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MULTI-AUTHOR REVIEW

Proline-rich antimicrobial peptides: converging to a non-lytic mechanism of action

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Abstract Proline-rich antimicrobial peptides are a group of cationic host defense peptides of vertebrates and invertebrates characterized by a high content of proline residues, often associated with arginine residues in repeated motifs. Those isolated from some mammalian and insect species, although not evolutionarily related, use a similar mechanism to selectively kill Gram-negative bacteria, with a low toxicity to animals. Unlike other types of antimicrobial peptides, their mode of action does not involve the lysis of bacterial membranes but entails penetration into susceptible cells, where they then act intracellularly. Some aspects of the transport system and cytoplasmic targets have been elucidated. These features make them attractive both as anti-infective lead compounds and as a new class of potential cell-penetrating peptides capable of internalising membrane-impermeant drugs into both bacterial and eukaryotic cells

Keywords Antimicrobial peptide · Proline-rich peptide · Antibiotic · Mode of action · Non-lytic mechanism

Abbreviations

AMP	Antimicrobial peptide
CPP	Cell-penetrating peptide
LPS	Lipopolysaccharide
PR-AMP	Proline-rich AMPs
HDP	Host defence peptide

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Introduction

The term 'antimicrobial peptide' (AMP) is used to describe a diverse group of innate immune effector molecules utilized by multicellular organisms to prevent or combat microbial infections. Endogenous AMPs, as distinct from artificial ones, are also referred to as host defense peptides (HDPs). Several categories of AMPs have been described based on common structural features or conserved sequence motifs. These molecules recur throughout the living world and have been extensively reported in numerous reviews (for examples, see [1–3]).

AMPs typically do not target specific microbial molecules, but rather interact directly with and rapidly permeabilize microbial membranes—in other words, they act principally via 'lytic' mechanisms. Their amino acid compositions lead to structural properties, in terms of amphipathicity, cationic charge, shape and size, which favor interaction with the microbial surface, insertion into the lipid bilayer and induction of membrane lesions. Several different models have been proposed to depict the consequences of this mode-of-action, including the 'barrel-stave' and 'toroidal-pore' mechanisms, the formation of 'aggregate channels' and the detergent-like 'carpet' effect (reviewed in [2]). 'Lytic' AMPs generally have broad spectrum activity covering both Gram-positive and Gram-negative bacteria as well as fungi, while their selectivity for microbial with respect to host cells is proposed to derive from distinctive features of microbial membranes [4].

Some AMP classes, and in particular the proline-rich antimicrobial peptides which are the focus of this review, do not conform to this predominant lytic mode of action. Proline-rich AMPs (PR-AMPs) are a group of peptides of widespread natural origin, whose quite diverse sequences show some common characteristics: (1) the unusually high

content of proline residues, (2) a net cationic charge due mainly to the presence of arginine residues, and (3) the fact that they act without extensive membrane damage, mostly targeting Gram-negative bacteria [5–10]. Furthermore, unlike 'lytic' AMPs for which the all-*D* enantiomers generally display the same activity as the natural all-*L* counterparts, all-*D* PR-AMP analogues are much less active or completely inactive [7, 8, 11]. These observations, together with the fact that some PR-AMPs have been shown to penetrate into bacterial cells by translocating across the membrane [7, 12], suggest a stereospecific mode of action involving interactions with a transport system followed by inhibition of specific intracellular target(s), rather than a non-specific disruption of membrane integrity. This hypothesis is consistent with the narrower activity spectrum.

Diversity of proline-rich antimicrobial peptides

PR-AMPs were first reported from organisms as diverse as honeybees [5] and cattle [10], and were subsequently found in a number of other insect and mammalian species [9, 13] and also in amphibians [14], crustaceans [15–17] and molluscs [18]. There are sufficient differences in origin, gene structure and primary sequence to suggest that the different families are the product of convergent evolution, although they are all characterized by a high content of proline (typically from 25 to 50%), as well as of arginine (conferring a net positive charge), often arranged in short recurrent motifs.

All mammalian PR-AMPs belong to the cathelicidin group of host defence effectors, which with the defensins represent the two most widespread families of vertebrate HDPs. Cathelicidins are characterized by a well-conserved N-terminal pre-proregion and a highly variable C-terminal domain, corresponding to the AMP, which becomes active after proteolytic release [19–21]. To date, mammalian proline-rich cathelicidins have been found only in artiodactyls. The first to be identified, Bac5 and Bac7, were isolated in our laboratory from bovine neutrophils (see Table 1) [10, 22], later followed by a putative pseudogene containing the sequence of a third bovine PR-AMP, Bac4 [23]. Their orthologues have been identified in other bovids, including sheep and goat [24], while additional members have been detected or purified in sheep, including OABac11 and OABac6 [25]. Pig leukocytes express other PR-AMPs, PR-39 [26] and prophenins [27]. Sequence analyses indicate these peptides have an active and complex evolutionary history, with some duplication events preceding and others following species radiations. Thus, while an evolutionary relationship likely exists between PR-39 and the bovine and ovine PR-AMPs, despite quite divergent sequences [28], the primary structure of the ~80 residue prophenins, formed from ten-residue repeats, appears to have no counterpart in the other artiodactyls [27].

Despite all belonging to the cathelicidin family of precursors and having the characteristic high content of Pro and Arg residues, mammalian PR-AMPs have quite different lengths and primary sequences, and are characterized by the presence of different types of repeated tetramer motifs of the type PPRX or PRPX, where X is most often a bulky hydrophobic residue or Gly (see Table 1).

Apart from cathelicidins, other types of proline-rich AMPs are apparently not common in mammals. A 1,905-Da cationic peptide, named basic proline-rich peptide or SP-B, was isolated as the main component of porcine salivary gland granules, being present as multiple repeated units in a large polypeptide precursor [29]. This weakly cationic peptide (net charge +1) is extraordinarily rich in proline residues (see Table 1), and was found to possess antifungal activity, but negligible antibacterial activity. It is unclear if similar proline-rich peptides from the salivary glands of other mammals, including cattle and primates, act in a similar manner. Regarding other vertebrates, a proline-rich peptide named PR-bombesin has been isolated from the skin of the toad *Bombina maxima* (see Table 1). Despite sharing features with the well-known bombesin family of α-helical amphibian AMPs, an 8-residue, N-terminal segment comprises four proline and three basic residues, including the Pro-Arg-Pro (PRP) motif considered to play a relevant role in some insect PR-AMPs. PR-bombesin displays a moderate antimicrobial activity, but with a wider spectrum than mammalian PR-AMPs, and may adopt a loose beta-hairpin structure, normally only observed in AMPs stabilized by one or more disulfide bonds [14].

