Amino Acids (2014) 46:2287–2294
DOI 10.1007/s00726-014-1820-1

MINIREVIEW ARTICLE

Proline-rich antimicrobial peptides: potential therapeutics against antibiotic-resistant bacteria

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Received: 25 July 2014 / Accepted: 28 July 2014 / Published online: 21 August 2014

Abstract The increasing resistance of pathogens to antibiotics causes a huge clinical burden that places great demands on academic researchers and the pharmaceutical industry for resolution. Antimicrobial peptides, part of native host defense, have emerged as novel potential antibiotic alternatives. Among the different classes of antimicrobial peptides, proline-rich antimicrobial peptides, predominantly sourced from insects, have been extensively investigated to study their specific modes of action. In this review, we focus on recent developments in these peptides. They show a variety of modes of actions, including mechanism shift at high concentration, non-lytic mechanisms, as well as possessing different intracellular targets and lipopolysaccharide binding activity. Furthermore, proline-rich antimicrobial peptides display the ability to not only modulate the immune system via cytokine activity or angiogenesis but also possess properties of penetrating cell membranes and crossing the blood brain barrier suggesting a role as potential novel carriers. Ongoing studies of these peptides will likely lead to the development of more potent antimicrobial peptides that may serve as important additions to the armoury of agents against bacterial infection and drug delivery.

Keywords Proline-rich antimicrobial peptides · Non-lytic mechanism · Membrane disruption · Immunostimulation · Cell penetration

Abbreviations

AMPs	Antimicrobial peptides
PrAMPs	Proline-rich antimicrobial peptides
LPS	Lipopolysaccharide
CPPs	Cell penetrating peptides
BBB	Blood brain barrier

Introduction

The development of antimicrobial resistance to conventional antibiotics is a major global health issue and, despite the availability of new classes antibiotics launched in recent years, this trait of antibiotic resistance in microbes is growing (Butler and Blaskovich 2013). The period of adapting resistance to newly released conventional antibiotics is also becoming increasingly short (Hede 2014; Hsueh 2012; Zhang et al. 2006) (Fig. 1). The resistance rates of *E. coli* and methicillin-resistant *S. aureus* have accelerated beyond country borders via human activity and avian migration (Kanthor 2014). The widespread feeding of livestock with various antibiotics to aid their growth has also contributed to the enhancement of antibiotic resistance genes, not only to the specific antibiotic used but also to the whole

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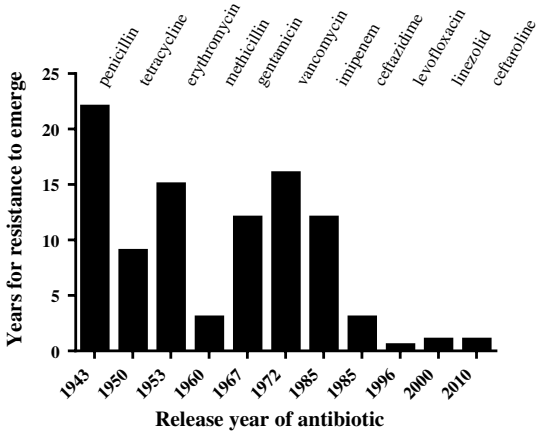

Fig. 1 The period for resistance to emerge for conventional antibiotics after their release. These include penicillin, tetracycline, erythromycin, methicillin, gentamicin, vancomycin, imipenem, ceftazidime, levofloxacin, linezolid, and ceftaroline. This figure is based on that from Hede (2014)

antibiotic class to which it belongs (Cully 2014; Zhu et al. 2013). Consequently, considerable research is focused on investigating the mechanism of antibiotic resistance and in developing new antibiotics possessing various mechanisms of action to combat resistance development (Cannon 2014; Gammon 2014).

Antimicrobial peptides (AMPs) are distributed in all living organisms as part of the host innate immunity (de Souza Cândido et al. 2014; Zasloff 2002). They have rapidly emerged as an important alternative to conventional antibiotics due to their potency and differing modes of action (Fox 2013; Hancock et al. 2012; Peters et al. 2010) which lead to broad-spectrum activity against various microorganisms (Brogden 2005; Zhang and Falla 2006). Generally, AMPs have a net positive charge due to the presence of several lysine/arginine residues, and display amphipathic properties, which are crucial for the interaction with the microbial membrane and membrane binding (Fernández-Vidal et al. 2007; Hancock 1997; Powers and Hancock 2003). However, the composition and structure of AMPs vary considerably leading to different mechanisms of action. There are a number of reviews detailing their features, including toxicity, secondary structure and biological activity (Chen and Luo 2009; Giuliani et al. 2007; Jenssen et al. 2006; Splith and Neundorf 2011).

