New Antimicrobial Frontiers

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Abstract: New antimicrobials able to counteract bacterial resistance are needed to maintain the control of infectious diseases. The last 40 years have seen the systematic tailoring and refinement of previously identified antibiotics, to produce a multitude of semi-synthetic derivatives that share their mechanism of action with the original molecules. The major limit of this approach is the emergence of multi- and cross-resistant bacterial strains, favoured by the selective pressure inherent to the targeting of specific enzymes. The most promising new strategies aim to the development of molecules that, targeting essential bacterial structures instead of specific enzymatic activities, achieve infection control without enforcing a selective pressure on bacteria. This review, based on the consultation of the up-to-date literature, deals with antimicrobial peptides and some antivirulence factors.

Keywords: Antimicrobial peptides, antivirulence factors, bacterial targets, inhibitors of adhesion, inhibitors of colonization, phage therapy, toxin inhibitors.

INTRODUCTION

Multidrug-resistant bacteria are emerging as a major public health threat. The decreasing efficacy of antibiotic-based antibacterial therapy is remarked by the recent reckoning that in US hospitals more people die of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) infections than of AIDS and tuberculosis combined [1]. Other multi- or pan-drug-resistant bacteria belong to the species *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Clostridium difficile* and *Mycobacterium tuberculosis* [2]. It has been estimated that in European Union, Iceland and Norway in 2007 approximately 25000 patients died from bloodstream infections due to MRSA, VRE, penicillin-resistant *Streptococcus pneumoniae, E. coli* and *K. pneumoniae* resistant to third-generation cephalosporins and carbapenems, and *P. aeruginosa* resistant to carbapenems [3]. Bacterial resistance complicates the management of infections in more vulnerable individuals, such as organ transplant and AIDS patients, haemodialysis patients and those with various types of cancer. The rise of bacterial resistance is prompted by the selective pressure inherent to the mechanism of action of classic antibiotics, that usually target a specific bacterial enzyme, and by their wide-spread use for medical and non-medical purposes, such as veterinary and food-preservation use. The different mechanism of action of new antibacterial molecules, such as antimicrobial peptides (AMPs), should bypass these obstacles [4]. However, AMP development comes up against the cost and difficulty to identify, characterize and licence these new molecules. The strategies and the challenges inherent to the discovery of new antibacterials are subject to extensive discussion [5-7]. Whereas for approximately four decades (from the 1940s up to the 1970s) the pharmaceutical industry provided a steady flow of new molecules that by exploiting novel mechanisms of action circumvented the problem of resistance to earlier agents, in the last 40 years only few antibiotics with a novel mechanism of action have reached the market: synercid (quinupristin/dalfopristin), a streptogramin antibacterial agent for intravenous administration, active against VRE; linezolid, a oxazolidinone that inhibits protein synthesis, indicated for skin and soft tissues infections and hospital acquired pneumonia; daptomycin, a cyclic lipopeptide that depolarizes cytoplasmic membranes of Gram-positive bacteria, and retapamulin, a member of the new class of pleuromutilins, derived from the fungus *Clitopilus passeckerianus* [8]. Retapamulin inhibits protein synthesis by binding a region of the P-site and peptidyl transferase centre of the 50S ribosomal subunit that is different from the site targeted by macrolides, tetracyclines and aminoglycosides [9, 10]. Because of this distinct mechanism of action, target-specific cross-resistance with other antibiotics does not occur, but retapamulin use is limited to skin infections caused by *S. aureus* and *Streptococcus pyogenes*. Considering that the above mentioned molecules are active against Gram-positive cocci, and linezolid and daptomycin are the only ones belonging to new antibiotic classes, it is a fact that the great majority of antibiotics presently in use for systemic infections derives by synthetic tailoring from a limited number of dated molecular scaffolds [2]. The rapid emergence of multi- and cross-resistant bacterial strains makes the identification of new scaffolds the most suitable and promising approach. The research on AMPs, antivirulence factors and phage-related molecules can take advantage of the possibility to exploit a huge range of natural products, and is recognized as a very promising field [11-13].

This review deals with the new perspectives opened by recent developments in this fields, focusing on the innova-

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tive mechanisms of action of antibacterial molecules that differ from classic antibiotics.

ANTIMICROBIAL PEPTIDES

AMPs are small amphiphilic peptides highly conserved in a wide range of species that includes bacteria, fungi, plants, arthropods, fishes, mammals and non-mammalian vertebrates [14, 15]. In plants and insects, that lack the adaptive immune response, AMPs provide the main protective mechanism against infections [16]. In mammals, AMPs are present in neutrophils and in skin and mucosal surfaces, where they participate to the innate immune response against bacteria, fungi, parasites and viruses [17-19]. The antimicrobial activity may be direct and/or mediated by angiogenic, immunomodulatory or anti-inflammatory activity [20-22]. The concept of using AMPs as therapeutic tools was first introduced in the 1990s [23, 24], however none of them has yet reached the market, due to the still unsolved problems related to toxicity, short *in vivo* half life, limited tissue distribution and high production cost [25, 26]. The identification of molecular structures conferring antimicrobial activity is the first step to design synthetic mimics that could overcome these problems [27]. According to their electrical charge, AMPs can be divided into anionic and cationic peptides. Anionic AMPs (AAMPs), found in vertebrates, invertebrates and plants, are active against bacteria, fungi, virus, nematodes and insects. Their net negative charge ranges from -1 to -7, and their length from 5 to about 70 amino acid residues. Most AAMPs have an amphiphilic structure that facilitates their interaction with cell membranes, but the mechanism of their antimicrobial action so far has not been elucidated. In comparison with cationic AMPs (CAMPs), AAMPs have received relatively little attention in the literature. For an outline of AAMP characteristics, the interested reader is referred to the exhaustive review by Harris *et al*. [28]. CAMPs should be more appropriately defined "cationic host defence peptides", because some of them are not significantly microbicidal *in vivo*, but perform a strong antimicrobial activity by modulating the host immune response [29-31, 22]. Certain CAMPs also exhibit selective direct cytotoxic activity against different types of human cancer cells. A comprehensive overview on the anti-cancer activity of these compounds is that of Mader and Hoskin [32]. Typically, CAMPs are 12-50 amino acid long with a net positive charge of +2 to +9, due to an excess of basic arginine and lysine residues, and have approximately 50% hydrophobic amino acids [33]. Based on their molecular and conformational structure, CAMPs can be divided into four classes: cysteine-rich α-sheet structures with one or more disulphide bonds (defensins from humans); linear α-helical peptides without disulphide bonds (cecropins, magainins and dermaseptins); loop-structured peptides (microcins from *Enterobacteriaceae*), and extended tryptophan-rich peptides (cathelicidins, indolicidin) (Fig. **1**) [34]. Another AMP class includes peptidoglycan recognition proteins (PGRPs), that have been first identified in the silkworm and subsequently in insects, humans ad mice [35, 36], but are not currently being developed as antibacterial drugs. A broad review of the fifteen hundred AMPs identified over the last 20 years is beyond the scope of this review, that will focus on some of the best studied and most promising molecules. An updated AMP database is available on line at: http://aps.unmc.edu/AP/main.php [37].

