

The sources of antimicrobial peptides against Gram-positives and Gram-negatives: our research experience

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SUMMARY

Antibiotic resistance of Gram-positive and Gram-negative bacteria is becoming increasingly prevalent. For this reason, the search for new molecules that can overcome current resistance and also recover antibiotics that are no longer effective is becoming increasingly urgent. Our research group at the 'Polytechnic University of Marche' managed to study the effectiveness of certain antimicrobial peptides (AMPs). We decided to review our experience with AMPs by classifying them according to their origin and evaluating their effect on Gram-negative and Gram-positive bacteria. AMPs can derive from mammals, amphibians, microorganisms, and insects. In conclusion, our research experience shows that the richest source of AMPs are amphibians. However, the studies done are mainly in vitro or in animal models, requiring further human studies to assess the efficacy and safety of these molecules. AMPs may be a new therapeutic option for infections sustained by multi-resistant micro-organisms and for overcoming the mechanisms of resistance to antibiotics currently used.

In particular, combining AMPs with antibiotics, in-

cluding those with limited antimicrobial activity due to antimicrobial resistance, has often shown a synergistic effect, increasing or restoring their efficacy. The possibility of using manageable and relatively safe antibiotics again is crucial, considering the widespread increase in bacterial resistance in hospitals and the community. Despite a plethora of research on AMPs and their application as potential treatment on infectious diseases, this area needs further exploration. There is evidence that the characteristics of AMPs can seriously improve through structural chemical modifications and different delivery systems to become alternatives drugs to conventional antibiotics. The aim is to provide an overview of the possible sources from which AMPs are extracted, evaluating their action exclusively on Gram-positive and negative bacteria. This is to determine, based on our experience, which might be the most promising sources of AMPs for future research as well.

Keywords: AMPs, antibiotic resistance, Gram positive bacteria, Gram negative bacteria.

■ INTRODUCTION

Antibiotic resistance of Gram-positive and Gram-negative bacteria is becoming increasingly prevalent, partly due to the widespread use of antibiotics not only in the medical field, but also in agriculture and animal husbandry [1]. For this reason, the search for new molecules that can overcome current resistance and also enable the

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recovery of antibiotics that are no longer effective is becoming increasingly urgent. Consequently, our research group at the 'Polytechnic University of Marche' managed to study the action of certain antimicrobial peptides (AMPs), evaluating their action against different microorganism *in vitro* or in animal models of sepsis, prosthetic/device infections, and skin wound infections. Our experience regards mainly *in vitro* studies and studies conducted on animals, not observational clinical studies. We decided to review our experience with AMPs by subdividing and classifying them according to their origin and evaluating their effect on Gram-negative and Gram-positive bacteria. AMPs can derive from mammals (especially human defense peptides), amphibians, microorganisms, and insects, as inferred from the antimicrobial peptide database [2]. It is important to note that AMPs are active not only against bacteria, but also against fungi, allowing resistance to traditional therapies to be overcome [3-9]. This is why the research field of AMPs is very promising for the future, especially considering that associations with traditional antibiotics have often proved to be synergic. A limitation of our experience is that no studies have been conducted on humans, but only on animal models or *in vitro*. However, we believe that the potential of some AMPs may also be useful in humans, offering an overview of available molecules that could in the near future also be used in clinical studies in patients.

■ MATERIAL AND METHODS

We performed a narrative review of studies from our research group at the Università Politecnica delle Marche, consisting of the dermatology clinic and the infectious diseases clinic. We searched on Pubmed for the following keywords in combination: sources, AMPs, Gram-positive, Gram-negative, mammalian, insects, amphibians, and synthetic peptides. Only English-language studies, produced by our research group, and in which AMPs were evaluated *in vivo* in an animal model or *in vitro*, were included. The aim was to provide an overview of the possible sources from which AMPs are extracted, evaluating their action exclusively on Gram-positive and Gram negative bacteria. This is to determine, based on our experience, which might be the most promising sources of AMPs for future research as well.

■ AMPS DERIVED FROM MAMMALS (Table 1)

Mammalian-derived AMPs are components of the innate immune system, mainly represented by cathelicidins and defensins. Most of these AMPs have been isolated from humans, but also from cattle, sheep, and other vertebrates [10]. Furthermore, cathelicidins and defensins can act directly against pathogens, but also appear to be able to regulate the immune response, apoptosis and wound healing [11].

Bovine myeloid antimicrobial peptide (BMAP)-28

BMAP-28 is a bovine antimicrobial peptide of the cathelicidin family, showing the ability to kill antibiotic resistant bacteria *in vitro*. Moreover recent *in vivo* studies have demonstrated BMAP-28 efficacy in reducing mortality in different infections [12, 13].

The efficacy of BMAP-28 was assessed on both central venous catheter (CVC) and urethral stent in two different studies. Some bacteria tend to develop biofilms on the surfaces of medical devices such as long-term silicone catheters, such as CVCs. Biofilms are structured microbial communities that occur as surface-attached communities or suspended aggregates which are able to start the production of an extracellular polysaccharide matrix. Antibiotic penetration within the biofilm is scarce and various components work together within a biofilm to reduce, or fully prevent, antibiotic effectiveness. In addition sessile bacteria living in a biofilm can exhibit a 10 to 1,000-fold increase in antibiotic resistance compared to planktonic bacteria.

Cirioni et al. described the potential of BMAP-28 pre-coating in the treatment of *S. aureus* central venous catheter-associated infections [14]. The authors demonstrated, using an *in vivo* murine model, that catheters pre-treated with BMAP-28 or high-dose antibiotics have a lower bacterial load compared to catheters with standard-dose antibiotics or without BMAP-28 (from 10^7 to 10^3 colony-forming unit (CFU)/mL and bacteremia from 10^3 to 10^1 CFU/mL). A further significant reduction in bacterial load (from 10^7 to 10^1 CFU/mL) was observed when catheters were impregnated with BMAP-28 and then treated with a higher dose of antibiotics. *In vitro* studies confirm these results [14].

