

Silver Nanoparticle Conjugated Marine Invertebrate Antimicrobial Peptides (AgNPs - Amps) against Gram-Negative *ESKAPE* Pathogens

M. Manohar¹ & Muhammad Musthafa^{2*} & K. Gopianand²

¹Sadakkathullah Appa College, Tirunelveli, Tamil Nadu, India.

²Vivekanandha Arts & Science College for Women, Namakkal, Tamil Nadu, India.

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ABSTRACT: Due to a couple of reasons like increasing bacterial resistance to the commercial antibiotics, re-emerging bacterial diseases, identification of new infectious agents, challenges in the anti-tumor therapy etc., the scientific world is searching for new antibiotics with significant action modes. A group of bacteria, notorious for 'escaping' the actions of antibiotic and forming resistance to them are identified and described as 'the *ESKAPE* pathogens'. They are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. They pose a new threat in pathogenesis, transmission and drug and antibiotic resistance through newly acquired mechanism. The antimicrobial peptides (AMPs) are such molecules of a new promise with multiple mechanisms of action as antibiotics, antifungals, anti-biofilm agents, anti-fouling agents, anti-tumor agents etc. and are the key agents of the only innate immune system of marine invertebrates, which are exposed to a large number of pathogens. On the other hand, the metal nanoparticles which have proved their activity against multidrug resistant bacteria can be conjugated with AMPs to improve the potentiality of action against the MDR pathogens. The present review analyses the chances of conjugating silver nanoparticle (AgNPs) with the antimicrobial peptides (AMPs) to get the anti-MDR bacterial activity multiplied.

Key Words: Multidrug resistance; *ESKAPE* pathogens; marine invertebrates; antimicrobial peptides; silver nanoparticle

1. Introduction

Over the past two decades the global emergence and spread of antimicrobial resistant strains of commonly encountered pathogens has been observed [1] and their alarming increase has become a serious global health issue threatening the achievements of modern medicine [2]. The World Health Organization warns that the 21st century may be seeing the beginning of a pre-antibiotic era, posing the biggest threat to health, food security and development [3]. Fatalities from antibiotic-resistant infections are predicted to rise into the millions by 2050. [4]. This trend of antimicrobial resistance (AMR) is more serious among bacterial pathogens [5] and a survey of hospital acquired infections (HAIs) in the United States in 2011 reported a total of about 722,000 cases, with 75,000 deaths associated with nosocomial infections [6]. A study conducted in 2002 had already estimated that, taking all types of bacterial infections into the account approximately 1.7 million patients suffered from HAIs, which contributed to the deaths of 99,000 patients per year [7].

The antibiotic resistance can affect anyone of any age, in any country and it leads to longer hospital stays, higher medical costs and increased mortality [3]. Infections in hospital-born babies were estimated to account for up to 56% of all neonatal deaths in some under-resourced countries. *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas* spp., *Acinetobacter* spp. and *Staphylococcus aureus* were the most frequent causative pathogens of neonatal sepsis; 70% of these isolates would not be eliminated by an empiric regimen of ampicillin and gentamicin. Many infections could be untreatable in resource-constrained environments, as a recent report demonstrated that 51% of *Klebsiella* spp. were extended-spectrum β -lactamase (ESBL) producers, 38% of *Staphylococcus aureus* strains were methicillin-resistant (MRSA) and 64% were resistant to co-trimoxazole [8].

The most serious, life-threatening infections are caused by a group of drug-resistant bacteria are named by the Infectious Diseases Society of America (IDSA) as *ESKAPE* pathogens, as they effectively escape the effects of antibacterial drugs. The six *ESKAPE* bacteria are *Enterococcus faecium* (E), *Staphylococcus aureus* (S), *Klebsiella pneumoniae* (K), *Acinetobacter baumannii* (A), *Pseudomonas aeruginosa* (P) and *Enterobacter* species/*Escherichia coli* (E). These bugs are responsible for two third of all healthcare associated infections (HAIs) [9]. They form the most common multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria [10], representing a serious threat to the patients who are frequently in an immunocompromised state (e.g. those undergoing transplantation, cancer and critically ill patients [5]).

The objective of the present review is to consider the clinical importance of emerging Gram negative members of the *ESKAPE* pathogens and to investigate the possibility of using marine invertebrate derived antimicrobial peptides (AMPs) conjugated with silver nanoparticles (AgNPs) as a promising tool in controlling these 'bad bugs'.

2. The *ESKAPE* pathogens

The *ESKAPE* pathogens will be of increasing relevance to antimicrobial chemotherapy in the coming years, not only due to the clinical threat they pose, but the anticipated honing of academic and industrial interests towards them [11]. The *ESKAPE* pathogens are known to 'escape' the effects of currently marketed antimicrobial agents. They are frequently multidrug-resistant (MDR) and are associated with poor patient outcomes because the patients infected with *ESKAPE* pathogens often receive inappropriate empirical antimicrobial therapy that leads to unfavorable clinical outcomes, high case fatality rates and opportunities for pathogen spread to other patients [12]. *ESKAPE* pathogens frequently present clinicians with serious therapeutic dilemmas because of their complex resistance profiles [13]. The following is a brief introduction to the group members.

2.1. *Enterococcus faecium*

Enterococci are gram positive, facultatively anaerobic, opportunistic pathogens frequently involved in Healthcare Associated Infections (HAIs) or nosocomial infections and can cause severe infectious diseases especially among immunocompromised patients [7, 14]. *Enterococcus* species were formerly classified as part of the genus *Streptococcus* [7]. They are distributed extensively in nature such as grounds, water, plants, food and are a part of the normal flora of human and animal enteric tract. [14]. Within the hospital environment, *Enterococci* have been described as 'triple-threat' pathogens displaying exemplary colonization of the gut and skin - with Enterobacteriaceae and *Staphylococcus aureus* respectively - alongside an environmental persistence characteristic of *Clostridium difficile* [15]

Some strains of the genus *Enterococci* are resistance to a broad number of antimicrobial drugs such as erythromycin, glycopeptides, tetracycline, vancomycin (vancomycin - resistant *Enterococci* - VRE, which have nine vancomycin resistance genes as *van A*, *B*, *C*, *D*, *E*, *G*, *L*, *M* and *van N*. [14]), aminoglycosides (high-level resistance - HLR), gentamicin (high-level resistance gentamicin - HLGR) and streptomycin (high-level resistance streptomycin - HLSR). These strains show inherent and acquired antimicrobial resistance. [16]. Also, the transmission of vancomycin resistant gene from *Enterococcus* spp. to *Staphylococcus aureus* has been detected in the laboratory. Since vancomycin has been considered as a drug of choice for treatment of Enterococcal and MRSA infections, the emergence of vancomycin- intermediate (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) can produce important problems to the treatment of *Staphylococcus aureus* [14].

Apart from the world widely reported nosocomial infections, various reports have demonstrated that *Enterococcus faecalis* and *Enterococcus faecium* can cause several infections including septicemia with higher toxicity rates in patient such as nephrotoxicity than monotherapy diet [17].

2.2. *Staphylococcus aureus*

Staphylococcus aureus - a Gram positive coccus - is a common representative of the skin microbiota and is most commonly isolated from the moist areas such as the anterior nares and the axillae. This was the first microorganism in clinical history to be shown growing as a biofilm. Nearly 25 - 30% of skin or noses of healthy people are colonized by *Staphylococcus aureus* [14, 18].

They show a wide category of resistance characteristics described as methicillin-resistant *Staphylococcus aureus* (MRSA - which are resistant to certain antibiotics such as methicillin, dicloxacillin, oxacillin, cloxacillin, nafcillin and closely related class of drugs such as cephalosporins) [14, 18], healthcare associated (HA-MRSA) or community-associated (CA-MRSA), vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) and so on [14]. Infected patients and health care providers may act as carriers and play a significant role in spreading and transferring this bad bug in hospitals. *Staphylococcus aureus* strains isolates from the hospital are named as hospital acquired *Staphylococcus aureus* (HASA) [14, 19, 20].

Staphylococcus aureus causes atopic dermatitis (AD) and induces skin inflammation by secreting super antigens following a chronic relapsing course and defects in innate and acquired immune responses resulting in a heightened susceptibility to bacterial, viral and fungal infections [14]. The high rate of *Staphylococcus aureus* colonization in the nasal cavity and skin lesions of AD patients has been reported, especially in ranges between 76–100%, compared to 2–25% in healthy people [14, 21].

2.3. *Klebsiella pneumoniae*

Klebsiella pneumoniae are non-fastidious, Gram negative bacilli belonging to the family Enterobacteriaceae. They are usually encapsulated and frequently cause lower respiratory tract infection and catheter-associated urinary tract infection [7]. Along with *Escherichia coli*, and together they lead a mounting pathological threat as they are globally prevalent in both the hospital and community sectors [22].

Klebsiella pneumoniae is one of the MDR organisms identified as an urgent threat to human health by the World Health Organization, the US Centers for Disease Control and Prevention and the UK Department of Health. *Klebsiella pneumoniae* infections are particularly a problem among neonates, elderly and immunocompromised individuals within the healthcare setting, but this organism is also responsible for a significant number of community-acquired infections including pneumonia and sepsis [23, 24].

