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Review Article

Anuran Antimicrobial Peptides: An alternative for the development of nanotechnological based therapies for multi-drug-resistant infections

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Abstract

Bioactive peptides from anuran venoms were designed by evolution in a microbe-host and predator-prey interaction scenario. Each new species studied reveals new molecules, homologous to hormones, neurotransmitters, antimicrobials, as well as several others with unknown biological activity. These secretions have also been reported as a rich source of multiple antimicrobial peptides against strains of bacteria, fungi, and protozoa, providing several instructive lessons for the development of new and more efficient nanotechnology based therapies for the treatment of potentially lethal infections. One of the greatest accomplishments of modern medicine has been the development of antibiotic therapies for infections by pathogenic microorganisms. Unfortunately, over the past two decades, the discovery and development of novel antibiotics has decreased while pathogen resistance to those currently available has increased. The emergence of multidrug-resistant microbes, increased use of immunosuppressive therapies, and the association with HIV co-infection, represent a serious public health problem. Several health specialists and scientists have warned that the antibiotic-resistant microorganisms strains, or superbugs, outstrip our ability to fight them with existing drugs. This situation requires drastic strategies in order to develop new therapies against superbugs. In this paper, the potential use of anuran antimicrobial peptides for the development of alternative antimicrobial therapies for fatal infections based on a nanotechnological approach against superbugs is presented.

Keywords: Amphibian, bioactive peptide, nanobiotechnology, antibiotic-resistance, superbugs.

1. Introduction

Anurans are the most diverse group of vertebrates, with more than 6,000 known species, a figure that is constantly increasing due to the discovery of new species. However, according to the International Union for the Conservation of Nature (IUCN), amphibians may be the group currently at highest risk of extinction (IUCN, 2011). The anuran skin presents morphofunctional and behavioral protective adaptations against a number of adverse factors in the terrestrial environment, in which the cutaneous glands play an essential role in the defence against infection by microorganisms on the body surface. These glands produce a secretion composed of a complex mixture of substances with diverse pharmacological effects, some of which are active against bacteria, fungi, protozoa, virus and cancer (Calderon et al., 2009, 2010, 2011). In spite of the large number of anuran species from different genera, the neotropical hylid frogs that belong to the subfamily Phyllomedusinae have been described as an excellent source of bioactive peptides. According to Vittorio Erspamer (1985) ‘‘No other amphibian skin can compete with that of the Phyllomedusae’’. These peptides have significant structural differences compared with species within this genus leading to an interesting molecular diversity, possibly associated with specific challenges presented in the specie niche during the evolution process, such as interactions with the environment, predators, and pathogens (Amiche et al., 2000).

2. Peptides as Weapons Against Pathogens

The complex chemical composition of anuran skin secretions constitutes a rich chemical warehouse of a wide number of bioactive peptides that are grouped into the Frog Skin Active Peptide (FSAP) family. The FSAP family can be classified into three main groups according to their primary biological activity: antimicrobial peptides (AMPs); smooth muscle active peptides; and nervous system active peptides (Calderon et al., 2011; Erspamer et al., 1981). The first group acts as a skin anti-infective passive defence barrier, composing of the innate immunity system of anurans against microbial invasion (Giuliani et al., 2008; Radek & Gallo, 2007; Zasloff, 2002). The second and third groups cause the disruption of the predator homeostasis balance (Calderon et al., 2011). AMPs are effective against multidrug resistant strains of bacteria, fungi, protozoa, and virus including cancer, and could provide instructive lessons for the development of new and more efficient nanotechnological-based therapies for fatal infections (Rinaldi, 2002; Zasloff, 2002; Radek & Gallo, 2007; Giuliani et al., 2008; Calderon et al., 2011; Calderon & Stábeli, 2011). Many AMPs possess a wide range of activity showing effectiveness against diverse microorganism strains. One example is the dermaseptin family of AMPs and their analogs from the skin of Phyllomedusinae species. Dermaseptins have in vitro lytic activity against a broad spectrum of free-living microorganism strains, including wall-less bacteria (*Acholeplasma laidlawii*, *Spiroplasma apis*, *S. citri*, *S. floricola*, and *S. melliferum*), Gram-negative bacteria (*Aerornonas caviae*, *Acholeplasma laidlawii*, *Acetobacter calcoaceticus*, *Escherichia coli*, *Neisseria gonorrhoeae*, and *Pseudomonas aeruginosa*), Gram-positive bacteria (*Corynebacterium glutamicum*, *Enterococcus faecalis*, *Micrococcus luteus*, *Nocardia spp*, *N. brasiliensis*, *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *S. uberis*), fungi (*Aspergillus fumigatus*, *Arthroderma simii*, *Cryptococcus neofonnans*, *Candida albicans*, *C. tropicalis*, *C. guilliermondii*, *Microsporum canis*, and *Tricophyton rubrum*), protozoa (*Leishmania major*, *L. mexicana, L. amazonensis*, and *L. chagasi* promastigote forms; *L. amazonensis* epimastigote form; *Plasmodium falciparum* trophozoite form*; and Trypanosoma cruzi* trypomastigote form), and virus (HSV-1 and HIV-1) (Calderon et al., 2011; Calderon & Stábeli, 2011). Despite amino acid sequence similarities, dermaseptins differ in their action efficiency (Nicolas & El Amri, 2009; Rivas et al., 2009). However, they present rapid and irreversible antimicrobial activity and low toxic effects in mammalian cells in vitro (Kustanovich et al., 2002; Navon-Venezia et al., 2002).