The efficacy of BMAP-28 alone and in combination with Vancomycin was assessed in the treat-

Table 1 - Mammalian-derived AMPs, summary of main studies.

AMPs derived from MAMMALS	Characteristics of the study	Main findings
<i>BMAP-28</i>		
Cirioni O. et al [14]	In vivo murine model, <i>S. aureus</i> infection, Pre-treated catheters with BMAP-28 vs high dose antibiotics vs standard dose antibiotics	BMAP-28 reduced bacterial load from 10^7 (no treatment) to 10^3 CFU/mL and bacteremia from 10^3 to 10^1 CFU/mL
Orlando F. et al [15]	In vivo murine model, ureteral stent infection <i>E. faecalis/S. aureus</i> BMAP-28 alone \pm Vancomycin	BMAP-28 reduced bacterial load from 8×10^6 to 5×10^4 <i>S. aureus</i> , from 8.7×10^6 to 6.4×10^4 against <i>E. faecalis</i> Enhanced effect of Vancomycin (no bacterial count)
<i>IB-367</i>		
Ghiselli et al. [18]	In vivo murine model, <i>S. aureus</i> and <i>E. faecalis</i> CVC infections IB-367 pre-coating, using the antibiotic-lock technique with linezolid.	IB-367 pre-treatment of CVC enhanced linezolid activity, with a significant reduction in bacterial load from 10^6 to 10^1 CFU/mL and absence of bacteriemia
Cirioni et al. [19]	In vivo murine model, MRSA wound infection IB-367 pretreatment \pm Daptomycin/ Teicoplanin	IB-367 plus Daptomycin reduced bacterial load by 4-logs, with IB-367 plus Daptomycin the most efficacy bacterial load reduction of $2.7 \times 10^3 \pm 0.3 \times 10^3$ CFU/mL
Simonetti O. et al [20]	In vivo murine model, skin wound infected with <i>P. aeruginosa</i> or <i>E. coli</i> MDR, IB-367 topical \pm intraperitoneal Imipenem/Colistin.	IB-367 plus Colistin resulted in the greatest inhibition of both bacterial strains, suggesting a strong efficacy
<i>LL-37</i>		
Simonetti et al. [23]	In vivo murine model, MRSA-infected surgical wounds, LL-37 (intraperitoneal and topical) vs Teicoplanin,	Topical and parenteral LL-37 demonstrated superior wound closure, stimulating granulation tissue, angiogenesis and collagen deposition vs Teicoplanin group. The combination of intraperitoneal/topical LL-37 reduced the bacterial $6.9 \times 10^2 \pm 0.4 \times 10^2$ CFU/g more than Teicoplanin alone $7.4 \times 10^4 \pm 1.0 \times 10^4$ CFU/g.
Cirioni O. et al. [24]	In vivo neutropenic murine model, <i>P. aeruginosa</i> septic shock Placebo, Imipenem, G-CSF, LL-37+G-CSF, and Imipenem+G-CSF	LL-37+ G-CSF the most effective, significantly reducing the apoptosis of neutrophils and preventing sepsis
Morrioni G. et al. [25]	in vivo mouse sepsis with <i>Escherichia coli</i> LL-37, polymyxin B, Imipenem, or piperacillin	LL-37 and polymyxin B resulted in a reduction of endotoxin and TNF- α No significant difference between LL-37 and polymyxin B considering antimicrobial and antiendotoxin activities
<i>Protegrin-1</i>		
Morrioni G. et al [29]	In vitro <i>Acinetobacter baumannii</i> strains from surgical infections	Minimum inhibitory concentration 2 mcg/ml and minimum bactericidal concentration 8 mcg/ml,

Notes: colony-forming unit = CFU; antimicrobial peptide =AMP; central venous catheter = CVC; methicillin resistant *Staphylococcus aureus* = MRSA; methicillin-susceptible and methicillin-resistant *Staphylococcus epidermidis* = MSSE and MRSE; multi-drug resistant = MDR; granulocyte cell stem factor = G-CSF; tazobactam-piperacillin = TZP; methicillin sodium-susceptible = MS; methicillin sodium-resistant = MR; RNAIII-inhibiting peptide = RIP; *S. epidermidis* glycopeptides-resistant intermediate = GISE; intraperitoneal = i.p.

ment of animal models of urethral stent infection induced by *Enterococcus faecalis* and *Staphylococcus aureus*. The study suggests that the BMAP-28-impregnated urethral stent has a lower rate of infection, in fact in vivo model revealed that BMAP-28 reduced bacterial load (from 8×10^6 to 5×10^4 against *S. aureus* and from 8.7×10^6 to 6.4×10^4 against *E. faecalis*) and enhanced the effect of Vancomycin (no bacterial count); and in vitro studies support these results [15].

Finally, BMAP-28 showed to reduce nitric oxide (NO) production and tumor necrosis factor (TNF)- α release. In murine models, similar lethality to antibiotics as Clarithromycin and Imipenem against *Staphylococcus aureus* was reported. Furthermore, it was superior to neutralize bacterial products of Gram-positives, improving severe staphylococcal infections when used in combination with other antibiotics [16].

IB-367

IB-367, known also as Isegran, is a synthetic pro-grin, cysteine-rich AMP, with bactericidal and fungicidal activity, isolated from porcine leukocytes [17]. Ghiselli et al. assessed the efficacy of IB-367 pre-coating in the treatment of CVC infections using the antibiotic-lock technique with linezolid in murine model. The study showed that IB-367 pre-treatment of CVC enhanced linezolid activity against *S. aureus* and *E. faecalis*, causing a significant reduction in bacterial load on CVC (from 10^6 to 10^1 CFU/mL) and absence of bacteremia [18].

Cirioni and colleagues investigated the activity of IB-367, exploring whether IB-367 pre-treatment could improve the efficacy Daptomycin and Teicoplanin against methicillin resistant *Staphylococcus aureus* (MRSA) wound infection in a mouse model. The study's primary outcomes were quantitative bacterial culture and analysis of natural killer (NK) cytotoxicity and leukocyte phenotype. While antibiotics alone showed comparable antimicrobial efficacy, their combination with IB-367 significantly enhanced their activity. IB-367 plus Daptomycin reduced bacterial load by 4-logs, demonstrating the most efficacy (bacterial load reduction of $2.7 \times 10^3 \pm 0.3 \times 10^3$ CFU/mL) [19].