Most known PR-AMPs have been isolated from insects (various species of Hymenoptera, Diptera, Hemiptera and Lepidoptera) and have been subdivided into two types: short-chain and long-chain peptides [30] (see Table 2). Their names reflect their origin rather than a systematic subdivision among the individual sequences [13]. Comparison of the primary sequences as well as the gene structure of vertebrate and invertebrate PR-AMPs suggests that they are not evolutionarily related. Rather, the presence of similar motifs comprising proline and arginine residues suggests the convergent evolution to a similar mechanism for both accessing and inactivating their microbial targets (see below).

Drosocin, a 19-residue peptide isolated from *Drosophila melanogaster,* contains three repeats of the PRP motif, evenly distributed along the sequence, and is also characterized by an O-glycosylation site in the middle (Thr11) [31] (see Table 2). Apidaecins are the earliest insect PR-AMPs to be described, having been isolated from lymph fluid of honeybees (*Apis mellifera*) infected with bacteria, with three isoforms present in a multipeptide precursor [5].

Table 1 Proline-rich antimicrobial peptides from vertebrate animals

Name	Origin	Sequence[a]	N° AA	Z[c]	Pro (%)	Repeated motifs
PR-39	pig	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP-NH2	39	+11	49	PPRX (× 4)
PR-39[b]	pig	RLRRQAFPPPIFPGPGFRPPIFPPPPFRPAPFGPPRFP-NH2	(38)[d]	+7	42	PPRX (× 1)
prophenin	pig	AFPPPNFPGPRFPPPNFPGPRFPPPNFPGPRFPPPNFPGPRFPPPNFPG PPFPPPIFPGPWFPPPPPFRPPPFGPPRFP-NH2	79	+7	53	PPPX (× 8)
Bac3.4	goat	RFRLPFRRPPIRIHPPPFYPPFRRFL-NH2	26	+8	31	PPXR (× 2)
Bac5	cow	RFRPPIRRPPIR---PPFYPPFRPPIRPPIFPPIRPPFRPPLGPFP-NH2	43	+10	47	PPXR (× 7)
Bac5	sheep	RFRPPIRRPPIR---PPFNPPFRPPVRPPFRPPFRPPFRPPIGPFP-NH2	43	+11	47	PPXR (× 7)
Bac5	goat	RFRPPIRRPPIR---PPFRPPFRPPVRPPIRPPFRPPFRPPIGPFP-NH2	43	+12	47	PPXR (× 8)
Bac4[b]	cow	RRLHPQHQRFPRER--PWPKPLSLPLPRPGPRPWPKPL-OH	(36)[d]	+6	33	PRPX (× 2)
Bac6	sheep	RRLRPRHQHFPSER-RPWPKPLPLPLPRPGPRPWPKPLPLPLPRPGLRPWPRPL-OH	53	+12	38	PRPX (×4)
Bac7	cow	RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRPL-OH	60	+17	47	PRPX (× 11)
Bac7	buffalo	RRFRPRRPRLPRPRPRPLPLPWPRPRPIPRPLPLPWPGPRPIPRPLPLPRPGPRPIPL-OH	58	+16	45	PRPX (× 8)
Bac7	sheep	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPQPRRIPRPILLPWRPPRPIPRPQPQPIPRWL-OH	60	+20	38	PRPX (× 7)
Bac7	goat	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPQPRRIPRPILLPWRPPRPIPRPQPQPIPRWL-OH	60	+20	38	PRPX (× 7)
Bac 11	sheep	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPKPRPIPRPLPLPRPRPKPIPRPLPLPRPR PRRIPRPLPLPRPRPRPIPRPLPLPQPQPSPIPRPL-OH	94	+28	45	PRPX (× 15)
PRP-SP-B	pig	APPGARPPPGPPPPGPPPPGP-OH	21	+1	67	PPPGP (× 3)
PR-bombesin	toad	ŒKKPPRPPQWAVGHFM [e]	16	+3	25	PP (× 2)

[a] Tandem repeats present in some sequences are underlined with alternating single and dashed underlines

[b] Sequence is from a putative pseudogene

[c] Net charge, His residues are considered neutral

[d] Putative peptide size. Charge and percentage of proline residues are based on this sequence

[e] Œ Pyroglutamic acid

They are the most prominent components of the honeybee humoral defence against microbial invasion [32]. Several more peptides belonging to this family have since been isolated from other bees, wasps and hornets belonging to the Apocrita suborder of Hymenoptera, as outlined in Table 2. They share a high degree of sequence identity with clearly conserved residues, notably an RP or PRP motif in the N-terminal portion and the eight-residue PRPPHPRL motif at the C-terminus (see Table 2) [33]. The central and N-terminal regions are more variable. Several different isoforms present on the same insect derive from a single precursor [32].

Pyrrhocoricin, a well-studied PR-AMP isolated from a hemipteran insect, the fire bug (*Pyrrhocoris apterus*) [34], presents some similarities to drosocin. A close pyrrhocoricin analogue from the bean bug (*Riptortus clavatus*), [35] is expressed as a multipeptide precursor with 14 tandem repeats. Twelve of these repeats (from the 2nd to the 13th) are identical and have an N-terminal stretch that closely resemble pyrrhocoricin, followed by a C-terminal stretch which may be an anionic spacer region. In the first repeat, however, the N-terminal stretch has the typical apidaecin eight-residue sequence (see Table 2). Metalnikowin, an inducible PR-AMP from a third hemipteran, the shield bug *Palomena prasina* [34], is also clearly related, albeit with a truncated sequence.

A distinguishing feature of pyrrhocoricin, in common with drosocin and the ant PR-AMPs formaecins [36], is the O-glycosylation of a conserved, centrally located threonine residue (see Table 2), which is relevant for the antimicrobial activity (see below).

