Preprint

Defensins: the natural peptide antibiotic

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Abstract

Defensins are a family of cationic antimicrobial peptides active against a broad range of infectious microbes including bacteria, viruses and fungi, playing important roles as innate effectors and immune modulators in immunological control of microbial infection. Their antibacterial properties and unique mechanisms of action have garnered considerable interest in developing defensins into a novel class of natural antibiotic peptides to fend off pathogenic infection by bacteria, particularly those resistant to conventional antibiotics. However, serious pharmacological and technical obstacles, some of which are unique to defensins and others are common to peptide drugs in general, have hindered the development and clinical translation of defensins as anti-infective therapeutics. To overcome them, several technologies have been developed, aiming for improved functionality, prolonged circulation time, enhanced proteolytic stability and bioavailability, and efficient and controlled delivery and release of defensins to the site of infection. Additional challenges include the alleviation of potential toxicity of defensins and their cost-effective manufacturing. In this review, we briefly introduce defensin biology, focus on various transforming strategies and practical techniques developed for defensins and their derivatives as antibacterial therapeutics, and conclude with a summation of future challenges and possible solutions.

Keyword

1 Introduction

To defend against the constant invasion of microbes such as bacteria, viruses and fungi, organisms from insects to humans have evolved their immune systems to deploy natural antimicrobial peptides as a major defense mechanism of innate immunity [1]. Since the discovery of melittin from honeybee venom – the first *de facto* antimicrobial peptide by Habermann in 1954 [2], thousands of antimicrobial peptides have been identified; some have undergone extensive pre-clinical and clinical tests with encouraging outcomes due to their broad-spectrum and unique mechanisms of action [3]. While their antiviral activity is mechanistically complex [4, 5], many antimicrobial peptides kill bacteria by perforating holes on the membrane, contrasting traditional antibiotics that achieve their functionality by targeting specific bacterial proteins or pathways [6]. The mechanistic multiplicity of antimicrobial peptides in general and their membranolytic activity in particular against bacteria and viruses render it difficult for microbes to acquire resistance [7], promising an attractive therapeutic paradigm for the treatment of various microbial infections.

Defensins, broadly defined as a family of 2-5 kDa, cationic and Cys-rich antimicrobial peptides, are found in plants, insects and mammals. Many excellent reviews have been published over the years on various topics of defensins [8-14]. In mammals, defensins of 18-45 amino acid residues are divided into three subgroups - α, β and θ, according to their tissue distribution and spatial arrangement of six cysteine residues [15]. Structurally, mammalian defensins are highly conserved despite high degrees of sequence variability, generally adopting a β-sheet framework stabilized by three disulfide bonds. Functionally, mammalian defensins are extremely diverse, playing multi-faceted roles in both innate and adaptive immunity against microbes [16-18]. In addition to their antimicrobial function [19-21], defensins have also been implicated in tumorigenesis [22-24].

The broad-spectrum antimicrobial activity of defensins against Gram-positive and -negative bacteria as well as enveloped- and non-enveloped viruses earns defensins a unique opportunity to be developed into a novel class of therapeutic agents to fend off infectious diseases. Important pharmacological hurdles remain, though, hindering their clinical application. First, defensins are relatively weak anti-infective agents that are subject to functional neutralization by divalent cations such as Mg^{2+} and Ca^{2+}, and

anionic substances such as polysaccharides and nucleic acids, among many other endogenous binding partners [25, 26]. Although being relatively weak against microbes may arguably be a feature intended by nature in order to make defensins "broadly active" in innate immunity, it does not debilitate defensins from mounting an effective defense mechanism *in vivo* as their expression is highly localized and at high concentrations when in action. Second, as small peptide therapeutics in general, defensins are also subject to proteolytic degradation *in vivo* by various proteases and fast renal excretion, resulting in poor bioavailability and efficacy [25, 27]. Third, defensins are generally difficult to produce in large quantities either chemically or recombinantly due in part to their peculiar folding behavior and bacterium-killing property [26], presenting an economical challenge to drug development. Finally, defensins, capable of acting on various host proteins and receptors, can chemoattract and activate immune cells [28], casting uncertainty on their safety profile as therapeutic agents for systemic use.

Many of these problems may be overcome with advanced delivery systems. Loading defensins onto rationally designed delivery systems help maintain their stability and activity, alleviates their susceptibility to proteolytic degradation, and delivers them with precision to desired pathological settings as needed with enhanced therapeutic efficacy. Various delivery systems are suitable for tackling the above-mentioned pharmacological hurdles as their structural, physiochemical, and functional properties can be easily manipulated [29, 30]. In recent years, the construction of nanoparticles from natural or chemically synthesized biocompatible components (such as biosurfactants, lipids, mesoporous silica, biopolymers, and metallic nanomaterials) has received considerable attention from scientists because of their favorable safety profiles and multifunctional properties. Nano-drug delivery systems are characterized by tiny and controllable particle size, chemically modifiable surface, as well as high surface area and loading capacity, all of which are beneficial for the *in vivo* transport and controlled release of peptide and protein therapeutics [31, 32], including defensins.

Recent advances in peptide drug discovery, development and delivery have alleviated, at least in part, some pharmacological hurdles facing the development of defensins and derivatives as anti-infective agents. In this review, we focus on various transforming strategies and practical techniques developed for defensins and derivatives as potential therapeutics for the treatment of bacterial infection, following a brief introduction to defensin biology. We conclude this review with a summation of

future challenges and possible solutions.

2   Classification and structural characteristics of human defensins

The mammalian defensin family consists of three distinct members: α-, β-, and θ-defensins. While α- and β-defensins are expressed in humans and other mammals, θ-defensins are only present in non-human primates such as rhesus macaques and Olive baboons [14, 33, 34]. The first α-defensin was discovered by Lehrer and colleagues from rabbit alveolar macrophages, named then macrophage cationic protein or MCP [35, 36]. It was not until 1985 when the same group discovered homologous antibiotic peptides from human neutrophils did Lehrer coin the term defensin [37, 38]. To date, six human α-defensins have been identified, also termed human neutrophil peptides 1-4 (HNPs 1-4) and human enteric defensins 5-6 (HD5 and HD6) [37]; many more human β-defensins exist in epithelial tissues [26, 39, 40].

HNP1-3 of 29-30 amino acid residues, rich in cysteine, arginine and aromatic residues, are abundant in azurophilic granules of neutrophils, accounting for 30%-50% of total granular protein [38, 41]. They differ by a single amino acid residue at the N-terminus, with an Ala in HNP1, an Asp in HNP3, and a deletion in HNP2. Despite the single-residue difference, HNP1 and HNP3 show different anti-microbial activities [42], indicative of the functional importance of charge. The six cysteine residues in HNP1-3 form three pairs of disulfide bonds, i.e., C1 to C6, C2 to C4, and C3 to C5 (Cn – the sequential number of a cysteine residue) to stabilize a three-stranded β-sheet structure [43-45]. Another member of them, HNP-4 of 33 amino acid residues was identified later with the same cysteine framework and structural similarity to HNP1-3 [46]. However, HNP4 significantly differs from HNP1-3 in amino acid sequence, which leads to a notable difference in function between them [47]. The remaining two members of the human α-defensin family, HD5 and HD6, are mainly expressed in intestinal Paneth cells [48, 49]. Although they have distinct residues composition, HD5 and HD6 share the same disulfide bond skeleton and similar three-dimensional structures with HNP1-4 [14, 50].

In addition to the six Cys residues, three other amino acid residues are also completely conserved in mammalian α-defensins, i.e., Arg^5, Glu^{13} and Gly^{17} (HNP1/HNP3 numbering). Structure and function relationship studies show that Arg5 and Glu^{13} form a salt bridge critical for defensin *in vivo* stability [51, 52], while Gly^{17}

as part of a "β-bulge" is essential for defensin folding and function [53, 54]. Despite being often referred to as "cationic" antimicrobial peptides, Arg residues in human α-defensins are NOT most consequential at the functional level. Rather, the C-terminal bulky hydrophobic residues such as Trp[26] in HNP1-3, Phe[26] in HNP4 and Tyr[27] in HD5 are often far more important than Arg [47, 55, 56], suggesting that hydrophobicity rather than cationicity imparts defensin function [14, 57]. The C-terminal hydrophobic residues in α-defensins also play a structural role in mediating functionally important defensin dimerization [58], which is the critical first step leading to defensin oligomerization and multimerization upon binding to other target molecules [59, 60], thus underlying defensins' structural "heterogeneity" and functional "versatility" [57].