In conclusion, IB-367 could be considered an interesting association with conventional antibiotics for the treatment of CVC and other medical devices and for the treatment of MRSA wound infection.

Considering Gram-negative bacteria, topical IB-367 showed interesting activity against multi-drug resistant (MDR) *P. aeruginosa* or *E. coli* [20]. In a mouse model of skin wound infection, IB-367 was administered topically alone and associated with intra-peritoneal Imipenem/Colistin. IB-367 plus Colistin resulted in the greatest inhibition of both bacterial strains, suggesting a strong efficacy [20]. In conclusion, IB-367 may be an excellent candidate for topical therapy of Gram-negative infected wounds in the future.

LL-37

LL-37 is a human antimicrobial peptide categorized as a cathelicidin, showing a broad-spectrum activity against a variety of pathogens, including Gram-positive and Gram-negative bacteria, viruses, and fungi [21]. Additionally, LL-37 demonstrated other biological activities, such as the regulation of inflammation responses and an important role in wound closure and angiogenesis [22]. Simonetti et al. compared the efficacy of LL-37 with that of Teicoplanin in MRSA-infected surgical wounds in mice. LL-37 had a greater effect than Teicoplanin on wound healing processes in MRSA-infected mice, although it had a lesser effect on bacterial culture growth. The administration of LL-37 alone, intra-peritoneally, reduced the bacterial count to $7.1 \times 10^5 \pm 0.6 \times 10^5$ CFU/g. The combination of intra-peritoneal and topical LL-37 further reduced the bacterial count to $6.9 \times 10^2 \pm 0.4 \times 10^2$ CFU/g. In comparison, Teicoplanin, when administered intra-peritoneally alone, produced a bacterial count of $7.4 \times 10^4 \pm 1.0 \times 10^4$ CFU/g. Furthermore, the administration of LL-37, both topically and parenterally, demonstrated superior wound closure by stimulating the formation of granulation tissue and promoting angiogenesis and collagen deposition, resulting in better organized epithelial reconstitution compared to the Teicoplanin group [23].

Considering Gram-negatives, LL-37 may be useful against *P. aeruginosa*. In a neutropenic murine model with *P. aeruginosa* septic shock, all therapy groups (Imipenem, granulocyte cell stem factor (G-CSF), LL-37+G-CSF, and Imipenem+G-CSF) were superior to placebo. LL-37+ G-CSF association was the most effective by significantly reducing the apoptosis of neutrophils and preventing sepsis [24]. Similar results were reported in mouse sepsis with *Escherichia coli* [25]. All treat-

ments (LL-37, polymyxin B, Imipenem, or piperacillin) reduced the death of mice, but exclusively LL-37 and polymyxin B resulted in a reduction of endotoxin and TNF- α plasma levels. Moreover, there was no significant difference between LL-37 and polymyxin B considering antimicrobial and antiendotoxin activities. In the light of its anti-inflammatory and immunomodulatory effect, LL-37 emerges as a candidate for the treatment of Gram-negative sepsis. Finally, LL-37 showed a good action against biofilm formation of *Escherichia coli* when combined with Colistin [26]. Similarly, another cathelicidin with activity against *P. aeruginosa* MDR is tritripticin, showing a complete inhibition of the procoagulant activity of lipopolysaccharides [27].

Protegrin-1

Protegrin-1 is a 18-amino-acid beta-hairpin AMP that belongs to the cathelicidin family. It showed a strong bactericidal action and synergy with Colistin as well in a murine model. On the other hand, no effect on biofilm was reported. Furthermore, resistance to PG-1 was not detected. For this reason, protegrin-1 may be an interesting future option for the treatment of Gram-negative MDR infections [28]. In particular, *Acinetobacter baumannii* represents a Gram-negative associated with multiple antibiotic resistance. The minimum inhibitory concentration and minimum bactericidal concentration of protegrin-1 were 2 mcg/ml and 8 mcg/ml, respectively, considering in vitro cultures of *A. baumannii* MDR [29].

■ AMPS DERIVED FROM AMPHIBIANS (Table 2)

Amphibians are a very important source of AMPs. In particular, frogs are the amphibians from which the most AMPs are derived. The skin secretion of frogs of the family Pipidae, including *Silurana*, *Xenopus*, *Hymenochirus*, and *Pseudhymenochirus*, is very rich in peptides that help to protect against infection [30]. In our research experience, amphibian-derived AMPs are in fact the most numerous and most studied.

Citropin 1.1

Citropin 1.1 is the major AMP produced by the green tree frog, *Litoria citropa*. It is a wide-spectrum amphibian antimicrobial peptide [31]. Cirioni et al. assessed the efficacy of Citropin 1.1 in

combination with minocycline and Rifampicin, in the treatment and prevention of *S. aureus* CVC-associated infection using the antibiotic-lock technique. In vitro studies show that biofilms were strongly affected by the presence of Citropin 1.1. Furthermore, this study demonstrated that Citropin 1.1 acts synergistically with both Rifampin and minocycline. In fact Citropin 1.1 acts synergistically with hydrophobic antibiotics, probably inducing a damage of the membrane allowing a maximal entry of the hydrophobic substrates [32]. Finally, Citropin 1.1 showed its efficacy against staphylococci and streptococci with a concentration ranging from 1 to 16 mg/L. When Citropin 1.1 was associated with antibiotics, such as Clarithromycin and doxyxline, synergy was reported [33, 34].