2.4. *Acinetobacter baumannii*

Acinetobacter baumannii is a typically short, almost round, rod-shaped (coccobacillus), Gram negative bacterium. It can be an opportunistic pathogen in human and is becoming increasingly common in intensive care units (ICUs), causing infections that include bacteremia, ventilator associated pneumonia, meningitis, urinary tract infections, central venous catheter-related infections and wound infections [22, 25].

The most important human pathogen belonging to this genus is *Acinetobacter baumannii*, which has a relatively long survival time on human hands, and can lead to high rates of cross contamination in nosocomial infections [26]. Many strains of *Acinetobacter baumannii* are highly resistant even to carbapenems and polymyxin/colistin in some instances and thus has arisen as a particularly worrisome threat for which there is no new antibiotic in development. Thus, the emergence of antibiotics-resistant *Acinetobacter baumannii* especially, multi-resistant strains seriously challenges the treatment of these infections [27].

2.5. *Pseudomonas aeruginosa*

This is a Gram negative, rod-shaped, facultatively anaerobic bacterium which is a part of the normal gut flora that has an intrinsic resistance characteristic. The outcomes of infection caused by MDR *Pseudomonas* spp. may be related to enlarged morbidity and mortality, which can cause narrow effective antimicrobial choices as resistance to at least three of the five classes of antibiotics, mainly carbapenems, antipseudomonal penicillins, cephalosporins, aminoglycosides and fluoroquinolones [7]. *Pseudomonas aeruginosa* is responsible for 9% of all healthcare-associated infections and the resistance to *Pseudomonas aeruginosa* is increasing [28]. *Pseudomonas aeruginosa* that infects Cystic Fibrosis patients has serious implications for infection control in the hospitals [7].

2.6. *Enterobacter* species

Enterobacter spp. are Gram negative rods that are sometimes encapsulated and are significantly responsible for urinary and respiratory tract infections. The emergence of antimicrobial resistance among *Enterobacter* spp. is of great concern worldwide in human medicine [29, 30]. They are also known to cause bloodstream infections and are becoming increasingly responsible for serious nosocomial infections, displaying broad MDR [7]. The resistance of *Enterobacter* spp. to extended-spectrum cephalosporins (ESC) is of particular concern [31]. They can cause opportunistic infections in immunocompromised, usually hospitalized patients and contain a wide range of antibiotic resistance mechanisms [7].

3. Antibiotic resistance in Gram negative *ESKAPE* pathogens

Out of the above mentioned MDR pathogens, the Gram-negative *ESKAPE* bacteria act as important reservoirs and transmitters of resistance, and are responsible for increased reporting of antimicrobial resistant nosocomial infections worldwide [12]. The increasing prevalence of carbapenem-resistance presents a significant challenge for physicians as carbapenems are conventionally used to treat persistent infections caused by Gram-negative bacteria [7, 32]. Even if several intensive infection control practices are used, outbreaks of carbapenemase-mediated multidrug resistant (MDR) strains are only reduced and cannot be completely eradicated [7].

3.1. *Klebsiella pneumoniae*

Klebsiella pneumoniae belongs to the extended-spectrum β -lactamase or ESBL strains and is increasingly multidrug-resistant (MDR) to a wide spectrum of antibiotics such as cephalosporin or ceftazidime [33]. In recent years, many *Klebsiella pneumoniae* strains have acquired a massive variety of β -lactamase enzymes which can destroy the chemical structure of β -lactam antibiotics such as penicillins, cephalosporins and carbapenems [7, 34]. The emergence of *Klebsiella pneumoniae* isolates producing carbapenemases (KPCs) has become a major obstacle in the last 5 years. Carbapenemases are able to destroy the carbapenems and cause resistance against a wide spectrum of antibiotics [14].

In addition, the emergence of the *Klebsiella pneumoniae* super enzyme, known as NDM-1 has increased the proportion of carbapenem-resistant *Klebsiella pneumoniae* isolates and may pose a threat to other antibiotics such as β -lactams, aminoglycosides and fluoroquinolones [7, 35]. As the carbapenems are conventionally used to treat persistent infections caused by Gram-negative bacteria, the increasing prevalence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP), with resistance encoded by *blaKPC* presents a significant challenge for physicians [7].

3.2. *Acinetobacter baumannii*

Acinetobacter baumannii is intrinsically resistant to antibiotics due to the protection afforded by a Gram-negative outer membrane, constitutively expressed active efflux pump systems and low-quantity expression of small-aperture outer membrane porins (which greatly reduce the permeability of antimicrobials) [7, 14, 22]. The epidemic potential and the clinical severity of *Acinetobacter baumannii* infections are primarily related to the capability to survive and propagate in healthcare settings and to expand resistance to a diversity of antimicrobial agents containing fluoroquinolones, broad-spectrum β -lactams and carbapenems [14, 36]. MDR *Acinetobacter baumannii* is resistant to 3 or more several classes of antibiotics containing β -lactams, aminoglycosides, fluoroquinolones and 3rd generation of cephalosporin [36]. Pan-drug resistance (PDR) *Acinetobacter baumannii* was described as the strains that were resistant to all examined antibiotics excluding colistin and tigecycline [14].

Recently, the emergence of carbapenemase producing *Acinetobacter baumannii* strains carrying imipenem metallo- β -lactamases, encoded by *blaIMP* and oxacillinase serine β -lactamases encoded by *blaOXA* has been reported. These strains show resistance to both colistin and imipenem, and the combination of resistance genes makes them capable of evading the action of most of the traditional antibiotic compounds [7, 37].

3.3. *Pseudomonas aeruginosa*

The reduced permeability of the Gram negative outer membrane, function of multidrug efflux pumps, mutations in targets of antibiotics, the horizontal gene transfer of antibiotic resistance determinants etc. make the *Pseudomonas aeruginosa* resistant to a range of antimicrobials [14, 38]. Many of the *Pseudomonas aeruginosa* strains show an intrinsic reduced susceptibility to several antibacterial agents as well as a propensity to develop resistance during therapy especially in carbapenem-resistant (chiefly imipenem) strains [14, 39]. The emergence of MDR in *Pseudomonas aeruginosa* containing resistance to β -lactams, aminoglycosides and fluoroquinolones is extremely problematic in the treatment of burn patient. Serious infections such as burn wounds due to *Pseudomonas aeruginosa* are treated through the combination of a β -lactam drugs and an aminoglycoside. The continuous increase of MDR isolates presents a complicated situation for antimicrobial therapy; however colistin is still effective in most cases [14, 7].

3.4. *Enterobacter* spp.

Many *Enterobacter* strains contain ESBLs and carbapenemases including VIM, OXA, metallo- β -lactamase-1 and KPC [7]. Furthermore, stable de-repression of the AmpC β -lactamases that can be expressed at high levels by mutation in this bacterial group is also notable. These MDR strains are resistant to almost all available antimicrobial drugs except tigecycline and colistin [7, 40]. Extended-spectrum β -lactamases (ESBLs) and carbapenemases have been identified in *Enterobacter* spp., exacerbating the issue of extended-spectrum cephalosporins (ESC) resistance. An even greater concern is that most ESC-resistant *Enterobacteriaceae* exhibit multidrug resistance, including fluoroquinolone resistance, mainly due to chromosomal mutations in the enzymes targeted by the drug and plasmid-mediated quinolone resistance (PMQR) [14, 40]. In recent years, these resistance mechanisms have been well documented among *Enterobacter* spp. isolates across several countries [7, 22].

Although few antimicrobials such as colistin and tigecycline are influenced by these resistant bacteria (and also against many of the other *ESKAPE* pathogens) there is little or no drugs in the 'pipeline', being remarkably effective in addressing this mounting health crisis [14]. In short, nosocomial *ESKAPE* bacteria represent paradigms of resistance, pathogenesis and disease transmission.

4. Mechanisms of antimicrobial resistance in Gram negative *ESKAPE* pathogens

There is a wide range of antimicrobial resistance mechanisms used by the nosocomial *ESKAPE* pathogens including enzymatic inactivation, modification of drug targets, changing cell permeability and mechanical protection provided by biofilm formation. Antimicrobial resistance genes may be carried on the bacterial chromosome, plasmids or transposons [41]. Mechanisms of drug resistance may be one or a combination of two or more of the following categories.

4.1. Drug inactivation or alteration

Many bacteria produce enzymes (that irreversibly modify and inactivate the antibiotics) such as β -lactamases, aminoglycoside-modifying enzymes or chloramphenicol acetyl transferases. The β -lactamases are highly prevalent and act by hydrolyzing the β -lactam ring which is present in all β -lactams like penicillins, cephalosporins, monobactams and carbapenems [42]. β -lactamases are classified using two main classification systems: the Ambler scheme (molecular classification) and the Bush-Jacoby-Medeiros system [43], out of which the former is being discussed here briefly.