In contrast with α-defensins, β-defensins have broad distributions, both in species and tissues. Up to now, they have been discovered in birds [61, 62], teleost fish [63] and nearly all of the mammals studied, including rabbit, cattle, rat, mouse, pig and human [64]. In humans, although more than 30 β-defensin genes have been identified, only several of them were studied at the protein level, including primarily the human β-defensins 1-4 (hBD1-4) [65]. hBD-1 was first discovered in the hemodialysis solution of a renal failure patient [66], and subsequently found in the gastrointestinal tract, urogenital tract and oral epithelium [67, 68]. hBD-2 and hBD-3 were discovered in damaged skin of psoriasis [69, 70]; the former also expressed in epithelial tissues of the respiratory tract, oral cavity and gastrointestinal tract [71-73]. While hBD-3 is mainly distributed in skin and tonsil [74], expression of both hBD-2 and hBD-3 can be induced by inflammatory irritation, which is transcriptionally regulated. The expression of hBD-4 was found to be limited to tissues such as testicles and antrum [75].

hBD1-4, rich in arginine residues, contain ten conserved amino acid residues including six cysteines [69, 76]. Among the four β-defensins, hBD-2 and hBD-3 share nearly 43% sequence identity, whereas hBD-4 shares only 20-25% sequence identity to hBD1-3 [77]. Distinct from α-defensins, β-defensins possess the topology of three disulfide bonds as C1–C5, C2–C4, C3–C6 [74]. β-defensins with 36 or more amino acid residues share the consensus sequence pattern x2–10Cx5–6(G/A)xCX3–4Cx9–13Cx4–7CCxn (x denoted as any amino acid, the numbers and 'n' mean the quantity) [26]. The β-defensin sequences significantly differ from those of the α-defensins, folding into a dissimilar three-stranded β-sheet structure that can be further divided into hydrophobic and charged domains. Table 1 and Fig. 1 provide a general overview of

the classification and expression, as well as the structures of human defensins.

Table 1. Overview of human defensins: classification, major cell sources and sequence.

Defensins	Name	Major cell sources	Sequence	references
α defensins	HNP1	neutrophils	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	[38]
	HNP2	neutrophils	CYCRIPACIAGERRYGTCIYQGRLWAFCC	[38, 45]
	HNP3	neutrophils	DCYCRIPACIAGERRYGTCIYQGRLWAFCC	[38]
	HNP4	neutrophils	VCYCRIPACIAGERRYGTCIYQGRLWAFCC	[46]
	HD5	intestinal Paneth cells	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR	[48]
	HD6	intestinal Paneth cells	AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL	[49]
β defensins	hBD1	epithelium	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK	[67, 68]
	hBD2	epithelium	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCK KP	[78]
	hBD3	epithelium	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRK CCRRKKI	[74]
	hBD4	epithelium	MQRLVLLLAVSLLLYODLPVRSEFELDRICGYGTARCRK KCRSQEYRIGRCPNTYACCLRKWDESLLNR	[77]

Abbreviations: hBD, human β defensin; HD, human enteric defensin; HNP, human neutrophil peptide;
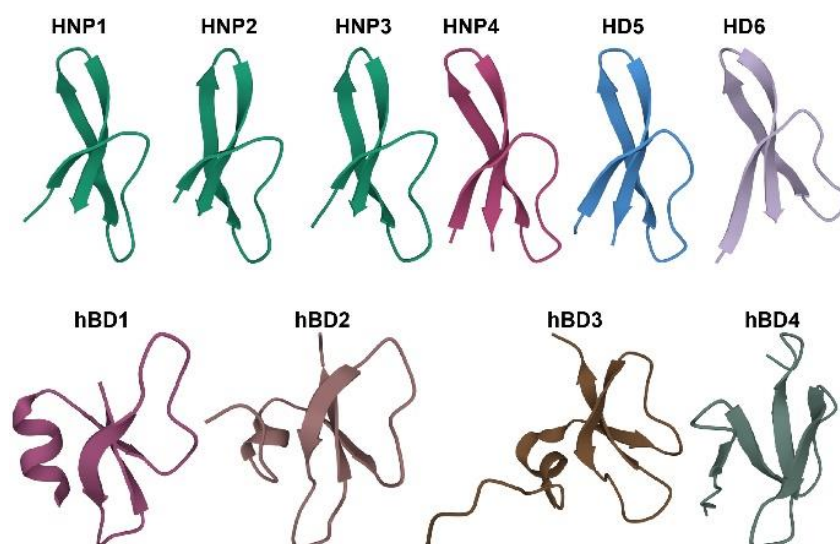

Fig. 1. Monomeric structures of human defensins, HNP1 (PDB ID: 2PM1), G16^{D}A-HNP2 (1ZMH), HNP3 (1DFN), HNP4 (1ZMM), HD5 (1ZMP), HD6 (1ZMQ), hBD1 (1IJV), hBD2 (1FQQ), hBD3 (1KJ6), and hBD4 (5KI9).

Different from α and β defensins, θ-defensin peptides of 18 amino acid residues exist only in non-human primates [79]. They were first identified from neutrophils of the rhesus macaques, termed rhesus theta defensin 1 (RTD-1) [33]. Immune-histochemical staining demonstrated the expression of θ-defensins in bone marrow-derived phagocytic cells, strongly positive in neutrophils, weakly positive in monocytes, but negative in lymphocytes and eosinophils [33]. The most striking feature of θ-defensins is that they form a naturally cyclic backbone structure without flanking sequences, where the six internal Cys residues pair in a ladder pattern to cross-connect two antiparallel β-strands, C3-C16, C5-C14 and C7-C12 [33]. This head-to-tail cyclic peptide is formed in a posttranslational reaction by two linear peptides of nine amino acid residues each (RTD1a and RTD1b), which are derived separately from their precursors [33]. Of note, the θ-defensin genes of humans contain a premature termination codon, consequently humans fail to produce endogenous θ-defensin peptides [79]. Lehrer and colleagues reconstructed humanized θ-defensins based on sequences encoded in the human genome and named them retrocyclins [80]. The cyclic structure entrusts θ-defensins with additional resistance to high salts and proteases that are abundant in inflammatory exudates, thereby explaining some of their potent antimicrobial activity [33, 34].

3   Antibacterial functions and mechanisms.

Defensins as antimicrobial peptides are active against a broad range of microbes including bacteria, viruses and fungi, and play as pleiotropic immune effectors multifaceted roles in inflammation, development and cancer [1, 5, 14, 19, 20, 81-85]. Although new functions and applications of defensins have aroused great interest in recent years, including the latest reports of defensin inhibition of SARS-CoV-2 [86], their bacterium-killing properties and mechanisms of action have been investigated the longest with both depth and breadth across the field, which is the focus of this part of our review (Fig. 2).
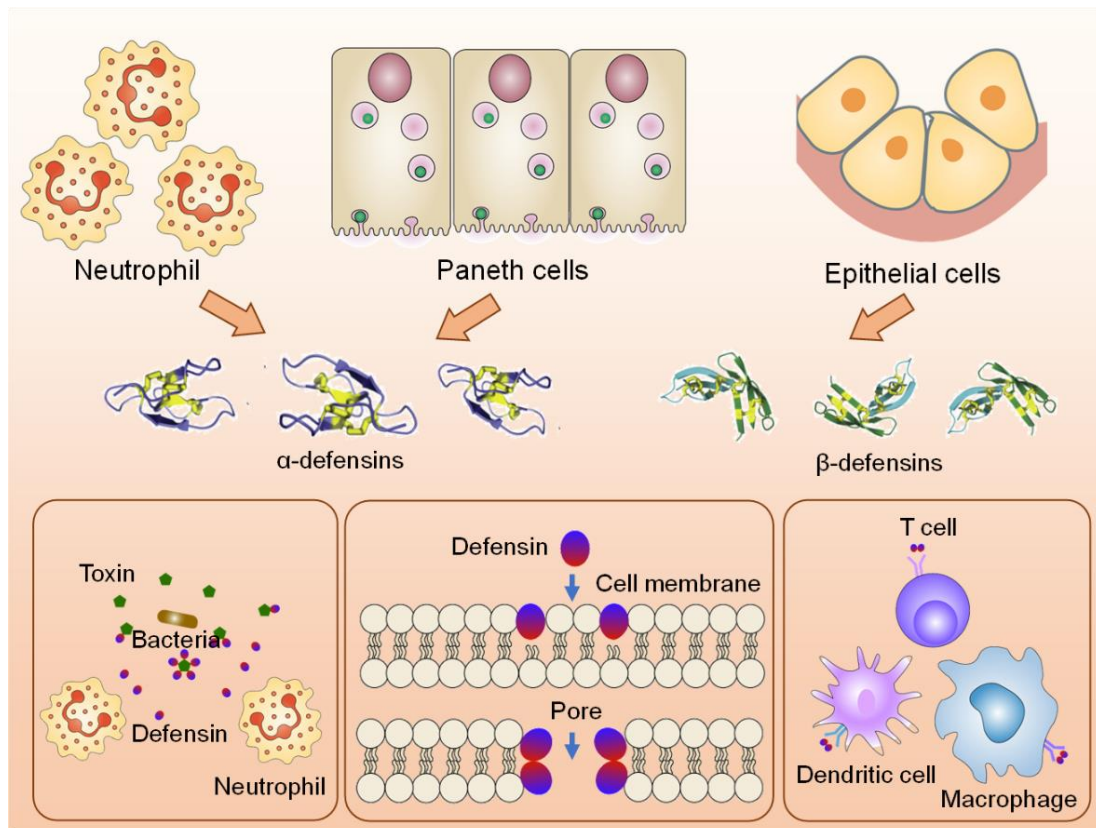
Fig. 2. The secretion and antimicrobial mechanism of human defensins.

