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New Horizons to Survive in a Post-Antibiotics Era

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Abstract

New antibiotics have not been developed since the late 80s. This situation poses a disadvantage to fight new multi-resistant emerging bacteria, thus limiting treatment possibilities for infected patients. The use of antibiotics revolutionized the world of modern medicine and allowed the development of fields such as agriculture and livestock. Additionally, the use of antibiotics indisputably led to cure multiple illnesses. Nevertheless, bacterial resistance to antibiotics in recent years has become a world health threat that calls for a coordinated action of many parts involved to address antibiotics resistance. Now a days, new research is being developed to find innovative alternatives to face this problem. In the present work we analyze new trends in the field of synthetic biology research focused on antimicrobial peptides, phage therapy and the use of gene editing tools CRISPR for controlling Multiple Drug Resistant pathogens (MDR).

Keywords: Antimicrobial Peptides; Microbial Resistance; Phage Therapy

Introduction

The infections caused by bacterial resistance to antibiotics have increased considerably in recent years and these are the reason for an important amount of morbidity and mortality worldwide, even in developed countries [1]. There are emerging Multidrug Resistant Organisms (MDRO) spreading such as *S. aureus* resistant to methicillin [2], *Enterococcos* resistant to vancomycin, and Gram negative bacteria resistant to third-generation cephalosporin's and carbapenems [3]. It has been estimated for the next decades that the number of deaths caused by multiresistant pathogens will be higher than deaths caused by cancer [4]. The steep rise of bacterial multiresistant pathogens to antibacterial agents has sped up dramatically in the last 10 years [5]. The prospects are so somber that even organizations such as the World Health Organization (WHO), the Centers for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control (ECDC) contemplate the possibility to consider infections caused by multiresistant pathogens (MDR) as a global illness emergency and a public health problem [6]. Antibiotics resistance is a natural phenomenon [7] that occurs in response to the strong evolutive selective pressure due primarily to the exposure of microorganisms to such compounds. Point mutations *de novo* in a susceptible bacterial population (for example, point mutations in union sites of ribosomes lends resistance to tetracycline) and the horizontal diffusion (transfer) of mobile genetic resistance determinants are probably the reason for the mass use of these drugs in clinics (hospitals) and even in the agroindustry. This hypothesis, is backed up by the low rates in the resistance percentages to the antibiotics in groups of pathogen bacterial strains before the antibiotic Age [8].

From a global point of view, to understand the evolution and impact of microbial resistance in 2007 the term "resistome" emerged for the collection of all the resistance genes to the antibiotics and their precursors in pathogenic and non-pathogenic bacteria; thus, to understand and study their origins, evolution and resistance manifestation [9]. The level of understanding of microbial resistance is constantly growing, given that resistance to antimicrobial is attributed to natural bacteria in the environment that do not cause diseases in humans [10,11]. These organisms have evolved during millions of years to interact, produce and

