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REVIEW

Host defence peptides from invertebrates – emerging antimicrobial strategies

Robert E.W. Hancock*, Kelly L. Brown, Neeloffer Mookherjee

Department of Microbiology and Immunology, University of British Columbia, 2259 Lower Mall, Vancouver, BC, Canada V6 T 1Z4

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Abstract

Cationic antimicrobial (host defence) peptides are found as potent components of the innate immune system of all invertebrates in which they have been investigated. They vary substantially in their amino acid sequences, secondary structures, inducibility, potency and antimicrobial activity spectra. This enormous diversity is providing templates for the design and development of both antibiotic peptides and peptides that selectively modulate innate immunity to increase protection against infections and sepsis.

Keywords: Antibiotics; Antimicrobial peptides; Cationic peptides; Invertebrate immunity; Innate immunity

Contents

Properties of cationic antimicrobial (host defence) peptides

Cationic amphiphilic peptides are found as a component of the innate immune system of all species of life, including invertebrates and vertebrates (Hancock and Diamond, 2000; Zasloff, 2002; Bulet et al., 2004; Iwanaga and Lee, 2005). Collectively they have been termed (cationic) antimicrobial peptides due to the observation that many have direct antimicrobial activity under physiological conditions, or cationic host defence peptides, reflecting their broader involvement in mammalian immunity, including an ability to neutralize bacterial endotoxins such as LPS (a property shared by many invertebrate peptides) and to modulate the activities of the innate and adaptive immune systems.

Cationic antimicrobial (host defence) peptides in nature comprise short sequences of amino acids ranging from around 12 to 50 amino acids in length. They have

*Corresponding author. Tel.: +1 604 822 2682.
E-mail address: bob@cmdr.ubc.ca (R.E.W. Hancock).

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net positive charges of +2 to +9 due to their lack or small number of acidic residues (glutamate or aspartate) and excess number of cationic (arginine or lysine and/or histidine) residues, and around 30–50% hydrophobic residues. Table 1 demonstrates a modest number of invertebrate peptides while Fig. 1 demonstrates some representative structures. A variety of modifications are observed in different peptides, but this is not common. Some peptides pre-form their secondary and tertiary structures in free solution, most notably those with two to four disulphide bridges, stabilizing a predominantly β-sheet or β-hairpin structure (although many of the larger β-stranded peptides have an additional α-helix). On the other hand, a large number of peptides have little or no structure in free solution, folding into an amphipathic or amphiphilic structure upon contact with a membrane target (Powers and Hancock, 2004). The most common of these are those which fold into α-helical structures, while other peptides which have an over-representation of particular amino acids (e.g. proline, tryptophan, histidine) can form more extended structures. While their three-dimensional structures might vary, most peptides in their final folded conformations have patches of hydrophobic and charged residues that permit the peptides to interact strongly with membranes. These interactions can be relatively selective for bacterial membranes due to the high proportion of anionic lipids at their membrane surface, a substantial electrical potential gradient oriented internal negative and a lack of rigidifying lipids like cholesterol. However, it is worth pointing out that there is a spectrum of toxicities ranging from relatively benign to extremely toxic and some of the most potent toxins, including scorpion charybdotoxin, bee venom melittin and wasp venom mastoparan, have strong antimicrobial activity and overlapping physical properties with the host defence peptides.

Antimicrobial peptides are an essential component of invertebrate innate immunity and consequently are found in every invertebrate in which they have been looked for. A full and detailed account of these peptides is beyond the scope of this review, and we refer the reader to the following reviews in which insect (Hetru et al., 2000) and marine invertebrates (Tincu and Taylor, 2004) are discussed in detail.