Long-chain PR-AMPs have been isolated from *Drosophila* (metchnikowin) [37] and moths (lebocins) [38], as well as from several hymenopteran species (abaecins) [39] (see Table 2). The fact that they, respectively, derive from dipteran, lepidopteran and hymenopteran species likely explains the relatively low sequence identity among them. Lebocins, like some short-chain PR-AMPs, are also glycosylated at a central threonine residue [38], as are the heliocins, PR-AMPs with putative analogues in several moth species. Heliocin-like peptides seem to derive from an upstream sequence in lebocin-like polypeptide precursors [40, 41]. Both types of PR-AMPs are principally active against, and their expression is induced by, Gram-negative bacteria.

PR-AMPs are also common in other types of arthropods, and several have been recently identified in different crustacean species [42]. The first of these to be isolated and partially sequenced, a 6.5-kDa peptide found in the hemocytes of *Carcinus maenas* (see Table 3), curiously shows a significant sequence similarity with the bovine Bac7 [15]. It has, however, yet to be fully characterized, and the known

Table 2 Insect proline-rich antimicrobial peptides

Name	Origin	Sequence[a]	N° AA	Z[b]	Pro (%)
short-chain					
Apidaecins	**honeybee**	GnnRPvyipqPRPPHPRl	18	+3	33
	bumblebee	anRPvyippPRPPHPRl	17	+3	41
	sphecoid wasp	nRPtyvppPRPPHPRl	16	+3	44
	vespoid wasp	snkPRPqqv-pPRPPHPRl	16	+3	44
	Ichneumon wasp	gkpnkPRPapi-kPRPPHPRl	20	+6	40
	hornet	gkpRPqqv-pPRPPHPRl	17	+3	41
Pyrrhocoricins	**fire bug**	VDKGSYLPRP**T**--PPRPIYNRN [c]	20	+3	25
	bean bug (putative)	VDKGGYLPRPT--PPRPVYRSR [d]	20[f]	+3	33
		GDKPVYLPRPT---PPRPIHPRL [e]			
Metalnikowins	**shield bug**	VDKPDYRPRP----wPRnm	15	+2	~30
Drosocin	**fruit fly**	GKPRPYSPRP**T**SHPRPIRV	19	+5	32
Formaecins	**ant**	GRPNPVNnKP**T**PhPRL	16	+3	31
long-chain					
Metchnikowin	**fruit fly**	HRHQGPIFDTRPSPFNPNQPRPGPIY	26	+2	27
Heliocins	**moths**	ŒRfIhP**T**yRPPpqprRPvImR	19-21	+3/4	~25
Lebocins	**moths**	DLRFlyPRgKLPvP**T**pPPFNPKPIYIDMGNRY	32	+2/3	25
Abaecins	**honeybee**	YVPLPNiPQPGRRPFPTFPGQGPFNPKIRWPQGY	34	+4	30
	bumblebee	FVPYNPPRPGQSKPFPTFPGHGPFNPKiQWPYPLPNPGH	39	+4	33
(putative)	**pteromalid wasp**	YVPPVQKPHPNGPKFPTFPGQGTWSGRPRRSPQKNG	36[f]	+3	~25
(putative)	**ant**	YqPPVKPPPPGGWKPFPTFPGQGPYNPKIRlpH	33[f]	+4	~35

[a] A representative sequence amongst several variants is shown. Residues in uppercase are highly conserved; those in lowercase are less conserved. Glycosylated residues are in bold. Gaps have been inserted to improve proline-rich motif alignment. Variants were obtained by blasting the respective type sequence [8, 31, 32, 34–36, 38, 39, 106–108] against the Uniprot database

[b] Net charge, His residues are considered neutral. Variants may have different cationicity as indicated

[c] O-glucosylated threonine residues are bold and underlined

[d] From repeats 2 to 13 of the *Riptortus clavatus* prosequence [35]

[e] From repeat 1 of the *Riptortus clavatus* prosequence [35]

[f] Putative peptide size. Charge and percentage of proline residues are based on this sequence

fragment might be part of a bipartite proline-rich/cysteine-rich AMP as found with other crustacean peptides. An abundant family of peptides, named penaeidins, has recently been described from several species of shrimp [42–43]. These 47- to 63-residue peptides display a proline-rich N-terminal domain, justifying their inclusion among the PR-AMPs, but also a C-terminal domain characterized by the presence of three conserved disulfide bonds, more typical of beta-sheet AMPs such as the defensins (see Table 3). The solution NMR structure of recombinant penaeidin-3a from *Litopenaeus vannamei*, in fact showed that the proline-rich domain (residues 1–28) was unconstrained, while the cysteine-rich domain (residues 29–58) displayed a well-defined structure stabilized by the three disulfide bonds [44]. Penaeidins are polymorphic, and three distinct classes (PEN2, PEN3, and PEN4) are expressed in the hemocytes of *L. vannamei* [45]. Unlike the insect peptides, however, these PR-AMPs have a broad spectrum of antibacterial activity covering fungi and Gram-positive bacteria. This characteristic is also reported for the 14-amino acid long astacidins, PR-AMPs isolated from different crayfish species, despite the absence of a Cys-rich domain. Astacidin sequences present a characteristic repeated RPxY motif (Table 3) [46].

The bipartite arrangement of Pro-Arg-rich and disulphide bridged domains is also found in arasins, recently identified in spider and mud crabs [47–48], and callinectin from the blue crab [49] (see Table 3). They both present a disordered N-terminal Pro-Arg-rich domain and a structured C-terminal domain, in this case stabilized by only two disulfide linkages. Arasin-1 is expressed as a