metabolize small molecules obtaining as a result the development of a broad spectrum of mechanism to modulate the activities of these compounds. The genes associated to this, often offer a greater selective advantage, easing the mobilization and horizontal transfer to other microorganisms that share a specific ecological niche [12]. The release of chemical agents (disinfectants, heavy metals and other contaminants) to the environment can accelerate the transfer of resistance genes to the surrounding bacterial population [13], which leads to an increment in the selective pressure and possible increase in the number of multiresistance isolates with evolutionary and adaptive advantages. Human beings have created ideal environments to bring together human infectious associated bacteria and microorganisms in the environment, for example, the use of waste water treatment plants [14], chemical production factories [15], and manure as row material for crops fertilization [16], create conditions that facilitate in great manner the mobilization and transfer of resistance genes. This translates into a collection of perfect scenarios for human bacterial pathogens and countless microbial generations to acquire a multitude of genetic elements that confer them adaptive and evolutionary advantages. Antimicrobial consumption is an important factor to consider in resistance generation, because there is a direct relation between the volumes and consumption patterns of antibiotics with the resistance variations to antibiotics in different countries [17]. The use in excess of antibiotics in humans and animals is greatly decreasing their activity and is one of the major threats to health worldwide, food safety and general sustainable development at the moment. The number of infections resistant to conventional treatments is on the increase; for example, tuberculosis [18], pneumonia [19], meningitis [20] amongst others, require more and more complex treatments combining various antibiotic compounds to fight them [21]. The multiple arsenal available to microorganisms to face antibiotics and protect themselves from their effects is broad and diverse. These go from mechanisms that include the enzyme inactivation of the antibiotic, modifications site-specific of the antimicrobial objective and mechanisms that eliminate toxic intracellular concentrations of the antibiotic by means of efflux pumps. Thus, showing the great genome plasticity that microorganisms have to adapt and survive. In spite of the urgent need of new antibiotics effective against resistant bacteria, very few compounds are being developed and most of them are similar to the different known types antibiotics [6]. Under this scenario, the urgent search of new strategies to combat persistent infections is an urgent need for the global public health.

Antimicrobial Peptides

Antimicrobial Peptides (AMPs) are active molecules produced by a broad variety of organism as the main component in their innate immunological response. The main function of the AMPs is based in the host defense by means of microbial death; and additionally, there is solid evidence about their immunomodulation capability in superior organisms [22]. The AMPs are considered to be an interesting strategy to fight microbial multiresistance [23]. Their extensive antimicrobial activity spectrum and bacteria selectivity over eukaryotic cells, makes them appealing candidates for new pharmaceutical compounds. In fact, attempts to exploit their potential have been carried out and some of the AMPs have been tested in clinical trials [24]. Antimicrobial Peptides (AMPs) are effective antibiotic agents present in plants, animals and microorganisms [25]. These molecules have a broad range of action against bacteria, funguses and viruses. The amphipathic structure common in AMP favors their interaction and the anionic cellular wall and phospholipidic membrane insertion in microorganisms [26]. The activity of AMPs is frequently the result of the cellular membrane alteration. Nevertheless, the AMP can operate on different cellular targets including DNA [27], RNA [28], and other proteins [29] as a promising alternative compared to conventional antibiotics [30]. The AMPs are an essential part of the innate immunity that evolved in most of the living organisms during 2.600 million years to fight the microbial challenge [31]. These small cationic peptides are multifunctional as innate immunity effectors on the skin and mucous surfaces [32]. These have proofed a direct antimicrobial activity against various bacteria, viruses, fungi and parasite species [30]. The AMPs have a broad range of secondary structures such as α-helix, β-pleated sheet with one or more disulfide bridges, loop and extended - strands structures [24].

Their multiple structural forms allows them a broad range of antimicrobial activity. Beside these properties, certain crucial factors such as size, charge, hydrophobic and amphipathic properties and specific interactions with the cellular membrane components are attributed to their broad activity spectrum [33]. One of the most notable features is their small size that eases a quick diffusion and secretion outside of the cells which is necessary to have an immediate defense against pathogens [34]. Most of the antimicrobial peptides are cationic in a physiological pH due to the high arginine and lysine type of residue in comparison to negatively charged amino acid residues like glutamic acid and aspartic acid [26]. This occurs generally in a substantial proportion of hydrophobic residues (≥30%) with a net charge of +2 and +9. Additionally, the cationic character is usually reinforced by an amide type of modification in the C-terminal of the sequence [35]. The AMPs commonly adopt amphipathic structures with hydrophobic faces and hydrophilic ends which grants them union properties to bacterial membranes by means of electrostatic interactions of the cationic lateral chains of the amino acids and the polyanionic surfaces of bacterial walls [36]. This is true for the teichoic and lipoteichoic acids in Gram positive bacteria or the lipopolysaccharides in Gram negative bacteria [37] allowing them to eliminate specific target cells without damaging the hosts cells [38]. AMP from different sources have been reported such as

insects, plants. Mammals, marine invertebrates and environmental libraries (Figure 1)