Functions of cationic peptides in invertebrate immunity

Invertebrates are devoid of adaptive immune responses which appeared in evolution some 450 million years ago (Hoffmann, 2004); in particular, invertebrates lack such critical elements of adaptive immunity as antibodies and lymphocytes, immunological memory and true self vs. non-self discrimination. Nevertheless, invertebrates can resist infections and can also transmit lethal diseases to humans and livestock, without necessarily succumbing to these infections. Thus, innate immunity, the most ancient first line of immune defence, is vital in invertebrate host defences, and indeed has become conserved throughout the animal kingdom. In their quest to understand how invertebrates survive without adaptive immunity, researchers discovered several aspects of immune responses which play a critical role in animal innate immunity, including the family of pathogen recognition receptors (Toll-like receptors; TLR), production of toxic oxygen and metabolites, and antimicrobial peptides. It is now understood that the immune system of invertebrates is complex and heterogeneous (Iwanaga and Lee, 2005), even though there are several elements in common, especially within related invertebrates. For example, microbial challenge of insects results in haemocyte-mediated cellular immune response including phagocytosis and encapsulation, and humoral defences which include production of antimicrobial peptides (Iwanaga and Lee, 2005).

Antimicrobial peptides in invertebrates came into focus in the 1980s (Steiner et al., 1981) and are now described as key effector molecules in antimicrobial host defences. The last decade has seen the identification of numerous antimicrobial peptides in invertebrates (Bulet et al., 2004). Gene-encoded, naturally occurring antimicrobial peptides found in the haemolymph, both in the plasma and haemocyte cells, are involved in both systemic as well as site-specific protection against microbial pathogens in invertebrates. They are produced in the phagocytic cells of invertebrates (Mitta et al., 1999; Iwanaga et al., 1998; Dimarcq et al., 1988), with tissues such as insect fat bodies being the main site of synthesis (Engstrom, 1998). Individual antimicrobial peptides are expressed either constitutively, for example in the haemocytes of shrimp, oysters or horseshoe crab (Bachère et al., 2004; Iwanaga and Kawabata, 1998), or are induced upon pathogenic challenge, such as in *Drosophila* where antifungal peptide synthesis is mediated by the Toll receptor (Lemaitre et al., 1996; Imler and Hoffmann, 2000). Upon entry of pathogens, the circulating haemocytes of invertebrates migrate through chemotaxis to the site of injury and release antimicrobial peptides, which in turn exert their antimicrobial effects, as well as potentially eliciting responses that modulate inflammation, thus creating a parallel scenario to that which is observed with the mammalian innate immune response.

One of the dramatic discoveries in insects that led to a paradigm shift in thinking about innate immunity was the discovery of pattern recognition receptors (TLR) in *Drosophila melanogaster* (Hoffmann, 2003). The use of similar receptors and pathways for innate immune