Table 3 Other invertebrate proline-rich antimicrobial peptides

Name	Origin	Sequence[a]		N° AA	Z[c]	Pro (%)
Penaeidins	shrimp		C_1-C_3,C_2-C_5,C_4-C_6[e]			
pen2[d]		yrGGyTgPipRPPpigrpp———(frpv)————Cna-CYrlsvSDarnCCikfGsCChlvK-NH2		23-24[b]	+3-5[b]	20-35[b]
pen3		qvYkGgYTRPiPRPpp—(fvrplpgggpigpyng)—CpvSCrgisfsQARsCCsrLGRCChvgKgys-NH2		21-31[b]	+3-5[b]	15-30[b]
pen4		hSSGYTRPLRKPSRPIFIRPIG————————CDV-CYGIPSSTARLCCFRYGDCCHl-NH2		22[b]	+4[b]	18[b]
Arasins	spider crab	SRWPSPGRPRPFPGRPKPIFRPRPCNCYAPPCPCDRW	(C_1-C_4,C_2-C_6)[f]	37	+7	33
Callinectin	blue crab	WNSNRRFRVGRPPVVGRPGCVCFRAPCPCSNY-NH2		32	+6	16
Bac6.5[g]	common crab	TSVPYPRPFPRPPIGPRPLPFPGGGRPFQS...				
Astacidins	crayfish	RPRPNYRPRPIYRP-NH2		14	+6	36
		RPAYRPAYRPSYRP-NH2		14	+6	29
Cg-PRP	oyster	DTGPIRRPKPRPRPRPEG-NH2[h]		18	+5	33

[a] A representative sequence amongst variants is shown. Residues in uppercase are highly conserved; those in lowercase are less conserved. Cys residues involved in disulfide bonds are in bold. For penaeidins, Pro-rich and Cys-rich domains, aligned separately, are connected by a stretch of different size, indicated as brackets. Variants were obtained by blasting the respective type sequence [15, 18, 42, 47–49, 109] against the Uniprot database

[b] Based on Pro-rich domain only

[c] Net charge, His residues are considered neutral

[d] The nomenclature of the PenBase penaeidins database is used (see [109])

[e] Disulfide bridging pattern

[f] Putative disulfide pattern

[g] Only the N-terminal sequence of a 6.5-kDa protein is known

[h] Putative mature peptide

64-residue propeptide with a 25-residue hydrophobic propiece. A synthetic version of mature arasin-1 has been confirmed to have antibacterial activity [48].

The fact that the crustacean peptides often show a bipartite Pro-rich/Cys-rich structure, and that the Pro-rich stretch on its own is active against Gram-positive microorganisms, indicates that these are a functionally different class of PR-AMPs to the mammalian and insect ones mainly targeting Gram-negative bacteria.

A first putative PR-AMP from a mollusc has been recently reported in the oyster *Crassostrea gigas* [18], consisting of a cDNA sequence encoding a 61 amino acid polypeptide precursor with a hydrophobic signal peptide/propiece. On release, the 37-residue mature peptide is composed of an acidic region and a cationic Pro-rich region. The antimicrobial activity is limited, although it seems to synergise with oyster defensins in killing bacteria.

Antimicrobial and other host defence activities of PR-AMPs

One of the more striking features of mammalian and insect PR-AMPs is a remarkably similar and selective activity spectrum directed against Gram-negative bacteria, and especially Enterobacteriaceae, being active in the low micromolar concentration range (see Table 4). At these concentrations, most Gram-positive microorganisms are not affected.

Susceptible microorganisms for mammalian PR-AMPs are *Escherichia coli*, *Salmonella enterica*, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*. Among the Gram-positive species, only those that are in any case known to be easily killed by AMPs in general, such as *Bacillus megaterium, Bacillus subtilis* and *Listeria monocytogenes*, are affected, albeit at higher concentrations [10, 24, 26, 50, 51]. PR-39 was found to be active against drug-susceptible and multi-drug-resistant clinical isolates of *Mycobacterium tuberculosis* [52], while Bac5 and Bac7 were reported to be active against various species of *Brucella* [53] as well as against spirochetes, such as *Leptospira* but not *Borrelia* species [54]. These PR-AMPs are ineffective towards *Burkholderia cepacia* [55], a species which is resistant to most tested AMPs. When tested against fungi, the functional N-terminal fragment Bac7(1-35) was active against collection strains and isolates of *Cryptococcus neoformans*, but not of *Candida albicans* [55].

A similar spectrum of activity has been reported for the ovine orthologues of Bac5 and Bac7, as well as their

Table 4 Antimicrobial activity of some representative PR-AMPs

MIC (μM)[a]				
Peptide	*E. coli* K12[b]	*E. coli* ATCC 25922	*S. typhimurium* ATCC 14028	
Bac7	1	1	1	[10, 55]
Bac7 (1-35)	0.5	0.5	0.5	[55, 80]
Bac5	1	0.5	1	[10, 55, 65]
PR-39[c]	nd	1	1	[50]
PR-39 (1-15)	0.5	nd	0.25	[51]
Apidaecin Ib[d]	8	8	4-8	[75, 80]
Pyrrhocoricin[d]	2	nd	nd	Unpublished

[a] MICs of the peptides were all determined using an identical protocol to allow direct comparison: the broth microdilution susceptibility test was carried out following the guidelines of the NCCLS with mid-log phase cultures. Serial twofold dilutions of each peptide were prepared in 96-well polypropylene microtiter plates in Muller–Hinton (MH) broth (final volume of 50 μL). A total of 50 μL of the adjusted inoculum ($1–5 \times 10^5$ cells/mL) in MH broth was then added to each well. The MIC value was defined as the lowest peptide concentration that prevented visible bacterial growth after incubation for 18 h at 37°C

[b] The following K12 strains were used: HB101 to test Bac7 (1-35), Bac5 and apidaecin Ib; MC4100 to test Bac7 (1-35) and pyrrhocoricin; D21 to test Bac7 and PR-39 (1-15)

[c] A slightly different broth microdilution method was used [50]

[d] MICs were determined as above described but with 50% Muller–Hinton (MH) broth in phosphate-buffered saline (PBS)

functional fragments. The concentrations required to kill Gram-negative bacteria such as *E. coli* and *S. enterica* are about two orders of magnitude lower than those needed to kill the Gram-positive bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Enterobacter faecalis* [56].

Insect drosocin, pyrrhocorycin and apidaecins have overlapping activity spectra directed towards *E. coli*, *S. enterica*, *K. pneumoniae*, and *Agrobacterium tumefaciens*, and are active in the sub- to low micromolar concentration range [11, 13] (see Table 4). Apidaecins are also effective against *Pseudomonas aeruginosa,* while drosocin-susceptible Gram-negative strains include *E. cloacae* and *Erwinia carotovora* [13]. As with the mammalian PR-AMPs, the only Gram-positive species that show some susceptibility are *Micrococcus luteus* and *B. megaterium*; otherwise, in their native forms, they do not appear to affect major Gram-positive bacterial or fungal pathogens [13].