The exact AMP mechanism of action has yet to be established but, according to the most accepted model, the initial phase is common and consists in an electrostatic interaction with the surface of the target cell. The difference between the anionic charge of bacterial membranes and the neutral charge of mammalian cell membranes explains the basic selectivity of action of CAMPs, that however varies among different molecules. Two kinds of subsequent mechanisms have been defined: the rapid disorganization of the cytoplasmic membrane, that takes seconds to minutes, and the binding to intracellular targets, that takes more time (3-5 hours). Several models, such as the toroidal pore and barrel stave models, which imply the formation of pores, or the carpet-like model, in which the cell membrane is disintegrated and/or micellized, have been proposed to explain the structures formed between membrane lipids and AMPs [38, 39]. None of them is receptor-based, consistent with the finding that D-peptides are generally as active as L-peptides [16]. Despite their variability, AMPs show a highly conserved amphiphilic topology, with the hydrophilic and hydrophobic side chains segregated into distinct opposing regions or faces of the molecule. This topology is essential for insertion into and disruption of bacterial cytoplasmic membranes, and numerous studies strongly support the view that physicochemical properties, rather than any precise amino acid sequence, are responsible for AMP activity [16]. Even non-peptidic compounds with amphiphilic structures, such as ceragenins, based on a lipidic scaffold, or polymers with phenylene ethynylene, polymethacrylate, β-lactam, or polynorbornene backbones, are active against Gram-positive and Gram-negative bacteria [40, 41]. These compounds are not currently developed for systemic therapeutic use, but their low cost, ease of production and non-toxicity for mammalian cells make them suitable for sterile clothing and biocompatible medical materials, such as catheters, sutures and indwelling devices [42].

Defensins

Defensins are structurally related peptides found in vertebrates, fungi, plants and insects. The presence of defensin-like peptides in the myxobacteria *Anaeromyxobacter dehalogenans* and *Stigmatella aurantiaca*, demonstrated by *in silico* analysis, suggests that eukaryotic defensins represent a mechanism conserved since early evolutionary steps [43]. Defensins work against bacteria, fungi, protozoa and enveloped viruses, and are generally thought to act by binding and disrupting microbial surface membranes. Some of them also have toxin-neutralizing and immunomodulatory properties [44]. Defensins are, with cathelicidins, the most documented AMPs in humans [45-47]. Human defensins are classified into α-defensins, produced by neutrophils, NK and Paneth cells, and β-defensins, produced by leukocytes and epithelial cells. Both α- and β-defensins have a similar triple-stranded β-fold structure, but differ in disulfide connectivity and peptide intervals between disulfide bonds [48].

Human α-defensin 1, 2, and 3 represent the most abundant bactericidal factors stored in the azurophilic granules of neutrophils, and can be considered the major components of