Regarding Gram-negative bacteria, in mice with *E. coli* sepsis Citropin 1.1 was administered alone or associated with tazobactam-piperacillin (TZP). Although all the treatment groups reduced the lethality compared with controls, cytropin 1.1 alone significantly reduced plasma endotoxins and inflammatory cytokines, while TZP exerted an opposite effect. The association of cytropin 1.1 and TZP was the best in reducing lethality, bacterial growth, and plasmatic oxidative stress parameters. Therefore, citropin 1.1 has not only antimicrobial but also immunomodulatory activity and may be an interesting option in case of severe Gram-negative infection [35]. The role of this molecule could be interesting in case of infections in patients with increased oxidative stress also due to other concomitant pathologies, such as psoriasis [36, 37].

Uperine 3.6

Uperine 3.6, a broad-spectrum AMP, was isolated from the amphibian *Uperoleia mjobergii*. Only 17 amino acids are included in its structure, and so it represents the smallest antibiotic peptide isolated from amphibians. Although most of the antibiotics tested were more effective than uperine 3.6, it was effective against both susceptible and multiresistant germs [38].

Temporin A

Temporin A is a peptide amide with an antimicrobial activity against a wide spectrum of microorganisms, including antibiotic-resistant Gram-positive cocci, found in the skin of the European red

frog, *Rana temporaria* [39]. The mechanism of action of Temporin A is still unclear, however there are different hypotheses: Temporin A could act inserting into the hydrophobic core of the cell membrane, troughs interaction with bacterial phospholipids, altering enzyme activities, binding to DNA [40].

Different studies investigated the role of Temporin A in the treatment of medical devices infections (vascular graft, polystyrene surfaces and central venous catheters) and of infected wounds.

Ghiselli et al. evaluated the efficacy of Temporin A as a prophylactic agent in a rat model of vascular graft infection from methicillin sodium-susceptible (MS) and methicillin sodium-resistant (MR) *Staphylococcus epidermidis*. According to in vitro studies both MR and MS were similarly susceptible to Temporin A. In vivo studies showed that the use of a Temporin A-soaked Dacron graft in vascular surgery can result in a consistent bacterial load reduction. The most effective treatment was the association of a Temporin A-soaked graft and intraperitoneal Vancomycin hydrochloride, which inhibited bacterial growth for both the methicillin-resistant (MR) and methicillin-sensitive (MS) strains [39].

Cirioni et al. tested the efficacy of topical Temporin A and RNAlII-inhibiting peptide (RIP) compared to Rifampicin in preventing *S. epidermidis* and *S. aureus* graft infection in a subcutaneous rat pouch model [41].

RIP is a heptapeptide isolated from culture supernatants of *S. xylois* and has a strong activity against *S. aureus* and *S. epidermidis*. RIP inhibits cell-cell communication, also known as quorum sensing, preventing bacterial adhesion and virulence [42]. This study showed that the use of Temporin A-soaked and RIP-soaked Dacron grafts induced a significant bacterial growth inhibition. The combination of RIP and Temporin A showed the lowest bacterial growth (negative quantitative cultures for Vancomycin intermediate *S. epidermidis* (VISE)4 and from 6×10^7 to 6.9×10^1 CFU/mL for VISA4). More specifically, Temporin A had a high antistaphylococcal activity, independent of the level of resistance shown by the isolates. RIP was more effective against staphylococcal strains when used alone than Temporin A or Rifampicin alone. In conclusion these molecules appear potentially useful for antimicrobial chemoprophylaxis in vascular surgery [41].

Another study investigated the ability of Tem-

Table 2 - AMPs derived from amphibians, summary of main studies.

AMPs derived from amphibians		
<i>Citropin 1.1</i>		
Cirioni et al. [32]	In vivo murine model, <i>S. aureus</i> CVC-associated infection (antibiotic-lock) Citropin 1.1 in combination with minocycline/Rifampicin	Citropin 1.1 acts synergistically with both Rifampin and minocycline
Giacometti A. et al. [35]	In vivo murine model with <i>E. coli</i> sepsis Citropin 1.1 alone or associated TZP.	cytropin 1.1 alone significantly reduced plasma endotoxins and inflammatory cytokine The association cytropin 1.1/TZP the best in reducing lethality, bacterial growth, and plasmatic oxidative stress parameters
<i>Temporin A</i>		
Ghiselli et al. [39]	In vivo murine model, vascular graft infection from MS and MR <i>S. epidermidis</i>	Temporin A-soaked Dacron graft result in a consistent bacterial load reduction The most effective treatment was the association of Temporin A-soaked graft + intraperitoneal Vancomycin hydrochloride
Cirioni et al. 41]	In vivo murine model, <i>S. epidermidis</i> and <i>S. aureus</i> graft infection Topical Temporin A and RIP vs Rifampicin	Temporin A-soaked and RIP-soaked Dacron grafts induced a significant bacterial growth inhibition RIP + Temporin A showed the lowest bacterial growth