Early studies by Lehrer and colleagues firmly established that defensins such as HNPs kill *Escherichia coli* (*E. coli*) by forming channels to permeabilize its lipid bilayer membranes [87, 88], a process initiated by electrostatic interactions between the positively charged peptide and the negatively charged microbial membrane. Systematic mutational studies indeed demonstrate that Arg residues in HNPs and HD5 dominate their antibacterial activity against Gram-negative strains of bacteria with hydrophobic residues playing a lesser functional role [14, 58]. Further, the more cationic human β-defensins are generally more active than their α-counterparts [44, 74, 78, 89]. Consistent with this tenet, the modification of phosphatidylglycerol with L-lysine, which reduces the negative charge on microbial membranes, confers resistance to defensin-mediated bacterial killing [90]. On the other hand, host cells largely escape from defensin assault due to their electrically neutral membrane [91].

This membranolytic mode of action of defensins has also been accepted for other classes of antimicrobial peptides as an overriding mechanism of bacterial killing, although opinions differ with respect to how antimicrobial peptides (AMPs)form membrane channels at the molecular level [92, 93]. Nevertheless, numerous studies

confirm that the ability of AMPs to self-associate (dimerize, oligomerize or multimerize) is correlated to their ability to form membrane pores and, thus, to kill bacteria [55, 58, 87, 94, 95]. Of note, the cyclic θ-defensins are not amphipathic, and their interactions with anionic membranes are somewhat different from HNPs [96, 97]. However, when it comes to the killing of Gram-positive bacteria, which have a much thicker bacterial cell wall than Gram-negative strains, the membranolytic mode of action of defensins becomes far less certain. In fact, the ability of α-defensins to permeabilize lipid bilayer membranes is poorly correlated to their bacterial killing activity against Gram-positive strains, in stark contrast to Gram-negative bacteria [98]. Growing evidence now suggests that defensins kill Gram-positive bacteria by targeting Lipid II to inhibit bacterial cell wall synthesis [98-100].

Defensin inhibition of bacterial infection does not always result from bacterial killing. Unlike human enteric HD5 or mouse intestinal α-defensins [101, 102], HD6 is of little bactericidal and membraneolytic activity *in vitro* [103]. Yet, HD6 prevents *Salmonella* infection of mice by forming a nanonets structure *in vivo* to entrap bacteria, thus inhibiting bacterial adhesion and invasion [104]. Defensins can also reduce bacterial infection through neutralization or inactivation of various secreted protein toxins [59, 105-107]. θ-Defensins can induce the release of cell wall lytic enzymes, along with membrane destruction, leading to the lysis of Staphylococci [108].

Defensins also work as an important modulator in the immune system to indirectly enhance their antibacterial effect. Recent studies have shown that the defensins can act as chemotactic factors, linking the innate and adaptive immune systems [16, 109]. For example, the β-defensins can stimulate the host adaptive immune response by recruiting dendritic and T cells to the site of microbial invasion; this effect depends on the chemokine receptor CCR6, preferentially expressed by immature dendritic cells and memory T cells [109]. hBD-2 can also induce the expression of co-stimulatory molecules that activate the Th1 response via Toll-like receptor (TLR)-4 [110], whereas hBD-3 has been reported to activate monocytes and induce macrophage chemoattraction via CCR2 [111, 112].

Furthermore, defensins can induce the expression of other cytokines and chemokines to fight off bacterial infection. For example, HNP1–3 can trigger macrophages to release tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), which

act in an autocrine loop to enhance the expression of CD32 and CD64, thereby augmenting phagocytosis of bacteria [113]. HNPs also up-regulate the expression of TNF-α and IL-1β in monocytes activated with *S. aureus*, thus modulating the inflammatory responses [114]. Defensins can also increase the expression of IL-8 in airway epithelial cells in the lung inflammatory processes [115]. Similarly, hBD2-4 can stimulate human keratinocytes for increased expression and protein production of IL-6, IL-10, monocyte chemoattractant protein-1, and macrophage inflammatory protein-3α, which participate in the skin immune activity against pathogens [116]. hBD-3 can also stimulate monocytes and macrophages for secretion of a variety of chemokines including macrophage-derived chemokine, the angiogenic factor and vascular endothelial growth factor [117]. Although not expressed by macrophages, defensins can contribute to the antibacterial function of the phagocytes by inhibiting phagosomal escape and intracellular multiplication of *Listeria monocytogenes* and *Mycobacterium tuberculosis* [118, 119]. Of note, HNP1 inhibits macrophage-driven inflammation in the aftermath of *Salmonella* infection, ensuring both pathogen clearance and inflammation resolution [120].

4 Delivery systems for defensins

Delivery vehicles are commonly utilized to improve the biological and pharmacological properties of therapeutic agents, including, but not limited to, biocompatibility, stability, and circulation time. They are particularly useful for defensins and defensin derivatives, which often display non-specific hemolytic activity against normal cells [121], short circulation half-life [122], susceptibility to functional impairment by their electrostatic interactions with salts (e.g., divalent cations such as Mg^{2+} and Ca^{2+}) and anionic substances (polysaccharides, nucleic acids, proteins, etc.), and to proteolytic degradation in body fluids [25, 27]. Here, we review delivery vehicles recently developed for the conjugation or encapsulation of defensins to combat bacterial infection, including lipidation, hydrogel, metal nanoparticles, silica nanoparticles, and polymeric nanoparticles (Fig. 3).
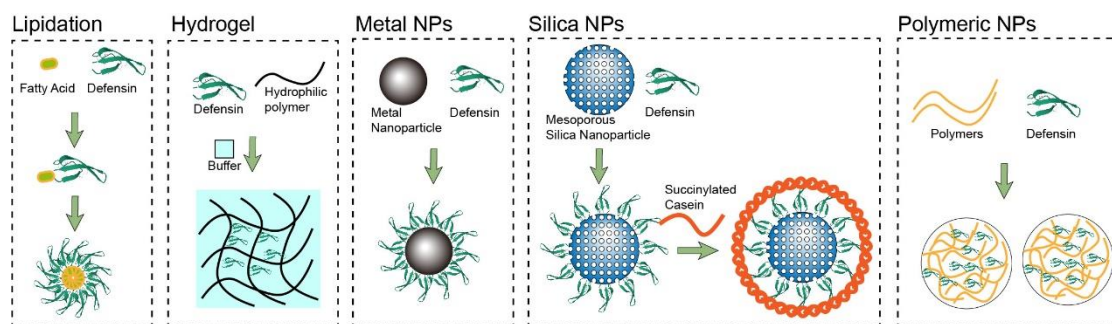
Fig. 3. Delivery systems for defensins.