It has been postulated that membrane-active AMPs selectively disrupt the cell membrane to form pores that allow efflux of essential ions or nutrients (Matsuzaki 1999; Zasloff 2002). Based on the Shai–Matsuzaki–Huang (SMH) model, most AMPs act via an interaction with the membrane resulting in a morphological change of membrane structure (Matsuzaki 1999; Shai 1999; Yang et al. 2000). Different models of pore formation have been proposed and described in several reviews (Brogden 2005; Jenssen et al. 2006; Reddy et al. 2004; Shai 1999) (Table 1), and include barrel-stave (Matsuzaki et al. 1998), carpet (Dagan et al. 2002), toroidal pore or wormhole (Matsuzaki et al. 1996) and aggregate mechanism models (Wu et al. 1999) (Fig. 2). However, there is increasing evidence that AMPs can kill microbes without causing membrane damage which suggests other potential intracellular targets and different mechanisms of killing (Bahar and Ren 2013; Brogden 2005; Otvos 2005) (Table 1). The intracellular targets can be divided into several groups, including inhibition of macromolecular (nucleic acid or protein) synthesis (Subbalakshmi and Sitaram 1998), inhibition of metabolic/enzymatic function (Otvos et al. 2000), and inhibition of cell-wall/membrane formation (Brötz et al. 1998) (Fig. 2).

Table 1 Examples of AMPs with pore forming or non-lytic mechanism against microbes

Pore formation mechanism	Barrel stave model	Alamethicin (He et al. 1996; Yang et al. 2001)
	Carpet pore formation	Dermaseptin S (Dagan et al. 2002; Ghosh et al. 1997), cecropin (Gazit et al. 1995; Shai 1995), melittin (Naito et al. 2000), aurein 1.2 (Fernandez et al. 2009; Wong et al. 1997), caerin 1.1 (Fernandez et al. 2009; Wong et al. 1997), ovispirin (Yamaguchi et al. 2001) and LL37 (Oren et al. 1999)
	Toroidal model	Magainin 2 (Ludtke et al. 1996; Matsuzaki et al. 1996), protegrin-1 (Yamaguchi et al. 2002), melittin (Smith et al. 1992; Yang et al. 2001), LL-37 (Henzler Wildman et al. 2003), MSI-78 (Hallock et al. 2003)
	Aggregate model	Dermaseptin (Pouny et al. 1992), magainin 2 (Matsuzaki et al. 1996)
Non-lytic mechanism	Inhibition of macromolecular synthesis	Indolicin (Subbalakshmi and Sitaram 1998), PR-39 (Boman et al. 1993), pleurocidin (Patrzykat et al. 2002), seminalplasmin (Scheit et al. 1979), tPMP-1 (Lehrer et al. 1989), aHNP-1 (Lehrer et al. 1989), microcin J25 (Delgado et al. 2001)
	Inhibition of cell-wall/membrane formation	Lantibiotic mersacidin (Brötz et al. 1998), PR-39 (Shi et al. 1996), PR-26 (Shi et al. 1996), indolicin (Subbalakshmi and Sitaram 1998), microcin J25 (Salomón and Farías 1992)
	Inhibition of metabolic function	Histatins (Kavanagh and Dowd 2004), pyrrhocoricin (Bencivengo et al. 2001; Chesnokova et al. 2004), drosocin (Chesnokova et al. 2004), apidaecin (Casteels et al. 1989), aiptercin (Chitnis et al. 1987, 1990)

