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Review

Biological characterization and modes of action of temporins and bombinins H, multiple forms of short and mildly cationic anti-microbial peptides from amphibian skin‡

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Abstract: Genetically encoded cationic anti-microbial peptides (AMPs) are essential components of the ancient and non-specific innate immune system, which is the principal defence mechanism of all species of life, with the primary role to kill infectious microorganisms. Amphibian skin is one of the richest natural sources of such molecules, which are produced by holocrine-type dermal glands and released upon stimulation. This review highlights the attractive and unique structural/functional properties of temporins and bombinins H, two families of short and mildly cationic peptides, isolated from the skin of frogs belonging to *Rana* and *Bombina* genera, respectively. Beside improving our knowledge on the role of AMPs in the regulation of the innate immunity, the biological significance of the existence of multiple forms of a prototypic peptide sequence within the same organism and the implication of short peptides in the endotoxin neutralization, these two classes of AMPs can be also considered as valid candidates for the design of novel anti-infective and anti-sepsis drugs. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: frog skin anti-microbial peptides; temporins; bombinins H; infectious diseases; membrane-active peptides; lipid–peptide interaction; synergism; innate immunity

INTRODUCTION

Ribosomally synthesized anti-microbial peptides (AMPs), comprising approximately 10–50 amino acid residues, are widely distributed in nature, being produced by unicellular microorganisms, plants and animals, including humans, as part of their first line of defence [1–4]. To date, hundreds of these gene-encoded peptides have been identified in different biological sources [5–7], proving their prominence in the innate immune system of all living organisms. In addition to their ability to rapidly protect the host against infections from a broad spectrum of pathogens and to limit the induction of microbial resistance [8], AMPs from higher eukaryotes also act as intercellular signalling molecules and coordinate the innate and adaptive host defence responses [9,10]. Therefore, they are also named 'host defence peptides' [11].

Despite substantial variations in their chain length and structure, most AMPs do possess: (i) a net positive charge and (ii) a potential to adopt amphipathic α-helix and/or β-sheet structures (i.e. structures with separate hydrophobic and hydrophilic faces), upon interaction with the phospholipid membrane of the target cell. These two attributes are crucial for their ability to interact with a biological membrane in order to exert their activity [12,13].

Mode of action studies have shown that several AMPs physically permeate the cell membrane, causing damage hard to fix, rather than acting via interaction with specific targets, as in the case of commonly used drugs, which make it easy for the pathogen to acquire resistance [14]. However, numerous peptides have been found to cause profound intracellular effects (e.g. inhibition of DNA, protein or cell wall synthesis and enzymatic activity) [3,15]) and to induce changes in the gene expression levels of microorganisms, when exposed to their sub-inhibitory concentrations [16]. Overall, AMPs are considered as excellent candidates to augment our knowledge on the organization of the innate immunity; in addition, they can also be used as promising templates for the development of novel small therapeutic agents, urgently needed because of the growing resistance of microbes to the available antibiotics [8,17]. It is therefore important to support and stimulate both scientific and application-oriented interests in such molecules.

ANTI-MICROBIAL PEPTIDES FROM FROG SKIN

Skin secretions of Anuran amphibians are an extraordinary rich source of biologically active substances,

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BIOGRAPHIES

Maria Luisa Mangoni was born in Rome on May 17, 1970. She took her degree in Biology with honours from the Sapienza University of Rome in 1996. From January to December 1998, she conducted research at the Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, under the supervision of Professor Hans G. Boman. In 1999, she was on a research contract at University of Chieti (Italy). She obtained her Ph.D. in biochemistry from the Sapienza University of Rome, and in November 2002 was appointed as Research assistant at the II Faculty of Medicine (S. Andrea Hospital) of the same University. Her research activities include: isolation and functional characterization of antimicrobial peptides from amphibian skin secretions; study of their mechanism of action; studies on the role of antimicrobial peptides in innate immunity using amphibia as an *in vivo* model system; and development of natural amphibian peptides as potential drugs. She is a member of the Italian Society for Biochemistry and Molecular Biology, the Italian Human Proteome Organization, the American Society for Biochemistry and Molecular Biology and the American Chemical Society.