Ambler class A enzymes consist of penicillinase, cephalosporinase, broad-spectrum β -lactamases, extended-spectrum β -lactamases (ESBLs) and carbapenemases. They can inactivate penicillins (except temocillin), third-generation oxyiminocephalosporins (e.g: ceftazidime, cefotaxime and ceftriaxone), aztreonam, cefamandole, cefoperazone and methoxycephalosporins (e.g: cephamycins and carbapenems). Class A enzymes can also be inhibited by β -lactamase inhibitors such as clavulanic acid, sulbactam or tazobactam [7]. The Ambler class A group contains a number of significant enzymes including ESBLs (eg: TEM-1 which is widespread among the members of the family Enterobacteriaceae like *Klebsiella pneumoniae*, *Enterobacter* spp. and in non-fermentative bacteria such as *Pseudomonas aeruginosa*). Another set of enzymes, the CTX-Ms have been identified in *ESKAPE* pathogens including *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species. Some of the highest prevalence and significant clinical impact are associated with the extended spectrum β -lactamases in *Klebsiella pneumoniae* [7, 44]) and KPCs (eg: KPC-1 which is prevalent in *K. pneumoniae* that imparts resistance to imipenem, meropenem, amoxicillin/clavulanate, piperacillin/tazobactam, ceftazidime, aztreonam and ceftriaxone [7]. The fast propagation of *Klebsiella pneumoniae* resistance is perhaps due to the *bla*-KPC gene carriage on plasmids. Plasmid-mediated imipenem-hydrolyzing enzyme (KPC-2) among multiple carbapenem resistant bacteria is increasing [45].

Ambler class B enzymes or group 3 enzymes include metallo- β -lactamases (MBLs), which require Zn^{2+} as a cofactor. Bacteria that produce these enzymes show resistance to all β -lactams including penicillins, cephalosporins, carbapenems and β -lactamase inhibitors except aztreonam. Genes encoding MBLs are found on plasmids; hence, they are easily transmitted to other microorganisms. The most common MBLs are imipenem-metallo- β -lactamases (IMP - found in *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacter cloacae*), veronaintegron encoded metallo- β -lactamases (VIM - mostly in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) and the newly described New Delhi metallo- β -lactamase-1 (NDM-1 identified in *Klebsiella pneumoniae* and *Enterobacter cloacae*) [7, 46].

The Ambler class C group consists of enzymes including penicillinase and cephalosporinase such as AmpC β -lactamase, which results in low level resistance to narrow-spectrum cephalosporin drugs. Chromosomally encoded AmpC are usually identified in *Pseudomonas aeruginosa* and bacteria in the Enterobacteriaceae family such as *Enterobacter* species where their production is typically in very low level and does not elicit any clinically relevant resistance but can be inducible during drug therapy. In *Enterobacter* spp., the extended-spectrum cephalosporins (ESC) resistance is most typically caused by the overproduction of AmpC β -lactamases, which is due to the de-repression of a chromosomal gene or the acquisition of a transferable AmpC β -lactamase [47].

Ambler class D consists of a variety of enzymes like oxacillin hydrolyzing enzymes (OXA). The most common members of this class such as OXA-11, OXA-14 and OXA-16 demonstrate ESBL properties and are normally found in *Pseudomonas aeruginosa* [7, 48]. Almost all of the OXA enzymes except OXA-18, are resistant to β -lactamase inhibitors [20]. Furthermore, OXA-type carbapenemases are commonly found in *Acinetobacter* spp. [7].

4.2. Modification of drug binding sites

Some resistant bacteria avoid recognition by antimicrobial agents by modifying their target sites. The mutation of gene encoding for penicillin-binding proteins (PBPs) results in the expression of unique penicillin-binding proteins. By changing the peptidoglycan crosslink target (D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser) encoded by a complex gene cluster (*Van-A*, *Van-B*, *Van-D*, *Van-C*, *Van-E* and *Van-G*), *Enterococcus faecium* and *Enterococcus faecalis* can increase their resistance to many of the glycopeptides in current clinical use [41].

4.3. Reduced intracellular drug accumulation

Reducing the amount of antibiotic able to pass through the bacterial cell membrane is one of the strategies used by bacteria to develop antibiotic resistance. This may be achieved by either of the following methods.

4.3.1. Porin Loss.

The outer membranes of Gram-negative bacteria contain proteins called porins that form channels which allow the passage of many hydrophilic substances including antibiotics. *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and MDR *Klebsiella pneumoniae* make use of this mechanism in particular cases [7, 49].

4.3.2. Efflux Pumps.

To increase the removal of antibiotics from the intracellular compartment some bacteria contain membrane proteins that function as exporters, called efflux pumps for certain antimicrobial agents. These pumps expel the drug from the cell at a higher rate and most efflux pumps are multidrug transporters that efficiently pump a wide range of antibiotics and thus contributing to multidrug resistance. This has been reported in different strains of *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* [7, 50].

4.4. Biofilm formation

Biofilms are complex microbial communities living as a thin layer on biotic or abiotic surfaces in a matrix of extracellular polymeric substances created by the members themselves [51]. The matrix of biofilms seems to provide a mechanical and biochemical shield that provides the conditions needed to attenuate the activity of the drugs (e.g. low O₂, low pH, high CO₂ and low water availability). Under these conditions it is difficult to eliminate bacteria using conventional antibiotics. Moreover, when the bacteria experience nutrient scarcity, they could become tolerant to antibiotics [7]. The most common Gram negative *ESKAPE* pathogens found in biofilms in a healthcare setting are *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* [52].

As mentioned elsewhere, members of the *ESKAPE* pathogenic group may achieve the drug resistance through a combination of two or more mechanisms described above. For example, the most common mechanism of imipenem resistance in *Pseudomonas aeruginosa* is a combination of chromosomal AmpC production and porin change [7, 53]. Also, *Pseudomonas aeruginosa* produces ESBLs and can harbor other antibiotic resistance enzymes such as *Klebsiella pneumoniae* carbapenemases (KPC), VIM encoded by *blaVIM* gene and imipenem metallo- β -lactamases. The combination of these enzymes leads to high rates of carbapenem resistance amongst *Pseudomonas aeruginosa* isolates and also to the emergence of fluoroquinolone-resistant strains as the corresponding mechanisms of resistance may be carried by the same plasmid [7].

5. Need for new antibiotics

The growing number of bacteria resistant to conventional antibiotics, reemergence of bacterial diseases, identification of new infectious agents, nosocomial and other dreaded infections by *ESKAPE* pathogens, limited number of available antibiotics, the similarities in the activity spectrum of the antibiotics [5, 54] etc. pose a major threat to the human life on earth [1] and has resulted in a major crisis in modern medicine which has stimulated major research efforts to develop novel antibiotic therapeutics [1, 55]. Unfortunately, the development of novel classes of antibiotics has been limited in the last 4 decades [5, 56]. While the development of new antibiotic drugs has been dramatically slow, the resistance rate has increased rapidly. The diversity of bacterial resistance mechanisms has largely outperformed our current classes of antibiotics [5]. Antimicrobial resistance causes major limits to treatment options in infected patients, particularly in isolates with carbapenems resistant [14, 57]. There is no current therapy that consistently provides bactericidal activity for critical infections due to vancomycin - resistant *Enterococci* (VRE) [14].

Nosocomial *ESKAPE* bacteria represent paradigms of resistance, pathogenesis and disease transmission. Antimicrobial resistance in these pathogens is a major menace to public health systems worldwide and seems likely to increase in the near future as resistance profiles change. Thus it remains as a challenge as there is no new antimicrobial agents with spectra of activity that reliably encompass multidrug-resistant and pan-resistant Gram negative *ESKAPE* isolates have not appeared in as a timely manner as hoped, and nosocomial infections remain a constant concern for patient health, particularly for critically ill inpatients as well as for patients requiring placement of invasive devices or surgical procedures [13, 37]. There are very few new antimicrobial agents in development to treat gram-negative *ESKAPE* pathogens despite the well-recognized need [12, 37].

As the rise of new diseases is outnumbering, the goal of the pharmaceutical research is to find a new drug or a novel therapeutic compound to fight against deadly diseases. Though there are an umpteen number of drugs commercially available in the market, their use could be restricted due to the limitations such as side effects, no selectivity, low specificity and toxicity etc. among others [58]. There is an urgent

need to develop novel antibiotics with less chance of resistance development to combat the threat from multidrug-resistant (MDR) pathogens [1]. The Infectious Diseases Society of America (IDSA) supported a program called 'Bad Bugs, Need Drugs; 10 × '20 Initiative', with the aim of developing ten new systemic antibacterial drugs by 2020 [37].

A lack of new antibiotics for treatment together with the appearance and persistence of multidrug-resistant bacterial strains, has led to demands that new antimicrobial strategies be developed and explored [59]. Thus, intensive nonclinical and clinical research is now invested into the identification of new and non-conventional anti-infective therapies, including adjunctive or preventive approaches such as antibodies targeting a virulence factor, probiotics and vaccines [5, 54, 60]. This results in the dearth of potential therapeutic agents in the pipeline that causes real concerns but should trigger research and development of new antibiotics or new approaches to control the infections they cause [7].