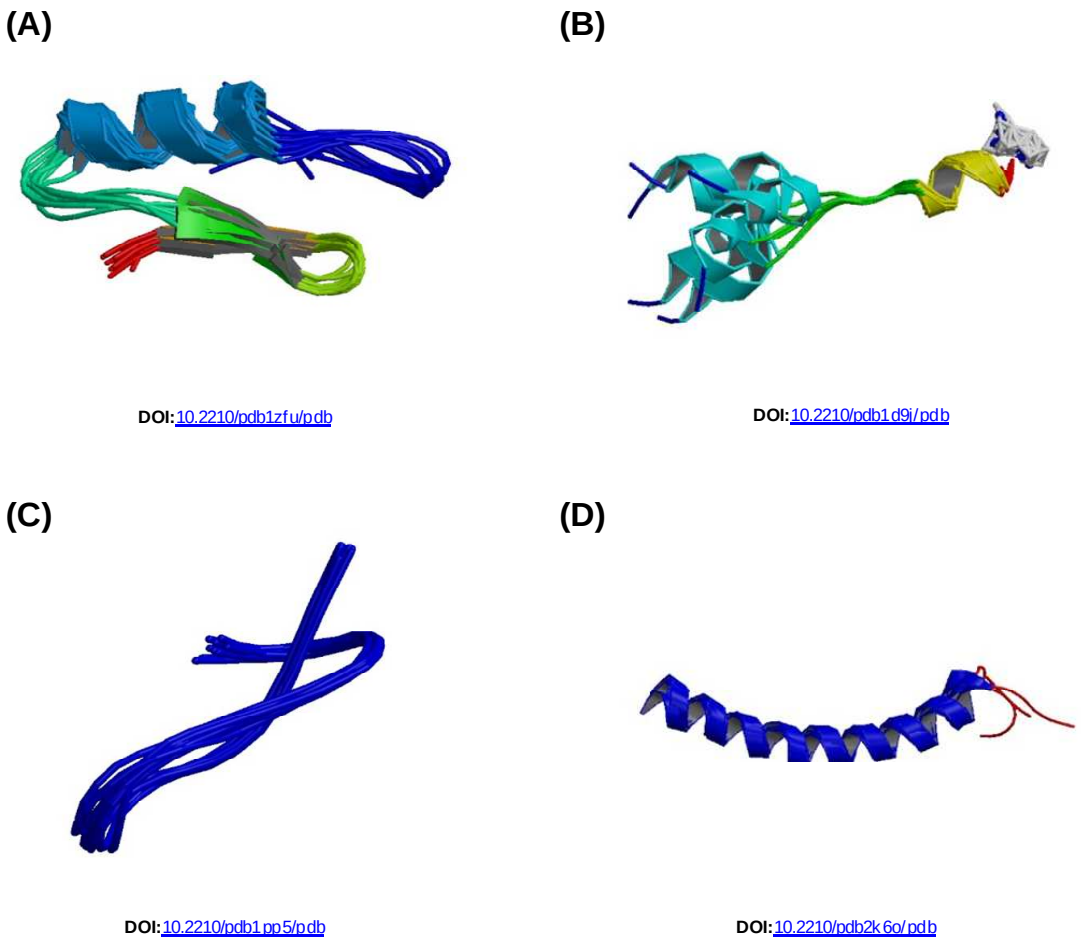

Fig. (1). Three-dimensional peptide representation of four CAMP structural classes, as determined by NMR spectroscopy. (**A**) Plectasin, a β-defensin [55]; (**B**) Cecropin A-magainin hybrid [171]; (**C**) Microcin J25 [172]; (**D**) Cathelicidin LL-37 [173]. RCSB Protein Data bank; http://www.rcsb.org [174].

an oxygen-independent mechanism involved in the intra- and extra-cellular killing of pathogens [49]. The development of these molecules for therapeutic use has been hindered by at least three kinds of problems: the difficulty of producing them at the scale and purity required for pharmaceutical products, their ability to stimulate the immune system, and their toxicity on mammalian cells [50-53]. Human α- and β-defensins are thought to contribute to maintain a stable commensal microbiota in the intestinal tract, preventing bacterial overgrowth [54]. Any alteration of their expression could be detrimental and shift the balance toward inflammation. In such a setting, the exogenous administration of natural human defensin could be problematic. To date, the most promising defensin is plectasin, a 40-amino acid residue peptide produced by the fungus *Pseudoplectania nigrella* [55]. This molecule shows good activity against a broad spectrum of Gram-positive bacteria, and low cytotoxicity on mammalian cells [55]. The selectivity is probably due to its recently clarified peculiar mechanism of action, that does not consist in the disruption of the cell membrane, but targets lipid II, a bacterial cell wall precursor [56]. Plectasin and one of its variants, the peptide NZ2114, are currently under development by Novozymes A/S as lead compounds to be used against vancomycin- and methicillin-resistant *S. aureus* [57].

A third class, that of θ-defensins, characterized by a cyclic backbone with a unique, ladder-like pattern of disulfide bonds, has been found in nonhuman primate leukocytes [48]. Rhesus θ-defensin protects mice from SARS coronavirus pulmonary infection [58]. Curiously, θ-defensin genes, normally expressed in nonhuman primates, are present in the human genome also, but as expressed pseudogenes. A premature termination codon in the signal peptide portion of human θ-defensin mRNA prevents its translation [48, 59]. However, the human θ-defensin genetic information has been used to produce retrocyclin (RC)-1, -2 and -3, that can be defined synthetic, humanized θ-defensin cyclic octadecapeptides. These molecules are active against HIV and herpes and influenza viruses, and neutralize anthrax toxin. RC can prevent the entry of HIV-1, but not of HIV-2 or SIV, into target cells by blocking the virus envelope-cell membrane fusion mediated by CXCR4 or CCR5 co-receptors. Following the observation that in eukaryotic cells aminoglycosides induce a low level of translational misreading, which suppresses the termination codon through the incorporation of an amino acid in its place, Venkataraman *et al*. utilized aminoglycosides to induce translational read-through of the θ-defensin pseudogene, which restored the expression of functional anti-HIV-1 retrocyclin peptides in human

cervicovaginal tissue models [59]. These authors suggest that the topical application of aminoglycosides to induce the production of endogenous retrocyclins by the vaginal mucosa might soon become an effective method to combat HIV-1 sexual transmission. In a screening test designed to assess the activity of four human α-defensins and six θ-defensins (RC-1-3 and rhesus θ-defensins 1-3), RC-2 resulted the most potent anti-HIV-1 factor, due to its exceptionally high affinity for gp120 and CD4 [60]. In other experiments RC-2, administered as an expression vector, protected both MDCK cells and chicken embryos from infection by the avian influenza H5N1 virus [61].

The issue of the still prohibitive RC production cost is being addressed by Lee *et al*. by the use of chloroplasts as bioreactors. These authors developed a technique based on the use of chloroplast transformation vectors that allows the production of RC-101, a non-haemolytic and minimally cytotoxic RC-1 analogue with good anti-HIV-1 activity, and of protegrin-1 (a 18-residue AMP discovered in porcine leukocytes, that showed potent antimicrobial activity against bacteria, fungi and yeasts) by tobacco chloroplasts [62]. According to these authors, the process allows the production of adequate quantities of purified peptides to be used in preclinical studies for topical protection against several sexually-transmitted diseases.