Ludovica Marcellini Hercolani Gaddi was born in Rome, Italy, on June 19, 1979. She obtained her Degree in Pharmacy from the Sapienza University of Rome in 2005. For her graduation thesis, she worked at the Department of Biochemical Sciences of the same University. In 2002 she got an Erasmus fellowship and moved to the University of Leiden (The Netherlands), Center for Drug Research, in the laboratory of Prof. Bob van de Water. During this time, she studied the effect of the anticancer drug cisplatin on the apoptosis of porcine renal epithelial cell lines and the cytotoxic effect induced by oxidation products of spermine on human osteosarcoma cell cultures. In December 2005, she passed the qualifying examination for pharmacist practise, at Sapienza University. In June 2006 she got a research fellowship from the Pasteur-Fondazione Cenci Bolognetti Institute and began working at the laboratory 'Advanced Molecular Diagnostic', S. Andrea Hospital, II Faculty of Medicine, Sapienza University of Rome, under the supervision of Dr. Maria Luisa Mangoni. She is now studying the activity of antimicrobial peptides from amphibian skin on different antibiotic-multiresistant microbes and their mechanism of action. In January 2007, she won a fellowship to attend a Masters Course on 'Methods for the Research and the Development of Novel Therapies'.

Maurizio Simmaco was born in Catanzaro, Italy, on February 1, 1961. In 1984, he took a degree cum laude in medicine discussing a thesis in biochemistry. In 1987, he obtained an advanced degree in clinical biochemistry. From 1988 to 1994, he was a Researcher at the Centro di Biologia Molecolare of the National Research Council, Italy. From 1994 to 2000, he was Full Professor of Biochemistry at the University G. d'Annunzio of Chieti, Italy. From 2000 to 2002 he was Full Professor of Biochemistry at the II Faculty of Medicine, Sapienza University of Rome. Since 2002, he has been Full Professor of Molecular biology. He is also the Coordinator of the Advanced Molecular Diagnostic Unit of the S. Andrea Hospital at the II Faculty of Medicine. His research interests include determination of the primary structure of proteins; setting up of methodologies for the identification of post-translational modifications; identification and structural characterization of phytotoxic lipodepsipeptides from *Pseudomonas syringae*; identification and structural/functional characterization of bioactive peptides from amphibian skin; molecular cloning, expression and characterization of genes coding for peptides of the innate immune system; and human proteome studies of lymphocytes from immuno-compromised patients with oncological or neurodegenerative diseases. He has published more than 60 articles in international refereed Journals.

many of which are very similar to mammalian neuropeptides and hormones, as indicated by the pioneering work of the Italian pharmacologist V. Erspamer [18–21]. The first report on the occurrence, in amphibian skin, of peptides with anti-microbial activities dates back to 30 years, when Csordas and Michl referred to bombinin [22]. Nevertheless, it has been only since the 1980s, when Michael Zasloff isolated magainins from *Xenopus laevis* [23], that many peptide antibiotics from different amphibian species have been isolated and examined in detail, so that nowadays amphibian skin is among the richest natural sources of such molecules (see an updated list at: http://www.bbcm.univ.trieste.it/~tossi/pag1.htm)

AMPs are produced and stored within granules of serous glands, which are located mainly in the skin of the dorsal region, and innervated by sympathetic fibres [24]. Adrenergic stimulation of myocytes surrounding the glands gives rise to a synchronous discharge of their content with a holocrine mechanism. As a result, secretions contain not only AMPs, but also cytosolic components and intact polyadenylated mRNAs encoding the peptides [25].

Similar to other AMPs of animal origin, amphibian AMPs are synthesized as large inactive precursors and then converted to the final product, upon suitable proteolytic cleavage. Earlier reports have demonstrated that AMPs from the granules of the serous dermal

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Table 1 Primary structure of temporins