4.1 Lipidation

Lipidation increases the antimicrobial activity of AMPs as a fatty acid added to their N- or C-terminus contributes to peptide embedding into lipid bilayers [123, 124], and induces peptide self-organization into micelles in bacterial membranes, thereby improving membrane permeability [125, 126]. Inspired by self-assembled nanobacterial agents [127], Lei et al. proposed a drug design strategy that combines lipidation of defensins with subsequent non-covalent self-assembly (Fig. 4A) [128]. The derivative (HD5-myr) was constructed by coupling via an amide bond myristic acid, a 14-carbon, straight-chain fatty acid, to the side-chain of a Lys residue appended to the C-terminus of human α-defensin 5 (HD5) (Fig. 4B). Compared with HD5, HD5-myr-assembled nanobiotics showed higher antibacterial activity even in the presence of sodium chloride or serum by disrupting bacterial membranes or cell wall structure (Fig. 4C and 4D). Additionally, the nanobiotics displayed fairly low toxicity in animals and protected mice from *Staphylococcus aureus* (MRSA) infection or *E. coli*-induced sepsis by decreasing bacterial burden and organ damage (Fig. 4E and 4F).
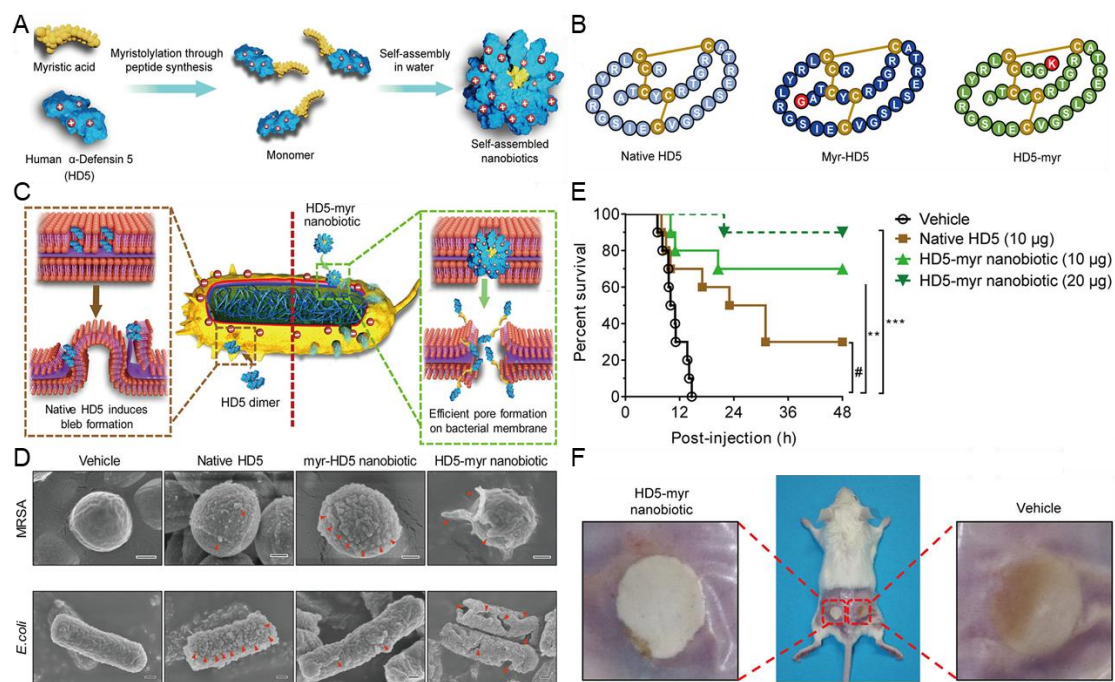
Fig. 4. Self-assembling myristoylated (myr) human α-Defensin 5 (HD5) against bacterial infections. (A) Engineering HD5 through peptide myristoylation and nanoassembly. (B) Amino acid sequences of HD5, myr-HD5, and HD5-myr. (C) Possible mechanisms by which the HD5-myr nanobiotics kill *E. coli*. (D) SEM images of MRSA and *E. coli* after different treatments. (E) Kaplan−Meier survival analysis of mice after different treatments. (F) HD5-myr nanobiotics significantly reduced cutaneous inflammation. Adapted with permission from ref. [128]. Copyright 2018 American Chemical Society.

To explore whether lipidation enhances the antibacterial activity of C-terminal cationic segments of hBD1-3 (Phd1-3), Krishnakumari et al. synthesized N-terminally myristoylated Phd1-3 peptides, i.e., MPhd1-3, and demonstrated their improved antibacterial activity against *S. aureus* as compared to non-myristoylated forms [129]. In fact, in the presence of 150 mM NaCl, MPhd1-3 peptides were active against *S. aureus*, whereas Phd1-3 showed no activity. MPhd1-3 peptides also were more effective in neutralizing the surface charge of bacteria than Phd1-3, indicating that myristoylation contributes to the interaction of peptides with the bacterial membrane. The anchoring of fatty chains into the bacterial membrane is thought to facilitate stronger electrostatic interactions between the peptides and the negatively charged bacterial cell surface, thus conferring the activity of MPhd1-3 in the presence of salt.

4.2 Hydrogel

Hydrogels are three-dimensional networks with high water content formed by synthetic or natural polymers [130]. While the environment provided by hydrogels protects peptides from denaturation and helps maintain their activity, the polymeric network of hydrogels significantly reduces the enzymatic degradation of loaded peptides [131]. In recent years, aided by a better understanding of diverse properties of hydrogels (e.g., polymer density, charge, mesh size, and crosslinking density), researchers have constructed carriers for the delivery of antimicrobial agents through modulation of the encapsulation and release of impregnated materials [132, 133]. To investigate the therapeutic efficacy of nanobiotics in wound healing and to further facilitate their topical application and controlled release at the wound site, Luo et al. designed an HD5-derived nanodefensin (ND) peptide and loaded it into the copolymer Pluronic F-127 hydrogel (NDEFgel) (Fig. 5A and 5B)[134]. Compared with the parent peptide HD5, ampicillin and kanamycin, ND showed stronger bactericidal activity against MRSA *in vitro* and significantly induced cell migration of fibroblasts and their transformation into myofibroblasts. Four days after excisional murine wound treatment, NDEFgel treatment resulted in a higher wound closure rate and lower cutaneous bacterial burden compared with an ND solution or Prontosan (a clinical hydrogel dressing) treatment (Fig. 5C-E). Additionally, NDEFgel-treated skin exhibited significant deposition of collagen, high expression of cytokeratin 14, enrichment of vascular endothelial cells, and lower macrophage infiltration; these results suggest that NDEFgel contributed to angiogenesis, re-epithelialization, and anti-inflammatory response. Mechanistically, ND promoted fibroblast migration and facilitated their transformation into α-SMA-positive myofibroblasts for skin repair in a Rac1-dependent manner.

To protect a fragment of hBD3 (D1–23) from environmental degradation and facilitate its delivery and controlled release on the tooth surface, Aida et al. designed a liquid crystalline system (LCS) composed of Carbopol® 971P, Carbopol® 974P, polyoxypropylene-(5)-polyoxyethylene-(20)-cetyl alcohol, and oleic acid [135]. When sufficiently spiked by artificial saliva to enhance viscosity and bioadhesiveness on the tooth surface, D1–23-loaded LCS showed time-dependent inhibitory activity against *S. mutans* biofilm and little cytotoxicity as compared with free D1–23, showcasing its potential as a safe prevention and treatment tool for dental caries.

4.3 Metal nanoparticles

Metallic nanomaterials have been developed as antimicrobial agents with the significant advantage of multiple antimicrobial mechanisms such as impaired cell membrane function, reactive oxygen species production, protein dysfunction, and loss of enzyme activity [136]; the lack of conventional therapeutic targets makes it difficult for pathogens to develop resistance against them [137, 138]. Metallic nanomaterials incorporating antimicrobial peptides have been reported to amplify the antimicrobial effects of both, resulting in a synergistic behavior. Among many metal derivatives such as ferrocene (Fc), bismuth (Bi) and nickel (Ni), gold (Au) and silver (Ag), gold/silver nanoparticles have received more attention because of their easily adjustable size and shape, enhanced local density of defensins, and potential for application in clinical practice [139, 140].