Cathelicidins

Cathelicidins comprise a large number of antimicrobial peptide precursors found in mammals, chickens and fishes. They are made of a heterogenic C-terminal antimicrobial domain of 12-100 residues linked to an evolutionary conserved N-terminal cathelin-like domain of 99-114 residues [63]. The C-terminal peptides express direct and/or indirect antimicrobial activity following their cleavage from the holoprotein [64]. The only member of the cathelicidin family identified in humans, also defined human cationic antimicrobial peptide-18 (hCAP18), has been isolated from neutrophils in 1995, and its expression has been successively observed in leukocytes, skin, mucous epithelia, wound and blister fluid, and in seminal plasma [64]. The coding gene is located on chromosome 3, and its expression is both constitutive (sweat gland cells) and inducible by vitamin D3, LPS and butyric acid (colonic epithelial cells) [47, 65]. Unlike neutrophil defensins, which are fully processed to mature peptides before storage in the azurophil granules, human cathelicidin is present as propeptide in the specific granules and is cleaved after secretion to generate the antimicrobial peptide LL-37, a cationic 37 a.a. AMP bearing tandem N-terminal leucine residues. There is evidence that within the same organism cathelicidins are processed by different proteases in different physiological contexts: in humans, the activation of neutrophil-derived hCAP18/LL-37 is carried out by the serine protease proteinase 3, whereas epididymal-derived hCAP18 in seminal plasma is cleaved by the prostate-derived protease gastricsin (pepsin C) in the presence of vaginal fluid at low pH [64]. LL-37 has a stable α-helical structure and can kill both Gram-positive and Gram-negative bacteria by cell membrane disruption. Moreover, it binds LPS with high affinity, inhibiting LPS-induced cellular responses, and prevents macrophage activation induced by lipoteichoic acid and lipoarabinomannan [66]. LL-37 can also inhibit mycobacteria and induce a Toll-like receptor-mediated killing of *M. tuberculosis* by monocytes [67]. It has been shown that LL-37 is expressed by human epithelial cells, alveolar and monocyte-derived macrophages and neutrophils following in *vitro* infection by *M. tuberculosis*, through stimulation of TLR-2, TLR-4 and TLR-9 [68]. However, native LL-37 is haemolytic and toxic to human leukocytes. *In vivo*, LL-37 cytotoxic effects are inhibited by its binding to plasma proteins, but the binding also lowers antimicrobial efficacy [69]. Considering LL-37 multifunctional activity, further investigation is needed to better define its biological properties and its possible therapeutic applications in the fields of immunomodulation and bacterial control [70]. At present, we can envisage that the future of cathelicidins relies on the ability to design synthetic variants to optimize antimicrobial efficacy and contemporarily limit harmful effects [71]. Significant achievements in this field could be not too far, considering that a synthetic 13-amino acid peptide, IDR-1, conceptually based on LL-37, with no direct antimicrobial activity, protects against bacterial infections *in vivo*, by inducing chemokine production and enhancing leukocyte recruitment [72]. An IDR-1 derivative, IDR-1002, showed stronger protective activity *in vitro* and in mouse models of infection with *S. aureus* and *E. coli* [73]. Other promising new molecules currently under investigation are novicidin, a linear cationic α-helical AMP derived from ovispirin, that is a cationic peptide originated from the ovine cathelicidin SMAP-29 [74], and a 34 amino acid residue cathelicidin derived from the king cobra (*Ophiophagus hannah*) that showed low cytotoxicity and good antibacterial activity against *E. coli*, *P. aeruginosa*, and *S. aureus* [75].

Cecropins

Cecropins are lytic peptides that possess antibacterial activity *in vitro*, originally isolated from the haemolymph of the giant silk moth *Hyalophora cecropia* [76]. The killing is mediated by membrane permeabilization, with a detergent-like effect accompanied by pore formation [77]. Cecropin specificity of action relies upon the differences in the composition and physicochemical properties of germ and host cell membranes. Pore formation is easily achieved in bacterial membranes rich in anionic phospholipids, but not in animal cell membranes, rich in neutral phospholipids and further stabilized by cholesterol. Cecropins are considered worth of further development because they show a well demonstrated biological activity and consist of a single polypeptide chain well suited for economical production through recombinant DNA technology or peptide synthesis [78]. Cecropin-like peptides are currently being developed following different strategies to improve antimicrobial and anticancer activity and diminish cytotoxicity [79, 80]. Based on the assumption that lysozyme is inactive on Gram-negative bacteria because it cannot reach the peptidoglycan layer, and that cecropin may disrupt the outer membrane of Gram-negatives, giving the enzyme access to peptidoglycan, a novel hybrid protein combining *Musca domestica* cecropin with human lysozyme has been expressed in *E. coli*. This chimeric protein showed an improvement of antibacterial activity and spectrum compared to its single original components [81]. Another chimera, the cecropin AD peptide, composed by the first 11 residues of *H. cecropia* cecropin A and

the last 26 residues of *H. cecropia* cecropin D, has been produced in a *Bacillus subtilis* expression system by Chen *et al.* [82]. The potent antimicrobial activity against *S. aureus* and *E. coli* of the recombinant product, and the low cost of the production process, with a yield of 30.6 mg of pure recombinant protein obtained from 1 litre of culture supernatant, make this molecule a suitable option for veterinary and medical applications. Cecropins have properties similar to those of melittin, a peptide that is the major component of the *Apis mellifera* venom [38]. Some melittin analogues showed a drastic cytotoxicity reduction though maintaining comparable bactericidal activity. Two recombinant cecropin A- and cecropin B-melittin hybrid peptides CA(1-7)-M(4-11) and CB(1-7)-M(4-11) have been recently expressed in the yeast *Pichia pastoris*. Both chimeric peptides showed strong antibacterial activity against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus subtilis, Bacillus thuringiensis*, and *Salmonella derby* [83]. The efficacy of a cecropin A-melittin hybrid peptide CA(1-8)M(1-18) and shorter derivatives against pan-resistant *Acinetobacter baumannii* has been tested both *in vitro* and in a mouse sepsis model. The peptide showed an *in vitro* good activity, that was not affected by the presence of capsule [84]. However, *in vivo* the peptides showed bacteriostatic activity only, and PD_{50} was not achieved with non-toxic doses [85].

Magainins, Dermaseptins and Temporins

Amphibian skin is an important source of AMPs characterized by highly variable sequences. It is estimated that there may be as many as 10^5 different peptides produced by the known 5000 species of anuran amphibians [86]. Therefore, the main work still concerns the screening and identification of the most useful molecules. Here we focus on three families of representative peptides, namely magainins, dermaseptins and temporins.