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Giacometti A. et al [43]	In vivo murine model, vascular graft infection GISE. Temporin A soaking and intraperitoneal linezolid	Temporin A/linezolid both showed high activity against GISE No clinically significant side effects
Cirioni O. et al [28]	In vitro model, MRSA biofilm on polystyrene surfaces (PSS) and (CVC) Temporin A, Citropin 1.1, CA(1-7)M(2-9)NH ₂ , and Pal-KGK-NH ₂	Combination of AMPs showed strong activity in inhibiting biofilm formation The eradication of preformed biofilms was more difficult and took 24 hours
Simonetti et al. [45]	in vivo murine models, MRSA-infected surgical wounds. Allevyn, Temporin A-soaked Allevyn, Allevyn with i.p. Teicoplanin, and Temporin A-soaked Allevyn with i.p. Teicoplanin	Temporin A+Allevyn reduced bacterial load 1.7×10 ³ CFU/ml. Temporin A-soaked Allevyn and i.p. Teicoplanin demonstrated the highest inhibition of bacterial load (0.85×10 ¹ ± 0.1×10 ¹ CFU/mL). Temporin A-soaked Allevyn (±Teicoplanin) had higher levels of collagen deposition and granulation tissue formation Teicoplanin
<i>Buforin II and ranalexin</i>		
Cirioni et al. [47]	In vivo murine model, vascular graft infected by glycopeptide-resistant intermediate <i>S. epidermidis</i> buforin II, ranalexin, Vancomycin, and Teicoplanin	buforin II and ranalexin when bound to the Dacron graft had a stronger activity than Vancomycin and Teicoplanin. Only buforin II was able to inhibit bacterial growth completely
Giacometti et al. [48]	In vivo murine model, vascular graft infected by MSSE and MRSE buforin II, ranalexin, Cefazolin and Rifampin	a strong in vivo antibacterial efficacy buforin II + ranalexin-coated grafts with Cefazolin showed higher activity against MRSE than Rifampicin-coated grafts and Cefazolin
<i>Distinctin</i>		
Giacometti et al. [50]	In vivo murine model <i>S. aureus</i> CVC-associated infection Distinctin, glycopeptides and betalactams	Distinctin pretreated CVC + antibiotics showed biofilm bacterial load further reduced from 10 ⁶ to 10 ¹ CFU/mL, no evidence of bacteriemia
Cirioni O. et al [51]	in vivo murine neutropenic model infected with <i>S. aureus</i> Distinctin and other antibiotics	Distinctin + Vancomycin and Teicoplanin produced the lowest lethality
Simonetti et al. [52]	In vivo model cutaneous MRSA wound infections topical Distinctin, topical Teicoplanin, i.p. Teicoplanin; topical Teicoplanin and i.p. Teicoplanin; topical Distinctin and i.p. Teicoplanin	Topical Distinctin + i.p. Teicoplanin inhibited bacterial growth to 4.7×10 ¹ ± 1.6×10 ¹ (levels comparable of uninfected animals), Wounded areas of Distinctin-treated animals showed more mature granulation tissue, a more organized and denser type of connective tissue, and a significant reduction in fibrinous exudation
<i>Magainin II</i>		
Cirioni O. et al [55]	In vivo murine model, glycopeptides intermediate <i>S. aureus</i> sepsis	Magainin II/Vancomycin shows a reduction of lethality
Cirioni O. et al [56]	In vivo murine model infected with <i>P. aeruginosa</i> MDR Magainin II, cecropin, Rifampin	Magainin II significantly reduced plasma endotoxin and TNF-alpha vs Rifampicin magainin II and cecropin combined reduced mortality

Notes: colony-forming unit = CFU; antimicrobial peptide =AMP; central venous catheter = CVC; methicillin resistant *Staphylococcus aureus* = MRSA; methicillin-susceptible and methicillin-resistant *Staphylococcus epidermidis* = MSSE and MRSE; multi-drug resistant = MDR; granulocyte cell stem factor = G-CSF; tazobactam-piperacillin = TZP; methicillin sodium-susceptible = MS; methicillin sodium-resistant = MR; RNAIII-inhibiting peptide = RIP; *S. epidermidis* glycopeptides-resistant intermediate = GISE; intraperitoneal = i.p.

porin A soaking and intraperitoneal linezolid to prevents vascular graft infection in a rat model with *S. epidermidis* glycopeptides-resistant intermediate (GISE). Temporin A and linezolid both showed high activity against the GISE clinical strain without clinically significant side effects, representing a valid opportunity of prophylaxis in vascular surgery [43].

Temporin A was also studied in combination with Citropin 1.1, CA(1-7)M(2-9)NH₂, and Pal-KGK-NH₂ for their synergistic activity against MRSA biofilms developed on polystyrene surfaces (PSS) and central venous catheters (CVC). Combination of AMPs showed strong activity in inhibiting biofilm formation on PSS and even on CVC where is common the formation of MRSA biofilm. The eradication of preformed biofilms, on the other hand, was more difficult and took 24 hours [28]. In addition, Temporin A inhibited the production of NO, TNF- α , and IL-6 by macrophages in mouse models, and was effective against antibiotic-resistant staphylococci and streptococci. In particular, it demonstrated particularly high efficacy against *S. aureus* 6 hours after injection. The most effective antibiotic used in combination was Imipenem (lethality rates of 25% Temporin A, 20% Imipenem, 10% Temporin A+Imipenem). Temporin A is able to facilitate the passage of Imipenem through the bacterial membrane by destructuring it while both go on to act on peptidoglycan [44].

Simonetti and colleagues conducted a study to examine the impact of using Temporin A topically in murine models with MRSA-infected surgical wounds. The mice were divided into several groups: those treated with drug-free Allevyn, Temporin A-soaked Allevyn, drug-free Allevyn with daily intraperitoneal Teicoplanin, and Temporin A-soaked Allevyn with daily intraperitoneal Teicoplanin. Results indicated that the combination of Temporin A and Allevyn reduced bacterial load to 1.7×10^3 CFU/ml. However, the group that received Temporin A-soaked Allevyn and i.p. Teicoplanin demonstrated the highest inhibition of bacterial load ($0.85 \times 10^1 \pm 0.1 \times 10^1$ CFU/mL). Furthermore, histological examination revealed that infected mice treated with Temporin A-soaked Allevyn (\pm Teicoplanin) had higher levels of collagen deposition and granulation tissue formation compared to the other groups. There was also a significant increase in serum VEGF expression

observed in mice receiving Temporin A topically with or without intraperitoneal Teicoplanin [45].

Buforin II and ranalexin

Amphibian tissues are the source from which Buforin II and Ranalexin are derived. They are polycationic peptides with antimicrobial activity on the cytoplasmic membrane. Buforin II was derived from Buforin I, a peptide from the stomach of a toad (*Bufo bufo gargarizans*), while Ranalexin was derived from the skin of a bullfrog (*Rana catesbeiana*) [46].

These two peptides were evaluated by Cirioni et al. to prevent infection of the vascular prosthetic graft by glycopeptide-resistant intermediate *Staphylococcus epidermidis*.

In vivo studies demonstrated that Buforin II and Ranalexin when bound to the Dacron graft had a stronger activity (from 4.9×10^6 to 1.9×10^2 CFU/mL) than Vancomycin (from 4.9×10^6 to 6.2×10^3 CFU/mL) and Teicoplanin (from 4.9×10^6 to 5.1×10^4 CFU/mL). In particular, only buforin II was able to inhibit bacterial growth completely [47].