Temporin	Frog species	Sequence	Reference
A	*R. temporaria*	FLPLIGRVLSGIL-NH_2	42
B	"	LLPIVGNLLKSLL-NH_2	42
C	"	LLPILGNLLNGLL-NH_2	42
D	"	LLPIVGNLLNSLL-NH_2	42
G	"	FFPVIGRILNGIL-NH_2	42
H	"	LSP—NLLKSLL-NH_2	42
K	"	LLP—NLLKSLL-NH_2	42
L	"	FVQWFSKFLGRIL-NH_2	42
Ala	*Amolops loensis*	FLPIVGKLLSGLSGLL-NH_2	43
1Ara	*R. areolata*	FLPIVGRLISGLL-NH_2	44
1Aua	*R. auroraaurora*	FLPIIGQLLSGLL-NH_2	45
1Bya	*R. boylii*	FLPIIAKVLSGLL-NH_2	46
1Ca	*R. clamitans*	FLPFLAKILTGVL-NH_2	47
1Cb	*R. clamitans*	FLPLFASLIGKLL-NH_2	47
1Ec	*R. esculenta*	FLPVIAGLLSKLF-NH_2	48
1Ga	*R. grylio*	SILPTIVSFLSKVF-NH_2	49
1Gb	"	SILPTIVSFLSKFL-NH_2	49
GH	*Hylaranaguentheri*	FLPLLFGAISHLL-NH_2	50
1Hka	*R. heckscheri*	SIFPAIVSFLSKFL-NH_2	51
1Ja	*R. japonica*	ILPLVGNLLNDLL-NH_2	52
1La	*R. luteiventris*	VLPLISMALGKLL-NH_2	41
1Lb	"	NFLGTLINLAKKIM-NH_2	41
1Lc	"	FLPILINLIHKGLL-NH_2	41
1M	*R. muscosa*	FLPIVGKLLSGLL-NH_2	53
1Oa	*R. ornativentris*	FLPLLASLFSRLL-NH_2	54
1Od	"	FLPLLASLFSGLF-NH_2	54
1Ola	*R. okaloosae*	FLPFLKSILGKIL-NH2	51
1Olb	''	FLPFFASLLGKLL-NH_2	51
1P	*R. pipiens*	FLPIVGKLLSGLL-NH_2	41
1Pla	*R. palustris*	FLPLVGKILSGLI-NH_2	55
1Pra	*R. pirica*	ILPILGNLLNGLL-NH_2	56
1Tga	*R. tagoi*	FLPILGKLLSGIL-NH_2	57
1TSb	*R. tsushimensis*	FLPLLGNLLNGLL-NH_2	58
1Va	*R. virgatipes*	FLSSIGKILGNLL-NH_2	59
1Vb	"	FLSIIAKVLGSLF-NH_2	59
1VE	*R. versabilis*	FLPLVGKILSGLI-NH_2	60

Basic and acidic residues are indicated by red and blue letters, respectively. Gaps (-) are inserted to maximize identities.

glands of *Rana* and *Bombina* genera are inducibly expressed in response to microbial challenge [26], and that their promotor regions are regulated by the NF-κB/IκBα machinery [27], which is tightly conserved throughout the zoological phyla from insects to mammals [28–30].

Altogether, frogs are a good model system to study the *in vivo* role of AMPs in vertebrates, and various studies have highlighted their functional importance in guarding the producer-hosts from infections and in keeping control of their natural flora [26,31]. All frog species are endowed with their own unique set of AMPs, constituting families of 2–50 closely related members [32], but very little is known about the biological significance of the existence of multiple forms of a prototypic peptide sequence within the same organism. The main families of structurally similar peptides encompass bombinins and bombinins H from the European toads *Bombina variegata* and *Bombina orientalis* [33–36]; magainins from the African clawed frog *Xenopus laevis* [23], dermaseptins from the South American arboreal frog *Phyllomedusa sauvagii* [37,38], and those from the *Rana* genus (e.g. brevinins, ranalexins, ranatuerins, esculentins and temporins) [32,39–42]. Do the homologous peptides work in concert? The answer to this question will be addressed in the next paragraphs.

With the aim to give a contribution to better understand how Nature equipped each animal with an accurate fast-operating protection mechanism, and to open additional roads for the future design of new anti-infective compounds with expanding properties, this

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review focuses on the two attractive families of small-sized and mild cationic AMPs from frog skin: temporins and bombinins H, isolated from *Rana* and *Bombina* species, respectively.

TEMPORINS

Structural Characteristics

Initially identified in 1996 in the skin secretion of the European red frog *Rana temporaria* [42], temporins were then purified from frogs of Northern American and Eurasian origin. Temporins are among the smallest amphipathic α-helical AMPs found in nature to date (10–14 amino acids, except the 16-residue temporin-ALa, from the Chinese torrent frog *Amolops loloensis* [43]) and with a low net positive charge at neutral pH, ranging from 0 to +3 (Table 1). Indeed, besides a few isoforms devoid of basic or acidic amino acids (net charge +1, due to the free *N*-terminal amino group) and temporin-1Ja, bearing an aspartic acid (net charge 0), all the remaining members contain only a single or a double (in temporins L, -1Lb and -1Lc) positively charged residue (Table 1).

Unlike the majority of Ranidae AMPs such as brevinins, ranalexins, ranatuerins and esculentins [24,32], temporins lack the *C*-terminal heptapeptide ring, stabilized by a disulphide bridge, and are amidated at their carboxyl end, as a result of a post-translational enzymatic reaction [61].