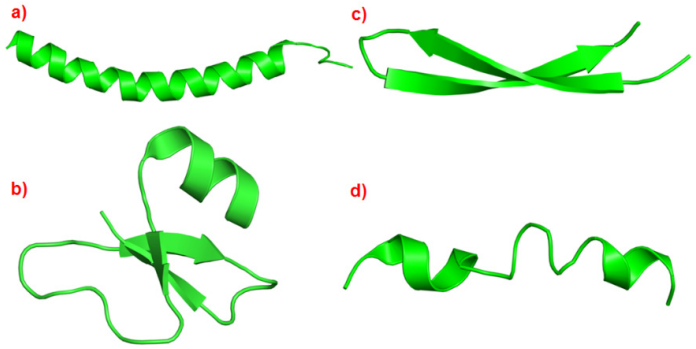

Figure 1: NMR structures of antimicrobial peptides obtained from different origins. **a)** Human cathelicidin LL-37 (PMID 18818205 ;), **b)** Human β- defensin-1 (PMID 17071614 ;), **c)** Arenicin-2 (*Arenicola marina* PMID 17585874 ;), **d)** Cecropin A (*Hyalophora cecropia* PMID 10424354 ;)

Mechanisms of Action

The size of AMPs can vary from 12 to 50 (or more) amino acids, which are cationic in general due to the excess of lysine and arginine amino acids. Approximately 50 % of the amino acids in the sequence are hydrophobic [39] prompting their quick action; they have a quick effect and a broad range of activity that includes Gram positive bacteria [40], Gram negative bacteria [41], fungi [42], encapsulated viruses [43], parasites [44]and even cancer cells [45]. In contrast to the antibiotics, the AMP´s mechanism of action is very different because the latter act as specific inhibitors for essential pathways in microbial cells; for example, inhibition of the cell wall synthesis (ß-lactam antibiotics: Penicillin and derivatives), inhibition of nucleic acids (quinolones: fluoroquinolones) or the inhibition of important metabolic routes (trimetroprim-sulfametoxazol: Inhibition of the tetrahydrofolate synthesis route) amongst others. The AMPs due to their broad structural variety, do not directly address cellular targets like enzymes or receptors but they address common characteristics of bacterial membranes [46-48]. There is evidence that the induced membrane permeation by peptides is the result of their interaction with the lipid matrix of the cells membrane [49,50]. In Gram negative bacteria the external and internal membrane have anionic molecules facing towards the outside of the cell whilst most of the peptides are cationic [37]. These peptides interaction with negatively charged phospholipids would explain their specificity for bacterial membranes and not for the zwitterionic lipids of the extracellular layer of eukaryotic cells [51]. In regard to the mechanism by which AMPs destroy a membrane, it is possible that they induce a complete rupture of the bacteria or a disruption in such a way that allows the release of essential cell components at the same time that the membrane potential is diminished. The initial rupture mechanism consists of phospholipids recognition through electrostatic interactions.

Once the peptides are united to the membrane, they undergo a structural reorganization (reconfiguration) that goes from a denaturalized state to an amphipathic structure. The latter stabilized by the lipid interphase in water [39]. It is thought that due to this kind of interactions the membrane increases it permeability; this mechanism has not been established yet and for this reason the following five main models have been established (Table 1):

Mechanism of Action for AMPs

Mechanism of action for AMPs	**Folder Model**	**Membrane reduction**
	The peptides are not inserted into the membrane but remain linked to the external surface; once they reach a critical point they transformed into a carpet like shape capable of weakening the membrane and collapsing into a mycelium configuration.	The AMPs are inserted only on one side of the lipid bilayer. They can create a space in between the lipid molecules in the chain area. This space in between creates a force that pulls the other neighbor lipid molecules in order to fill it.
Reference	[52,53]	[54,55]