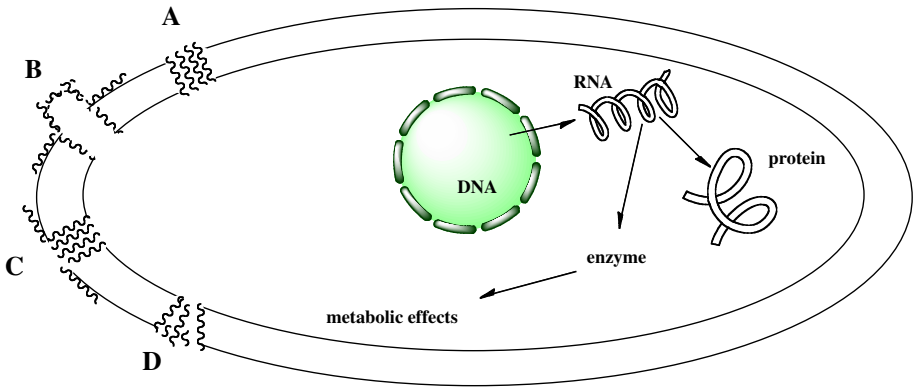

Fig. 2 Lytic and non-lytic mechanisms of AMPs: *A* barrel stave model, *B* carpet pore formation, *C* toroidal model, *D* aggregate model; inhibition of macromolecular synthesis, inhibition of cell-wall/membrane formation, and inhibition of metabolic function

Many AMPs have been isolated from invertebrates, insects, plants and fungi, which can be divided into two groups, cationic AMPs and non-cationic AMPs each with a diversity of structure motifs including linear peptides assuming α-helical, or β-strand stabilized structures that may be aided by one or more disulfide bonds (Dimarcq et al. 1998; Pushpanathan et al. 2013). Based on their side-chain arrangement and structural motif features, these AMPs display potent antimicrobial activity and specificity to the microbial target. AMPs can be further categorised based on their amino acid content and proportion, e.g., some have a high content of one or two amino acids such as proline (Otvos 2002), arginine (Chan et al. 2006; Dong et al. 2012) and/or glycine (Baumann et al. 2010; Ilić et al. 2013). Among these different types of AMPs, proline-rich AMPs (PrAMPs) have attracted particular attention due to their wide distribution in insects and unique mechanism of killing bacteria without cell membrane disruption (Otvos 2000). Several reviews have discussed specific characteristics of PrAMPs, including isolation (Otvos 2000), non-lytic mechanism (Scocchi et al. 2011), engagement with intracellular targets (Otvos 2005; Otvos et al. 2000) and synergistic function (Cassone and Otvos 2010).

Mechanism of action

Previously, PrAMPs have been isolated from various species, including vertebrates (Agerberth et al. 1991; Gennaro et al. 1989, 2002; Shamova et al. 1999), crustaceans (Destoumieux et al. 2000; Stensvag et al. 2008), *anellidae* (Cho et al. 1998) and especially insects (Bulet et al. 1993, 1999; Casteels et al. 1989; Otvos 2000). While the non-lytic mechanism of PrAMPs had been investigated extensively (Gennaro et al. 2002; Otvos 2002; Scocchi et al. 2011), additional PrAMPs exhibiting different modes of actions have been both isolated and chemically synthesized. PP30, isolated from *Apocrita* and similar to abaecin, acts against microbes causing morphological changes and membrane damage (Shen et al. 2010). The designed PrAMP, A3-APO and arasin 1, exhibits a dual mode of action with both membrane disruption and probable intracellular target inhibition (Paulsen et al. 2013; Rozgonyi et al. 2009). More interestingly, membrane destruction is concentration dependent indicating that membrane permeabilization shifts to a membrane disruptive effect at high concentration (Hernandez-Gordillo et al. 2014; Paulsen et al. 2013; Podda et al. 2006; Végh et al. 2011). These findings will aid the development of PrAMPs bearing a range of selective modes of action, thus increasing potency and reducing potential resistance while increasing the spectrum of activity.

The intracellular mode of action of PrAMPs has been shown by the inhibition of the 70 kDa bacterial heat shock protein, DnaK, resulting in protein misfolding and aggregation and subsequent bacterial death (Otvos 2005). Furthermore, two binding sites on bacterial DnaK were revealed by molecular modeling calculations and solving crystal structure of *E. coli* DnaK with PrAMPs (Kragol et al. 2001; Rozgonyi et al. 2009; Zahn et al. 2013, 2014). Although the PrAMP mechanism of action has been shown to be via DnaK inhibition, there are indications that they also have novel modes of action against the targeted bacteria. PrAMPs generally penetrate the outer membrane of Gram-negative bacteria and translocate into the cytoplasm via a permease/transporter-mediated uptake, but there is no increase in activity after engineered increases with the AMP cell penetration (Berthold and Hoffmann 2014). Furthermore, apidaecin IB not only depresses the GroEL/GroES chaperone team, but also results in the unbalanced biosynthesis of lipopolysaccharide (LPS) and phospholipids in *E. coli* (Zhou and Chen 2011). Particularly encouraging is the ability of PrAMPs to inactivate bacterial toxins (through inhibition of their proper folding) because these are frequently as lethal as bacteremia (Otvos et al. 2014). Moreover, Ls-Stylicin 1, which has a proline-rich N-terminal region, displays potent LPS-binding activity in vitro, suggesting potential selective action against