Magainins are 23 amino acid residue peptides with α-helical structure, isolated from the skin of the African clawed frog (*Xenopous laevis*) in 1987 [87]. Following the observation that magainin-2 possesses broad spectrum antibacterial and antifungal activity, many synthetic analogs have been developed in order to maximize antimicrobial effects and minimize cytotoxicity. Magainin-A, a magainin-2 analog, underwent preclinical evaluation studies on *Macaca radiata* monkeys as local contraceptive, showing good spermicidal, antibacterial and antifungal activity [88], but has not been further developed. So far, the only magainin derivative that entered phase III clinical trials is MSI-78, or Pexiganan [89]. This peptide was assayed for the topical treatment of diabetic foot ulcers, but in 1999 the FDA denied its approval requiring additional clinical trials for further consideration. A new peptide that is not a magainin, but is often included in the magainin family, is PGLa, that has been also isolated from *X. laevis* skin [90]. This 21 amino acid residue peptide, like melittin, possesses an amidated C-terminus that provides good resistance to proteases. It showed good anti-fungal activity, further augmented by the use in combination with magainin-2 [90].

Dermaseptins, a family of 5 structurally and functionally related peptides originally isolated from the skin of frogs belonging to the *Phyllomedusinae* subfamily, consist of a characteristic polypeptide chain of 24-34 amino acids with 3-6 lysine residues and a highly conserved tryptophan residue in the third position from the C-terminus residue [91]. Dermaseptin antimicrobial activity is currently being characterized and analogs are being developed [91]. Dermaseptin S4 analogs are active against *Neisseria gonorrhoeae* [91] and 15 analogs of dermaseptin S1, synthesized by our group, showed variable activity against *Trichomonas vaginalis*, *Herpes simplex virus-1* and human *Papillomavirus 16* [92, 93]. These properties, coupled with the already demonstrated spermicidal activity of dermaseptins S, suggest that dermaseptins, as well as magainins, alone or even better in combination, could be used as topical contraceptives and microbicides to contemporarily prevent unwanted conceptions and sexually transmitted diseases [87].

The first molecule belonging to the temporin family was identified in the skin of the Asian frog *Rana erythraea* [94]. Subsequently, Simmaco *et al*. identified a family of similar peptides with antibacterial and antifungal properties from the skin secretion of *Rana temporaria* and termed them temporins [95, 96]. They have some interesting peculiar characteristics: while some of them reach a length of 17 amino acid residues, most temporins are among the shortest amphipathic α-helical AMP found, with a single 10–14 amino acid chain. Temporins are also among the most highly variable of all AMP and no single amino acid residue is invariant. All temporins so far isolated are C-terminally α-amidated and contain a prevalence of hydrophobic amino acids and basic residues (generally Lys, alternatively His and Arg) that give them a net charge ranging from 0 to +4 at physiological pH. Temporins are the largest family of AMPs, with more than 100 isoforms. They mainly act on Gram-positive bacteria, including methicillin-resistant strains. Interestingly, temporin-1Tl has a higher and broader spectrum of activity than the other isoforms, being active against fungi and Gram-negative bacteria such as *P. aeruginosa* and *E. coli*, but it disrupts human erythrocytes at microbicidal concentrations [97]. Temporins-1Ta, Tb, and Tl have been shown to neutralize the toxic effect of LPS derived from various species of *E. coli*, by complexing with it and making it unavailable for interaction with macrophage receptors to stimulate the production of TNF-α, considered to be a primary mediator of endotoxemia [98]. Owing to these characteristics, temporins are considered worth of further development. In this perspective, by studying the structure-activity relationship of a library of Tl derivatives, Mangoni and co-workers identified novel analogues with better properties that could be used for future developments [97].

Microcins and Bacteriocins

Microcins are a peculiar class of antibacterial hydrophobic peptides with molecular mass below 10 kDa, secreted by enterobacteria (mostly *E. coli*), involved in the regulation of microbial competition within the intestinal microbiota [99]. These molecules are secreted under conditions of nutrient depletion, are remarkably resistant to heat, extreme pH and proteases, and exert potent antibacterial activity in nanomolar concentrations, usually against a narrow spectrum of closely related species. Their mechanism of action has been defined as a "Trojan horse" behaviour: they are recognized

as siderophores by the outer membrane receptors of susceptible bacteria, and as such internalized; once inside they bind essential enzymes or interact with the inner membrane killing the bacterium [99]. In order to protect themselves from the effects of their own microcins, producing cells co-express a set of resistance factors that concur to form the so called "immunity system". Gene clusters encoding the precursor peptide, the posttranslational modification enzymes, the transport proteins, and the self-immunity are most often carried by plasmids, and to a lesser extent, by the chromosome [100]. At present microcins are still into the characterization phase, and despite their potent antibacterial activity, they are not being developed as antibacterials [101]. Microcin E492, a pore-forming molecule produced by *Klebsiella pneumoniae*, beyond exerting antibacterial activity on related strains, has been shown to induce apoptosis of malignant human cell lines [102]. Microcin B17, produced by various *E. coli* strains harbouring the 70-kb single-copy, conjugative pMccB17 plasmid, is a potent inhibitor of DNA gyrase, whereas microcin J25, the best-studied member of the lasso peptides, inhibits RNA polymerase (for review, see [99, 103]).