6. The importance of marine source

The ocean covers 71% of the surface of the earth and contains approximately half of the total global biodiversity with estimates ranging between 3 and 500×10^6 different species [61]. They constitute almost 80% of the world biota and the marine macro fauna alone comprise 0.5 to 10×10^6 species [62]. It is a wide and largely unexplored environment [59] with genetic uniqueness and diversity (in the tropical zones there are almost 1000 different species per square meter) [63] and has the presence of large chemical diversity structures (which is only rarely seen in terrestrials) [64]. Out of the 33 known animal phyla, 32 are present in the aquatic environment & 21 are exclusively marine [61, 65].

Since marine organisms live in complex habitats and are exposed to extreme conditions such as salinity, pressure, temperature and illumination, they produce a wide variety of secondary metabolites that cannot be found elsewhere [66]. The marine organisms also have special structures that imparts different bioactivities like antioxidant activity, antimicrobial activity, anticancer activity, antihypertensive activity, anti-inflammatory activity and so forth [67].

The marine environment is a potentially prolific source of highly bioactive secondary metabolites (in organisms such as fish, shellfish, molluscs, univalves, cephalopods, crustaceans and echinoderms, which significantly contribute to economic and research development [66] that might represent useful leads in the development of new pharmaceutical agents [61]. The first attempt to locate antimicrobial activity in marine organisms was initiated in 1950s. Since then, a large number of marine organisms from a wide range of phyla have been screened for antimicrobial activity. Many of these organisms have antimicrobial properties although most of the antibacterial agents that have been isolated from marine sources have not been active enough to compete with the classical antimicrobial activity [68].

However, utilization of marine organisms as sources of bioactive metabolites started seriously at the end of 1960s [69] with the isolation of prostaglandin derivatives from the Caribbean Gorgonian - *Plexaurahomomalla*. In the 1980s effective collaborations were established between marine chemists and pharmacologists and the investigations were focused on central nervous system membrane active toxins, ion channel effectors, anticancer and anti-viral agents, tumor promoters and anti-inflammatory agents [70].

7. Drugs from marine invertebrates

Invertebrates comprise approximately 60% of all marine animal diversity [62] and most of them belong to the phyla porifera (sponges), annelida, arthropoda, bryozoa, cnidaria, echinodermata, mollusca and chordata. Several studies addressing marine invertebrates also include these groups of organisms [70].

Marine invertebrates have proven to be the rich sources of bioactive compounds with activities ranging from antimicrobial to antitumor. [72]. This is better explained by the fact that, they lack an acquired, memory-type immunity based on T-lymphocyte subsets and clonally derived immunoglobulins, which means that their defense relies solely on innate immune mechanisms that include both humoral and cellular responses which differs from the vertebrate immune system [61].

The marine invertebrates are known for the presence of potential antimicrobial compounds in the blood and plasma. Their survival in an environment with invading microorganisms, numbering up to 10^6 bacteria/ml and 10^9 virus/ml of seawater, some of which are also present in anthropic environments [73] suggests that their innate immune system is effective and robust [74].

Marine natural sources as potential anticancer agents were reviewed in 2011 which mentioned 39 marine-derived potential anticancer agents, and among them 18 compounds were from sponges with different mechanisms of action. Interestingly, from the 16 marine natural products that are currently under preclinical trials as new drug candidates, most are derived from invertebrates [70]. Invertebrates - mainly sponges, tunicates, bryozoans or mollusks provided the majority of the marine natural products involved in clinical or preclinical trials [75]

The discovery of marine natural products has accelerated over the last two decades with the number of new compounds discovered annually increasing from 20 to more than 200 [76]. It has been estimated that by 2010 more than 15,000 new marine natural products (NMNP) had been discovered with 8368 new compounds recorded for the decade between 2001 and 2010. This constitutes over half of all the compounds discovered since 1951. [70]. Out of the NMNPs from invertebrates, 33% from sponges, 18% from coelenterates (sea whips, sea fans and soft corals) and 24% from the representatives of other invertebrate phyla such as ascidians (also called tunicates), opistho-branch mollusks (nudibranchs, sea hares, etc), echinoderms (starfish, sea cucumbers etc.) and bryozoans (moss animals) [77].

TABLE 1. The most important bioactive compounds isolated from marine non-chordates.

Marine sources (Major phylum)	Major bioactive compounds	Disease prevention
Porifera	Manzamine, phenolic or quinoid, alkaloids, terpenoids, brominated tryptamines,	Antimalarial, antiviral especially AIDS, antibacterial, antifungal, anticancer
Coelenterata	Prostaglandins, proteins, enzymes, steroids, terpenoids, brominated alkaloids, macrolides and ceramides	antibacterial, antifungal, antialgal, cardiac and nerve muscle relaxation, antitumour, anticancer, antineoplastic
Annelida	Peptides, Arenecins, hedistins, antimicrobial peptide (AMP),	Arthritis, osteoporosis, bone cancer, antimicrobial, antibacterial, antifungal
Arthropoda	Lectin viz. limulin and carnoscorpin, thiol ester protein, fatty acids, triglycerides, carotenoids and lipids,	Antibacterial, anti cancer, antioxidant, antiproliferative, antimutagenic, antiinflammatory, immune response
Mollusca	Dolostatin, lectin, steroid, terpenoids, acetylenic compounds, dollstains, polysaccharides	Antileukemic, immune response, hypotension, relaxation smooth muscle, antinicotinic activity, antiviral especially HIV virus inhibiting compound
Echinodermata	Saponins, sterol derivatives, terpenoids, glycoproteins, cerebroside, pyrimidine nucleosides, thymine deoxyriboside and uracil deoxyribose, polysaccharides, β -carotene	Hemolytic, antibacterial, antifungal, antineoplastic, antitumor, antiviral especially anti HIV activity, antiinflammatory, anti-cancer, anti-allergic

8. Antimicrobial peptides (AMPs) – the ‘natural antibiotics’

AMPs are the major component of the innate immune defense system in marine invertebrates with a broad spectrum of antimicrobial activities against bacteria, viruses and fungi [77]. They are also termed as "natural antibiotics" and are evolutionarily conserved [78]. Alternative terms for AMPs have also appeared which include better descriptive terms such as "host defense peptides," "alarmins" and even "defensins" (used in a broad context instead of the gene family) [79].

AMPs are relatively short (commonly consist of 6–100 amino acid residues, display an overall positive charge ranging from +2 to +11 and contain a substantial proportion (typically 50%) of hydrophobic residues [80]. They are mostly ribosomally produced peptides with less than 10 kDa in mass and provide an immediate and rapid response to invading microorganisms and are post-translationally activated by proteolytic cleavage [61].

AMPs have been found virtually in all the organisms including bacteria, fungi, protozoa, parasites and in vertebrates as a host defense mechanism against invading microorganisms and they display remarkable structural and functional diversity [81]. Even the human skin secretes some AMPs that act as a barrier against the invasion of microorganisms. [82]. Besides the direct antimicrobial activity, AMPs carry immunomodulatory properties [83], which make them especially interesting compounds for the development of novel therapeutics or food additives [59]. The ubiquitous presence of AMPs (AMPs) in nature attests to their overall importance in building the defense strategies of most organisms. They are

considered part of the humoral natural defense of invertebrates against infections. Their enhanced expression, due in part to various stress factors such as infective organisms, has been directly linked to the quick and efficient innate immune response of their hosts [59].

Sources of AMPs range from single celled microorganisms such as bacteria (bacteriocins) to invertebrates. Many AMPs exhibit relatively non-specific bactericidal activity against both Gram-positive and Gram-negative species and selectively kill prokaryotic rather than eukaryotic cells. Their value in innate immunity lies in their ability to function without either high specificity or memory. Moreover, they are synthesized without dedicated cells or tissues and they can rapidly diffuse to the point of infection [84, 85].

Since their discovery in the 1980s, AMPs have been considered to be promising candidates for therapeutic uses in humans, animals and plant health. During, more than the last 30 years, AMPs have been considered as a potential source for the development of new therapeutic molecules to control infectious diseases owing to their significant specificity for micro-organisms with low toxicity for mammal cells [58]. Due to the fact that they exhibit broad spectrum antimicrobial activity, possess selective toxicities and are less prone to microbial resistance [2], AMPs represent an exciting class of bioactive compounds that could potentially provide major reprieve for mankind in the efforts to curb/control infections [59].