Table 2 Proline-rich AMPs modulation of the immune system via cytokine activity or angiogenesis or proteasome inhibition

Cytokine activity	
Penaeidin	Acts as cytokine (Li and Song 2010)
Bac7(1–35)	Activates defence response (Pelillo et al. 2014)
Cathelicidin OaBac5mini	Decreases the production of cytokine interleukin-12 (Yu et al. 2010)
A3-APO	Cytokines interleukin-4/interleukin-10 (Ostorhazi et al. 2011; Otvos et al. 2014)
Angiogenesis	
PR39	Proteasome inhibition/syndecans induction/angiogenesis stimulation (Anbanandam et al. 2008; Gaczynska et al. 2003; Gallo et al. 1994; Gennaro et al. 2002)

Gram-negative bacteria via cell wall interaction (Avitabile et al. 2014; Rolland et al. 2010). Together, these findings indicate additional intracellular targets, beyond DnaK binding and inhibition, and that PrAMPs have novel modes of action including potential macromolecular biosynthesis inhibition and LPS-binding activity.

Immunostimulation

Native host defence peptides, produced in innate immune cells, display antimicrobial activity against bacteria and fungi associated with anti-inflammatory/immunomodulatory activity (Hancock et al. 2012). These peptides have multiple mechanisms of action and possible additional therapeutic applications against antibiotic-resistant bacteria (Hilchie et al. 2013; Wilson et al. 2013). Several PrAMPs have been shown to modulate systemic immunostimulation (Table 2). For instance, the proline-rich domain of penaeidin imparts a pro-inflammatory cytokine-like property and modulates the immune system of the wound-induced inflammation response via autocrine activity (Li and Song 2010). Another PrAMP, Bac7(1–35), was shown to reduce the mortality of *S. typhimurium* infected mice by not only direct killing of the bacteria but also modulating the defence response of macrophages after phagocytosis of the bacteria (Benincasa et al. 2010; Pelillo et al. 2014). Further, the cathelicidin-derived OaBac5mini AMP has been shown to suppress the inflammatory responses induced by *S. aureus* (Yu et al. 2010). In addition, the designed PrAMP A3-APO has been shown to have greater in vivo (mouse infection model) than in vitro bactericidal efficacy, and this enhanced antimicrobial activity was demonstrated to be via deactivating bacterial toxins as well as up-regulating the expression of anti-inflammatory cytokines, interleukin (IL)-4 and IL-10 (Ostorhazi et al. 2011; Otvos et al. 2014; Szabo et al. 2010). As well as having antimicrobial activity, the PrAMP PR 39 acts as a proteasome inhibitor, and inducer of syndecans and angiogenesis to assist cell proliferation or new blood vessel growth (Anbanandam et al. 2008; Carmeliet 2000; Gaczynska et al. 2003; Gallo et al. 1994; Gennaro et al. 2002). These studies indicate that PrAMPs have diverse biological functions leading to microbial death. A3-APO and its monomeric analog accelerate wound healing with or without bacterial over-infection (Ostorhazi et al. 2013). By exploiting these actions of direct killing, modulation of immune cell function, cytokine secretion and up-regulating the wound repair process, novel strategies in the design of PrAMPs with enhanced immunomodulatory and antimicrobial activity can be developed.

Drug delivery properties of PrAMPs

As well as having direct antimicrobial activity, PrAMPs may also have potential in drug delivery. A common challenge for drug development is the in vivo and cellular distribution in the treatment of disease. Cell penetrating peptides (CPPs) have shown promise for the delivery of drugs to both bacterial and eukaryotic cells (Stewart et al. 2008), as well as for crossing the blood brain barrier (BBB) (Lalatsa et al. 2014). Although CPPs are attracting attention as potential drug delivery candidates, they can display cytotoxicity depending on cargo used and cargo coupling position (El-Andaloussi et al. 2007). However, PrAMPs are, in the majority, translocated across cellular membranes without inducing lysis or causing damage and display much less toxicity to mammalian cells. Thus, these peptides may have significant potential as CPPs or transport systems to deliver drugs to the target cells but may also be used to enhance antimicrobial activity (Otvos 2002; Scocchi et al. 2011) (Table 3). For example, based on the penetration ability of native PrAMPs, designed pyrrhocoricin analogues conjugated with class I major histocompatibility complex epitope successfully passed through bacterial and mammalian cells (Otvos 2002; Otvos et al. 2004). Furthermore, because of the dual function of cell permeability and antimicrobial action, bactenecin 7 or its fragments might represent a novel vector to deliver protein and phosphorescent oxygen-sensitive probe into various mammalian cells (Dmitriev et al. 2010; Sadler et al. 2002). Similarly, the PrAMP, PR39, as a complex with siRNA followed by translocation into breast cancer cells, was able to inhibit