Bacteriocins are peptides of molecular mass ranging from 2.5 to 6 kDa, secreted by both Gram-positive and Gram-negative bacteria. Those produced by lactic acid bacteria are the most widely investigated and are classified into two classes: class I bacteriocins, commonly called lantibiotics (lanthionine-containing antibiotics), and class II, or non-lanthionine-containing bacteriocins [104]. Lantibiotics occur naturally in food and in the gastro-enteric tract of mammals and some of them, such as nisin and lacticin, are widely used as antibacterial agents by the food and agricultural industry of more than 50 countries [105, 106]. Lantibiotics are synthesized with a N-terminal leader sequence that is believed to keep the peptides inactive while inside the producing cell. Many of these peptides are extremely potent antibacterial agents with minimum inhibitory concentrations in the nanomolar range [107]. Lantibiotics are active against several very common food spoilage organisms (for example, *Listeria monocytogenes* and *Clostridium botulinum*) and show very promising activity against resistant *S. aureus* and enterococcal infections [108]. In the last few years some bacteriocins have been considered for human health and medical purposes: nisin A, the prototype lantibiotic produced by *Lactococcus lactis*, is highly efficient against Gram-positive bacteria and has no human toxicity. It was discovered in 1928 and has been accepted by the Food and Drug Administration (FDA) as a food additive in 1988. Its 34-amino acid residue structure contains five macrocyclic rings stabilized by thioether bonds [109]. Nisin inhibits the growth of vegetative Gram-positive bacteria by binding to lipid II, so disrupting cell wall biosynthesis and facilitating pore formation. Nisin also inhibits the outgrowth of bacterial spores, including *Bacillus anthracis* spores [110]. However, natural nisin A is unsuitable for medical uses, being unstable and poorly soluble in neutral or basic conditions and easily inactivated by thiols such as cysteine and glutathione [111]. Nisin A derivatives obtained by amino acid substitution are being developed and evaluated as anti-mycobacterial drugs [112]. Lacticin 3147, another lantibiotic produced by lactic acid bacteria, is more stable than nisin and is active against MRSA and VRE at nanomolar concentrations [113]. Lacticin 3147 consists of a 2-peptide (lacticin A1 and A2) system: lacticin A1 binds lipid II, and the complex binds lacticin A2, that induces pore formation in the bacterial membrane. To the class I bacteriocins also belongs thuricin CD, another 2-component peptide system produced by *Bacillus thuringiensis* and selectively active against *Clostridium difficile* [114]. A problem inherent the current antibiotic treatment of *C. difficile*-associated bowel disease is that large-spectrum antibiotics can perturb the gut flora to the point to interfere with recovery and in same cases even to promote recurrences. These problems could be avoided by the use of thuricin CD that, according to extensive tests against a broad range of Gram-positive and Gram-negative bacteria, targets a restricted spectrum of spore-forming Gram-positive bacteria [114]. Class II bacteriocins act by inducing membrane permeabilization, and are known for their strong activity against *Listeria monocytogenes*, but their structure-function analysis is still in progress [115, 116].

Indolicidin

Originally isolated from bovine neutrophils, indolicidin is a 13-residue cationic peptide rich in tryptophan and proline residues. It has been shown that indolicidin has a significant leishmanicidal activity, mediated by the disruption of *L. donovani* promastigotes and induction of autophagic cell death [117]. It is also a potent antibacterial, active against Gram-positive and Gram-negative bacteria, fungi, and HIV, but its cytotoxicity barred a therapeutic use [118]. However, less toxic novel derivatives showing promising pharmaceutical potential are currently under development. Omiganan, a synthetic indolicidin homologue, has demonstrated *in vitro* activity against a wide range of Gram-positive and Gram-negative bacteria and fungi. It is now in phase III clinical trials for the prevention of infections arising from short-term central venous catheters, for the prevention of surgical wound infections in contaminated wounds, and for the treatment of acne and rosacea [119]. It has been also used as reference control to test the activity of recently synthesized short (5- to 11-residue) antimicrobial peptides [120].

Lipoglycopeptides

Dalbavancin, oritavancin and telavancin are semisynthetic lipoglycopeptides active against multi-drug-resistant Gram-positive pathogens [121]. These molecules share a heptapeptide core that affects cell wall synthesis by inhibiting transglycosilation and transpeptidation, and contain lipophilic side chains that facilitate binding to cell membranes and increase antibacterial activity. Lipophilic residues also prolong *in vivo* half life, that is of 147-258 h for dalbavancin, 393 h for oritavancin and 12-24 h for telavancin. These molecules must be administered i.v. and are indicated for patients with complicated skin and skin-structure infections (SSSI) resistant to vancomycin [121]. Telavancin has been approved for this indication by FDA in September 2009 and is on the market with the registered name of Vibativ.

Dalbavancin, a teicoplanin derivative, has a long half life that allows for once weekly dosing. In published clinical trials, a dose on day 1 and 8 of treatment provided 14 days of antimicrobial activity. In clinical trials, dalbavancin has

demonstrated non-inferiority as measured by safety and efficacy for the treatment of uncomplicated SSSI, catheter-related bloodstream infections, and complicated SSSI [122].

Oritavancin, still in phase II trials, acts by depolarizing and permeabilizing the membrane of Gram-positive bacteria; these effects are attributable to the 4'-chlorobiphenylmethyl group of the molecule [123].

ANTIVIRULENCE FACTORS

Traditional antibiotics target processes essential for *in vitro* growth, with the implicit assumption that they are also essential for *in vivo* growth, but recent studies on fatty acid biosynthesis inhibitors evidence that in some cases there is a potential disparity between the requirements for *in vivo* and *in vitro* bacterial survival [124]. Most bacterial functions that concur to disease fall into two categories: those required for *in vivo* survival, that in some cases may be also essential for *in vitro* survival, and those that cause tissue damage.

Compounds that target bacterial virulence have at least three advantages over classic antibiotics: first, they do not exert a selective pressure on bacteria, because they do not target genes essential for bacterial viability *in vitro*, but only affect the host/pathogen interaction; second, their effect, being very specific, does not affect resident non-virulent bacteria [13]; third, the host, being exposed to harmless but intact bacteria, will develop a sound immune response against the wild-type virulent form [125]. The main obstacle standing in the way of the screening for antivirulence factors is the requirement of standardized functional *in vitro* assays that should mimic the *in vivo* conditions of the infectious process, considering that by definition virulence inhibitors will not kill bacteria *in vitro* [124]. Moreover, the clinical use of such drugs would rely on rapid diagnostic methods, because the specificity of action is linked to a limited spectrum of activity. However, the recent availability of many thoroughly sequenced bacterial genomes makes it possible to select antivirulence factors active against conserved targets common to different bacterial species. The relevance of virulence factor targeting is underscored by the remarkable amount of correlated research, that is producing the first practical results, like, for example, the recent development of a new simple assay to screen compounds affecting *S. aureus* virulence gene expression [126].