AMPs were crafted by evolution into an extremely diversified array of sequences and folds sharing a common amphiphilic 3-D arrangement (Giuliani et al., 2008). This feature is directly associated with a common mechanism of action which is predominantly based on the interaction of AMPs with cell membranes (Giuliani et al., 2008). The action mechanisms of AMPs in microbial membranes are complex and relatively little is known about them, however this isn't a barrier in order to use AMPs as a promising and attractive new antimicrobial therapy (Calderon et al., 2009, 2011). Interestingly, it is known that the mechanism of interaction between AMP and microbial membrane prevents the fast adaptation of parasites, because a fundamental change in its membrane structure or composition is necessary, demanding a great metabolic effort during a short period of time, in contrast to drugs currently used (Phillips, 2001).

Despite their obligatory interaction with the plasmatic membrane, some AMPs translocate across the membrane and affect cytoplasmic processes, including inhibition of macromolecular synthesis, particular enzymes, cell division or the stimulation of autolysis (Marr et al., 2006). Furthermore, AMPs are not hindered by the resistance mechanisms that occur with currently used antibiotics (Zhang et al., 2005) due to the skill of the AMPs to eliminate multidrug resistant strains of microorganism by a mechanism unlikely to induce antibiotic-resistance by the action on the microbial cell membrane and physically damage the membrane structure, therefore reducing the likelihood of microbes developing resistance (Engler et al., 2012). Indeed, elimination can occur synergistically with other AMPs and conventional antibiotics, helping to overcome several barriers that resistant bacteria have against currently used antibiotics (Marr et al., 2006).

Nowadays, the emergence, increased prevalence and rapid spread of extreme multidrug resistant pathogenic microorganisms commonly known as "superbugs" and associated with the increased use of immunosuppressive therapies together with HIV co-infection have resulted in several fatalities, and represents a serious challenge for public health systems. A lack of therapeutic options against fatal infections has stimulated research into new antimicrobials from the biodiversity as a source of more efficient therapies with low toxicity and major potency for infection control (Calderon et al., 2009; Vaara, 2009).

The interest in the development of new anti-infective therapies based on AMPs from anuran skin has been increased (Rinaldi, 2002; Xiao et al., 2011). Thus, they are likely to be active against pathogens and even those that are resistant to conventional drugs, with few exceptions, such as *Salmonella typhimurium* (Gram-negative bacteria), *Mycoplasma gallisepticum* (wall-less bacteria) and *M. mycoides* (fungi) that have shown resistance to dermaseptin B9 from *P. bicolor* (Fleury et al. 1998). More than 500 AMPs isolated from amphibians have been shown to be effective against multi-drug resistant pathogens (Xiao et al., 2011), an insignificant number when compared to all those represented by the amphibian global fauna, that is composed of much more than 6,000 species which potentially represents at least 6,000 kinds of poison together with hundreds of thousands of new bioactive molecules to be discovered (Jared & Antoniazzi, 2009).

