to conventional antifungal drugs (Kavanagh and Dowd, 2004).

Plectasin is composed of α-helices and β-sheets and exhibits antibacterial, antiviral, and antifungal activity. Plectasin has a novel antimicrobial mechanism; it targets bacterial cell wall precursors and interferes with cell wall synthesis (Li *et al.*, 2017). It is effective against *Streptococcus pneumoniae* and does not target host cells such as murine fibroblasts, normal human epidermal keratinocytes, and erythrocytes. This indicates the considerable selectivity of plectasin towards bacterial cells *in vitro* (Mygind *et al.*, 2005).

IDR-1 is a 13-amino acid peptide that shows antimicrobial activity against MRSA, VRE, and other Gram-positive and Gram-negative pathogens. IDR-1 does not target bacteria directly but regulates inflammation pathways and induces the expression of cytokines and chemokines, which are components of the innate immune system. Due to its indirect way of targeting microbes, IDR-1 possesses a low possibility of inducing resistance (Scott *et al.*, 2007).

Phase II

PAC-113 is the active segment of histatin 5 in human saliva. The α-helical structure of PAC-113 disrupts membranes and modulates the innate immune system. Clinical trials for the treatment of oral candidiasis with PAC-113 are ongoing, but they have shown only slight efficacy and potentially serious side effects such as drug resistance and multiple drug interactions (Greber and Dawgul, 2017).

CZEN-002 is a synthetic 8-mer peptide derived from α-melanocyte-stimulating hormone. CZEN-002 targets and kills Gram-negative and Gram-positive bacteria and pathogenic fungi, and inhibits human immunodeficiency virus-1 replication (Catania *et al.*, 1998; Grieco *et al.*, 2003). It has now been examined for the treatment of *Vulvovaginal candidiasis* and shown positive results in Phase I/II trials (Zhang and Falla, 2006).

Novexatin (NP213) is a synthetic cyclic AMP. Due to its water-solubility, the treatment of nails and skin with a water-based formula of Novexatin exhibits a rapid inhibitory effect against fungal infections (Mercer *et al.*, 2020).

hLF1-11 is an 11-mer peptide derived from the N-terminus of human lactoferrin. *In vivo* introduction of hLF1-11 intravenously showed an effect on MRSA-infected mice. In addition to antibacterial activity, this AMP also shows antifungal activity by energizing the mitochondrion and producing high levels of reactive oxygen species, which results in increased plasma membrane potential and permeability. In addition, hLF1-11 also exhibits activity against allogeneic bone marrow stem cell transplantation-associated infections (Nibbering *et al.*, 2001; Lupetti *et al.*, 2003; Faber *et al.*, 2005).

EA-230 is a β-human chorionic gonadotropin (β-hCG)-derived tetrapeptide (AQGV). The β-loop fragment of hCG exerts an immunological effect by inhibiting severe inflammation and preventing renal failure in patients with diabetes (Khan and Benner, 2011). The most effective derivative of the fragment is EA-230, which was developed by Exponential Biotherapies. It has completed a phase II trial studying its effects on systemic inflammatory response syndrome in 2018, and phase II/III clinical trials were performed in patients with renal failure in 2016.

C16G2 is a specifically targeted antimicrobial peptide (STAMP) that only targets *Streptococcus mutans* to treat dental caries. The precursors of this AMP are CSP_{C16} and G2, which are truncated forms of the competence stimulating peptide (CSP) pheromone of *S. mutans* and the broad-spectrum AMP novispirin G10, respectively. These parts are joined together by a linker (Kaplan *et al.*, 2011). C16G2 exhibits selective membrane disruption in *S. mutans*. C3J Therapeutics proceeded with a clinical trial using this AMP but discontinued the phase II trial in 2020.

Glutoxim (NOV-002) is a hexapeptide stabilized with a disulfide bond. It exerts an anticancer effect by stimulating myeloproliferation, which increases the populations of circulating monocytes, lymphocytes, natural killer cells, and T-cells. A combination of NOV-002 with carboplatin/paclitaxel, which are conventional anticancer drugs, exhibits significantly enhanced anti-tumor effects than the individual drugs. NOV-002 oxidizes thiols in cell surface proteins, leading to reduced membrane stability (Townsend *et al.*, 2008).

LTX-109 (Lytixar) is a synthetic antimicrobial peptidomimetic (SAMP), developed by Lytix Biopharma that targets the bacterial membrane and degrades it (Saravolatz *et al.*, 2012). This peptide underwent phase I/II clinical trials for the treatment of MRSA infections and phase II clinical trials for the treatment of skin and soft tissue infections, but both were discontinued in 2015.