Aggregation	**Toroidal pores**	**Barrel model**
In this model, the peptide merges with the membrane and at the right concentration it reconfigures to form mycelia like structure that stretches out across the bilayer in a lipid-peptide complex. These random lipid-peptide transmembrane aggregates in water form a canal that releases ions and produces cellular death due to the loss of cytoplasm content.	The peptides merge with the membrane when reaching a limit concentration that makes the lipids bend, forming a canal defined by the head of the lipidic groups (associated) to the peptides. These form a mixed canal of the peptide and the lipids from the membrane.	Once the AMPs interact with the membrane and reach a critical level of peptide and lipid, the peptides reconfigure in a perpendicular fashion forming a palisade with the side of the hydrophobic chains facing the hydrophobic center of the membrane and its polar chains and face the center forming a hydrophilic pore.
[56,57]	[58,59]	[32,60]

Table 1: Proposed activity mechanism for AMPs.

New Peptides Production

In the same way natural AMPs derivate largely from codified sequences in genes, bioinformatic methods have been used to create data bases of known AMPs as well as tools to predict specific AMPs from non-registered genomes. From the publication of the APD data bases 15 years ago [61] and ANTIMIC [62], various data bases have been created to emphasize certain features of AMPs, grouping them in different categories for example: natural, synthetic or recombined peptides (circular peptides, defensins and thiopeptides) [63,64]. There are data bases grouping AMPs based on their origins (human, bacteria, plants, insects and amphibian) [65,66]. The DADP data base amphibian peptides only [67].

More recently, the YADAMP [68], CAMP [69] and LAMP [70] data bases were created. A significant amount of research is focusing currently in the development of new AMPs for biomedical, therapeutic and biotechnological applications; the current methods to look for new functions and features of AMPs in the almost limitless known and predicted peptide sequences (empirically or computationally) are continuously evolving. Three clear known research approaches in this field can be distinguished: known AMP sequence modifications (so-called templates), biophysical modeling to understand the peptide activity and virtual screening [70]. The current methodologies used for AMPs library construction have advantages and disadvantages regarding the design of sequences, for example, the length of the sequence or the size to the library. Technics based on Polymerase Chain Reaction (PCR) such as saturation site-directed mutagenesis [71,72] DNA shuffling [73], were the randomly generated nucleic acid libraries that codify AMPs are expressed in the biological host have great complexity and the peptide length is not restricted in most cases because the mutations are randomly introduced. Nevertheless, to control of the sequence design by the user is very limited in these technics. On the other hand, the combinatory synthetic methods allows a sequence design customizing a variety of features in the sequences *perse*. This reason, the latter methodology has been implemented successfully to generate combinatory libraries of AMPs [74-76]. However, these methods still are limited by the size of the peptide sequence (optimal length up to 20 amino acids), as well as the large library due to the hard work in production and high costs associated to the complex chemical synthesis [77]. Build libraries that codify AMPs from a combination of oligonucleotides is comprised of 5 steps (Figure 2); the first three steps and the last step are essential for the technic, nonetheless the forth step can vary based on the appropriate expression host election for the library of interest.

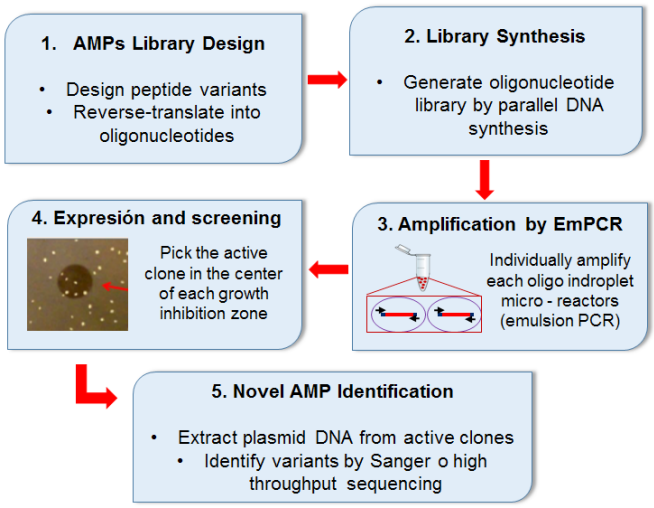

Figure 2: Building process and AMPs library election. Image modified by [78].

