Proline-Rich Antimicrobial Peptides: Multiple Potential Therapeutics Against Antibiotic Resistant Bacteria

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Abstract

The increasing resistance of pathogens to antibiotics is causing a huge clinical burden that is placing 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 possess 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.

The trend of antimicrobial resistance development to conventional antibiotics has increased more rapidly, despite the availability of new classes antibiotics launched in recent years (Butler et al. 2013). The period of adapting resistance to these antibiotics is also becoming increasingly short (Hede 2014; Hsueh 2012; Zhang et al. 2006) (Figure 1). The resistance rates of *Escherichia coli* and methicillin-resistant *Staphylococcus 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 (Cully 2014; Zhu et al. 2013). Consequently, much research is ongoing to investigate the mechanism of antibiotic resistance and seeks to develop new antibiotics possessing various mechanisms of action (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 microorganism (Brogden 2005; Lijuan Zhang 2006). Generally, AMPs have a net positive charge due to the presence of several lysines/arginines, 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) (Figure 2). However, there is increasing evidence that AMPs can kill targeted cells 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) (Figure 1).

Many AMPs have been isolated from invertebrates, insects, plants and fungi, which are divided into groups, cationic AMPs and non-cationic AMPs with diversity of structure motifs including linear peptide of α -helices, β -strand stabilized via one or more disulfide bonds (Dimarcq et al. 1998; Pushpanathan et al. 2013). Based on their structural motif features, these AMPs display potent antimicrobial activity and specificity to the targeted cells. Beyond them, there are also some AMPs with high content of one or two amino acids, such as proline (Otvos 2002), arginine (Chan et al. 2006; Dong et al. 2012) and glycine (Baumann et al. 2010; Ilić et al. 2013). Among these different types of AMPs, proline-rich AMPs (PrAMPs) have attracted special attention due to their wide distribution in insects and unique mechanism of killing bacteria without cell membrane disruption (Otvos 2000). There are several reviews relating to the characteristics of PrAMPs, including isolation (Otvos 2000), non-lytic mechanism (Scocchi et al. 2011), 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; Gennaro et al. 2002; Shamova et al. 1999), crustaceans (Destoumieux et al. 2000; Stensvag et al. 2008), *annelidae* (Cho et al. 1998) and especially insects (Bulet et al. 1993; Bulet et al. 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 possessing different 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, exhibit 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 more potent PrAMPs bearing a range of selective mechanisms of action.

Several reviews have discussed the action of intracellular inhibition in which PrAMPs show inhibition of the 70 kDa bacterial heat shock protein, DnaK, resulting in protein misfolding and aggregation and subsequent bacterial death (Otvos 2005). Furthermore, the binding site of inhibition was revealed *via* crystal structures of *Escherichia coli* Hsp70 chaperone DnaK (Otvos et al. 2000; Rozgonyi et al. 2009; Zahn et al. 2013; Zahn et al. 2014). However, there were additional indications of novel modes of action against the targeted bacteria. PrAMPs generally penetrate the outer membrane of Gram-negative bacteria and translocate *via* a permease/transporter-mediated uptake, but there is no increase in activity following engineered increased of the AMP cell penetration (Berthold and Hoffmann 2014). Furthermore, apidaecin IB was found not only to depress the GroEL/GroES chaperone team, but also resulted in the unbalanced biosynthesis of *Escherichia coli* lipopolysaccharide (LPS) and phospholipids (Zhou and Chen 2011). Moreover, Ls-Stylicin 1, which has a proline-rich N-terminal region, displayed potent LPS-binding activity *in vitro*, suggesting potential selective action against Gram-negative bacteria *via* cell wall interaction (Avitabile et al. 2014; Rolland et al. 2010). These findings indicate additional intracellular targets, beyond DnaK binding and inhibition, and novel modes of action including macromolecular biosynthesis inhibition and LPS-binding activity to attack targeted cells.