The biological activities of the amino acid residues of AMPs are based on their composition and sequence [67]. Recently, much attention has been paid to unravelling the structural, compositional, and sequential properties of bioactive peptides [86]. Many marine organisms are exposed to more extreme conditions than that on land, which make the marine bioactive peptides have significant different amino acid compositions and sequences from land bioactive peptides; besides, the species and amounts of marine bioactive peptides are more than that of land bioactive peptides. Moreover, marine bioactive peptides can be obtained from various marine animals, plants and lower organisms. Each is unique as a species, considering its great taxonomic diversity and special characteristics, marine bioactive peptides have better bioactivity in some areas than land bioactive peptides [67]. Despite these responses, additional care has to be taken when assessing the origin of the antimicrobial content in marine organisms [59]

The cationic charge of the AMPs promotes selectivity for negatively charged microbial cytoplasmic membranes over zwitterionic mammalian membranes and hydrophobic residues facilitate interactions with the fatty acyl chains. Many eukaryotic peptides act on bacterial membranes or other generalized targets, in contrast to most antibiotics, which usually target specific proteins. This creates an advantage for AMPs as the development of microbial resistance by gene mutation is less likely to occur [59, 87]. New vistas on AMPs suggest that they are capable of playing multifunctional roles that extend beyond their capacity to act as gene-encoded antibiotics. Thus, some of these peptides have not only been shown to display anticancer activity [88], but also act effectively to stimulate the immune system by favoring cytokine release, chemotaxis, antigen presentation, angiogenesis, inflammatory responses and adaptive immune induction [89, 59]

AMPs are a potential source of novel antimicrobial agents currently being extensively investigated and are considered attractive alternatives to conventional antibiotics in the fight against MDR pathogens [2]. The combination of a broad spectrum antimicrobial activities targeted at non-protein cellular components with localized, high-level expression at the site of infection, makes AMPs highly effective antimicrobial agents with significant potential as a source of new antimicrobial drugs such as new more effective anti-tubercular agents active against multidrug resistant (MDR) and extensively drug resistant (XDR) *Mycobacterium tuberculosis* complex pathogens [90, 91].

In many organisms AMPs are produced as inactive precursors requiring proteolytic cleavage to become active [92]. Their regulation is therefore not only dependent on their own expression but also on the abundance of appropriate proteases [93]. In multicellular organisms, some AMPs are constitutively expressed, stored at high concentrations as inactive precursors in granules and released locally at infection and inflammation sites, whereas the expression of others is induced in response to pathogen-associated molecular patterns (PAMPs) or cytokines [3, 93]. Criticality emerges in the case of deeper skin burn patients prone to nosocomial bacterial infections, their medical treatment becoming a major problem. [82]

Only a few AMPs have reached phases of clinical and preclinical pipelines [82, 95]. They have limitations in therapeutic development due to their poor enzymatic stability and low permeability across biological barriers [2, 96]. Chemical modifications such as peptide cyclization, the use of non-protein amino acids, peptidomimetics, lipidation, etc. are often used to overcome these drawbacks, particularly to enhance enzymatic stability [2].

There are encouraging examples of AMPs already introduced into the market, and many AMPs are currently being tested in clinical trials [97], which provide are as on for optimism for introduction of novel AMP-based drugs in several indication areas [60]. An updated database (Jan. 2012) shows 1773 AMPs and

are distributed as: antiviral (5.8%), antibacterial (78.56%), antifungal (31.19%) and antitumor (6.14%). Means, some of them function against more than one type of pathogens [58], in short, as a class, natural and synthetic AMPs exhibit a very high potential as new therapeutic agents because of their novel mechanism of antimicrobial activity, coupled with the difficulty of bacteria to develop resistance to them. 19–21 AMPs have also been shown to be key components of the innate immune response [60, 98]

Considering the diverse functions that AMPs exert, much of the research in this field has been focused over the past decade on the bioprospecting of novel antimicrobial compounds capable of exhibiting a broad spectrum of activity against a wide range of microorganisms, which include Gram-positive and Gram-negative bacteria, yeasts, fungi, viruses, protozoa and parasites such as nematodes [77]. More recently, bioinformatics tools have also helped to develop and/or remodel preexisting AMPs, driving their synthesis toward more selective and effective drugs [59]. Several databases exist for natural AMPs, today covering more than 2000 peptides [99]. Thus, interestingly, the AMPs (AMPs) have rapidly captured attention as novel drug candidates [60] and are currently being used for drug development due to their activity as immune modulators, which give them clinical potential beyond the treatment of antibiotic-resistant strains [100].

9. Marine invertebrate derived AMPs (AMPs) - a new promise

When considering marine invertebrates which are thought to account for 30% of all animal species, comprising 20 different phyla, it is noteworthy that they should have retained an ancestral, nonspecific innate immune-defense system mainly composed of AMPs. In such animals, critical elements such as antibodies and lymphocytes, immunological memory and true self vs. non-self-discrimination are absent. [59]. The cellular component of marine invertebrate immunity – as mentioned elsewhere in this article, is mediated by hemocytes, motile cells that phagocytose microbes, secreting soluble antimicrobial and cytotoxic substances in the hemolymph. For this reason, most of the AMPs reported in different groups of invertebrates have been isolated from hemocytes, which represent an interesting natural source of AMPs [59, 65].

AMPs with potential interest for biopharmaceutical companies have been isolated from marine invertebrates. Some AMPs, such as mollusc defensins, have very low MICs in the nanomolar range against Gram-positive bacteria [101]. Their fungal homologue – plectasin - is considered to be a major candidate for therapeutic use [100]. The AMPs characterized in marine invertebrates include those from arthropods, molluscs and cnidarians. They are cationic and hydrophobic, and target essential components of microbial cell walls and membranes, which determines their spectrum of activity [102]. In recent years, researchers have isolated AMPs from mud crab (*Scylla paramamosain*), oyster (*Crassostrea gigas*), sponge (*Trichoderma* sp.), the marine snail (*Cenchrismuricatus*) etc. [66]. Among AMPs from marine invertebrates, ALF-derived peptides have been shown to modulate the inflammatory response in murine macrophage cell lines and display anti-tumour activity against HeLa cells through the alteration of the cell membrane. Those novel activities may open the way to future drug developments [100]. LL37, a cationic AMP produced by the innate immune system exhibits antimicrobial activity and has a direct effect in wound healing, neovascularization and angiogenesis [103].

Approximately 75% of AMPs contained in the different databases are of animal origin [16], among which invertebrates represent over 80% of the Animal Kingdom and a huge majority of them are marine derived AMPs from invertebrates have been extensively studied with particular interest in marine invertebrates in the context of bioprospecting research for natural products [58]. Likewise, the value of invertebrate AMP research is not only focused on their capacity to kill microorganisms but also on their potential as insecticides, as in the case of peptides found in toxins and venoms of insects and their natural enemies [58, 104]. Thus, the marine invertebrates represent a potentially rich source for pharmacologically useful AMPs as presumably share antimicrobial tools effective against related pathogenic microorganisms present in vertebrate and invertebrate organisms [59]. Thus, the AMPs from marine organisms have safe, natural, inexpensive and high bioactivity properties [66].

10. Classification of AMPs

AMPs can be classified based on different criteria. Based on biosynthesis they can be grouped into i) non-ribosomally synthesized peptides (that contain at least two moieties acquired from amino acids are mostly produced by bacteria and significantly modified and ii) ribosomally synthesized peptides are produced by both prokaryotic and eukaryotic organisms [60].

Based on their structure they belong to any one of the four following classes.

(i) α -helices peptides: This group is composed of α -helical peptides such as magainins, cecropins and LL-37 and are often unstructured in aqueous solution, but adopt an amphipathic helical structure in contact with a biological membrane [80]. Two of the most studied peptides in this group are LL-37, which is present in neutrophils and epithelial cells and the human lactoferricin, which is derived from the proteolytic cleavage of the antimicrobial and immunomodulatory iron-binding glycoprotein lactoferrin, present in milk and exocrine secretions [105].

(ii) β -sheet peptides

The second group includes cationic peptides that contain two to four disulfide bridges that form β -sheet structures including β - defensins, plectasin and protegrins [2]. Due to their rigid structure the β -sheet peptides are more ordered in aqueous solution and do not undergo as drastic conformational change as helical peptides upon membrane interaction [60]. The best-studied β -sheet peptides are the defensins - a large group of AMPs, which are produced as inactive precursors in neutrophils, macrophages and epithelial cells [80, 89].

(iii) Loop peptides: They are the smallest group of AMPs that form loop structures due to interlinking by at least one disulfide bridge such as the dodecapeptides 59 and tachyplesins [60, 106]

(iv) Extended peptides: This group contains cationic peptides which are rich in proline, tryptophan, arginine or histidine [106]

Based on the general mechanisms of action, the AMPs can be divided into two classes - the membrane disruptors and non-membrane disruptors. Based on the biological activity AMPs are classified as antibacterial, antifungal, antiviral, antitumoral, antiparasitic, spermicidal, insecticidal etc. They have also been classified on the basis of charge, length and hydrophobic residue contents [106, 107]

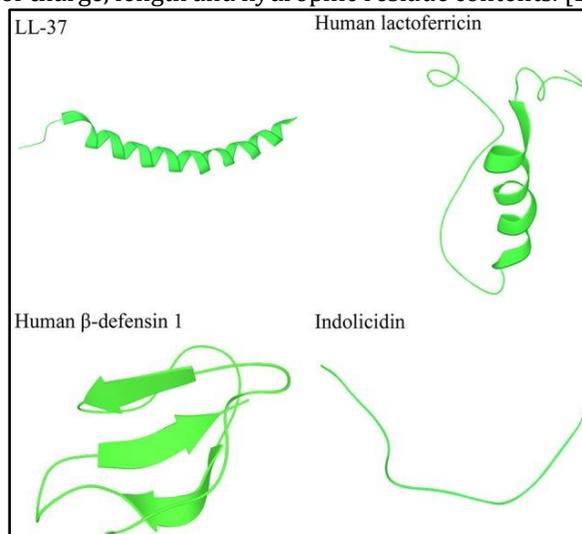


FIGURE 1. Peptides representing the three main categories of the secondary structures of AMPs. LL-37 and human lactoferricin represent α -helical peptides, human β -defensin 1 represents β -sheet peptides, and indolicidin represents extended/random-coil structures. Structures are from Protein Data Bank Europe