The abundance of AMPs structures from frog skin is remarkable and constitutes a wonderful source for the design of new pharmaceutical molecules. Unfortunately, several anuran species have become extinct due to the events related to the amphibian decline before their bioactive molecules have had a chance to be discovered, such as the golden toad *Bufo periglenes* (Bufonidae) (Calderon & Stábeli, 2011).

3. Anuran Antimicrobial Peptides and the Resistance Crisis

According to Huber et al. (2008), ever since the first therapeutic use of penicillin as an antibiotic in the early 1940s, life threatening bacterial infections has generally been considered to be a thing of the past. However, bacteria have mechanisms that allow them to adapt with ease, and by 1950 almost half of all hospital samples of the *Staphylococcus aureus* had became resistant to penicillin and 60 years after, some public health specialists and researchers are warning that antibiotic-resistant infections produced by superbugs have reached unprecedented levels, outstripping our ability to fight them with existing drugs (Calderon & Stábeli, 2011). In 2007, approximately 25,000 patients died in the European Union, Iceland and Norway from fatal infections due to antibiotic-resistant bacteria able to outsmart even the up to date antibiotics, such as *Staphylococcus aureus*, methicillin resistant (MRSA); *S. aureus*, vancomycin intermediate resistant and vancomycin resistant (VISA/VRSA); *Enterococcus* spp. (e.g. *Enterococcus faecium*), vancomycin resistant (VRE); *Streptococcus pneumoniae*, penicillin resistant (PRSP); Enterobacteriaceae (e.g. *Escherichia coli*, *Klebsiella pneumoniae*), third-generation cephalosporin resistant; Enterobacteriaceae (e.g. *K. pneumoniae*), carbapenem resistant; and non-fermentative Gram-negative bacteria (e.g. *Pseudomonas aeruginosa*), carbapenem resistant (European Centre for Disease Prevention and Control/European Medicines Agency [ECDC/EMEA] Joint Working Group, 2009). In addition, infections due to any of these antibiotic-resistant bacteria resulted in approximately 2.5 million extra hospital days and extra in-hospital costs of more than EUR 900 million (ECDC/EMEA Joint Working Group, 2009).

The emergence and rapid spread of extremely multi-resistant pathogenic microorganisms endowed with a new and very efficient antibiotic resistance mechanisms, such as *New Delhi metallo-beta-lactamase-1* (NDM-1), an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics, including the carbapenem family (except aztreonam), one of the last resort for many bacterial infections, such as *Escherichia coli* and *Klebsiella pneumoniae* (Kumarasamy et al., 2010; Nordmann et al., 2011; Richter et al., 2011). This gene has been identified in strains that possess other resistance mechanisms, which contribute to their multidrug resistance patterns, in turn making them difficult to treat successfully (Raghvendra et al., 2011). Most of the strains isolated with NDM-1 enzyme are resistant to all standard intravenous antibiotics for treatment of severe infections (Health Protection Agency [HPA], 2009a,b). It has been recently extensively reported from the United Kingdom, India and Pakistan and, to a lesser extent, from other countries (Nordmann et al., 2011).

The emergence of superbugs together with a immunosuppressant agent represents a serious public health problem with high mortality and morbidity rates, such as those produced by *Cryptococcus*, *Cryptosporidium* and *Leishmania* (Abu-Raddad et al., 2006; Pukkila-Worley & Mylonakis, 2008; Rivas et al., 2009; Vaara, 2009). The issue represented by limited therapeutic options for increasing multidrug resistance in Gram-negative bacteria, in particular *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, have forced infectious disease clinicians and microbiologists to reappraise the clinical application of the antibiotic polymyxin, an old cyclic peptide discovered in 1947 (entered into clinical use in 1960) (Li et al., 2006; Giuliani et al., 2008; Pirri et al., 2009). Polymyxin is usually active *in vitro* (though not *vs. Morganella morganii*, an intrinsically resistant species), however, its clinical efficacy, especially in pneumonia, is uncertain due to poor lung penetration.

One of the greatest accomplishments of modern medicine has been the development of antibiotic therapies for infections of pathogenic microorganisms. Unfortunately, over the past two decades, the discovery and development of novel antibiotics has decreased while pathogen resistance to those currently available has increased, allowing the re-emergence of potentially fatal infections (Li et al., 2006).