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 acts as a pro-inflammatory cytokine and modulated the immune system of the wound-induced inflammation response *via* autocrine activity (Li and Song 2010). Another PrAMP, Bac 7 (1-35), was shown to reduce the mortality of a pathogen infected mice model and modulated the defence response of macrophages after phagocytosis. As well, the cathelicidin-derived OaBac5mini showed the ability to suppress the inflammatory responses induced by pathogens (Pelillo et al. 2014; Yu et al. 2010). Furthermore, the designed PrAMP A3-APO showed more efficient *in vivo* than *in vitro* bactericidal abilities, which had been demonstrated in an infection model mouse with immunostimulatory properties *via* deactivating bacterial toxins, as well as upregulating the expression of anti-inflammatory cytokines, interleukin-4 and interleukin-10 (Ostorhazi et al. 2011; Otvos et al. 2014; Szabo et al. 2010). On the other hand, PR 39 not only acted as the inhibitor proteasome, but as the 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). PrAMPs have also been shown to modulate the expression of inflammatory cytokines and upregulate the wound repair process, which attack pathogens synergistically, suggesting a novel strategy to design and develop potent PrAMPs with enhanced immunomodulatory activity.

Drug delivery properties of PrAMPs

A common challenge for drug development is its distribution for treatment of disease. Cell penetrating peptides (CPPs) have shown promise as a means to deliver 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). As discussed above, the mechanism of PrAMPs may not only involve membrane damage but also membrane penetration into cells. This indicates that much peptides have significant

potential as CPPs or transport systems to deliver drugs with antimicrobial activity (Otvos 2002; Scocchi et al. 2011) (Table 3). For example, based on the penetration ability of native PrAMP, designed pyrrolicin 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, battenecin 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 cell invasion and immigration (Tian et al. 2012). Furthermore, a recent report showed that several PrAMPs, including oncocin, apidaecin (Apl137), drosocin and drosocin (Pro5Hyp), could cross the BBB to selectively target brain cells (Stalmans et al. 2014). This indicated the PrAMPs would not only be potential therapeutics for cerebral infections, but also novel potential carriers for brain drug delivery.

Mechanism resistance of 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, compared with other cationic AMPs, the designed PrAMP, A3-APO, displayed considerably less propensity to induce antimicrobial resistance or genetic alteration within its intracellular target, which highlighted its potential as an alternative to replace antibiotics (Cassone et al. 2009). In general, PrAMPs entered the bacteria through the inner membrane *via* the translocation of permease/transporter-mediated uptake mechanism involved membrane protein SbmA (Runti et al. 2013). However, partial antimicrobial resistance of PrAMPs has been observed with co-cultured *Escherichia coli* mutants with the deletion of *SbmA* gene (Narayanan et al. 2014). This indicates a potential induced-resistance

mechanism and the need to further develop PrAMPs with multiple targets to better combat antibiotic resistance.

Summary

In the fight against multi-drug resistant microbes, PrAMPs display potent activity when supplementing conventional antibiotics. Even though mutant *Escherichia coli* lacking the *SbmA* gene have shown the onset of resistance to PrAMPs, these peptides showed various modes of action against microbes. Furthermore, their mechanism shifts from non-disruption to membrane damage depending on concentration and can modulate the immunity system and affect different potential intracellular targets. This in turn could lead to 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 could lead to a new strategy for drug delivery as a potential drug carrier.

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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), caerin 1.1 (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), pyrrolicorin (Bencivengo et al. 2001; Chesnokova et al. 2004), drosocin (Chesnokova et al. 2004), apidaecin (Casteels et al. 1989), aiptercin (Chitnis et al. 1987; Chitnis et al. 1990)

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)

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)	

FIGURE LEGENDS

Figure 1. **The period for resistance to emerge for conventional antibiotics after their release.**

These includes penicillin, tetracycline, erythromycin, methicillin, gentamicin, vancomycin, imipenem, ceftazidime, levofloxacin, linezolid, ceftaroline. This figure has been modified with permission from Hede (2014).

Figure 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.

FIGURE 1.

FIGURE 2.