Recent studies have shown that Au/Ag nanoparticles can be coupled with various AMPs, including but not limited to defensins. Rai et al. chemically immobilized the AMP cecropin-melittin on gold-nanoparticle-coated surfaces, which quickly permeabilized *E. coli* membrane even in the presence of serum [141]. Yeom et al. developed DNA-aptamer-conjugated AuNPs for the delivery of AMPs, which protected mice from *S. typhimurium* colonization [142]. Ruden et al. combined AgNPs and polymyxin B, a cyclic polycationic antimicrobial peptide, and reported their synergistic antimicrobial effects against gram-negative bacteria [143]. Besides, Pal et al. linked Odorranain-A-1 (OA1), an AMP derived from the skin of Chinese odorous frogs, to AgNPs via cysteine interactions, and obtained an AgNP-OA1 conjugate with enhanced stability and bactericidal activity [144].

As a major member of the antimicrobial peptide family, defensins can also be delivered using the above strategies. Based on the specific mode of action of HNP-1 against mycobacteria [145], Sharma et al. conjugated an antimicrobial motif (Pep-H) of HNP-1 to AuNP and examined its potential in inhibiting *Mycobacterium tuberculosis* infection (Fig. 5F-H) [146]. Pep-H at concentrations of 5-20 µg/mL suppressed *Mycobacterium tuberculosis* grown both in monocyte-derived macrophages and *in vitro*, and this antimicrobial activity was further enhanced by Pep-H conjugation to AuNPs (Fig. 5I). Similar to free Pep-H, Pep-H-AuNPs showed little effect on the erythrocyte integrity and viability of monocyte-derived macrophages even at a higher concentration of 100 µg/mL.
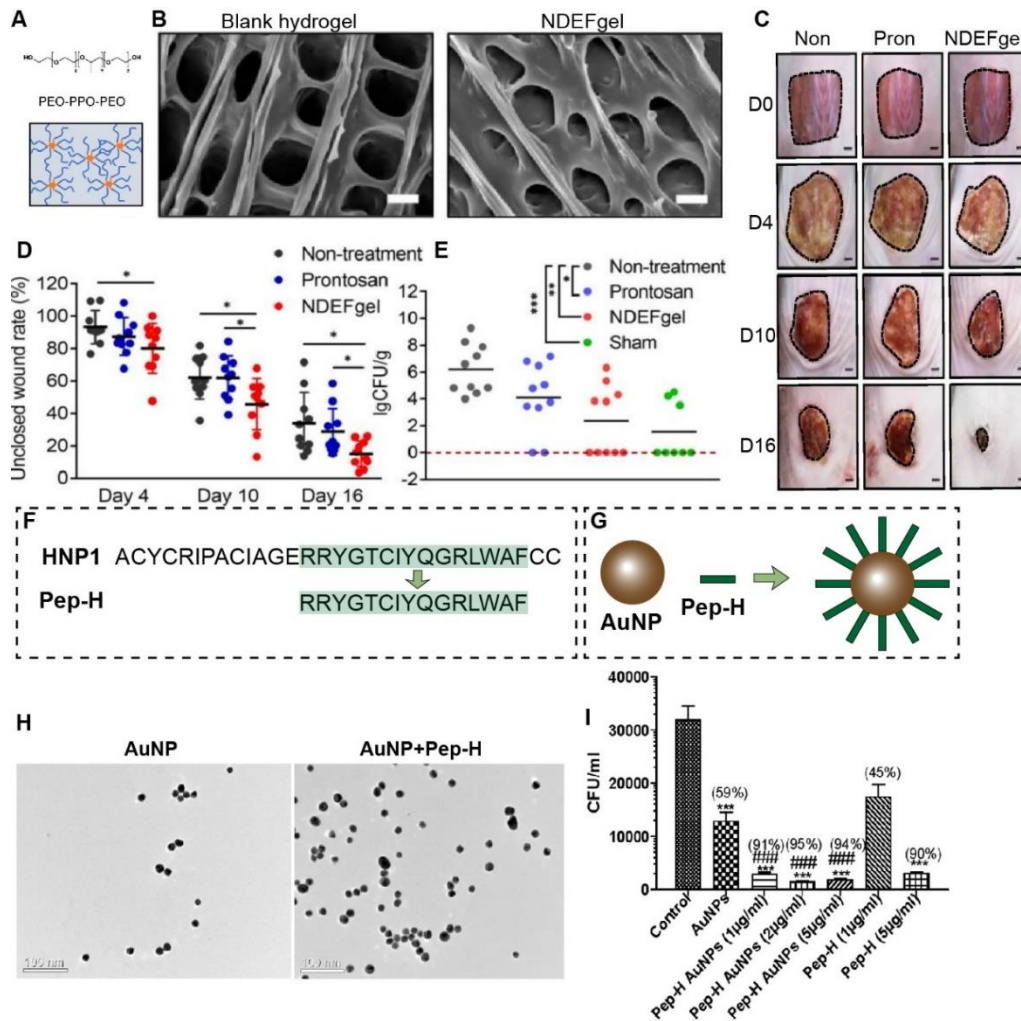
Fig. 5. HD5 hydrogel treatment helps wound healing and decreases the bacterial burden in murine full-thickness wound models. (A) Illustration of the copolymer Pluronic F-127 hydrogel. (B) Scanning electron microscopies of the blank hydrogel and the hydrogel loaded with the HD5-dirived nanodefensin (NDEFgel). (C) Macroscopic images of the wounds after surgery (D0), and after 4, 10, or 16 days of treatment. (D) Quantification of unclosed wound rates in (C). (E) Bacterial burden after 16 days of treatment. Adapted with permission from ref. [134]. (F-I) Pep-H-AuNP nanoparticles exhibit enhanced bactericidal activity against *M. tuberculosis*. Pep-H is an antimicrobial motif of HNP-1 (F), and conjugated to AuNPs (G). (H) Transmission electron microscopies of the AuNPs and Pep-H-AuHNPs. Scale bars, 100 nm. (I) Compared with AuNPs and free Pep-H, Pep-H-AuNPs shows significantly enhanced antimicrobial activity against *M. tuberculosis*. Adapted with permission from ref. [146].

4.4 Silica nanoparticles

As nanocarriers with a hollow structure, mesoporous silica nanoparticles (MSNs) have adjustable pore size, chemically modifiable surface, and high surface area and loading capacity; they also exhibit good biocompatibility and physiochemical stability [147]. These special properties make MSNs promising drug carriers [148, 149]. hBD-1 and HNP-1 have been reported to permeate biofilms and further lyse microorganisms, thus possessing broad-spectrum antimicrobial activity [150]. Bolatchiev et al. examined the inhibitory effect of hBD-1/HNP-1 encapsulated in MSNs on methicillin-resistant MRSA [151]. Pharmacodynamic tests demonstrated that after treatment with hBD-1/HNP-1-containing MSNs, MRSA-infected rats showed high wound healing rates, which could be attributed to the anti-staphylococcal activity of and induction of cell migration and angiogenesis by hBD-1/HNP-1 [152-154].

HD5 is an enteric antibiotic candidate, but its medicinal development is hampered by the shielding effect of salt ions [103, 155]. Peptides containing a high proportion of positively charged residues have salt-tolerant antimicrobial activity [89, 156]. Wang et al. attempted a charge-reversal substitution to optimize HD5 activity by replacing Glu21 with an Arg residue [157], which increased the antimicrobial activity of HD5 and improved its ability to penetrate microbial membranes (Fig. 6A). Additional mutational studies of the three electroneutral residues Gly^{24}, Ser^{23} and Thr^7 resulted in T7R/E21R-HD5, a highly active defensin analog against *E. coli* and *S. aureus* (Fig. 6B and 6C). This potent bactericidal activity was retained by the T7R/E21R double mutation even in saline (150 mM NaCl) and 10% serum solutions compared with HD5. Zhao et al. subsequently used MSNs as a carrier for T7R/E21R-HD5 to examine its therapeutic potential in treating intestinal infection (Fig. 6D and 6E) [158].