Various AMP have been developed successfully for pharmaceutical and commercial proposes [79]. Information about the structure and sequence of approximately 2846 AMPs from different sources can be found in databases all over the world [61]. Representative AMPs currently undergoing clinical trials are shown in (Table 2). Nevertheless, for this compounds to fulfil their therapeutic purpose and surpass clinical impasses more studies are necessary to understand their mechanism of action and reduce the potential of undesired cytotoxicity while conferring them more resistance to protease degradation, improving the half-life in peripheral blood and establishing a reliable and profitable mass production process.

Anti-microbial peptides in clinical trials or in development									
Name	AMP derivative	Description	Company / Location	Structure	Administration	Indication	Phase	Clinical Trials Identifier	References
Pexiganan acetate (MSI-78)	Magainin-2 (*Xenopus* frog skin)	Linear antimicrobial Peptide with 22-amino-acid, formulation named Locilex	Dipexium Pharma (White Plains, New York)	α-Helix	Cream	Skin and soft tissue infections and diabetic ulcers	1 and 3	NCT01590758	("Homepage of dipexium pharmaceuticals. Available from: https://plxpharma.com/dipexium-plx-merger/," n.d.)
Omiganan (MBI-226)	Indolicidin	Synthetic cathionic peptide analog of indolicidin (bovine)	Microbiologix Biotech Vancouver, BC (Canada)	α-helical	Cream	Rosacea	2	NCT00608959	(Sader, Fedler, Rennie, Stevens, & Jones, 2004)
OP-145	LL37	Synthetic 24-mer peptide binding to lipopolysaccharides or lipoteichoic acid	OctoPlus; Leiden University, The Netherlands	α-Helix	Eardrops	Chronic bacterial middle			
ear infection	2	NCT01071902	(Malanovic et al., 2015)						
Novexatin (NP213)	Fungicidal Active Pharmaceutical Ingredient (API)	Cationic antifungal peptide that has been formulated as a brush	NovaBiotics (Aberdeen, UK)	Cyclic arginine-based heptamer	Topical	Treatment onychomycosis	1 and 2	NCT02343627	("Homepage of NovaBiotics. Available from: http://www.novabiotics.co.uk/pipeline/novexatin-np213," n.d.)
Lytixar (LTX-109)	L-Arginamide	Broad spectrum synthetic antimicrobial peptidomimetic	Lytix Biopharma (Oslo)	N/A	Topical	Nasal decolonisation of MRSA	2	NCT01158235	("Homepage of Lytix Biopharma. Available from: http://www.lytixbiopharma.com/news/152/252/Successful-Proof-of-Concept-for-topical-antimicrobial-drug-Lytixar-LTX-109.html," n.d.)

NVB302	Posttranslationally modified peptides (lantibiotics)	Type B lantibiotic (lanthionine-containing antibiotics) selectivity *Clostridium difficile*	Novacta (Welwyn Garden City, UK)	N/A	Oral	Treat hospital-acquired *Clostridium difficile* infection	1	NVB302/001	(Boakes & Dawson, 2014)
MU1140	Lantibiotics	22-amino acid lantibiotic produced by *Streptococcus mutans*	Oragenics (Tampa, Florida)	N/A	N/A	Treat resistant *S. aureus* and resistant *Enterococcus faecalis*	Preclinical	N/A	(Kang, Liao, Wester, Leeder, & Pearce, 2010)
Arenicin	Lugworm *Arenicola marina*	21 amino acids Antimicrobial peptide , with two disulphide bonds							
bridging	Adenium Biotech Copenhagen	N/A	N/A	Multiresistant Gram-positive bacteria	Preclinical	N/A	(Panteleev, Bolosov, Balandin, & Ovchinnikova, 2015)		
Iseganan (IB-367)	Protegrin (pig leukocytes)	Antimicrobial peptide under development for the prevention of oral mucositis	Intrabiotics Pharmaceuticals, Inc. Mountainview, CA	Peptide containing two disulfide bonds	Oral/ aerosol	Mouthwash	3	N/A	(Giles et al., 2017)

Table 2: Anti-microbial peptides in clinical trials or in development.