According to ECDC and EMEA with contributions from the international network Action on Antibiotic Resistance (ReAct), there is a need for further initiatives in the development of new antibiotics that are effective against multidrug-resistant bacteria. The ECDC/EMEA Joint Working Group, using data from the *European Antimicrobial Resistance Surveillance System* (EARSS) and two commercial databases of antibacterial agents in clinical development worldwide (Adis Insight R&D and Pharmaprojects) concluded that there is a serious gap between the burden of infections due to multidrug-resistant bacteria and the development of new antibiotics to fight them and conclude that a global strategy to solve this gap is urgently needed (ECDC/EMEA Joint Working Group, 2009).

Limited therapeutic options against these pathogens demand the urgent bioprospection of new bioactive molecules from the biological diversity as a source for more efficient (low toxicity and major potency) mechanisms of microorganism elimination (Calderon et al., 2009; Vaara, 2009). The discovery of new lead compounds is necessary in order to subsidize the development of new chemicals with structural characteristics for large-scale production by the pharmaceutical industry at a feasible cost. Sources from biodiversity, such as the skin of several amphibians and other vertebrate/invertebrate animals, plants and microorganisms, have proved to be a rich source of AMPs with a broad spectra of activity (Calderon et al., 2009), especially against drug-resistant pathogens as described, in which AMPs could be used in the development of a therapeutic application (Gomes et al., 2007; Hancock, 1997; Hancock & Lehrer 1998; Koczulla & Bals, 2003). One example of this is the cationic AMP Ascaphin-8 from the skin secretion of the anuran *Ascaphus truei* (http://www.uniprot.org/uniprot/P0CJ32) (Conlon et al., 2004). This AMP shows a broad-spectrum antibacterial activity against clinical isolates of beta-lactamase producing bacteria such as *Escherichia coli* (MIC=1.5-6 μM) and *Klebsiella pneumoniae* (MIC=12.5-25 μM), as well as a group of miscellaneous beta-lactamase producing strains of *Citrobacter*, *Salmonella*, *Serratia*, and *Shigella* spp (Eley et al., 2007). According to Eley et al. (2007), Ascaphin-8 also shows toxicity to human erythrocytes ($LC_{(50)}$= 55 μM), however, L-lysine-substituted analogs Lys10, Lys14, and Lys18 also displayed potent antibacterial activity while showing very low hemolytic activity ($LC_{(50)}$> 500 μM). This result shows that peptide engineering could reduce toxicity of haemolytic AMPs, which makes possible the use of this engineered AMP with drug delivery systems in order to improve the efficiency of Ascaphin-8 analogs as a therapeutic peptide antibiotic against multidrug-resistant pathogenic microorganisms (Calderon & Stábeli, 2011).

4. Nanotechnological Approaches Against Superbugs

According to Marr et al. (2006), therapeutic peptide antibiotics could have several advantages over conventional antibiotics due to their diverse potential applications, such as single antimicrobials or in combination with other antibiotics in order to obtain a synergistic effect (Zasloff, 2002). Compared with conventional antibiotics, these bacteria-killing peptides are extremely rapid, attacking multiple bacterial cellular targets (Brogden, 2005). Many efforts have been made in order to utilize AMPs in the development of new infection-fighting drugs applicable to nosocomial and multidrug-resistant infection therapy (Amiche et al., 2000). According to Veerapandian & Yun (2011), several research groups have reported that nanoparticles composed of a self-assembling amphiphilic peptides have potential antimicrobial activity against a wide range of bacteria, yeasts, and fungi (Lihong et al., 2009). At the end of the antibiotic age, new antimicrobials (based on AMPs), make possible the development of new weapons to be used in the war against multidrug-resistant microorganisms (Alanis, 2005; Arias & Murray, 2009; Nordmann et al., 2011).

Even with the expected advantages in the use of AMPs as new antimicrobials, several impediments to therapeutic peptides have arisen. According to Marr et al. (2006), the main problem is the commercial production cost, which is economically unfeasible given the amounts of AMPs needed compared to other antibiotics, so preventing the clinical use of AMPs as a common antibiotic. Other issues include: shortage of studies thoroughly examining systemic peptide pharmacodynamic and pharmacokinetic issues; peptide aggregation problems; in vivo half-life of peptides (and particularly their susceptibility to mammalian proteases; and the required dosing frequency (Marr et al., 2006).