Giacometti et al. investigated the efficacies of the same polycationic peptides comparing their activity to that of Rifampicin in the prevention of methicillin-susceptible and methicillin-resistant *Staphylococcus epidermidis* vascular prosthetic graft infections. This study found that these peptides have a strong in vivo antibacterial efficacy, in fact their polycationic activities against *Staphylococcus epidermidis* were not significantly different from that of Rifampin. The combinations of buforin II and ranalexin-coated grafts with Cefazolin showed higher activity against the methicillin-resistant strain (no infection detected) than that of the combination of Rifampicin-coated grafts and Cefazolin [48].

Polycationic peptides appear potentially useful for future topical antimicrobial treatments, such as peri and preoperative chemoprophylaxis in prosthetic surgery.

Distinctin

Distinctin is an AMP derived from *Phyllomedusa distincta* with a heterodimeric structure consisting of two different polypeptide chains linked by a disulfide bond. It has a lytic activity on unilamellar vesicles, suggesting their possible action on bacterial membranes [49].

Giacometti et al. assessed the efficacy of Distinctin in the treatment of *Staphylococcus aureus* CVC-as-

sociated infection. The study evaluated the ability of Distinctin in inhibiting the attachment of *S. aureus* to CVCs and in increasing its susceptibility to glycopeptides and betalactams once it is adherent. The *in vitro* study showed a strong activity of Distinctin on the biofilm and the ability to enhance the efficacy of antibiotics when used in combination. When antibiotics were used in Distinctin pretreated CVC, biofilm bacterial load was further reduced from 10^6 to 10^1 CFU/mL with no evidence of bacteremia [50].

Furthermore, in neutropenic mice infected with *Staphylococcus aureus*, this molecule demonstrated efficacy when administered intravenously, alone or in combination with other antibiotics. Specifically, the combination with Vancomycin and Teicoplanin produced the lowest lethality rate in this murine model [51]. Distinctin displays potential as an adjunctive agent to antibiotics in the treatment of CVC-related infections.

Simonetti et al. studied the efficacy of Distinctin in the management of cutaneous MRSA wound infections. Mice were treated with topical Distinctin, topical Teicoplanin, intraperitoneal Teicoplanin; topical Teicoplanin and daily intraperitoneal Teicoplanin; topical Distinctin and daily intraperitoneal Teicoplanin. Topical Distinctin combined with *i.p.* Teicoplanin inhibited bacterial growth to $4.7 \times 10^1 \pm 1.6 \times 10^1$ (levels comparable with those observed in uninfected animals), but the combination of topical and *i.p.* Teicoplanin proved to be the most effective treatment in reducing bacterial counts. In addition, wounded areas of Distinctin-treated animals showed more mature granulation tissue, a more organized and denser type of connective tissue, and a significant reduction in fibrinous exudation [52].

Aurein 1.2

Aurein 1.2 is a 13 amino acid, amphipathic peptide. Its action against Gram-positive cocci occurs at concentrations between 1 and 16 mg/litre. Clarithromycin and minocycline have shown synergistic activity with aurein 1.2. In particular, the latter promotes the entry of hydrophobic substrates by increasing membrane permeability and altering membrane organisation [53].

Magainin II

Magainin II is an alpha-helical AMP with *in vitro* activity and *in vivo* efficacy against glycopeptides

intermediate *Staphylococcus aureus*. More specifically, the combination of Magainin II/Vancomycin shows a reduction of lethality in murine models with staphylococcal sepsis (death of 1/20 vs 6/20 Vancomycin vs 10/20 Magainin II). This peptide inserts itself into the cytoplasmic membrane, activating murine bacterial peptidoglycan hydrolases and so destroying the pathogen [54].

Magainin II was also effective against Gram-negative bacteria. In a mouse model infected with *P. aeruginosa* MDR, Magainin II significantly reduced plasma endotoxin and TNF-alpha levels compared to the control group and those treated with Rifampicin. In addition, Magainin II and cecropin A were combined in this study, showing a synergistic effect against *P. aeruginosa* MDR, reducing mortality [55].

■ AMPS DERIVED FROM INSECTS

Antimicrobial peptides are mainly synthesized in fat bodies and blood cells of insects, which is one of the main reasons for insects' strong adaptability to survival [56]. Cecropin, an alpha-helical antimicrobial peptide, is the most famous family of AMPs from insects, and it can be found in guppy silkworm, bees, *Drosophila*. Cecropin A shows activity against different inflammatory diseases and cancers. Furthermore, it demonstrated *in vitro* and *in vivo* efficacy against *Staphylococcus aureus* in comparison with Vancomycin and Magainin II in murine models [57].

Tachypleisin III

Tachypleisin III is a peptide that shows activity against *P. aeruginosa* MDR when associated *in vitro* with Colistin and beta lactams [58]. In a murine model with *P. aeruginosa* MDR sepsi tachypleisin III, Colistin, and Imipenem were evaluated [59]. Combination therapy of Tachypleisin III and Imipenem resulted in the highest significantly lower levels of bacteremia than groups with a single agent, or other combinations. Furthermore, the efficacy was evaluated considering plasma levels of lipopolysaccharide, tumour necrosis factor alpha, and interleukin-6, showing the best reduction in Tachypleisin III and Imipenem group. From this study we can highlight that combination therapy with AMPs and traditional antibiotics may be a new therapeutic option. Similarly, mice treated with Tachypleisin III in combination

with Piperacillin/tazobactam had a reduction of *P.aeruginosa* growth by 1000-fold compared to monotherapy [60].

Finally, Tachyplesin III and Clarithromycin were evaluated against *Escherichia coli* sepsis in a murine model. Tachyplesin III, administered 1 mg/kg intraperitoneally alone, obtained a greater reduction in bacterial growth and a lower level of endotoxin and TNF-alpha plasma concentration compared to the control and Clarithromycin alone. The association of Tachyplesin III and Clarithromycin was the most effective for all considered parameters [61].