11. The role of AMPs on the innate immune response

AMPs have been vastly studied with a special importance to marine invertebrate organisms that lack an adaptive immune system. AMPs are a part of the innate immune response with a relevant role in the first line of defense against microorganisms. In addition to their direct antimicrobial activity, some AMPs possess different immunomodulatory functions [106]. Some of the most relevant are:

i) Chemotactic activity: In this case, they act as chemo-attractants capable of recruiting immune cells to the site of infection. In other words, AMPs induce the expression of a broad range of chemokines [106, 108]

ii) Anti-endotoxin activity: AMPs possess the capacity to dampen production of endotoxin-induced pro-inflammatory mediators such as tumor necrosis factor alpha (TNF- α) by blocking or modulating toll like receptor (TLR) signaling pathways [106].

iii) Immune cell differentiation: AMPs appear to have a direct link inducing cell differentiation and activation thereby bridging the innate and adaptive immune responses [106, 109]

iv) Wound healing and angiogenesis: Wound healing involves the re-growth of epithelial layers and the formation of new blood vessels (angiogenesis). AMPs act directly on the epithelial and endothelial cells

inducing promoting re-epithelialization and angiogenesis. AMPs also induce wound healing indirectly through their chemotactic effects.[109]

Due to these immunomodulatory and antibacterial properties, AMPs are excellent candidates for infection treatment as they can also control inflammation at the site of infection.

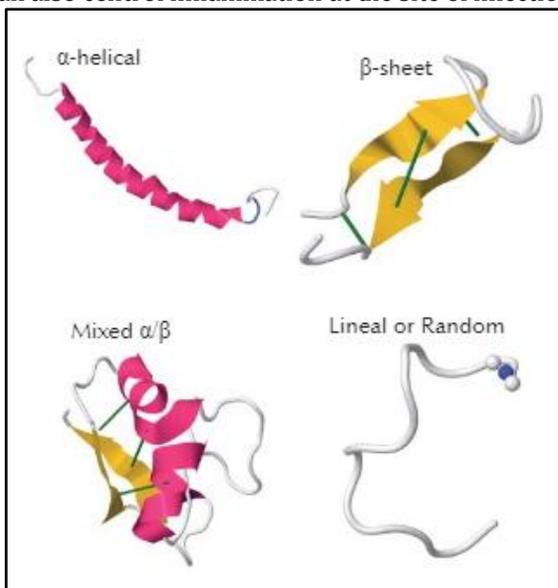


FIGURE 2. Representative secondary structure of AMPs. α -helix (Morcin), β -sheet (Hepcidin-20), mixed α -helix/ β -sheet (Hydramacin-1) and lineal or random (Lf11). α -helix structures are shown in magenta, β -sheet in yellow, and green lines represent the disulfide bridges.

12. Mechanisms of AMPs action

The mechanism of action of the AMPs is completely different to common antibiotics. These are very primitive molecules which have been functioning as a chemical shield of defense over millions of years in plants and animals [110]. Now, at this time of escalation in multi-drug resistant pathogens or superbugs, the scientific world is enthusiastically analyzing the possibilities to make these molecules an alternative for combating diseases, including those by Gram negative ESKAPE pathogens. The mechanism by which these peptides act make them very difficult to generate bacterial resistance, becoming a promising future as more durable therapeutic agents. Besides, knowing the role of peptides as modulator of the innate immune response, they can be helpful as guide for developing immunomodulatory therapies [110]. The mechanism of action of APMs may be by one or a combination of two or more of the following.

12.1. Interaction with bacterial membrane

The primary step in the direct antimicrobial activity of AMPs is the membrane interaction [60]. Many AMPs display a direct and rapid antimicrobial activity by causing disruption of the physical integrity of the microbial membrane and/ or by translocating across the membrane in to the cytoplasm of bacteria to act on intracellular targets [111]. Electrostatic forces between the cationic AMPs and the negatively charged bacterial surface are critical determinants for this interaction between peptides and microbial membrane [60].

The cytoplasmic membranes of both Gram-positive and Gram-negative bacteria are rich in the phospholipids phosphatidylglycerol, cardiolipin and phosphatidylserine, which have negatively charged head groups, highly attractive for positively charged AMPs. The teichoic acids which is present in the cell wall of Gram-positive bacteria and lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria provide additional electronegative charge to the bacterial surface [60, 89]

12.2. Membrane disruption and intracellular targets in bacterial cells

As the outer membrane of Gram-negative bacteria constitute permeability barriers to the cytoplasmic membrane by cations like Ca^{2+} and Mg^{2+} (which bind with the inner layer of LPS), AMPs have to pass through the outer membrane and are proposed to be translocated through this outer membrane via so called self-promoted uptake [60]. This model suggests that, due to greater affinity for the LPS, AMPs displace the divalent cations and bind to the LPS. By being bulky, the AMPs then cause transient cracks and permeabilize the outer membrane, thereby permitting passage of the peptide itself across the membrane. In

contact with the cytoplasmic membrane, the AMPs form an amphipathic secondary structure essential for interaction with the cell membrane. The charged domains of the peptide allow for interaction with the hydrophilic head groups of the phospholipids, while the hydrophobic domains of the peptide interact with the hydrophobic core of the lipid bilayer, thereby driving the AMP deeper into the membrane [112]

Several models have been proposed describing the next events occurring at the bacterial cytoplasmic membrane, which ultimately lead to membrane permeabilization. According to the “barrel-stave model,” the peptides insert perpendicularly into the bilayer while recruitment of additional peptides subsequently results in formation of a peptide-lined transmembrane pore. In this pore, the peptides are aligned with the hydrophobic side facing the lipid core of the membrane and the hydrophilic regions facing the interior region of the pore. According to the “toroidal-pore model,” insertion of peptides forces the phospholipid to bend continuously from one leaflet to the other, resulting in a pore lined by both peptides and the head groups of the phospholipids. Finally, in the “carpet model,” accumulation of peptides on the membrane surface causes tension in the bilayer that ultimately leads to disruption of the membrane and formation of micelles. [60, 113]

Membrane permeabilization by AMPs is suggested to initially lead to leakage of ions and metabolites, depolarization of the transmembrane potential with subsequent membrane dysfunction (e.g., impaired osmotic regulation and inhibition of respiration) and ultimately, membrane rupture and rapid lysis of microbial cells [60, 112]. Besides leading to membrane dysfunction and disruption, membrane permeabilization is important for translocation of certain AMPs into the cytoplasm, where they target key cellular processes including DNA/RNA and protein synthesis, protein folding, enzymatic activity, and/ or cell wall synthesis [60, 114, 115].

The microbial death caused by AMPs could be a result of multiple and complementary actions, referred to as multi-hit mechanism. This strategy helps to increase the efficiency of AMPs and to evade resistance development [60]. It is likely that the mode of action of individual AMPs varies depending on parameters such as peptide concentration, target bacterial species, as well as tissue localization and growth phase of the bacteria [60, 116]. The membrane disruptor peptides are the most predominant and much of them have α -helix structures, which directly act at the plasma membrane level, altering the cell permeability or lysing cells through pores formation [100].

The non-membrane disruptor AMPs use show a variety of mechanisms like binding to nucleic acids, interference with the synthesis of nucleic acids, inhibition of the synthesis of proteins, inhibition of the enzymatic activity, inhibition of the synthesis of cell wall, blocking some virulence factors, such as flagella, proteases, secretion systems, effector proteins etc. [100, 117]

12.3. Immunomodulatory activities

Along with the anti-bacterial activities, the immunomodulatory actions of AMPs are also well documented [60, 117]. They include stimulation of chemotaxis, modulation of immune cell differentiation and initiation of adaptive immunity. All these contribute to the bacterial clearance of the host. The activities under this mechanism also include suppression of toll-like receptors (TLR), cytokine-mediated production of pro-inflammatory cytokines and anti-endotoxin activity, together preventing excessive and harmful pro-inflammatory responses including sepsis [60].