The activity of crustacean PR-AMPs, and in particular the penaeidins, is instead quite different: Pen-2 and -3a have broad spectrum antifungal properties and their antibacterial activities are essentially directed against Gram-positive bacteria, with a strain-specific inhibition mechanism [16]. This quite different specificity does not seem to be dependent on the presence of the cysteine-rich domain, as the proline-rich domain on its own maintains activity against Gram-positive species [46, 57].

In addition to their antibacterial action, some PR-AMPs exert other functions within the immune system of the producing animals that contribute to host defence. PR-39 seems to have a plethora of such functions, among which the capacity to up-regulate the expression of cell surface proteoglycans in fibroblasts [58], to induce chemotaxis of neutrophils [59], to promote angiogenesis [60] and to alter macrophage viability by inhibiting apoptosis [61], as has been reviewed in detail elsewhere [9]. In addition to this, in a mouse model of endotoxaemia, PR-39 induced elevated levels of NO in liver [62] and was effective in improving survival of animals following a septic episode [63]. At variance with the wealth of information on the effects of PR-39 on host cells, relatively little is known about the role(s) of other proline-rich AMPs besides their direct antimicrobial activity. The functional fragment Bac7(1-35) [64] and Bac5 [65] are rapidly internalized into 3T3 and U937 cells by a nontoxic energy- and temperature-dependent process, apparently via the concurrent contribution of macropinocytosis and direct membrane translocation [64]. On cell penetration, they may have stimulatory functions, such as enhancing S phase entry of 3T3 cells, but whether this correlates with host defence functions has as yet to be determined.

PR-AMPs are well tolerated by eukaryotic cells, and generally display a very low toxicity. Peptides based on both mammalian and insect PR-AMPs have been used in animal model studies, at concentrations well above the antimicrobial ones, without showing adverse effects [66, 67].

Conformation and structure–activity relationships

For many mammalian and insect PR-AMPs, over 30% of residues are proline, an amino acid with unique effects on secondary structural elements, and which is incompatible

with α-helical or β-sheet conformations. Proline-rich sequences tend to adopt the poly-L-proline type II helical conformation (PP-II helix), an extended structure with three residues per turn. The circular dichroism spectrum of porcine PR-39 in water is indeed compatible with this conformation, and is not affected by the presence of liposomes, indicating that interaction with membranes does not markedly modify its structure [68]. CD or NMR spectroscopic studies of Bac5 and its functional fragments, in aqueous solution and in the presence of lipid vesicles, led to similar conclusions [69–71]. Apidaecins and drosocin are also reported to form the PP-II helix [32, 72]. This suggests that the PP-II helix may be the biologically active conformation for all these peptides. NMR studies of penaeidins Pen-3 and Pen-4 provided a direct visualization of the unconstrained proline-rich domain, which is extended and contrasts markedly with the cysteine-rich domain that has a similar fold to that observed for defensins [73].

Although structural aspects for mammalian and insect PR-AMPs are mostly inferred from low resolution methods such as CD, PR-AMPs of different origin clearly seem to assume similar extended, unconstrained conformations which, in contrast to other types of AMPs, are not markedly altered in the presence of biological membranes or environments that mimic them. This is compatible with a mode-of-action that does not involve major rearrangement of microbial membranes or their permeabilization.

Most of what is known about the mechanism of action of PR-AMPs comes from studies on the insect peptides pyrrhocoricin, apidaecin and drososcin, and from the mammalian cathelicidins Bac7, Bac5 and PR-39. These studies often made use of overlapping synthetic peptide fragments covering the whole sequence of the natural peptides, to dissect the role of different regions (reviewed in [9]). From these studies it is evident that the antimicrobially active domains generally correspond with specific segments of the peptides.

The three cathelicidin-derived peptides Bac5, Bac7 and PR-39 can be shortened from the C-terminus until a minimum length of 15–16 residues, while maintaining a substantial antimicrobial activity [51, 55, 74]. Shortening from the N-terminus, the most cationic region in all three peptides, is instead deleterious, and fragments comprising the central or C-terminal regions such as Bac7(29-56), Bac5(19-43) and PR-39(11-26) are poorly active or inactive, despite having a considerable residual cationicity [9, 55]. This trend has also been observed in naturally occurring N- and C-terminal fragments of the sheep bactenecins OaBac5 or OaBac7.5 [56].

The N-terminal arginine residues appear to be particularly important for activity. Bac7(1-23) and (1-35) are fully functional fragments of Bac7, but N-terminal truncation to Bac7(5-23) and Bac7(5-35), removing the RRIR stretch, greatly reduced antimicrobial activity. Conversely, adding this stretch to the inactive fragment Bac7(29-56) partially restored activity [55]. Substitution of the three N-terminal arginines in the functional PR-39(1-15) fragment with three polar uncharged Asn residues also resulted in a poorly active molecule [51]. Even substitution of the RRIR stretch of Bac7(1-35) with the equally charged KKIK resulted in a reduced activity [9]. This suggests that specific features of arginine, apart from its charge, are important for antimicrobial activity. Recent studies in our laboratory with Bac7(1-23), using various charged or neutral arginine analogues, indicate that both the stereochemistry and H-bonding capacity of the side-chain are relevant (in preparation).

It would thus appear that the bactericidal activity of the mammalian PR-AMPs is concentrated in the N-terminal portion, while the rest of the peptide sequence (which can be quite long) seems to have an accessory function, at least as far as the direct cidal activity is concerned. Apart from the N-terminal residues, however, others at specific positions within the sequence can be important determinants for activity. For instance, altering the sequence of the functional PR-39(1-15) fragment in the region comprising residues 9–13 (LPRPR in the native PR-39), by permutations to LPPRR or LRPRP, or by single substitutions such as L9 W or P10 N, resulted in 4- to 32-fold reduction of antibacterial activity, depending on the target bacterial test strain [51].