Some of the major approaches being taken to target virulence have been reviewed by Clatworthy *et al*. [127].

Inhibitors of Adhesion

The main early steps in host colonization involve pathogen adhesion and replication. Bacterial adhesion has been thoroughly studied in relation to *E. coli* urinary tract infections. Prevalence and degree of bacterial adhesion to uroepithelial cells are closely associated with the clinical category of urinary tract infections: among strains isolated from patients with pyelonephritis or bacteremia, 70 to 100% adhere to uroepithelial cells, compared with 50 to 60% of strains among cystitis isolates, 22 to 36% of strains among asymptomatic bacteriuria isolates, and 10 to 36% of faecal strains [128]. Adhesion and colonization result from a complicate interplay between germ virulence factors and the host early immune response. Bacterial adhesion to host cell is mediated by bacterial surface proteins or by proteinaceous short filaments called pili or fimbriae protruding from the bacterial surface. It is interesting to note that the three major streptococcal human pathogens, namely, *S. agalactiae*, *S. pyogenes* and *S. pneumoniae* possess pili, whose protein subunits have been shown to elicit protective immunity against the corresponding pathogen in mouse models of infection, making them potential vaccine candidates. Such strategies have particular promise for *S. agalactiae*, where pilus protein conservation across a large number of clinical isolates has been shown to be relatively high [129]. Other Gram-positive bacteria, such as *Staphylococci*, possess many different cell wall-anchored proteins that mediate bacterial binding to the host cells, acquire essential nutrients, and circumvent the immune response [130]. The mechanism involved in the anchoring of these proteins to the cell-wall is conserved in almost the entire class of Gram-positive bacteria, and is mediated by a class of cysteine transpeptidases called sortases, that are considered an attractive potential target for specific inhibitors. A number of different strategies, such as screening natural products and small compound libraries, or synthesizing rationally designed peptidomimetics, have been employed to search for sortase inhibitors. Recently Suree *et al*. have identified two promising small molecules that inhibit *S. aureus* SrtA sortase but do not impair bacterial growth *in vitro* [131]. Pili, the more efficient mediators of bacterial adhesion, are produced by many Gram-negative bacteria, including *Neisseriae* and fermenting and non-fermenting rods. The genome sequencing of meningococci led to the discovery of several other adhesins, which are expressed normally at low levels *in vitro* and can be up-regulated *in vivo*, but their potential role in pathogenesis remains to be fully defined [132]. In this section we deal more in detail with *E. coli* pili and their inhibitors, because this system is the most investigated and the most promising. *E. coli* adhesion can be prevented by the inhibition of pili formation or of pili binding to the surface of mammalian cells. Pili are multi-protein fibers assembled in the periplasmic space *via* a highly conserved mechanism called the chaperone-usher pathway, common to different Gram-negative species such as *E. coli*, *P. aeruginosa*, *Yersinia enterocolitica*, *Haemophilus influenzae*, and *Bordetella pertussis* [125]. Beyond type 1 pili uropathogenic *E. coli* (UPEC) produce type P pili, more frequent in pyelonephritis-associated strains, and curli, i.e. extracellular amyloid fibers that are major components of the bacterial extracellular matrix [133]. It has been demonstrated that pili- and curli-expressing *E. coli* adhere to uroepithelial cells better than non-piliated or non-curliated strains do [134]. Small synthetic compounds of the family of N-substituted amino acid derivatives and substituted bicyclic 2-pyridones, called pilicides, have been developed on the basis of the known molecular details of the interaction of pili subunits with the chaperone proteins [125]. By competitively inhibiting the chaperone protein function, pilicides dose-dependently decreased type 1 pili production by UPEC. Pilicides share a common chemical lineage with FN075 and BibC6, two ring-fused 2-pyridones that inhibit curli biogenesis by preventing the polymerization of the major curli subunit protein CsgA. Biological evaluation showed that the reduction of pili consistently correlated with the loss

of UPEC's ability to colonize bladder cells and to form biofilms [125], and curli reduction significantly attenuated UPEC virulence in a murine model of urinary tract infection [135]. Another possibility to inhibit fimbriated *E. coli* adhesion involves the use of glycodendrimers, a new family of well defined small macromolecules, that mimick glycans present on the surface of mammalian cells and prevent adhesion by binding to pili [136]. The potential clinical applications of these approaches remain to be explored.

Inhibitors of Colonization

In many instances the successful establishment of an infection depends upon the ability of the involved bacteria to form biofilm, i.e. large colonies of bacteria that adhere to biotic or abiotic surfaces and behave as an organized community reacting to small diffusible signal molecules termed autoinducers or quorum sensors [137]. Gram-negative bacteria mainly produce acyl-homoserine lactone (AHL) autoinducers, whereas Gram-positive bacteria use a two-component sensory system and oligopeptide autoinducers [138]. Quorum sensors are constitutionally produced and secreted by bacteria, and when their concentration reaches a threshold value that depends upon bacterial population density (quorum), they interact with species-specific receptors belonging to the LuxR family of response regulators. LuxR homologues contain two domains, an AHL-binding domain and a DNA-binding domain, that upon ligand binding acts as a transcriptional activator. The whole system is termed quorum sensing (QS).

QS systems generally offer three points of attack: the signal generation process, the signal molecule, and the signal receptor. During the last 20 years, many natural and synthetic agents belonging to the categories of small non-peptide molecules, peptides, and enzymes, have been identified as QS inhibitors [139]. The field is rapidly evolving, and an exhaustive analysis is beyond the scope of this paper. For review, see [140-146].

Inhibitors of Toxin Production and Secretion

The induction of specific antibodies by means of anti-toxic vaccines used for mass pre-emptive vaccinations, available for a limited number of infections, is unsuitable for the treatment of sporadic single cases of infections. Novel approaches consider targeting toxin transcription, expression, and function. The efficacy of the inhibition of toxin transcription has been demonstrated in a murine model of cholera infection by Hung *et al*., who observed that virstatin, an inhibitor of cholera toxin and toxin-coregulated pilus expression, blocked intestinal colonization by *Vibrio cholerae* [147].