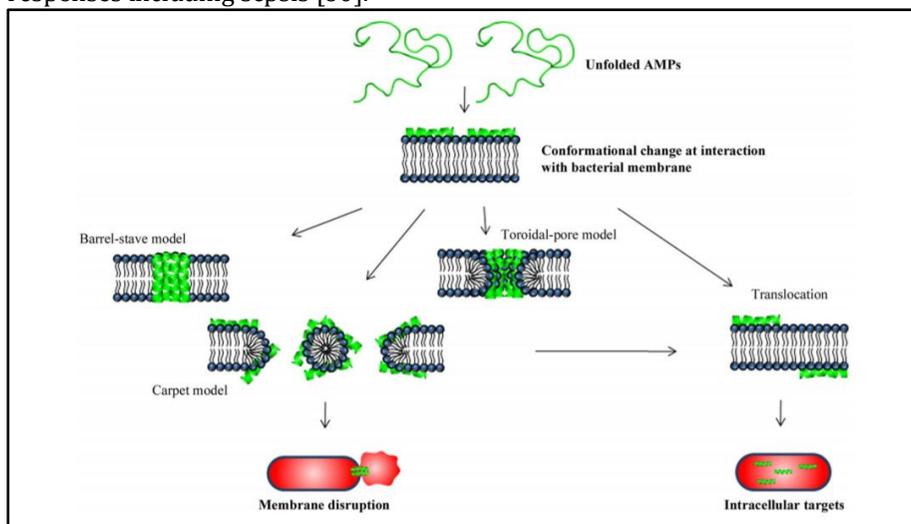


FIGURE 3. Schematic illustration of bacterial killing mechanisms by AMPs.

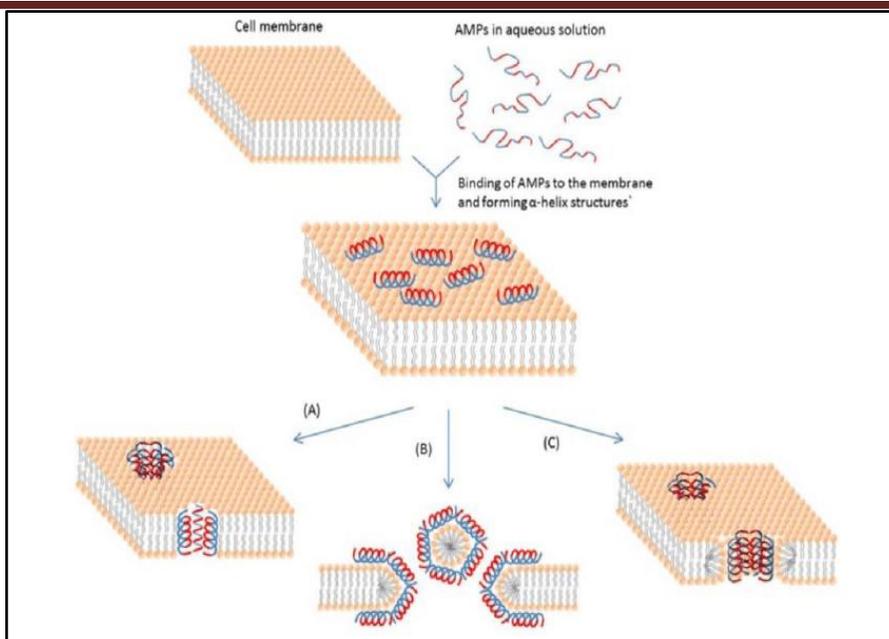


FIGURE 4. Schematic models of action mechanisms of membrane disruption by AMPs. A. Barrel stave pore. Peptides on the membrane surface are aggregated and inserted inside the membrane; the hydrophobic regions are aligned with the central lipid region of the bilayer, while the hydrophilic regions form the pore interior. B. Toroidal pore. Peptides are aggregated and induce that the lipid monolayer are continuously curved forming a pore; the pore interior is covered of peptides and also of polar heads of lipids. C. Carpet or detergent type. Peptides cover the membrane surface forming a carpet; toroidal pore are transiently formed, which permit the entry of peptides and the membrane is starting to be disaggregated by the formation of micelles. Hydrophobic regions are represented in black and the hydrophilic in blue.

12.4. Differentiation between mammalian and bacterial cells

The basic differences in the net charge and chemical constitution between the microbial and mammalian membranes protect the latter from the action of AMPs [60]. The cytoplasmic membrane of mammalian cells is rich in the zwitterionic phospholipids phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, providing a membrane with a neutral net charge [60]. There is also an asymmetric distribution of phospholipids in mammalian membranes, with the zwitterionic phospholipids being present in the outer leaflet, while phospholipids with negatively charged head groups, if present, are localized in the inner leaflet facing the cytoplasm [60, 93]. So, the interactions between AMPs and mammalian cell membrane occur mainly via hydrophobic interactions, which are relatively weak compared to the electrostatic interactions taking place between AMPs and bacterial membranes. The high cholesterol content of mammalian cell membranes is proposed to reduce the activity of AMPs via stabilization of the phospholipid bilayer [60]

The differences in the mechanisms of action of AMPs, in many cases, determine the type of cells on which they act. For example, mollusc defensins, which are essentially active against Gram-positive bacteria, bind to lipid II, the precursor of peptidoglycan [37]. Arthropod anti-lipopolysaccharide factors (ALFs) and mollusc bactericidal/permeability-increasing protein (BPI), which are essentially active against Gram negative bacteria, bind to lipopolysaccharide (LPS) [38–40]. Finally, crustacean PvHCT, which is strictly antifungal, permeabilizes the fungal plasma membrane [41].

13. AMP databases

As the number of bacterial strains and other pathogens developing resistance against conventional antibiotics increase, the search for natural compounds with novel modes of action reached in extraction and purification of many new AMPs [91]. The AMPs have achieved scientific and medical relevance by the identification of cecropins (in insects), magainins (in amphibians) and defensins (in humans) in 1980s. As an outcome of the extensive studies, various groups of AMPs have been described such as the cecropins and

defensins in insects, the clavanins in tunicates, the penaeidins and crustins in crustaceans, the caenopores in nematodes [58] etc.

As mentioned, thousands of AMPs have been isolated from plants, animals, fungi and several microorganisms [58, 119] and obtained through a conventional chemistry approach or designed using AMPs as models to produce peptides with improved selectivity and potency [58]. This expanse of data has been compiled in approximately 23 specialized databases that facilitate extraction of information and provide bioinformatics tools to rationalize the design of new AMPs [120]. To mention few examples for such databases are CAMP, APD, YADAMP, DRAMP, PhytAMP (for plant AMPs), BACTIBASE (for bacterial AMPs), DADP (for amphibian AMPs), MilkAMP (for AMPs of dairy origin), InverPep (for AMPs from invertebrates) [58, 91] etc. AMSdb, Defensin knowledgebase, Peptaibol Database, SAPD, AMPer, CyBase, BAGEL, Minicope (the Innate immunity defense peptides MiniCOPE Dictionary) CAMP, RAPD, Dragon Antimicrobial Peptide Database (DAMPD, <http://apps.sanbi.ac.za/dampd>) etc. are other examples [58, 91, 121, 122]. Each of them contains details of hundreds and thousands of AMPs arranged based on different criteria such as taxonomy, species, AMP family, citation, keywords and a combination of search terms and advanced search fields.

14. Nanoparticles: candidates for conjugation to AMPs

Particles that are having at least one dimension ranging from 1nm to 100 nm are considered as nanoparticles (NPs). Richard P. Feynman (1959) introduced the concept, and it was Norio Taniguchi who coined the term “nanotechnology” in 1974 [2]. Feynman’s idea about the possibilities of manipulating matter at the atomic scale led to numerous revolutionary developments in multidisciplinary science. Thus, nanotechnology is emerging as a useful tool for various applications in biomedical devices, waste management, material science, and electronics [123]

Nanoparticles provide another potential solution to combat multidrug resistant pathogens. The large surface area to volume ratio of Nanoparticles provides a high loading of coated molecules.97 Nanoparticles by themselves (e.g. silver, other metal oxides such as titanium, copper, zinc and iron etc.) have been known to possess antimicrobial activities and work through numerous modes of action [2]. Nanoparticles can disrupt the bacterial cell membrane, causing cell penetration, react with intracellular target and cause toxicity [2, 124].

The area of bioconjugation in general and the conjugation of peptides and nanoparticles in particular is of growing interest and would attain great heights in the near future.90. When peptides are conjugated to nanoparticles, the resulting conjugate will have new critical properties like enhanced potency, site of action targeting capability, less toxicity, etc. that are directly acquired from the peptides but not previously possessed by the nanoparticles.[82] Nanoparticles are excellent candidates for transporting drugs to their targets [2]

The use of nanoparticles in combination with antibiotics makes it possible to decrease the toxicity from both agents toward human cells because of the synergistically enhanced antimicrobial activities and the reduced requirement for high dosages [125]. Nanoparticles as antibiotic carriers to the site of infection are emerging as a promising strategy in antibiotic therapy. The results from recent research in which nanoparticles have been combined with AMPs are promising and show an increasing trend toward a safer profile of the conjugates toward mammalian cells [2].

The linkage between nanoparticles and peptides should be stable and be responsive to other foreign agents and physical factors 99 for the effective release of drugs (peptides) from nanoparticles to the site of action. The density or ratio of the peptide to nanoparticles is also important for efficient uptake. The four strategies that are proposed to conjugate peptides and nanoparticles100 are, (a) the electrostatic interaction (between oppositely charged nanoparticles and peptides), (b) the direct interaction (binding of the peptides with the nanoparticle surface with high affinity), (c) the secondary interactions (like the biotinylation where the biotin on the peptide would mediate directional assembly) and (d) covalent attachment linkages (which utilize EDC-based coupling of amines to carboxylic acids and N-hydroxysuccinimide- and maleimide-mediated couplings to amines and thiols) [2, 82]

The examples for some peptides commonly used for the therapeutic delivery of nanoparticles are TAT and TAT-like peptides, RGD peptides, the pep-I peptide, organelle-specific peptides, neuropeptides, the rabies virus-derived peptide etc. Several recent studies have shown that the nanoparticles provide an efficient way to kill bacterial pathogens, including drug-resistant bacteria and have low probability for resistance development by the pathogens [2, 126]. The peptide conjugated nanoparticles have found numerous applications in various fields including intracellular delivery, drug delivery, cancer therapy, neurology and many others.