Table 1. Examples of invertebrate cationic antimicrobial peptides

Group	Order	Genus species	Peptide	Structure	Size (amino acids)	Charge	% Hydrophobic residues[a]	Sequence[b]
Tunicate (Sea Squirt)	Pleurogona	*Styela clava*	Styelin C	α	31	+5	61	GWFGKAFRSVSNFYKKHTYIHAGLSAATLLG
	Pleurogona	*Styela clava*	Clavinin A	α	23	+2	65	VFQFLGKIIHHVGNFVHGFSHVF
Crustacean (crab)	Decapoda	*Panaeus vannemi*	Paneidin 1	β-3	50	+8	56	YRGGYTGPIPRPPPIGRPPLRLVVCACYR LSVSDARNCCIKFGSCCHLVK
Chelicerate (horseshoe crab)	Xiphosura	*Tachyplesin tridentatus*	Tachyplesin I	β-2	17	+7	41	KWCFRVCYRGICYRRCR
	Xiphosura	*Limulus polyphemus*	Polyphemusin I, II	β-2	18	+8	39	RRWCFRVCYRGFCYRKCR
	Xiphosura	*Tachyplesus tridentatus*	Big defensin	β-3	79	+7	53	NPLIPAIYIGATVGPSVWAYLVALVGAA AVTAANIRRASSDNHSCAGNRGWCRSK CFRHEYVDTYYSAVCGRYFCCRSR
Mollusk	Mytiloida	*Mytilis edulis*	Mytilin A	β-4	34	+11	35	GCASRCKAKCAGRRCKGWASASFRGRCCYCKCFRC
	Cyrtodontida	*Mytilus galloprovincialis*	Defensin MGD1	β-3	37	+7	38	GFGCPNNYQCHRHCKSIPGRCGGYCGGCHRLRCTCYRCG
Fruit fly	Diptera	*Drosophila melanogaster*	Metchnikowin	extended	52	+2	46	MQLNLGAIFLALLGVMATATSVLAEPH RHQGPIFDTRPSPFNPNQPRPGPIY
Flesh fly	Diptera	*Sarcophaga peregrina*	Sarcotoxin IC	α	39	+6	52	GWLRKIGKKIERVGQHTRDATIQVLGIAQQAANVAATAR
	Diptera	*Sarcophaga peregrina*	Sapecin B	β-3	34	+4	35	LTCEIDRSLCLLHCRLKGYLRAYCSQQKVCRCVQ
Moth	Lepidoptera	*Hyalophora cecropia*	Cecropin	α	35	+6	57	RWKIFKKIEKVGQNIRDGIVKAGPAVAVVGQAATI
	Lepidoptera	*Heliothis virescens*	Heliomycin defensin	β-3	44	+2	41	DKLIGSCVWGAVNYTSDCNGECKRRG YKGGHCGSFANVNCWCET
Beetle	Celeoptera	*Acrocinus longimanus*	ALO1	β-3	34	+2	35	CIANRNGCQPDGSQGNCCSGYCHKEPGWVAGYCR
Spider	Araneae	*Acanthoscurria gomesiana*	Gomesin	β-2	18	+7	22	ZCRRLCYKQRCVTYCRGR

[a]Glycine was included in the count of hydrophobic amino acid residues even though it is not hydrophobic.
[b]Gomesin is pyroglutamate.

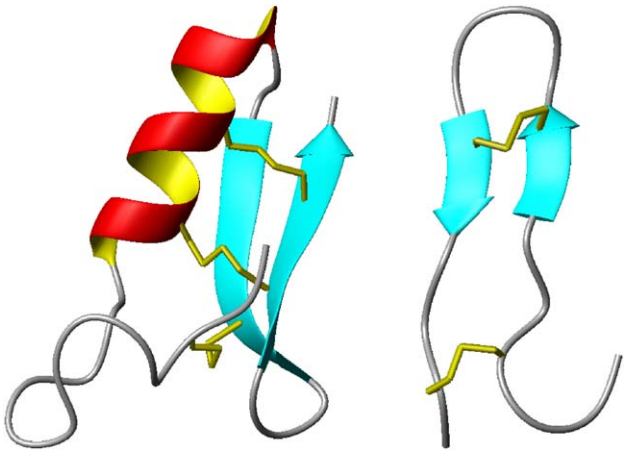

Fig. 1. Structures of two cationic antimicrobial peptides, sapecin (left) and polyphemusin (right).

responses in insects and mammals suggests that they have developed similar molecular mechanisms to eliminate pathogenic challenge and thus the effector molecules, including antimicrobial peptides, may have similar functions in both invertebrates and mammals. The *Drosophila* Toll pathway is initiated by Gram-positive bacterial peptidoglycan or fungal β-glucan which finally activates a serine protease that in turn cleaves Spätzle that binds to and activates the pattern recognition receptor Toll. This then leads to the receptor-mediated triggering of a signal transduction pathway resulting in phosphorylation of an IκB-like inhibitor Cactus causing it to dissociate from the NF-κB-like transcription factor DIF, which in turn translocates to the nucleus and activates numerous antimicrobial genes (Belvin and Anderson, 1996; Hoffmann and Reichhart, 2002). Similarly, the IMD pathway in *Drosophila* responds mainly to Gram-negative bacterial peptidoglycan, through a distinct pattern recognition receptor PGRP-LC, leading to activation of another kinase-based signal transduction pathway causing the phosphorylation and consequent translocation into the nucleus of another NF-κB-like transcription factor Relish, which ultimately regulates the transcription of immune-related genes (Hoffmann and Reichhart, 2002). Thus, these two distinct signalling cascades in *Drosophila* modulate complex transcriptional response to different pathogenic microbes.