P113 (Demegen) is C-terminus of an amidated fragment of histatin-5. It forms a stable complex with zinc (II) and copper (II). While binding with metals, it is active against streptococci, staphylococci, *Pseudomonas aeruginosa,* and *Candida albicans* (Porciatti *et al.*, 2010).

Omiganan (CLS001) is an analog of indolicidin. It has effects against the infections caused by *S. aureus* (Niemeyer-van der Kolk *et al.*, 2020). It is now undergoing phase II clinical trial to the patients with mild atopic dermatitis and phase III for rosacea, but failed for the treatment in catheter-related infection at phase III clinical trial.

Phase III

D2A21 (Demegal) is a 23-amino-acid amphipathic peptide that shows antifungal activity against various fungi, including *C. albicans*, *Aspergillus niger*, and *Trichophyton mentagrophytes*. It is also active against infectious bacteria, including *P. aeruginosa* and *S. aureus* (Chalekson *et al.*, 2003).

Xoma-629 is a synthetic peptide derived from bactericidal/permeability-increasing protein; it exerts immunomodulatory effects and exhibits antibacterial activity against MRSA (Jenssen and Hancock, 2010). The peptide developed by Xoma Ltd. had been under phase II clinical trials for the treatment of impetigo; these trials was suspended after 2008. Preclinical trials for the treatment of acne and mycoses using this peptide were discontinued in 2006.

Discontinued AMPs

Many AMPs failed before and during clinical trials.

Talactoferrin-α is a recombinant derivative of human glycoprotein, lactoferrin; it is known to possess an immunomodulatory effect (Vincent *et al.*, 2015). Talactoferrin also shows properties similar to lactoferrin, such as the ability to activate the innate and adaptive immune systems. Further, it has been reported to exert beneficial effects against sepsis. Talactoferrin, developed by Agennix, Inc., was under phase II/III clinical trials for the treatment of sepsis and phase III trials for the treatment of non-small cell lung cancer, but all trials were discontinued in 2012 for the recommendation of Data Safety Monitoring Board (DSMB), and the reason remains unclear (Martin *et al.*, 2015).

Pexiganan (MSI-78) is a 22-mer synthetic cationic peptide and its mode of action is disturbing the permeability of cell membrane or cell wall. Its effects were examined in diabetic patients with infection-related foot ulcers; however, it was rejected by the FDA because it exhibits lower efficacy than conventional antibiotics, ofloxacin (Lamb and Wiseman, 1998), and no significant differences were observed between pexiganan and ofloxacin treated group.

Iseganan (IB-367) is a synthetic analog of protegrin I obtained from pig protegrin. A clinical trial studying the effects of Iseganan is aiming to treat patients with oral mucositis (Trotti *et al.*, 2004). However, the patients who had been treated with Iseganan via mouth rinse form experience clinical adverse, such as vomiting, nausea, fatigue, and increased mortality that led to fail in phase II clinical trial (Giles *et al.*, 2004; Kollef *et al.*, 2006).

Opebacan ($rBPI_{21}$, Neuprex) is an α-helical peptide that kills bacteria and neutralizes bacterial endotoxins. It received orphan drug status for *Meningococcal* infection from the European Union in 2006, but discontinued in treatment for haemorrhagic trauma due to insufficient efficacy in antibiotic activity than the most potent antimicrobial peptides (Hancock, 2000).

Limitations of using AMPs as clinical substances

The first natural antibiotic AMP, gramidicin, which is non-ribosomally synthesized, possesses an antibacterial effect only when injected intraperitoneally into infected mice. On the other hand, an intravenous injection of gramicidin is toxic to cells (Dubos, 1940; Rammelkamp and Weinstein, 1942). However, AMPs have several advantages and can be considered as a substitute for antibiotics. The most remarkable characteristic of AMPs is their non-specific mechanism, due to which they can target a broad spectrum of microorganisms (Bahar and Ren, 2013; Kumar *et al.*, 2018). They target and disrupt the integrity of the cell membrane and the biomolecules that exist in every microorganism, and the complexity of the mechanism underlying their action makes it difficult for microorganisms to develop resistance to AMPs (Shai, 2002). Due to these strengths, some AMPs are now undergoing clinical trials, but there are still hurdles that need to be overcome. AMPs are generally sensitive to salt, serum, and pH, and are susceptible to proteolysis. These reasons hinder the efficiency of AMP-based therapies (Papo and Shai, 2005; Hilchie and Hoskin, 2010; Gaspar *et al.*, 2013). Although AMPs can distinguish cancer cells from normal cells based on membrane charge and material composition, it is not safe to rely on this ability (Papo and Shai, 2005).