15. Silver nanoparticle – antimicrobial peptide (AgNPs - AMPs) conjugation

The interaction between proteins and silver nanoparticles (AgNPs) has been demonstrated to play a pivotal role in the nanomaterial's biocompatibility and ultimately, its antimicrobial performance. Proteins, peptides and free amino acids can be used to control the structure of AgNPs during synthesis and improve their stability under a variety of conditions. However, the mechanism(s) that underlie such stabilization as well as the exact role of various amino acid moieties remains elusive [127]. It is being increasingly recognized that nanoparticles, especially silver nanoparticles have good antimicrobial properties and their conjugation with AMPs have been proposed to enhance the activity. The bio-functionalized AgNPs could remove endotoxin thus maximizing their potential in various applications [82].

Silver nanoparticles are known to be toxic to mammalian cells above a certain concentration and one way to reduce its toxicity is to conjugate it with peptides [128]. The conjugate is less toxic to mammalian cells as revealed by the MTT assay compared to AgNP alone, up to a concentration of 96 $\mu\text{g}/\text{mL}$ concentration. The conjugation through weak interactions between the AMPs and the nanoparticles is sufficient to reduce the toxicity of the AgNP, which in turn may be due to controlled release of Ag^+ [82].

The combination of AgNPs with the CSG-LL37 peptide (LL37@AgNP) has been used as the antimicrobial and anti-biofilm agent by [129] and they found that the LL37@AgNP exhibited very low MIC values against both Gram-positive and -negative bacteria. Also, LL37 capped AgNPs were not cytotoxic at the MIC levels or even at double the MIC level and they did not hinder cell proliferation at this level. The conjugate was found to be more biocompatible and more stable. Apart from that, the conjugate the ability of this conjugate to was able to prevent the biofilm formation by *Pseudomonas aeruginosa* [82, 129]

The activity of the cell penetrating peptide GGGRRRRRRYGRKKRRQRR (G3R6TAT) as the stabilizer and reductant to produce AgNPs and the screening of the same against Gram positive *Bacillus subtilis*, Gram negative *Escherichia coli* and the yeast *Candida albicans* showed that the conjugate dramatically improved the biocide efficacy as much as 50 times against *Bacillus subtilis*, at least 10 times against *Escherichia coli* and 3.3 times against *Candida albicans* with a very low MIC value. The SEM results have also shown the breakdown of bacteria and the conjugate showed low hemolytic activity at effective concentrations. Hence, this platform could provide an alternative therapy for topical or systemic infections [82, 130]. Also, it has been shown that the AgNPs with 20 nm diameter exhibit more cytotoxicity than the 40 nm diameter. Also, the AgNP-based hydrogels showed high antimicrobial efficiency against MDR *Pseudomonas aeruginosa* [131] It is expected that this Ag releasing biomaterial offers great potential for application in wound healing, particularly after surgery, and in the treatment of chronic and large surface wounds, such as diabetic ulcers or severe burns, due to its potential to reduce inflammation and prevent infection [82]. The antimicrobial and hemolytic properties of AgNPs in combination with different AMPs found that the need for high dosages can be reduced by the synergistic action of antimicrobial agents that can also bring down the side effects [82, 132]. Thus the silver nanoparticle – antimicrobial peptides (AgNPs - AMPs) conjugates bring a new hope of ray to the ongoing war against MDR pathogens including the Gram negative ESKAPE members.

The peptide exhibits a dynamic exchange from the surface of the AgNPs without undergoing a significant conformational change. The weak interaction is useful particularly in the case of AMPs where the charge and structure of the peptide is important for their activity [133] and hence too strong an interaction with Ag nanoparticles through the positively charged residues will reduce their activity. Very strong interactions can also perturb the structure of the AMPs resulting in reduced activity. Thus, the balance of strong interaction/stability vis-à-vis the activity is a key feature to be considered for design of new conjugates [133, 134]. Simulations predict high affinities of Arg, Cys, and Met residues for the Ag facet. However, cooperative effects over larger portions of the peptide sequence have long been known to be essential in determining affinity for metal surfaces and peptides selective for Ag surfaces [135] further demonstrate that the affinity is not determined solely by individual amino acids but also by the conformational statistics of the peptide as determined by its sequence [127].

The stability of the AgNP-AMP conjugate originates from steric repulsion. It has been shown that stability is enhanced if AgNPs are conjugated with polypeptides, where the steric repulsion between proteins prevent Nanoparticles from approaching each other at close distance, thereby preventing their aggregation [136]. The nature of interaction is closely associated with stability of the system. As the AgNPs are generally unstable in solution an AgNP-AMP system having long-term stability with enhanced activity is preferable. Conjugating a cysteine containing peptide with AgNP is expected to have a favorable effect on the strength and nature of nanoparticle-peptide interactions. However, for designing AgNP-AMP conjugates with increased stability and antimicrobial activity, it is important to understand the nature of interactions between the AMP and AgNP. [134].

16. Conclusion

It has been more than two decades that spread of the 'bad bugs', which are resistant to a wide spectrum of conventional antibiotics bothering the healthcare systems globally. The emergence of new infectious agents, infections that emerge out of surgical devices pose, the never lasting threat of nosocomial infections and other dreaded incidents by bacteria including the Gram negative members of *ESKAPE* group are serious threats which account for very high morbidity in hospitals [6]. In 2014, the World Health Organization (WHO) first looked at the antimicrobial resistant data globally; the data on the issue also revealed the serious and worldwide threat on the public health [137]. It is at these circumstances that the scientific world turned back to the nature in search for new antibiotics with novel modes of action.

Because the ocean occupies almost 70% of Earth's surface, it offers unlimited potential for biological and chemical diversity. Marine ecosystems comprise a continuous resource of immeasurable biological activities and vast chemical entities. The invertebrates, as the biggest group of the animal kingdom, are the principal source of Antimicrobial peptides with validated antimicrobial activity. Especially the marine invertebrates that rely solely on innate immune mechanisms for host defense is a spectacular resource for the development of new antimicrobial compounds [61] and is mainly composed of AMPs, which are endogenous and are exciting candidates as new antibacterial agents due to their broad antimicrobial spectra, highly selective toxicities, and the difficulty for bacteria to develop resistance to these peptides [58]. Thus the AMPs offer promising alternatives to standard therapies as anti-infectives and immunomodulatory agents with mechanisms of action which are less prone to resistance induction compared to conventional antibiotics. Although challenges in translating nonclinical candidate AMPs into successful clinical products are well recognized, the discovery and commercial development of next-generation therapeutic peptides and peptide mimetics predicted to be accelerated by recent advances in overall understanding of their mechanism of action, resistance patterns, and smart formulation strategies. With several AMPs currently undergoing latest age clinical development in different therapeutic areas, the next years hold a promise to confirm the therapeutic benefit of these novel candidates and lead to market authorization of several new AMP-based drugs [60]. It is apparent that many peptide drugs have entered the market and several are in the pipeline and few are in various stages of preclinical and clinical trials [82]. Thus, peptide-based molecules present a promising future in drug discovery programmes.

It is at this stage that, the nanoparticles emerge as the bridge to fill this gap since these are endowed with unique properties and potential therapeutic applications. Their small size and high surface-to-volume ratio make them attractive both in therapeutic and biomedical fields. Further, these materials can also be used in theranostics, which incorporate both therapeutic and diagnostic moieties in a single species. This is possible because nanoparticles can be tethered with differently functionalized entities. Taking the advantage of this unique feature, there are pieces of evidence wherein nanoparticles are conjugated to different biologically active molecules and one among them is the peptides. These are considered as the "value added" constructs that will have the properties from both the moieties in-connection [58].

A wide variety of nano-technological devices for the treatment of infectious diseases have been developed, including microemulsions, vaccines and metallic, inorganic, lipid and polymeric-based Nanoparticles (Nanoparticles) [87]. Metallic Nanoparticles such as silver (AgNP) and gold (AuNP) show unique and considerably distinct physical, chemical and biological properties due to their high surface-to-volume ratio, with which surfaces can be modified with ligands containing functional groups, providing an electrostatic or steric stabilization [138].

The antibacterial activity of Ag nanoparticles against a broad spectrum of bacteria is well-known. Ag nanoparticles have been conjugated to different molecules with antibacterial activity in order to obtain synergic effects, such as poly (ethyleneimine), amoxicillin, polysaccharides, peptides, surfactants and polymers [87, 128]. Thus, the silver nanoparticles conjugated antimicrobial peptides open a window to the new horizon of hope in the fight against the 'bad bugs'

Conflict of Interest

Authors declare no conflict of interest.

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