Clostridium difficile, emerging as a leading cause of nosocomial infections, produces two exotoxins, TcdA and TcdB. The toxic domains of these molecules are released by an autocatalytic cysteine protease that is part of the toxin and is activated by binding to intracellular phytic acid, and inhibitors of this protease are currently under development [148]. A novel synthetic inhibitor of bacterial methionyl-tRNA synthetase, REP3123, active against *S. aureus* and *S. pyogenes*, inhibits *C. difficile* toxin and spore formation [149], and improved survival in a hamster model of *C. difficile* infection [150].

The recognition of *Bacillus anthracis* as a potential low cost bioweapon prompted the research on anthrax toxin inhibitors. Lethal factor (LF), a secreted zinc-dependent metalloprotease, that directly kills host cells, is one of the two toxins involved in anthrax pathogenesis. A hydroxamate synthesized at Merck Research Laboratories inhibits LF protease activity *in vitro*, and protected mice from a challenge with lethal doses of LF or *B. anthracis* spores [151]. This molecule has been further modified and extensively tested in pharmacological and animal model studies, and several other groups have successfully developed potent LF inhibitors, but none has yet reached clinical trials [152].

BACTERIOPHAGES AND PHAGE-DERIVED MOLECULES

Since the pioneer work of d'Herelle [153], several studies demonstrated that bacteriophages can be successfully used in the therapy of animal and human bacterial infections [154, 155]. Phages are already used in the agricultural, food-processing and fishery industries, and for the treatment of human bacterial infections in Georgia and Eastern Europe [156]. Recent experiments performed by Fu *et al*. on the efficacy of a bacteriophage cocktail to prevent the formation of *P. aeruginosa* biofilms on catheters in an *in vitro* model showed a 99.9% reduction of the number of bacteria [157]. The human use of phages in Western countries has been hindered so far by cost, safety concerns about phage injection into the bloodstream, and by the sometimes inconsistent outcome of the treatments, due to the poor characterization of bacteriophage preparations. Moreover, the *in vivo* pharmacokinetics of phages are complex [158], being influenced by the host immune system-mediated phage clearance rate and by the possible insurgence of bacterial resistance due to lysogeny or mutations concerning metabolic steps or surface receptors. However, phage therapy is considered a potential treatment for some selected infections, such as multidrug resistant *P. aeruginosa* lung infection in cystic fibrosis patients [159] and chronic otitis [160].

A different approach overcoming some of the above-mentioned problems involves the use of purified phage products as anti-infective agents. Bacteriophage endolysins are mureine-degrading enzymes, originally studied and developed to control mucous membrane infections [161] and are also denominated "enzybiotics" [162]). They only work on Gram-positive bacteria because the outer membrane of Gram-negative bacteria prevents direct lysin-peptidoglycan interaction [163]. To this end, a recent paper from Briers *et al*. reports that the use of endolysins in conjunction with outer membrane permeabilizers resulted in strong lytic activity against *P. aeruginosa*, with a reduction of more than four log units of viable bacteria in 30 min [162]. Endolysins, some of which have been found active against *B. anthracis* [164], *S. pneumoniae* [165] and *S. agalactiae* [166], alone or in combination with conventional antibiotics or lysozyme, have a short half-life (15-20 min), but their action is so rapid that nanogram quantities kill sensitive Gram-positive bacteria in seconds after contact [167]. Moreover, they are *per se* non toxic and, unexpectedly, not easily inactivated by anti-

bodies [168]. Considering that the endolysin target, peptidoglycan, is not present in eukaryotic cells, it can be anticipated that they will also be well tolerated by humans. Experiments performed on a murine model of pneumococcal pneumonia showed that an endolysin with muramidase activity, Cpl-1, protected 100% of mice when administered by intraperitoneal injections starting 24 hours after pulmonary infection [169]. These results suggest that Cpl-1 and related molecules could provide a new therapeutic option for pneumococcal pneumonia. The issue of the possible toxic effect due to the massive release of proinflammatory molecules by lysed bacteria has also been addressed. Circulating endotoxin, teichoic and lipoteichoic acids, and peptidoglycan could result in septic shock and multiple organ failure, but so far no side-effects related to lysin-induced bacteriolysis have been reported [161]. According to experiments performed on a murine model, lysins may also cure already established infections [169]. Endolysin applications include the elimination of bacteria from mucous membranes, the treatment of bacterial infections, and the biocontrol of bacteria in food.

CONCLUSIONS

The decline in new antibiotic approvals over the past 20 years is due to multifactorial causes involving technical and economical issues. The high-throughput target-based screening of chemical libraries, made possible since 1995 by the availability of hundreds of bacterial genomic sequences, did not fulfil the expectations. Major technical challenges bound to new targets are target essentiality and spectrum prediction, identification of spontaneous bacterial resistance, and evaluation of the probability of emergent resistance [7]. The more recent and promising approach consists in the systematic search for natural products, to be found in the so called "parvome", i.e. the group of biologically active, low-molecular-mass (< 5 kDa) compounds produced by defined biosynthetic pathways in bacteria, yeast, plants and other organisms [170]. This natural and so far just tapped reserve can offer both novel class antibiotics that work in the classic way killing pathogens, and molecules that only affect bacterial pathogenicity, some of which have been outlined in this review. However, the need for long-term, huge investments, and the prospect to see the novel drug indications limited to the small number of cases in which no other existing molecule is working, made many large pharmaceutical companies to quit antibiotic discovery for more profitable therapeutics [170]. The political, medical and public concern about the rising innovation gap in 2009 prompted the U.S. and European Community presidencies to establish a Transatlantic Task Force to address antimicrobial resistance, and the Infectious Diseases Society of America called for a global commitment to develop 10 novel antimicrobials by 2020 [7]. We can envisage that in the near future the synergy between new technical developments and public-private industrial partnerships will bring into being a new harvest of badly needed novel antimicrobials.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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