The negative charge on the MSN surface (-14.6) supported electrostatic attraction of T7R/E21R-HD5 with a net positive charge (+7), ensuring an efficient loading of the peptide at close to 70% as confirmed by BCA protein assay. In addition, T7R/E21R-HD5 partially aggregated on the material surface, leading to an enhanced local density of hydrophobic and charged residues. MSNs significantly enhanced the penetration of T7R/E21R-HD5 into the outer and inner membranes, and upon MSN binding to bacteria, T7R/E21R-HD5 was released from the pores of MSNs at a higher local concentration than free peptide. To reduce the denaturation/degradation of T7R/E21R-HD5 by pepsin in the stomach, succinylated casein (SCN), which can be degraded by intestinal proteases such as trypsin, was fabricated on the surface of MSNs. In the

presence of trypsin but not pepsin, MSN@T7R/E21R-HD5@SCN effectively eliminated multidrug-resistant *E. coli*, indicating that the SCN coating controls the T7R/E21R-HD5 release in the intestine. The resulting product MSN@T7R/E21R-HD5@SCN showed stronger antibacterial activity against multidrug-resistant *E. coli* than T7R/E21R-HD5 (Fig. 6F) and no toxicity to host cells. Further, MSN@T7R/E21R-HD5@SCN killed multidrug-resistant *E. coli in vivo,* and effectively controlled intestinal infection.
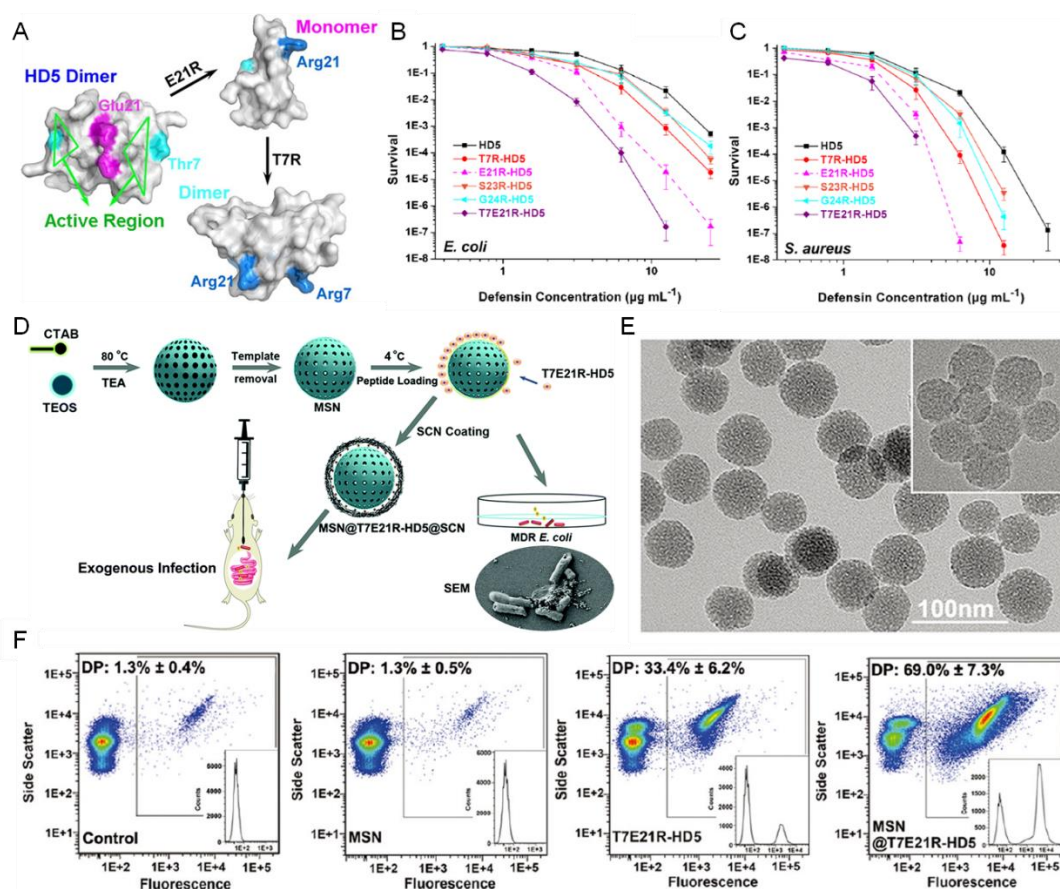

Fig. 6. HD5-derived antibiotic peptide T7E21R-HD5 loaded by mesoporous silica nanoparticles (MSNs) against intestinal bacterial infection. (A) Optimization strategies focusing on the active region of HD5. Antibacterial activity of HD5-derived antibiotics against *E. coli* (B) and *S. aureus* (C). Adapted with permission from ref. [157]. Copyright 2015 American Chemical Society. (D) MSN@T7E21R-HD5 preparation and functional verification. (E) TEM micrographs of MSNs. (F) Flow cytometry analysis of membrane potential of multidrug-resistant *E. coli* treated with bactericides. Adapted with permission from ref. [158]. Copyright 2019 The Royal Society of Chemistry.

4.5 Polymeric nanoparticles

Biodegradable and biocompatible polymers have been widely used for drug delivery. Generally, synthetic water-soluble polymers enhance solubility and prolong the circulation of the payload [159]. For defensin delivery, various polymeric structures have been investigated, including linear polymers, dendrimers (allyl-functional polyesters), and polymeric nanoparticles (Poly (lactic-co-glycolic acid) (PLGA)).

Liang et al. prepared neutrophil β-defensin-5 (B5)-loaded PLGA nanoparticles (B5-NPs) using a water-oil-water solvent-evaporation method [160], and then investigated their immunoregulatory and antibacterial activity against *Mycobacterium bovis*. Under physiological conditions, the defensin B5 was slowly released from the B5-NPs. Treatment with B5 or B5-NPs reduced the concentration of TNF-α in bronchoalveolar lavage fluid or serum and enhanced the therapeutic effect of rifampicin against *Mycobacterium bovis*. Further, B5-NPs effectively increased the secretion of TNF-α and interleukin-10 in macrophages compared with B5, indicative of its ability to induce pro- and anti-inflammatory cytokine responses.

Plectasin, a defensin isolated from a fungus, has an inhibitory effect on the growth of Gram-positive bacteria [161]. Water et al. used the double emulsion solvent evaporation method to encapsulate plectasin into PLGA nanoparticles (PLGA-P) for the treatment of bacterial infection of epithelial cells [162]. PLGA-P showed high encapsulation efficiency of plectasin (71-90%), which was released over 24 h. Compared with free plectasin, PLGA-P significantly inhibited *S. aureus* infection of bronchial epithelial cells with little toxicity to eukaryotic cells at the concentrations measured. These results promise plectasin as a potential therapeutic for the treatment of bacterial airway infections *in vivo*.

To improve the pharmacological properties of mouse β-defensin-14 (mBD-14) Yuan et al. endowed polyether ether ketone with a three-dimensional porous structure (SP) through sulfonation, on which mBD-14 was loaded to make antimicrobial coating (SP-mBD) [163]. Compared with blank SP, SP-mBD showed durable and potent antibacterial activity against both *P. aeruginosa* and *S. aureus*. Consequently, a significant amount of new bone formed around SP-mBD in the femurs of both infected and uninfected rats, and no evidence of bacterial survival was found. These data demonstrate SP-mBD's superior bactericidal and osteointegrative properties and its

potential application in the clinical management of periprosthetic joint infection.

4.6 Other specific delivery systems

In addition to the commonly used strategies, some other specific delivery systems were also developed to facilitate the application of defensins. To accelerate osteogenic differentiation and reduce the risk of infection associated with osseous grafts, He et al. constructed an artificial bone scaffold for the delivery of hBD3 and bone morphogenetic protein-2 (BMP2), allowing for postoperative antibacterial effects and directional bone induction [164]. hBD3 is antibacterial, whereas BMP2 induces osteogenesis. A mixture of hBD3, BMP2, and phytohemagglutinin was prepared into a porous structure scaffold (BMP2-hBD3-PS) by a 3D printer at low temperature. Approximately 35% of hBD3 in the scaffold was slowly released over 30 days with sustained antibacterial activity against *E. coli* and *S. aureus*, promising these synthetic biomaterials as potential antimicrobial bone implants.

Since hBD-2 improves the clearance of non-typeable Haemophilus influenzae (NTHi) in the middle ear cavity, Woo et al. developed adenoviral vectors carrying hBD-2 gene as an alternative therapy for experimental otitis media (OM) [165]. The resulting adenoviral vector Ad-DEFB4 enhanced the expression of hBD-2 gene in human middle ear epithelial cells (HMEEC) in a time- and dose-dependent manner. Ad-DEFB4 infection facilitated the production and release of hBD-2 in HMEEC cells, which, in turn, inhibited the adhesion of NTHi to HMEEC cells. Upon inoculation into the middle ear cavity of mice, Ad-DEFB4 alleviated high-grade effusion in the ears of NTHi-infected mice and enhanced the clearance of NTHi from the middle ear cavity, demonstrating the feasibility of adenoviral vector-carried hBD-2 gene therapy for experimental OM.