It is well established that each invertebrate that has been intensively studied can produce a range of peptides, many of which have excellent direct antimicrobial activity and thus provide templates for commercial development of a new generation of antibiotics. In addition, mammalian cationic host defence peptides have been proposed to directly stimulate certain innate immune functions and selectively modulate inflammatory responses to endotoxin (Scott et al., 2002). The selective modulation of immunity is mediated by the alteration of TLR-agonist-induced NF-κB-mediated responses (Mookherjee et al., 2006). Since this has been proposed as a new therapeutic approach for treating infectious diseases (Finlay and Hancock, 2004) and since the Toll and IMD pathways in *Drosophila* share similarities with the IL-1/TLR and TNF-α pathways that regulate transcription factor NF-κB in mammals, we also discuss this in detail below.

Peptide antibiotics

Invertebrate antimicrobial peptides, like their mammalian counterparts, can protect rodents from bacterial challenge/sepsis, providing evidence that antimicrobial peptides play a key role in the innate immune defence to pathogenic assault. The insect-derived cationic antimicrobial peptide CEMA (a cecropin–melittin hybrid) protects neutropenic mice from *Pseudomonas aeruginosa* infection (Gough et al., 1996). Mice transgenic for the synthetic cecropin-like peptide, Shiva 1a, are resistant to infection by *Brucella abortus*, the causative agent of abortions in cattle and relative of the pig pathogen *Brucella suis* (Reed et al., 1997). A synthetic peptide analogue of heliomycin, a peptide from tobacco budworm (*Heliothis virescens*) (Lamberty et al., 2001), provided protection to mice challenged with *Candida albicans* even when administered 48 h post-infection, and is being developed by Entomed SA for antifungal treatment.

Numerous reports demonstrate direct in vitro killing activity of antimicrobial peptides against bacterial, fungal or viral pathogens, implicating this direct antimicrobial activity of antimicrobial peptides in host defence (Hancock and Diamond, 2000; Zasloff, 2002). The most active antimicrobial peptide with which the authors have ever worked is the horseshoe crab peptide polyphemusin which has minimal inhibitory concentrations against the most feared nosocomial bacterial and fungal pathogens of less than 2 μg/ml, when assessed by standard minimal inhibitory concentration assays (Zhang et al., 2000) and which is known to have antiviral activity against human immunodeficiency virus (Masuda et al., 1992).

Interestingly, the antifungal activity exhibited by certain antimicrobial peptides from coleopteran insects *Holotrichia diomphalia* and *Tenebrio molitor* is strongly influenced by ionic conditions (Lee et al., 1996). Furthermore, as concentrations of peptides in invertebrates can become quite high in some tissues, the mere presence of a direct killing activity in a peptide does not predict its potential usefulness as a template for an antibiotic. Indeed individual peptides vary from highly active to rather modestly active, and may have quite

complex structures including multiple disulphide bridges, chemical modifications or large numbers of amino acids, making them very expensive to produce for therapeutic use. Thus while invertebrates are a useful source of new templates for antibiotic peptides, it is worth considering the balance of efficacy, toxicity, cost of goods and stability in choosing an appropriate template molecule. Having stated this, methods for improving the efficacy of peptides, ranging from rational to random design (Hancock and Patrzykat, 2002; Hilpert et al., 2005), are available.