Many of the AMPs have been tested in prophylactic therapy and therapeutic agents against biofilm formation in *in vitro* and *in vivo* [80,81]. Given the AMPs capability to act quickly on a broad spectrum of bacteria, including slow growing bacteria and non-growing bacteria [82], their effect on the different stages of the biofilm formation and the few selection of resistant strains are attractive features for their use as an alternative amongst the current low efficient antibiotics. On the other hand, the use of AMPs as immunomodulation agents have drawn great interest due to the role that cationic AMPs could perform in the innate immune modulating response, boosting the infection resolution by stimulating the host's own immunity [83] while controlling the potential pro-inflammatory damage.

Non-conventional Therapeutic Alternatives

There has been a new interest in non-traditional antimicrobial agents, especially in those generated by means of genetic engineering and synthetic biology. This due to the concerning rate growth of multiresistant bacterial pathogens specifically in hospitals in the last decades, as well as the gradual decline of new antibiotic compounds discoveries [84]. The search for new alternatives to the conventional antibiotics has become an important research goal. Two current alternatives in constant development are described below.

Bacteriophage Against Resistant Bacteria - Medicines as a Customized Therapy

Phage therapy refers to the use of bacteriophage (or simply phages, viruses that infect bacteria) to treat bacterial infections [85]. Bacteriophage are very abundant [86] and it is believed that each bacteria has its own specific virus that could be used as an antibacterial agent [86-88]. Historically, phages were used therapeutically at the beginning of the 20th century [89]. Nevertheless, the discovery of highly effective antibiotics slowed down the development of phage therapy in western countries and only when the antibiotics started to fail the old tool resume its development [90]. However, this second comeback of phage therapy phases challenges related with strict regulations and the development of an effective therapeutic practice [91-92]. Nonetheless, phage therapy provides an evolutive sustainable

alternative to conventional antibiotics [93], if we could only adjust our regulations and procedures to meet the special requirements of phage based medicine [94-95]. It is important to highlight that phages infect bacteria in a very selective manner. The narrow spectrum of hosts is often considered an advantage over traditional antibiotics because phage treatment con focus with accuracy on the pathogen without damaging the commensal intestinal flora [91]. Bacteria can quickly develop phage resistance as well and consequently the antibacterial effect could only be transitory [96]. When a group of different phages is used simultaneously in a phage cocktail, resistance development becomes less likely [97], but it is difficult to obtain an effective phage group against all variations of a specific pathogen [98]. There can be a compensation between the spectrum of bacterial targets and the therapeutic efficiency of a phage cocktail for a specific bacteria species. This happens whenever the number of phages in a cocktail are increase in an attempt to broaden the number of bacterial targets, the number of phage for a specific microbial strain can be reduced [99]. Therefore, the phage specificity to microbial cells, though benefic in theory, poses a practical problem when it is combined to treat resistant phenotypes that quickly emerge. For this reason, the therapeutic use of phages is considered a possible alternative to conventional antibiotics. Bacteria add foreign DNA or RNA inside their own genetic code and promote gene dispersion from a species to another through the phage translation, transformation or the connective plasmids and increases antibiotic resistance by Horizontal Gene Transfer (HGT). This allows bacteria to adapt to an ecologic variety and protect it against environmental pressures such are the antibiotics.

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Competing Interests

The authors declare that they have no competing interests.

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