Table 3 Proline-rich AMPs able to penetrate cell membranes and cross blood brain barrier as novel potential carriers for drug delivery

Cell penetrating peptides (CPP)	
Pyrrhocoricin	Delivery of peptidic/epitope based cargo (Otvos et al. 2004; Rappocciolo 2004)
Bactenecin 7	Carrier for protein cargo and phosphorescent oxygen sensor (Dmitriev et al. 2010; Sadler et al. 2002)
PR39	Delivery of siRNA into 4T1 cells (Tian et al. 2012)
Blood brain barrier (BBB) crossing	
Oncocin	Mostly destined for endothelial cells (Stalmans et al. 2014)
Apidaecin (Api137)	Mainly trapped in the brain parenchyma (Stalmans et al. 2014)
Drosocin	
Drosocin (Pro5Hyp)	

cell invasion and immigration (Tian et al. 2012). Furthermore, a recent report showed that several PrAMPs, including oncocin, apidaecin (Api137), drosocin and drosocin (Pro5Hyp), could cross the BBB to selectively target brain cells (Stalmans et al. 2014). This indicated that the PrAMPs would not only be potential therapeutics for cerebral infections, but also novel potential carriers for brain drug delivery. It needs to be added that passive transport (in our case using the abundant positive charges) has high capacity but low affinity as opposed to active (receptor-mediated) transport (Fasano 1998). In turn, the presumably increased antibacterial peptide and cargo load required for successful cell penetration of the complex after intracellular processing may lead not only to hemolysis due to the AMP part, but also offset effects of the abundant cargo.

Bacterial resistance and PrAMPs

Even though AMPs have significant potential as alternatives to current small molecule antibiotics (Marr et al. 2006), there are reports of development of bacterial resistance against them. They may be due to proteolytic cleavage, external inactive-molecules, specific exporters and electrostatic repulsion (Kraus and Peschel 2006). However, because PrAMPs possess multiple targets within bacterial cells, it is believed that bacteria are less likely to become resistant to them. In general, PrAMPs enter into bacteria through the inner membrane via the translocation of permease/transporter-mediated uptake mechanism involved membrane protein SbmA (Runti et al. 2013). Recently, partial antimicrobial resistance of PrAMPs has been observed with co-cultured *E. coli* mutants with the deletion of *SbmA* gene (Narayanan et al. 2014). While this finding suggests that caution is required when evaluating PrAMPs as potential therapeutics, the designed PrAMP dimer, A3-APO, has not yet been shown to display a propensity to induce antimicrobial resistance. Its monomeric variant does, but alterations in the genes encoding neither its intracellular target DnaK nor the SbmA transport protein have not been implicated, which highlights the potential of A3-APO and its monomer as alternatives to replace or supplement antibiotics (Cassone et al. 2009).

Summary

In the fight against multi-drug resistant microbes, PrAMPs display potent activity when supplementing conventional antibiotics. These peptides show various modes of action against microbes and, furthermore, their mechanism can shift from non-disruption to membrane damage depending on the AMP and concentration and can modulate the immune system and affect different potential intracellular targets. By understanding the mechanisms and rationally altering PrAMPs synthetically, it may in turn lead to the development of more effective antimicrobial alternatives. In addition to their antimicrobial activities, PrAMPs also display the ability to penetrate cell membranes and to cross the BBB which may afford exciting new opportunities for drug delivery as a potential drug carrier.

Acknowledgments We acknowledge partial support of the studies undertaken in the authors' laboratory by the Australian Research Council (DP150103522) to MAH and JDW and the National Health and Medical Research Council (NHMRC) Grant APP1029878 to NMOBS. JDW is an NHMRC (Australia) Principal Research Fellow. Research at the FNI was supported by the Victorian Government's Operational Infrastructure Support Program.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard The manuscript does not contain clinical studies or patient data.

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