Structure activity studies in insect PR-AMPs followed similar lines, but also needed to take into account the fact that some of these are glycosylated. Analogously to mammalian PR-AMPs, deletion of 5 N-terminal residues in drosocin completely abolished its antimicrobial activity [11]. Furthermore, removal of the disaccharide Gal(βl → 3)GalNAc(αl → O) from the central threonine residue, or replacement with a monosaccharide, considerable reduced its potency. SAR studies with apidaecins indicated that the conserved C-terminal region was responsible for the general antibacterial capacity, while the variable N-terminal region was responsible for the antibacterial spectrum [32]. Replacement of a single arginine/leucine residue in apidaecin 1b with the N-substituted glycine analog (peptoid), to improve resistance to proteolysis, has shown that the antibacterial efficacy depended markedly on the position of the peptoid residues. At the N-terminus, these modified residues increased stability without affecting activity, but when moved into in the C-terminal end of the molecule, and especially into the conserved PRPPHPRL motif, activity was progressively reduced. Substitution of the C-terminal Leu with its peptoid analog also abrogated activity [75].

A common conclusion coming from SAR studies with mammalian and insect PR-AMPs, or their functional

fragments, was that they did not act in a lytic manner, as there was no indication of membrane permeabilization. Rather, a significant body of evidence points to a mechanism involving cellular internalization and binding to a cytoplasmic target. It is rather interesting that studies on the unrelated vertebrate and invertebrate PR-AMPs should lead to a quite similar outcome.

Mode of action for antimicrobial activity

The first clues suggesting that PR-AMPs might act through a non-lytic mechanism came from experiments showing that light scattering by an *E. coli* cell suspension treated with either insect apidaecin or bovine PR-39 remained unchanged at incubation times at which bacteria were killed, indicating that microorganisms were not lysed [5, 6]. The absence of membrane permeabilization was subsequently confirmed at concentrations exceeding lethal doses by four orders of magnitude, while on the other hand, apidaecin-resistant mutants showed undiminished sensitivity to pore-forming peptides [8]. Similar observations were made for pyrrhocoricin [76] and Bac7 fragments [7]. Another indication that proline-rich peptides have a different mechanism of action than lytic peptides is their relatively slow bacteria-killing kinetics (from several minutes to some hours) compared to lytic AMPs, which are in general very fast killers (often a reduction of viable cells of several logs is observed within a few minutes exposure).

The relevance of peptide stereochemistry to the antimicrobial activity is another feature that further convincingly points to a different mechanism of action for PR-AMPs. It has been extensively established that the all-*D* enantiomers of lytic AMPs generally display the same activity as the natural all-*L* counterparts, consistent with a non-stereospecific mode of interaction with the membrane [77, 78]. Conversely, the all-*D* enantiomers of PR-AMPs generally show a marked loss of activity [7, 8, 13]. All-*D* apidaecins could associate with bacterial cells as rapidly as the natural all-*L* counterparts, but unlike these could then be recovered almost entirely by exhaustive washing, indicating they did not internalize [12]. Uptake appeared to be energy-driven and irreversible and could be partially antagonized by free proline, in a stereospecific fashion, which supports a model with a permease/transporter-mediated process as part of the antimicrobial mechanism. Similarly, the uptake of fluorescently labeled all-*D* Bac7(1-35) was significantly reduced with respect to the all-*L* analogue [79]. A membrane protein involved in uptake of both cathelicidin-derived PR-AMPs and apidaecin has now been identified in our laboratory (see below) [80].

Regarding the putative internal target for PR-AMPs, Otvos et al. reported that pyrrhocoricin, drosocin and apidaecin could all bind the 70-kDa bacterial heat shock protein DnaK (Hsp70) in a specific manner, and could also bind to the 60-kDa bacterial chaperonine GroEL, but in a non-specific manner. It was suggested that peptide binding to DnaK could be correlated with antimicrobial activity [81]. DnaK assists a large variety of protein folding processes in the cell by transient association of its substrate binding domain with short hydrophobic peptide segments within the substrate proteins [82]. Deletion of the *dnaK* gene may severely affect bacterial cells [83], so that DnaK inhibition could be the ultimate mechanism of PR-AMPs activity. Active *L*-pyrrhocoricin was in fact found to diminish the ATPase activity of recombinant DnaK [84], while the inactive all-*D*-pyrrhocoricin enantiomer, as well as membrane-active antibacterial peptides cecropin A or magainin 2, did not. In addition, the refolding function of DnaK was reduced upon incubation with *L*-pyrrhocoricin and drosocin but not with *D*-pyrrhocoricin, with the lytic AMP magainin 2, or with the non-lytic helical AMP buforin II [84]. The mechanism of inhibition is as yet still unclear, although it has been proposed that the binding of drosocin or pyrrhocoricin to DnaK prevents the movement of the multi-helical lid over its peptide-binding pocket, permanently closes the cavity, and thus inhibits chaperone-assisted protein folding [84]. Others suggest that these peptides interact with DnaK by binding to its conventional substrate-binding site so that their antimicrobial activity is a consequence of the competitive inhibition of bacterial DnaK [85]. A dual-mode of inhibition has also been proposed, based on the latter competitive mechanism and interference with an allosteric mechanism that determines the lid-mediated regulation of the chaperone cycle [86].

With respect to the mammalian PR-AMPs, in a search for bacterial cytoplasmic interactors using affinity resins functionalized with *L*-Bac7(1-35), it is striking that the only protein specifically retained with high affinity from *E. coli* protein lysates was DnaK, while the all-*D* enantiomer failed to retain it. In addition, the peptide was found to inhibit in vitro the protein refolding activity of the complete DnaK/DnaJ/GrpE/ATP molecular chaperone system, in a dose-dependent manner [87].

The mechanism by which insect and mammalian PR-AMPs inactivate susceptible bacteria might thus depend on their ability to inhibit protein folding and refolding by binding to DnaK. Significantly, the homologue of DnaK in *S. aureus*, a Gram-positive species not susceptible to PR-AMPs, does not bind pyrrhocoricin. It cannot be excluded, however, that PR-AMPs inactivate bacteria by binding to other internal targets apart from DnaK that have as yet to be identified. In this respect, the in vitro sensitivity of DnaK-deficient *E. coli* strains to Bac7(1-35), under growth permissive conditions, was not decreased significantly compared to the wild-type strains, supporting our

impression that, apart from DnaK, other vital targets for the proline-rich AMPs are present in susceptible bacteria.