5 Defensin mimics

As a strategy to enhance the activity and stability of antimicrobial peptides, scientists design mimetic peptides to retain the biological functions of their parent molecule with or without structural similarity. These mimetics can be chemically constructed by extracting active fragments from naturally occurring AMPs and further modifying the functional moieties [166].

As a defensin that protects the small intestine from invasion by various enteric pathogens, HD6 lacks significant bactericidal activity [103]. Rather, HD6 self-assembles, upon binding to bacterial surface proteins, into structurally ordered nanonets that entrap bacteria to prevent infection [104]. Inspired by this "encircle without attacking" antimicrobial strategy of HD6 [167] and the dynamic process of peptide self-assembly (Fig. 7A) [168], Fan et al. constructed a human defensin-6 mimic peptide (HDMP) [169]. HDMP comprises three modules: (1) the amino acid sequence RLYLRIGRR that specifically binds lipoteichoic acid in the cell membrane of Gram-positive bacteria, (2) sequence KLVFF for β-sheet structures to mimic HD6 fibrous networks, and (3) aromatic bis-pyrenes (BP) and fluorescent groups that maintain HDMP in a particulate form (Fig. 7B). Mechanistically, HDMP self-assembled into nanoparticles (HDMP NPs), bound to the cell wall of Gram-positive bacteria, and transformed into nanofibers (NFs) stabilized by π-π interactions and hydrogen bonding (Fig. 7C). Bacteria were entrapped in fibrous networks and, consequently, infection was inhibited. The therapeutic efficacy of HDMP was demonstrated in a mouse model inoculated with *S. aureus*, where NFs formed on the surface of *S. aureus* at the inoculation site as verified by fluorescence microscopy. Of note, HDMP NP treatment resulted in a survival rate of up to 100% in MRSA-infected bacteremia mice, surpassing the efficacy of vancomycin with little or no damage to major organs (Fig. 7D and 7E).
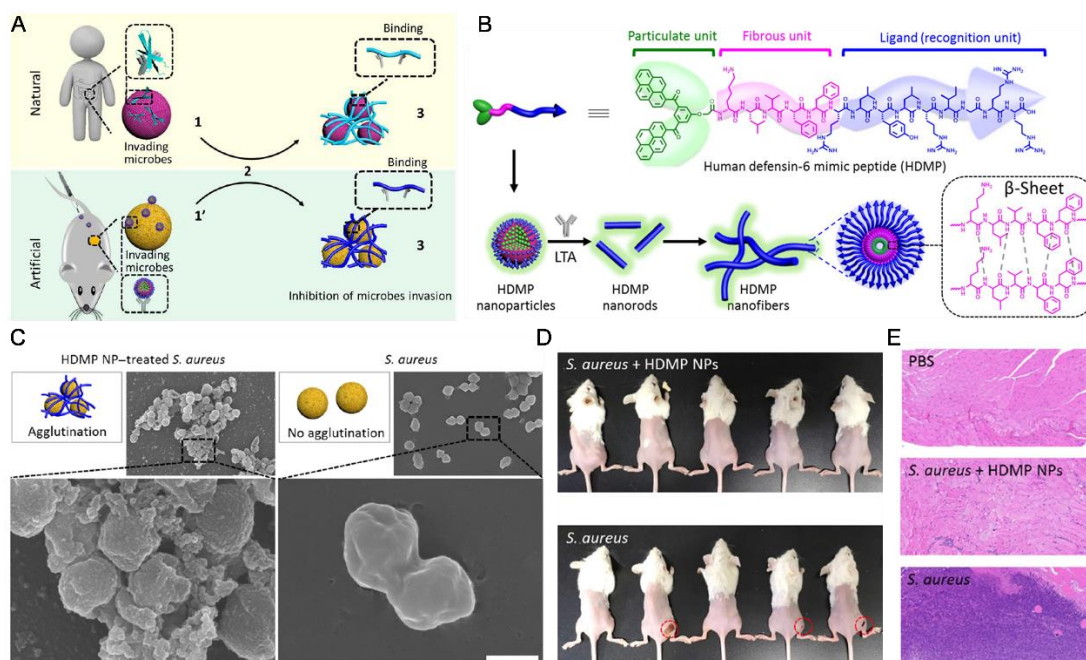

Fig. 7. A human defensin-6 mimic peptide (HDMP) for recognizing and trapping

bacteria. (A) Antimicrobial mechanism of natural HD6 and artificial HDMP. (B) Molecular structure of HDMP. (C) SEM images of *S. aureus* treated with HDMP NPs and bare *S. aureus*. (D) Images of *S. aureus* inoculated in mice receiving HDMP NPs. (E) Histological analysis of the leg muscle tissue of mice. Adapted with permission from ref. [169]. Copyright 2020 The American Association for the Advancement of Science.

The cyclic backbone in peptide molecules can eliminate their susceptibility to amino- and carboxyl-peptidases compared with linear counterparts, thus increasing their proteolytic stability and systemic circulation time [170]. Besides, backbone cyclization eradicates terminal charges that may hamper functional peptide-membrane interactions [171]. Cyclic θ-defensins have been prepared chemically with ease, showing a wide variety of antimicrobial activities *in vitro* and *in vivo* [79, 172, 173]. Given that enzyme-catalyzed reactions are economical and efficient for peptide macrocyclization [174], Nguyen et al. examined the ability of butelase 1, an Asn/Asp-specific ligase, to cyclize θ-defensin [175]. The D-amino-acid-containing θ-defensin was modified with a C-terminal tripeptide NHV, which is the recognition site for butelase 1. The cyclization yield was determined to be greater than 95% by mass spectrometric analysis. The cyclized D-θ-defensin correctly formed three intra-molecular disulfide bonds in the presence of dimethyl sulfoxide, and the resulting peptide showed a wide spectrum of antibacterial activity against both drug-sensitive and drug-resistant clinical strains of bacteria.

6 Challenges

While the potential therapeutic value of defensins and their derivatives in treating infectious diseases is manifested by numerous studies, challenges impeding their clinical translation still remain. A growing body of evidence suggests that defensins can function as a "double-edged sword" in host immunity [23]. Although their antiviral properties are well documented [5, 20], defensins have also been shown to promote viral infection in certain biological settings [176-182]. For example, HD5 and HD6 can promote HIV infection *in vitro* by targeting the virion for enhanced viral attachment to cells [5], whereas HNP1 can promote HIV traversal across epithelial barriers by disruting epithelial integrity [181]. Infection-promoting activities have also been

reported for HNP1 and HD5 with adenovirus, mouse α-defensins with HIV, and equine β-defensins with herpesvirus in cell cultures [177, 180, 183]. Of note, a recent *ex vivo* study using enteroids expressing mouse α-defensins and the mouse pathogen adenovirus 2 demonstrates that defensin-enhanced viral infection *in vitro* may be physiologically relevant *in vivo* [184].