Due to the specific characteristics of the AMPs, that differentiate them from other antibiotics, the development of new strategies that make the therapeutic use of AMPs in medicine feasible are necessary in order to reduce the amount of AMPs necessary to promote efficiency at very low concentrations and negligible toxicity (Marr et al., 2006). According to Veerapandian & Yun (2011), functionalization of AMPs with nanoparticles could produce a stable and highly active biomolecule–nanoparticle hybrid system with significant enhancing of the antibacterial function of these biomolecules. The nanotechnological approach has become an efficient and viable alternative to promote the therapeutic application of AMPs through the use of nanocarriers in order to: protect the AMP from degradation; enhance AMP absorption by facilitating diffusion through epithelium; modification of pharmacokinetic and tissue distribution profile; and/or improving intracellular penetration and distribution. According to Couvreur & Vauthier (2006), over the past 30 years, the explosive growth of nanotechnology has led to innovations in pharmacology, which is in the process of revolutionizing the delivery of biologically active compounds (Calderon & Stábeli, 2011).

The application of nanotechnology in cancer and infectious diseases therapy has revolutionised the pharmacology of these diseases by the development of several approved forms of drug-targeting systems (Couvreur & Vauthier, 2006). One of the most important examples is the Ambisome®, a lipossomal formulation of amphotericin B (NeXstar now Gilead, Foster City, CA, USA). The toxicity of the non-nanostructured leading compound against leishmaniasis and fungus is 50- to 70-fold higher (Adler-Moore & Proffitt, 1993). Nowadays it is considered the most efficient treatment for leishmaniasis and fungal infections (Dupont, 2002; Ringden, 2002; Croft & Coombs, 2003).

Nanotechnology also seems to be a promising alternative to overcome the problems of the administration of AMPs. Nanotechnological based drug-targeting system carrying AMPs can be targeted to a precise location which would make the AMP much more effective, reducing the amount necessary to promote the antimicrobial action, as well as the chances of possible side-effects and production costs, making the therapeutic peptide antibiotics feasible economically compared to other antibiotics (Calderon & Stábeli, 2011).

The structural and physicochemical properties of AMPs, such as the presence of a α-helix fold and distribution of positive charges along the chain have allowed their use as active material in the development of bio-nanostructures which together with the physicochemical properties of nanoparticles, interface/linking agents and selective biomolecules play a vital role in dictating the antimicrobial activity thus allowing its potential application in therapeutics by the pharmaceutical industry and diagnosis (Zampa et al., 2009; Veerapandian & Yun, 2011).

These nanostructures include cationic nanoparticles, formed by the conjugation of cholesterol and AMPs, able to cross the blood–brain barrier for treatment of fatal Cryptococcal meningitis in patients with late-stage HIV infection (Wang et al., 2010); Polymyxin B conjugates with Au nanoparticles and CdTe quantum dots with improved antimicrobial activity and reduced toxicity to mammalian cells (Park et al., 2011); nanostructured thin films with immobilized AMPs as an agent intended to combat and prevent infection and formation of Staphylococcus biofilm (slimelike communities) related to implant failure (Shukla et al., 2009).

The overall antimicrobial mechanisms from nanomaterials are hypothesized mainly due to reactive oxygen species (ROS) production and impacts on cellular membrane integrity and metabolic activity (Veerapandian & Yun, 2011). According to Veerapandian & Yun (2011), the understanding of the interaction mechanisms between functionalized nanomaterials and microorganisms/biosystems is in the embryonic stage and several research efforts are necessary in order to unravel the exact mechanism of action. It is believed that the knowledge of the mechanism of functionalized nanomaterials, together with appropriate biointerface phenomena, will permit researchers to create more standard design features for the development of advanced functional materials (Veerapandian & Yun. 2011).

The use of AMPs through nanotechnological innovation approach could provide a groundbreaking strategy in the prevention and therapy of fatal infections where the current antibiotics fail. However, nanotechnology has to drastically reduce the amount of AMPs necessary to obtain therapeutical efficiency by the improvement of cell, tissue, or organ's specific biodistribution together with the improvement of AMP potency by the association with nanotechnological structures. In the forthcoming years, nanotechnology could promote the emergence of new pharmaceutical products for therapy and prevention of superbug infection arising from the identification and analysis of AMPs from anuran amphibian biodiversity.

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