The mechanism underlying the antibacterial activity of Bac7(1-35) was extensively investigated against both *S. enterica* and *E. coli*. At 0.25–0.5 μM concentrations, the all-*L* enantiomer rapidly killed bacteria by a non-lytic, energy-dependent mechanism, being rapidly internalized into bacterial cells, while the inactive all-*D* enantiomer was excluded. At higher concentrations (≥64 μM), both *L*- and *D*-enantiomers of Bac7(1-35) killed bacteria by permeabilization of the cytoplasmic membrane [7]. This PR-AMP could thus inactivate bacteria via two different modes of action, depending on the concentration: a stereospecific mechanism based on uptake and target binding at concentrations near the MIC value, and an additional non-stereoselective membranolytic mechanism relevant only at concentrations several times the MIC values [7].

Subsequently, a genetic approach was set up with the aim of identifying the protein(s) involved in the transmembrane transport of PR-AMPs. This was based on mutagenesis to select bacterial mutants that were either more resistant or more susceptible to peptide action [88]. It allowed the identification of the sbmA gene, which, if mutated or deleted, conferred partial resistance to several PR-AMPs, including Bac7, PR-39, Bac5 and apidaecin, but not to representative α-helical membranolytic peptides [80, 89]. This gene codes for SbmA, a inner membrane protein predicted to be part of an ABC transporter, which is also necessary for the antimicrobial activity of the microcins J25 and B17, antibacterial peptides of bacterial origin [90, 91], as well as the glycopeptide antibiotic bleomycin [92]. Deletion or mutation of SbmA in *E. coli* markedly decreased the ability to internalize fluorescently labeled Bac7(1-35), and this correlated with a reduced susceptibility to PR-AMPs, indicating that SbmA is necessary for the transport process [80]. The oligomerization state of the transporter, and the other membrane or cytoplasmic interactors that may be required to constitute it, are under investigation.

Although *sbmA* is not an essential gene, at least in the growth conditions tested thus far, its importance in vivo can be inferred from the functions of its homologue *bacA* in the endosymbiont *Sinorhizobium meliloti* as well as in the intracellular parasite *Brucella abortus*. In these alphaproteobacteria, BacA is essential to establish a chronic intracellular infection in their respective hosts [91]. It is worth noting that both species are susceptible to some PR-AMPs [89] as are several Enterobacteriaceae, in which SbmA is present [89]. In contrast, those species in which *sbmA* has no close homologues, such as *P. aeruginosa*, are less susceptible to Bac7. SbmA/BacA may thus be part of a general transport system essential for the internalisation of PR-AMPs in bacteria and in making them susceptible to these AMPs.

Studies underway in our laboratory, aimed at dissecting the regions in Bac7 responsible for cell penetration, indicate that an N-terminal region of at least 15 residues is necessary for internalization (unpublished data). A reversed situation has found by others for pyrrhocoricin in which the N-terminal part (residues 2–10) is required for target binding and its C-terminal part for bacterial entry [93].

What has been learnt about the mode-of-action of mammalian and insect PR-AMPs, mainly from studies with pyrrhocoricin, Bac7, apidaecin and their analogs is collated in Fig. 1, and this unified scheme might be extendable to other PR-AMPs. These are transported into the bacterial cells at sub- to low micromolar concentrations, by a stereoselective transport system involving the membrane protein SbmA, and act intracellularly. At higher concentrations, they act on bacteria in a non-stereoselective

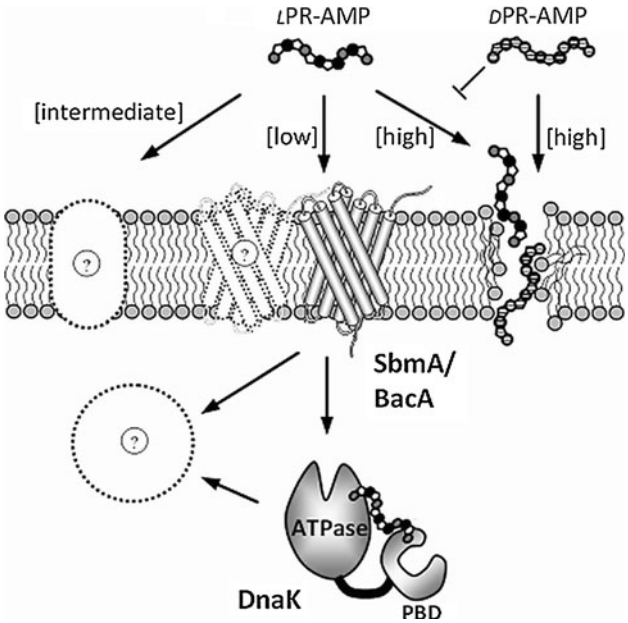

Fig. 1 Model for the mode-of-action of mammalian and insect PR-AMPs. PR-AMPs like Bac7 and apidaecin can penetrate into susceptible bacterial cells in a stereoselective manner using a transport system involving the membrane protein Sbma/BacA, likely part of an ABC transport system for which the oligomerization state and other interactors are as yet unknown. The natural all-*L* PR-AMPs are internalized by this system at sub- to low micromolar concentration, while the all-*D* enantiomers are not. At considerably higher concentrations, both *L*- and *D*-isomers of PR-AMPs like Bac7 are capable of killing bacteria via a membranolytic mechanism. Other, as yet unknown, transporters may internalize these peptides at intermediate concentrations. Once internalized, PR-AMPs such as pyrrhocoricin and Bac7 can interact with the bacterial chaperone DnaK, affecting the ATPase activity or its peptide binding domain (PBD) or both. There are likely also other intracellular interactors, alongside the chaperone, or downstream from it

membranolytic manner. It is feasible that they have other transport systems acting at intermediate concentrations, but this has to be verified. The only intracellular target identified to date is the bacterial chaperone DnaK, where they could inhibit the essential ATPase activity or protein folding activity or both. There are indications that other targets exist, and it may also be that events subsequent to DnaK binding play a relevant role in the killing action.