Solid-phase peptide synthesis of AMPs is expensive. To use AMPs as clinical agents, they need to be produced cost-effectively (Bommarius *et al.*, 2010). The recombinant approach using heterologous peptide production offers a more cost-effective means for large-scale peptide production than chemical synthesis. Thus, several *in vivo* methods have been developed for synthesizing AMPs using an *Escherichia coli*-based system. AMPs are often expressed as fusion proteins in *E. coli* to avoid their proteolytic degradation and their lethal effects on the host. Commonly used fusion partners enhance the solubility of AMPs, promote their aggregation, and contain self-cleavable carriers (Li, 2011). Aggregation-promoting carriers that have high inclusion body-forming tendency are frequently used to purify AMPs. For instance, expression of AMPs from the green fluorescent protein (GFP)-fusion construct prevented bacteriotoxic AMPs from affecting bacterial viability. When the AMPs are dissociated from GFP and purified, their activity was similar to that of chemically synthesized AMPs (Soundrarajan *et al.*, 2016).

Relatively poor host cell membrane permeability of the AMPs may also be troublesome when treating infections caused by intracellular bacteria (Papo and Shai, 2005). To overcome these drawbacks, multiple strategies have been discovered. One strategy is to substitute some of the L-amino acids in the AMPs with their respective D forms. This enhances the hydrophobicity, selectivity, and stability of α-helical AMPs in serum (Riedl *et al.*, 2011; Huang *et al.*, 2012). However, these technical developments can be a double-edged sword because enhanced toxicity to bacterial or cancer cells can also result in increased toxicity towards normal cells. Accordingly, new technical approaches need to be developed to deliver AMPs with more specificity and safety.

De novo methods for *in vivo* delivery of AMPs

Due to the lack of delivery systems for AMPs that protect them from rapid degradation and introduce them into cells across the cytoplasmic membrane, *de novo* introduction methods have been recently developed. Recent reports have shown that AMPs can be loaded onto gold nanoparticles (AuNPs) conjugated with DNA aptamers and delivered efficiently into cells (Yeom *et al.*, 2016; Lee *et al.*, 2017). In addition, AMPs delivered via AuNP-DNA aptamer conjugates exhibited increased stability and antimicrobial activity in mice, and decreased cytotoxicity against human cells (Rai *et al.*, 2016). The decreased cytotoxicity of the AuNP-AMP conjugates results from reduced membrane depolarization of human red blood cells by AMPs, which protects these cells from hemolysis (Pal *et al.*, 2011). However, because there are no conserved amino acid sequences among AMPs, DNA aptamers are conjugated to a hexahistidine-tag, which is additionally fused to the AMPs. Since the size of AMPs is usually small, ranging from 12 to 50 amino acids (Wu *et al.*, 2018), addition of the hexahistidine tag may alter their structure and/or function. One approach to circumvent this prob-

lem is to use a DNA aptamer specific to each AMP, which can be obtained by selecting the aptamer for each AMP using the systematic evolution of ligands by exponential enrichment (SELEX) method (Ellington and Szostak, 1990; Tuerk and Gold, 1990).

The silver-conjugated nanoparticle (AgNP)-mediated system may also be used to deliver AMPs. Interactions between AgNPs and AMPs are enhanced by cysteine residues in the peptide, and strengthened interactions result in the increased stability and biological activity of the AMPs in the serum (Pal *et al.*, 2016). Direct conjugation of AuNP with indolicidin was also shown to enhance antimicrobial activity at a 1,000-fold lower concentration compare to the peptide itself (Sur *et al.*, 2015). In addition, chitosan nanoparticles (CS-NPs)-based delivery of AMPs have been developed. Chitosan is proved to be a biocompatible, non-toxic, biodegradable molecule with antimicrobial activity (Hirano and Noishiki, 1985; Chandy and Sharma, 1990; Kong *et al.*, 2010). It has been shown that Temporin B loaded CS-NP increases antimicrobial activity of the peptide for a long-period, while reducing toxic potential of it (Piras *et al.*, 2015). In other case, AMPs interact with lipids; therefore, methods for the lipid-based delivery of peptides were developed. Thus far, AMPs have usually been administered by injection because of their low hydrolytic stability and poor permeability through the gastrointestinal (GI) mucosa when enterally administered (Li *et al.*, 2012). But the lipid-based approach allows peptides to show their complete activity because the lipid digestion starts from the stomach and is completed in the intestine, where the AMPs are absorbed and delivered through the blood stream to reach the target tissues. For instance, encapsulation of LL-37 within liposomes enhanced the bioactivity of the peptide and reduced its toxicity towards cells in culture (Ron-Doitch *et al.*, 2016).