■ SYNTHETIC ANTIMICROBIAL PEPTIDES

6.1 Pal-Lys-NH₂ and Pal-Lys-Lys

Pal-Lys-NH₂ and Pal-Lys-Lys are short bactericidal lipopeptides with a strong antimicrobial activity against a wide spectrum of Gram-positive cocci [62]. Lipopeptides are monomeric in solution while longer ones form oligomers and this feature can potentiate bacterial killing.

A study investigated their action alone or combined to Vancomycin in preventing prosthesis biofilm in a subcutaneous rat pouch model of staphylococcal vascular graft infection. The results of this study showed that Vancomycin (from 6.94 log to 3.65 log CFU/mL) and lipopeptides (from 6.94 log to 3.87 log CFU/mL for Pal-Lys-Lys NH₂ and from 6.94 log to 4.080 log CFU/mL for Pal-Lys-Lys) when used alone had similar significant bacterial growth inhibition. All combinations showed efficacies significantly higher than that of each single compound. The combination of Vancomycin with Pal-Lys-Lys-NH₂ had the strongest efficacy (from 6.94 log to 1 log CFU/mL). The in vitro study globally confirms the in vivo one [63].

Daptomycin

Daptomycin is a lipopeptide with a rapid bactericidal activity against staphylococci. The efficacy of this peptide was assessed in different studies to evaluate its action on biofilm, wound infections and enterococcal infections [64].

Cirioni et al. investigated the efficacy of Daptomycin and Rifampicin either alone or in combination in preventing vascular graft biofilm formation in a rat pouch model of Staphylococcal infection. The results of this study showed that when tested alone both Rifampicin and Daptomycin have

good efficacies without any toxicity and drug-related adverse effects.

Their combination showed higher efficacies than that of each single compound, this effect could be due to the Daptomycin mechanism of action, in fact, it binds and opens channels that can allow specific entry of Rifampicin [65].

In vivo efficacy of Daptomycin in the treatment of burn wound infections caused by MRSA was evaluated, in comparison to Teicoplanin, assessing the wound healing process through immunohistochemical and morphological analysis. The results showed greater antimicrobial activity of Daptomycin compared with Teicoplanin. In addition, there was better overall healing with significantly higher epithelialization and collagen scores than the other groups; these results were also confirmed by immunohistochemical data on EGFR and FGF-2 [66]. Moreover, in a previous study, Daptomycin showed synergy in its effect against MRSA when combined with other antibiotics such as tigecycline [67, 68].

Teicoplanin

Teicoplanin is a glycopeptide antibiotic structurally related to Vancomycin. It is produced from *Actinoplanes teichomyceticus*. Its spectrum of activity includes Gram positive bacteria [69].

Giacometti et al. investigated the efficacy of Levofloxacin, Cefazolin, and Teicoplanin in preventing vascular prosthetic graft infection induced by methicillin-susceptible and methicillin-resistant *Staphylococcus epidermidis*. The efficacy of Levofloxacin against the methicillin-susceptible strain did not differ from that of Cefazolin or Teicoplanin, but Levofloxacin (from 10⁶ to 10³ CFU/mL) showed slightly less efficacy than Teicoplanin (from 10⁶ to 10² CFU/mL) against the methicillin-resistant strain. The Levofloxacin-Rifampin combination proved to be similarly effective to the Rifampin-Teicoplanin combination and more effective than the Rifampin-Cefazolin combination against both strains. The most useful combination for the prevention of late-appearing vascular graft infections caused by *S. epidermidis* is Rifampicin-Levofloxacin (no infection detected), because it takes advantage of the good antistaphylococcal activity of both drugs [70].

Ghiselli et al. wanted to study the efficacy of Teicoplanin for the treatment of wound infection with *Staphylococcus aureus* in a mouse model,

comparing topical vs systemic treatment. Results showed a strong inhibition of bacterial growth in all groups treated with intraperitoneal Teicoplanin. However, the highest inhibition was obtained with Teicoplanin-soaked Allevyn and intraperitoneal Teicoplanin. Histologic examination showed that each treatment improved wound repair, but the best results were obtained with Teicoplanin-soaked Allevyn, associated with wound remodeling similar to that in uninfected mice, assessing microvessel density, VEGF expression, and granulation tissue formation [71]. VEGF is an endothelial cell-specific mitogen and an angiogenic inducer active in growth and development, in wound healing, and in various pathologic conditions [72-75].

Dalbavancin

Dalbavancin is a semi-synthetic lipoglycopeptide that exhibits activity against Gram-positive bacteria. It has been approved for treating acute bacterial skin and skin structure infections (ABSSSI) [76]. Simonetti et al. conducted a study to investigate the effect of Dalbavancin on wound healing compared to Vancomycin. They also determined the potential involvement of MMP-1, MMP-9, EGFR, and VEGF in Dalbavancin's therapeutic mechanisms. It was established a mouse model of MRSA skin infection, and mice were treated daily with Vancomycin or weekly with Dalbavancin on days 1 and 8. Both drugs effectively reduced the bacterial load ($8.71 \times 10^5 \pm 9.02 \times 10^5$ CFU/mL for Dalbavancin vs. $8.04 \times 10^6 \pm 7.96 \times 10^6$ CFU/mL for Vancomycin). Wounds treated with Dalbavancin exhibited well-organized granulation tissue with several blood vessels, though slightly fewer than those in the uninfected group. Immunohistochemical staining showed elevated EGFR and VEGF expression in both treated groups (more pronounced in Dalbavancin-treated mice), decreased MMP-1 and MMP-9 levels in uninfected tissue, and in both treated tissues compared to untreated infected wounds [77].

Laur-CKK-NH2

Laur-CKK-NH2 is a new compound synthesized by conjunction of lauric acid with the amino acids cysteine-lysine-lysine.