In vivo application of PR-AMPs and potential for development

Studies have been performed recently with selected PR-AMPs to explore their potential as lead compounds for development of novel anti-infective drugs. The interest for their exploitation is twofold: (1) as direct antimicrobials they combine a mode of action different to those of lytic AMPs or conventional antibiotics with a very low in vivo toxicity; and (2) as cell penetrating peptides they may be capable of internalising useful molecular cargo into either bacterial or host cells. Attempts to improve their potential are mostly aimed at increasing serum stability without affecting their antimicrobial or cell-penetrating properties.

Optimization of pyrrhocoricin led to designer peptides which entered bacterial cells and maintained their DnaK-binding ability combined with a high stability and low toxicity in mice [94]. The most promising of these, A3-APO, retained full antibacterial activity in the presence of serum and was effective for the treatment of systemic, Gram-negative bacterial infections in mouse models [95–97]. This peptide appears to have a dual mode of action, being capable of interacting with and lysing bacterial membranes, as well as interacting with the intracellular target DnaK. (reviewed in [98]). Interestingly, A3-APO derivatives would seem to have a greater in vivo than in vitro bactericidal efficacy, which might be explained by additional immunostimulatory properties of these peptides [97].

A synthetic peptide, oncocin (VDKPPYLPRPRPPR-RIYNR-NH2), was designed starting from a moderately active analogue of pyrrhocoricin (70% identity), isolated from the milkweed bug (*Oncopeltus fasciatus*) [99]. A number of substituted analogues were then optimized for treatment of Gram-negative pathogens [100]. These peptides were not toxic to human cell lines and could freely penetrate lipid membranes without lytic activity. Substitution of arginine residues with ornithine increased both the activity and the half-live in full mouse serum [100].

We have recently collected data supporting the potential of the mammalian PR-AMP Bac7 as an anti-infective drug for the treatment of salmonellosis and other Gram-negative infections. The fragment Bac7(1-35) was substantially active in murine serum or plasma even after 24 h incubation, and was able to significantly reduce the mortality of infected animals in a mouse model of typhoid fever. Results indicated that the peptide's efficacy could be substantially enhanced by decreasing its excretion rate or modifying the treatment schedule [67]. In addition to its anti-infective activity in vivo, it was shown that Bac7(1-35) also neutralized the effects of lipopolysacharide (LPS) in an experimental rat model of Gram-negative septic shock, with a potency comparable to that of polymixyn B [101].

The therapeutic potential of PR-AMPs was further consolidated by a series of experiments showing that transgenic mice constitutively expressing the pig peptide PR-39 showed increased resistance to group A *Streptococcus* skin infection, an effect that was not observed in transgenic mice overexpressing their only native cathelicidin (mCRAMP), which belongs to the α-helical, membranolytic group [102].

The other potential application of PR-AMPs is their use as cell-penetrating peptides (CPPs) for the intracellular delivery of impermeant drugs into both bacteria and eukaryotic cells. Fluorescently labeled Bac7(1-35) is rapidly detected in the cytoplasm of exposed Gram-negative bacteria using flow cytometry or confocal microscopy [79], indicating that it can efficiently internalize small molecules such as the fluorophores BODIPY or fluorescein. Even more striking results were obtained with eukaryotic cells, into which Bac7 fragments were capable of delivering a noncovalently linked protein [103]. More recently, a functionalized derivative of Pt(II) coproporphyrin I (PEPP0) was conjugated to the 15–24 fragment of Bac7 to make an oxygen-sensitive phosphorescent probe for intracellular use [104].

The capacity to enter both animal and bacterial cells has also been observed with insect PR-AMP-derived peptides. Fluorescently labeled oncocin homogeneously stained *E. coli* cells within 50 min [100]. Similarly, native pyrrhocoricin entered *E. coli* cells very efficiently and its more potent dimeric analogue A3-APO is reported to penetrate dendritic cells and fibroblasts [105].

The capacity of PR-AMPs to be both eukaryotic and prokaryotic CPPs is uncommon, and likely derives from quite different mechanisms: the concurrent contribution of macropinocytosis and direct membrane translocation in the first case, and use of a membrane transporter in the second. Overall, these results indicate that these proline-rich peptides represent a potentially new class of CPPs for intracellular delivery of impermeant molecular cargo.

Conclusions

Proline-rich antimicrobial peptides from both insects and mammals are a distinctive group of host defence peptides with a characteristic selectivity for Gram-negative bacteria

and a non-lytic mechanism of action. They do not conform to the widespread view that AMPs act principally at the microbial membrane, in a non-selective manner. Studies starting in the early 1990s demonstrated that they could inactivate susceptible bacterial cells via a stereoselective mechanism, and likely interfered with cytoplasmic targets. In the last few years, a concerted effort on peptides of both vertebrate and invertebrate origin has allowed to identify a part of the transporter system and a first specific target: respectively, the membrane protein SbmA/BacA and the chaperone DnaK. The selectivity of this type of PR-AMP can thus be explained by the fact that they target bacteria expressing both the transport system and a vulnerable internal target (e.g., a molecular chaperone). This is the first case where specific molecular targets are indicated for animal AMPs. Studies are underway to verify the presence of other transport systems for PR-AMPs, as well as further cytoplasmic targets.

From the point of view of the development of novel anti-infective agents, PR-AMPs have several advantages, including: (1) a selective but potent antimicrobial activity in vitro reflected by a reasonable potency in vivo; (2) a remarkably low toxicity and the possibility of being modified to reduce proteolytic degradation and increase activity in biological fluids; (3) specific druggable targets; (4) an accessory anti-LPS activity; (5) indications that additional immunostimulatory activities could augment the direct antibiotic action; and (6) the possibility of using them as a new class of cell-penetrating peptides capable of internalizing other drug cargo into both susceptible bacterial and host cells.

For all these reasons, it is likely that PR-AMPs will continue to attract both basic and applied research efforts in the future, and provide new insights into the perennial struggle between host and pathogen. In addition to their exploitation as drug candidates, the PR-AMPs deserve to be studied for more basic scientific questions, which include, for instance, the origin and biological significance of the repeats present in the structure of many PR-AMPs, whether they effectively play a multifunctional role in the producer organism beyond their direct antibiotic capacity, why in mammals they are found only in Artyodactila, and the reasons underlying the convergent evolution of vertebrate and invertebrate PR-AMPs.

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