Furthermore, coupling reactions between AMPs and metal substrates (Chen *et al.*, 2009; Costa *et al.*, 2011), matrix and immersion loading in the polymerization process (Chen *et al.*, 2009; Laverty *et al.*, 2012; Lee *et al.*, 2017), and electrostatic attraction between AMPs and polymers (Etienne *et al.*, 2004) have been shown to increase the stability of the peptides, with lower cytotoxicity.

Discovery of novel AMPs

After the initial discovery of lysozyme and gramicidin, many AMPs have been discovered in animals as components of host defense mechanisms against microbial infection. However, most AMPs have failed to exert their therapeutic potential because of the side effects described above.

Due to the diversity in the sequence and structure of AMPs, establishment of a database of AMPs is a significant problem. However, there are strategies to predict active AMPs using computational methods: 1) using amino acid sequences of AMPs and/or pro-peptide sequences, and 2) applying information related to AMP expression and processing. The first strategy compares the amino acid sequences of novel AMPs with those from the database. AMPs hardly share conserved sequences, but pro-peptides, which are unprocessed AMPs, have remarkably similar sequences. This finding has been used to identify AMPs from amphibians (Agerberth *et al.*, 1995; Sørensen *et al.*, 2001; Li *et al.*, 2007). In addition, polypeptide-modifying enzymes were used to identify novel AMP-like families of lantibiotics that could exhibit antimicrobial activity after undergoing post-translational modification using such enzymes. By screening for the existence of enzymes in the sequenced bacterial genome, the method can sort out strains that may produce the target AMPs (McClerren *et al.*, 2006; Begley *et al.*, 2009).

For the fast and precise analysis of the activity of novel AMP candidates, high-throughput screening methods were developed. The ability of putative AMPs to inhibit the growth of microbes in rich media can be measured (Wiegand *et al.*, 2008). In previous reports, an agar diffusion assay in which the AMP-containing material was spotted onto a bacterial culture-seeded nutrient agar plate was performed; this assay simultaneously showed the potency and solubility of AMPs (Wang, 2010).

The surface localized antimicrobial display (SLAY) system has been developed recently. It simultaneously generates and screens a large number of novel AMPs (Tucker *et al.*, 2018). This system can localize target peptides in the outer membrane of *E. coli* with a structured fusion protein. The method is based on the following principles: When the peptides in the library have an antibacterial effect, the *E. coli* expressing the fusion peptide will be depleted from the population. Then, the read of the corresponding nucleotide sequences will be decreased in the output library. This method enabled researchers to screen novel AMP candidates that have not been discovered in nature. Unlike known AMPs and their typical properties (cationic and hydrophobic peptides), this method can uncover a pool of putative peptides that are not limited by length, electric charge, and hydrophobicity. Thus, this method can provide active AMPs with a new range of characteristics. In addition, due to accumulating information about AMP structure and function, along with advances in computational methodologies, it has now become possible to predict novel AMP-ligand interactions (Shaker *et al.*, 2020).

Conclusion and Future perspectives

The occurrence of antibiotic-resistant pathogenic bacteria is one of the biggest threats to global health. AMPs have been recognized as essential components of a host's natural innate immunity against microbial challenge; they have recently emerged as substitutes for antibiotics. Although AMPs have many attractive properties and are being analyzed in diverse clinical trials, their frequent failures in clinical trials emphasize the need for the development of technologies to increase the stability and delivery of AMPs with lower cytotoxicity in living systems. In addition, their broad spectrum of antibiotic activity as well as anti-inflammatory and immunomodulatory functions need to be extensively investigated to better understand their pleiotropic properties and the mechanisms underlying their contribution to the host defense. The recent introduction of AMPs into the market indicates the potential of AMPs as a novel class of antimicrobial drugs. However, their efficacy remains to be proven.

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Conflict of Interest

We have no conflicts of interest to report.

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