An experimental study assessed both the in vitro and in vivo interaction between the Laur-CKK-NH2 lipopeptide and Daptomycin using two *En-*

terococcus faecalis strains with different patterns of susceptibilities. The in vitro studies demonstrated that the Laur-CKK-NH2 dimer prevents the emergence of Daptomycin resistance. The in vivo study showed the presence of a synergistic effect between the Laur-CKK-NH2 dimer and Daptomycin exhibiting the highest efficacy in measuring lethality and bacteremia. In conclusion this AMP could be a potential therapeutic option for severe enterococcal infections [78].

Pexiganan

Pexiganan is a 22-amino acid synthetic lysine-rich peptide, derived from magainin. In a mouse model with *A. baumannii* sepsis, it was reported effective. Considering the bacterial count, Pexiganan plus Colistin association resulted in the best reduction, with 90% of survival rate [79]. For this reason, Pexiganan may also be a future option to overcome MDR Gram-negative. Furthermore, Pexiganan showed synergic activity with tigecycline against *P. aeruginosa* in a mouse model, revealing a possible antibiotic that would not normally be effective against Gram-negatives. In fact, *Pseudomonas aeruginosa* is not reliably inhibited by tigecycline [80]. Considering *P. aeruginosa* urethral stents infections in a murine model, Pexiganan and Imipenem at sub-MIC concentration resulted in a marked reduction of adhesion and biofilm expression compared to untreated controls. The average reduction was of 34+8% and 27+4%, respectively [81].

AMPs derived from microorganisms

Some famous peptides obtained from microorganisms are nisin and Gramicidin from *Lactococcus lactis*, *Bacillus subtilis*, and *Bacillus brevis* [82].

Interestingly, there are some neoplastic conditions, in particular cutaneous lymphomas, in which it appears to be a dysregulation of the production of cutaneous AMPs. In these patients the risk of developing infections, even lethal, is higher [83-85]. However, some AMPs such as nisin showed an immunomodulatory action, enhancing the cytotoxic and apoptotic action of Rituximab in Burkitt's lymphoma [86].

Due to the high price of chemical synthesis of AMPs, the biological expression has attracted the increase of attention. Specific yeast species like *Pichia pastoris*, *Saccharomyces cerevisiae*, and bacteria like *Escherichia coli*, *B. subtilis*, and plants

have been used for expression systems [87], but it should be noticed that because of the toxicity, proteolytic degradation, and purification, AMPs are difficult to be produced in *E. coli*, which is necessary to take advantage of fusion tags [88].

Colistin

Colistin, derived from *Bacillus Colistinus*, allows the permeabilization of the *A. baumannii* outer membrane, facilitating the action of large size molecule as glycopeptide and lipopeptide, and achieving an improvement of the patients with *A. baumannii* MDR severe infections [89]. In fact, the bacterial count was $6.7 \times 10^4 \pm 1.1 \times 10^4$ Colistin alone, $5.0 \times 10^9 \pm 1.6 \times 10^9$ Daptomycin alone, $7.3 \times 10^9 \pm 1.8 \times 10^9$ Teicoplanin alone, $2.9 \times 10^2 \pm 0.4 \times 10^2$ Colistin + Daptomycin, and $3.1 \times 10^2 \pm 0.2 \times 10^2$ Colistin + Teicoplanin, respectively [90]. Furthermore, the association with Colistin and Pexiganan was more effective against Gram negative bacterial infection than single treatment [91]. This is an example of how an antibiotic not normally effective against Gram-negative bacteria can become effective with the help of an antimicrobial peptide [92].

■ CONCLUSIONS

In this narrative review of our research experience, as already anticipated in the study, we decided to focus more specifically on the sources of AMPs, with the aim of assessing which might be the best sources from which to draw new AMPs [93]. In conclusion, our research experience shows that the richest source of AMPs are amphibians. However, the studies done are mainly in vitro or in animal models, making further human studies necessary to assess the efficacy and safety of these molecules. A further limitation is the cost of some AMPs, although the possibility of selecting some extremely promising ones could encourage the search for manufacturing solutions to reduce their cost and make them more easily available.

However, AMPs may be a new therapeutic option for infections sustained by multi-resistant micro-organisms and for overcoming the mechanisms of resistance to antibiotics currently used. In particular, combining AMPs with antibiotics, including those with limited antimicrobial activity due to antimicrobial resistance, has often shown a synergistic effect, increasing or restoring

their efficacy. The possibility of using manageable and relatively safe antibiotics again is crucial, considering the widespread increase in bacterial resistance in hospitals and the community.

In the literature there are cases of AMP used on humans [94-98]. Specifically, with regard to Gram-negative infections, the most promising molecules are IB-367 and LL-37, showing a synergistic action with traditional antibiotic therapy. Regarding IB-367 (Isegran), a phase I clinical study on prophylaxis of oral mucositis post cytotoxic therapy showed that oral topical IB-367 was not absorbed and was well tolerated. As early as one hour after administration of 9 mg of IB-367, the density of Gram-positive flora was reduced 1000-fold, and Gram-negative bacteria had also been significantly reduced [94]. Some phase III studies have also been conducted. In particular, Isegran was studied in patients receiving stomatotoxic chemotherapy, underlining that topical Isegran HCl significantly reduces the total oral aerobic bacterial, streptococcal, and yeast load [95]. In addition, Elad et al. conducted a multicentre, double-blind versus placebo study on 2025 patients undergoing chemotherapy, mainly myeloablative. Isegran HCl 9 mg/3 ml was used topically 6 times daily for mucosal prevention. After one month, there was a statistically significant reduction in bacterial load and an unchanged MIC, confirming its efficacy in this type of patient [96]. Specific studies on MDR microorganisms are currently lacking, but the data reported on humans are promising. Furthermore, LL-37 showed in humans its action on wound marginalization of venous leg ulcers, proving to be safe and thus making it an excellent candidate for further studies [97-99].

Despite a plethora of research on AMPs and their application as potential treatment on infectious diseases, this area needs further exploration. There is evidence that the characteristics of AMPs can seriously improve through structural chemical modifications and different delivery systems to become alternatives drugs to conventional antibiotics.

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The authors have no competing interests to declare.

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