The basis for the direct antimicrobial activity of peptides has been well studied, and is described in passing here. All fairly active antimicrobial peptides are able to interact with membranes by folding into amphipathic three-dimensional structures, which insert into membranes at the membrane interface. This can then lead to a rearrangement of the peptides at sufficient concentration that has been described by four models termed the barrel stave, carpet, torroidal pore and aggregate models (Hancock and Chapple, 1999; Zasloff, 2002; Hancock and Rozek, 2002). If there is a substantial local perturbation of the cytoplasmic membrane bilayer of bacteria, in each of these models, ion-permeable channels are created in the lipid bilayer, leading to increased membrane permeability that is proposed to lead to cell death. A number of antimicrobial peptides, including polyphemusin, can traverse the membrane (as proposed by the aggregate model; Hancock and Chapple, 1999) and induce killing by effecting anionic intracellular targets, for example, by disturbing crucial molecules and processes required for growth (Kragol et al., 2001; Zhang et al., 2000; Patrzykat et al., 2002; Hong et al., 2003).

Immunomodulation

Although the antibacterial activities of mammalian cathelicidins are substantially suppressed by physiological concentrations of monovalent and divalent ions, the cathelicidins can confer protection to pathogenic challenge, possibly by modulating immune responses (Bowdish et al., 2005). This concept has been utilized to generate immune modulating peptides that are devoid of direct antimicrobial activity, but still protect against infections. Similarly the ability of cationic antimicrobial (host defence) peptides to protect against endotoxaemia in animal models, in the complete absence of an infecting organism, indicates that there are other potential routes for development of therapeutically useful peptides from invertebrate templates. Because even invertebrate antimicrobial peptides have immunomodulatory activities, we use the more expansive term host defence peptides in this section.

Mammalian host defence peptides have been shown to boost, inhibit or complement cellular functions such as chemotaxis, apoptosis, gene transcription, cytokine production and wound healing (Salzet, 2002; Finlay and Hancock, 2004; Mookherjee et al., 2006). These biological effects are often induced by inflammatory stimuli including conserved microbial components of endogenous or pathogenic origin acting on pattern recognition receptors. A high degree of similarity between invertebrate and vertebrate innate immune systems and host defence peptides (Lehrer and Ganz, 1999; Salzet, 2001a,b; Bulet et al., 2004) would suggest that the immunomodulatory functions observed in mammals likely exist in invertebrates and are not an evolved function of antimicrobial peptides in the mammalian innate immune system. Indeed although this general topic has not been well studied in invertebrates themselves, there are an increasing number of reports of the immunomodulatory effects of invertebrate host defence peptides on mammalian hosts, including neutralization of LPS, induction of signal transduction, gene transcription and release of reactive oxygen species.

In human peripheral blood mononuclear cells, a *Limulus polyphemus* anti-lipopolysaccharide factor (LALF)-derived peptide induced the release of antiviral and immunomodulating cytokines, IFN-α, IFN-γ, IL-2 and IL-13, but not TNF-α or IL-6 (Vallespi et al., 2000), and CEMA altered the expression of genes involved in cell proliferation, apoptosis and cell adhesion in murine RAW macrophages (Scott et al., 2000). Several synthetic peptides, derived from Sapecin B of the fleshfly *Sarcophaga peregrine*, had protective activity towards methicillin-resistant *S. aureus* (MRSA)-infected mice (Nakajima et al., 1997; Cho et al., 1999). However, the chemotherapeutic activity of these peptides could not be explained by their direct bactericidal effect, as the peptide with the highest antibacterial activity did not exhibit any chemotherapeutic activity. Those peptides with chemotherapeutic activity activated human neutrophils and induced superoxide anion (O_2^-) release in a Ca^{2+}-dependent manner. Together, these data provide evidence for multifunctional and immunomodulatory properties of invertebrate antimicrobial peptides.

Natural and synthetic peptides have been shown to neutralize the effect of LPS-induced gene expression and signal transduction. For example, the insect-derived peptide CEMA suppressed LPS-induced inflammatory gene expression (IL-1ß, IL-15, MIP-1, iNOS) and protein production (IL-1ß, NO) in murine RAW macrophages (Scott et al., 2000), and, in vivo, CEMA protected against endotoxaemia by neutralizing LPS-induced TNF-α levels in a murine model of sepsis (Gough et al., 1996).