More recently, Lu & colleagues have made a surprising discovery that the α-defensins HNP1 and HD5 promote *Shigella* infection by enhancing bacterial adhesion to and invasion of host cells [23, 185, 186]. *Shigella* is a Gram-negative pathogen causing bacillary dysentery and millions of deaths worldwide. *In vitro*, however, *Shigella* is poorly infectious as it lacks adhesion molecules on bacterial surface for effective host attachment -- an obligate step leading to the activation of its type 3 secretion system and subsequent invasion. Perplexingly, *Shigella* is highly infectious and contagious in humans, but does not readily infect other animals. For many years, the molecular basis underlying *Shigella*'s extraordinary infectivity and pathogenecity and its strict host selectivity remained elusive. HD5 is expressed in large quantities in the small intestine by Paneth cells and contributes to gut immunity and homeostasis [10, 101, 104]. The enteric α-defensin HD5 binds to *Shigella*'s outer membrane proteins OmpA and OmpC, clustering bacteria via defensin self-association or directly bridging bacteria and host cells for enhanced bacterial adhesion (Fig. 8A) [185]. This in turn augments bacterial invasion and infection, as evidenced by *in vitro*, *in vivo* and *ex vivo* studies, thereby explaining *Shigella*'s pathogenicity and host selectivity (Fig. 8B-D). Of note, HD5 also exacerbates the pathogenicity of *Shigella* in macrophages[187], where HNP1 can promote *Shigella* infection of epithelial cells [188].
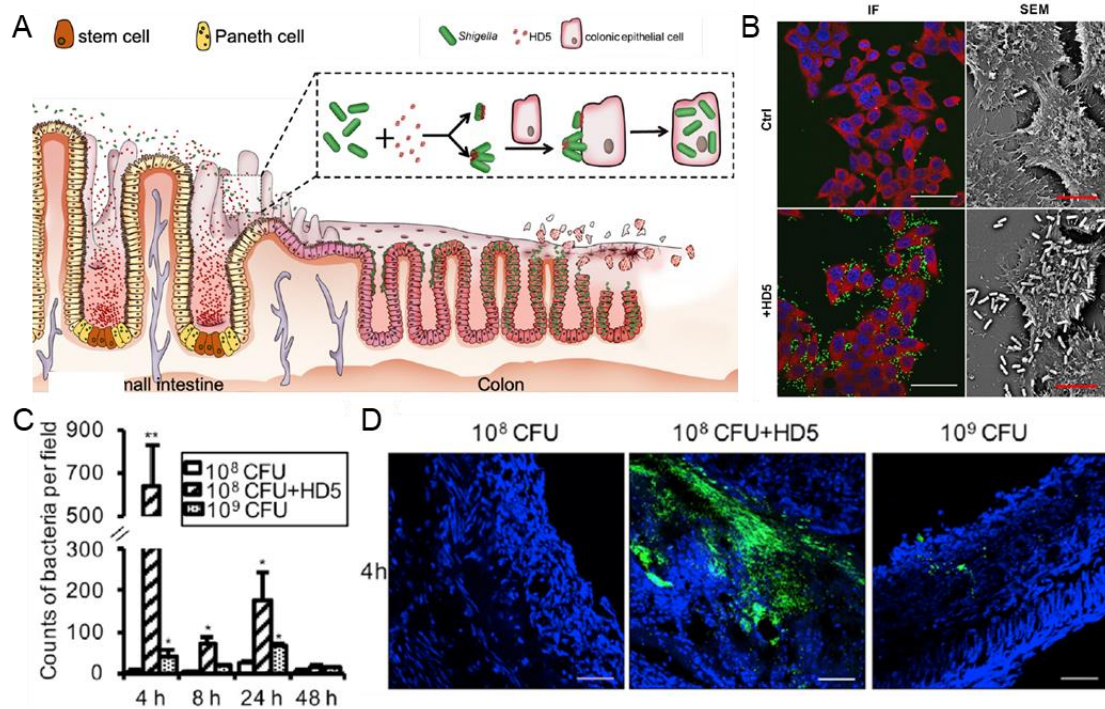
Fig. 8. HD5-promoted *Shigella* infection *in vitro* and *in vivo*. (A) HD5-promoted Shigella infection of the colonic epithelium. (B) Fluorescence microscopy and SEM analysis of Sf301 adhesion to HeLa cells with or without HD5. (C) Counts of bacteria per field and (D) confocal microscopy analysis of colon tissue after challenge. Adapted with permission from ref. [185]. Copyright 2018 Elsevier Inc.

Additionally, defensins have been known to be both anti-infammatory and pro-inflammatory [5, 189], and can be either tumor-proliferative or -suppressive depending on which defensin and cancer type are studied [22-24]. It has been reported that an increased concentration of defensins at sites of inflammation and infection may be a pathological cause of some diseases [190]. For example, the levels of both α- and β-defensins are elevated in patients suffering from conditions such as inflammatory lung disease. *In vitro* cytotoxicity data indicate that bronchoalveolar-derived HNPs can be cytotoxic to alveolar epithelial cells and macrophages [191, 192]. A mouse model experiment shows that high concentrations of HNP1-3 can cause a significant decrease in peripheral arterial oxygen saturation, increased lung permeability, impairment of mitochondrial function, and a significant influx of neutrophils [193].

Human defensins may also play an adverse role in host responses to implanted biomaterials. When neutrophils bind to biomaterials and acquire an activated state, they may produce high levels of extracellular HNPs, causing eukaryotic cytotoxicity and

reducing the anti-infective efficiency of neutrophils [194-197]. This is partly due to the dysregulation of neutrophils around the biomaterial and their diminished killing effect on pathogens [194, 195]. Further, since hBDs magnify pathogen-associated immune responses by facilitating the production of pro-inflammatory cytokines [198-200], and contribute to the survival of activated neutrophils, they may exacerbate the accumulation of pro-inflammatory and cytotoxic mediators around biomaterials [201].

Understandably, the possibility that defensins can be both host-protective and pathogenic calls into question their therapeutic value in controlling microbial infections in humans. While systemic use of defensins clearly warrants the exercise of caution, localized delivery with precision of defensins to the site of infection, which can be aided by advanced nanotechnologies and formulations, is not only possible but also of significant clinical value. Still, in-depth studies are needed to dissect the pros and cons of defensin activities at the molecular level so that pharmacologically viable derivatives can be designed with a clinically acceptable safety, pharmacokinetics and pharmacodynamics profile.

Designing defensin derivatives as therapeutics are important for several other reasons. First, the production of defensins can be costly. Although defensins can be produced in prokaryotes such as *E. coli* [202, 203], their sensitivity to proteolytic degradation, susceptibility to disulfide mispairing, and antimicrobial activity against the host limit the use of the recombinant technology [204]. Expression of defensins as fusion proteins can alleviate these problems to large extent. However, the necessity to chemically or enzymatically remove the fusion protein tag, which is significantly larger than the defensin itself, invariably leads to a low yield [205, 206]. Total chemical synthesis of correctly folded and highly pure defensins are feasible [89, 207-211], but the cost can be quite high, let alone the use of environmentally unfriendly chemistries and toxic materials. Second, defensins and human α-defensins, in particular, are known to bind "promiscuously" many different types of molecules such as lipids and microbial membranes, bacterial cell wall components, bacterial toxins and virulence factors, viral protein, carbohydrates and glycoproteins, cellular receptors and host proteins, nucleic acids, etc., thus significantly limiting their bioavailability [27, 212]. Moreover, divalent cations such as Mg^{2+} and Ca^{2+}, derived from most body fluids, can compete with defensins for binding on the bacterial surface (e.g. on lipopolysaccharide) [25, 190]. As endogenous peptides, defensins are susceptible to some extent to proteolytic

degradation [14, 25], further impacting their pharmacokinetic behavior and, ultimately, antimicrobial activity *in vivo*. Derivatization of defensins may be necessary to further improve their functionality and specificity for clinical translation.

Conclusion

Defensins have the potential to be developed into a novel class of highly effective therapeutic agents to fend off infectious microbes given their broad antimicrobial activity against bacteria, viruses and fungi, and their immunomodulatory property. Compared with other classes of antimicrobial peptides, however, defensins are mechanistically more complex, functionally more diverse, and can be potentially pathogenic in certain biological settings. These features along with other factors have complicated the therapeutic development of defensins for clinical use. Although important progress has been made in a number of areas such as structure-activity relationship studies of defensins for their functional improvement, and development of various nanotechnologies (e.g., micelles, metal, silica, or polymeric nanoparticles) for efficient and controlled delivery and release of defensins, some technical and pharmacological challenges still remain, including, but not limited to, sub-optimal therapeutic efficacy, potential toxicity, high manufacturing cost, etc. Synergistic use of defensins with conventional antibiotics likely presents a cost-effective solution to treating pathogenic infections for which antibiotic monotherapy is inadequate, which may also help reduce antibiotic resistance. With rapid research efforts on defensin biology and continued progress and innovation in peptide drug discovery and development, the future of defensins and their derivatives to be used as anti-infective therapeutics has never been brighter.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Abbreviations: hBD, human β defensin; HD, human enteric defensin; HDMP, human defensin-6 mimic peptide; HNP, human neutrophil peptide; mBD, mouse β-defensin; MRSA, Methicillin resistant *Staphylococcus aureus;* MSN, mesoporous silica nanoparticle; ND, nanodefensin; NF, nanofiber; RTD, rhesus theta defensin; SCN, succinylated casein; TLR, Toll-like receptor; TNF, tumor necrosis factor; IFN, interferon;

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