Antimicrobial host defence peptides appear to play a critical role in response to endotoxin in invertebrates

other than insects. For example, the circulating haemocytes of the Japanese horseshoe crab (*Tachypleus tridentatus*) are sensitive to bacterial LPS and on detection of LPS release, through rapid exocytosis, the contents of their L- and S-granules which include antimicrobial host defence peptides including LALF (also found in *L. polyphemus*) and defensins (Iwanaga and Lee, 2005). An LALF-derived peptide increased survival in mice following a lethal dose of *P. aeruginosa* that was correlated with diminished systemic TNF-α, and elevated mRNA synthesis of IL-2, IL-12 and IL-13, but not IL-4 and IL-10, in the spleen and liver (Vallespi et al., 2000, 2003).

Tachyplesin, a potent cationic antimicrobial peptide in *T. tridentatus* haemocyte granules (Morvan et al., 1997), is also released after contact with microbial endotoxins (Iwanaga et al., 1998), and can form a complex with bacterial LPS (Hirakura et al., 2002). Both LALF and tachyplesin neutralize LPS-induced activation of factor C, a serine protease zymogen required for haemolymph coagulation (Tanaka et al., 1982; Nakamura et al., 1988). Two high-affinity LPS-binding domains of factor C called Sushi1 and Sushi3 were identified. Synthetic peptides based on these domains blocked LPS-induced TNF-α secretion from human monocytic THP-1 cells, and protected galactosamine-sensitized mice from *E. coli* LPS-induced mortality (Tan et al., 2000a,b), showing promise as an immunotherapy for Gram-negative bacterial septicaemia. Similarly, another potent antimicrobial peptide polyphemusin from *L. polyphemus* haemocytes served as the basis for design of a more potent anti-endotoxic peptide that protected in mouse sepsis models (Zhang et al., 2000). Also, anti-LPS peptides were observed in the freshwater crayfish, *Pacifastacus leniusculus* and the solitary ascidian, *Halocynthia roretzi* (Iwanaga and Lee, 2005).

It has been suggested that several cationic and non-cationic peptides expressed in vertebrate, invertebrate and bacterial species act synergistically to improve immune responses (Luders et al., 2003). For example, in horseshoe crabs, the antimicrobial peptide tachycitin synergistically enhances the antimicrobial activity of big defensins against Gram-negative bacteria. The prophenoloxidase (pro-PO) system is considered a primitive non-self-recognition system in invertebrates that has also been linked to blood coagulation and killing of invading microbes. Serine proteinases of the pro-PO system, clotting factors, big- defensins and serine proteinases in *Drosophila* have common clip-domains in the N-terminal profragment. Conversely, the clip-domain from a freshwater crayfish serine proteinase was reported to have antimicrobial activity similar to the horseshoe crab big defensin and bovine neutrophil β-defensin-12 (Wang et al., 2001). Although not experimentally proven, there is potential for complementary or synergistic functions between host defence peptides and other components of the invertebrate immune system, such as the pro-PO system.

Conclusions

Functional similarities among the antimicrobial host defence peptides of distant evolutionary species indicate that the study of invertebrate peptides could permit the development of new design templates for anti-infectious agents in humans (Zasloff, 2002). Antimicrobial peptides are promising as therapeutics since they are relatively small (easy to synthesize), act rapidly with a broad range antimicrobial activity, provide defence by multiple mechanisms thereby making pathogen resistance unlikely, have limited immunogenicity but are immunomodulatory, and have a low toxicity for vertebrate cells (Hancock and Diamond, 2000; Zhang and Falla, 2004). Conversely there are hurdles to overcome including lability to proteases, toxicity in some cases (Ohashi et al., 1984) and the substantial expense of peptide synthesis. A more detailed analysis of invertebrate antimicrobial peptides structure and function will aid our understanding of antimicrobial peptides recognition and neutralization of pathogens, yielding a potentially large number of effective therapeutics.

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