Their precursors are characterized by a 22-residue signal peptide, which is remarkably similar to that present in the precursors of other AMPs from *Rana* genus [40,62] and in those of the opioid and anti-fungal peptides from the skin of frogs of the subfamily *Phyllomedusinae* [63]. The signal peptide is then followed by an acidic 'spacer', ending with a convertase processing domain [64], and preceding a single copy of the mature peptide. Presumably, all these molecules evolved through dissemination and mutations of a common ancestor gene.

For the time being, temporins represent the largest family of AMPs (more than 50 members [65]), and up to 10 isoforms have been extracted from a single specimen. The physiological relevance of their simultaneous presence is discussed below.

Functional Features

Temporins are particularly active toward Gram-positive bacterial strains, including methicillin- and vancomycin-resistant staphylococci and enterococchi (minimal inhibitory concentrations (MICs) ranging from 2.5 to 20 μM [32,66,67]) and *Candida* species, without being toxic on non-cancerous mammalian cells. However, an exception is given by temporin L (net charge +3), being highly active on Gram-positive and Gram-negative bacteria, erythrocytes and cancer cells [68] and capable of synergizing with β-lactam antibiotics [69] as well as temporin isomers (see below). Lately, also the longer temporin-ALa was shown to be equally effective on both the Gram-positive *Staphylococcus aureus* and the Gram-negative *Escherichia coli*, with MICs of 2 and 3 μg/ml, respectively [43]. Overall, both the peptide's net positive charge and length are critical factors in determining the anti-microbial efficacy of temporins [70]. Of benefit is the fact that two members of this family, temporins A and B, exhibit potent anti-parasitic activity on the insect (promastigote) and the mammalian intracellular stage (amastigote) of *Leishmania* protozoa, which are responsible for severe parasitic infections in vertebrates, including humans [71]. The two peptides have a similar effect against promastigotes, with an LC_{50} value (the peptide concentration required to inhibit 50% of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction compared to that in the untreated parasites) equal to 8.5 μM. Note that contrary to those few natural AMPs with anti-parasitic properties, temporins preserve activity against the more resistant morphological stage of the parasite, the amastigote, with temporin B being the strongest one. In addition, temporins do not damage the host cell for amastigotes (the macrophage) at doses that are lethal for the intracellular parasites [71].

Target selectivity of temporins, as well as that of many other AMPs, is not clear. It is governed not only by the physico-chemical characteristics of the peptide (sequence, charge distribution, oligomeric state, amphipathicity and helicity) but also by the type of the target cell surface and its metabolism. It is worthwhile to emphasize that a significant anti-bacterial activity against Gram-negative bacteria can be manifested also by temporins A and B, when each peptide is combined with a sub-inhibitory concentration of temporin L [72]. Interestingly, this synergistic effect is more pronounced against *Aeromonas hydrophila*, an opportunistic pathogen living in healthy frogs, but capable of causing high mortality in amphibian populations, by inducing diseases such as the natural outbreaks of 'red leg' [73]. This bacterium, which has been found to be resistant to several AMPs from frog skin, such as magainin I, magainin II, PGLa, CPF, ranalexin and dermaseptin [73] and to several conventional antibiotics [74], is also responsible for a variety of infections in humans, especially in immunocompromised individuals [75,76].

In light of the different target cell specificity and the synergistic action of temporins, the occurrence of structurally related peptides within the same organism should confer the animal a better shield to either combat a broad array of invading noxious

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microorganisms, or prevent infections from those microbes belonging to the natural flora, without harming the host. However, further non-anti-microbial functions might support the reason for the coexistence of homologue peptides; indeed, temporins are not only anti-infective molecules, as multiple biological properties related to host defence, which will not be presented in this paper, have been demonstrated [70].

Mechanism of Action Underlying the Anti-microbial Activity and the Synergistic Effect of Temporins

How do these peptides kill microbes? The selectivity of a huge number of cationic AMPs towards bacteria is generally attributed to their higher affinity to the negatively charged bacterial membranes [77], compared with those of eukaryotic cells, which are rich in sterols and zwitterionic phospholipids [13]. Peptide molecules initially bind electrostatically to the membrane, which stabilizes their amphipathic structure required for the subsequent membrane perturbation [12,13]. However, before reaching the cytoplasmic membrane, the peptides need to cross the microbial wall, which, in Gram-negative strains, is surrounded by a second membrane, consisting primarily of anionic lipopolysaccharides (LPS, or endotoxins) [78]. This outer membrane is a barrier against temporins and interferes with the uptake process of the peptide by inducing its oligomerization. This makes it difficult for the oligomers to reach the cytoplasmic membrane, as proposed for the two isoforms, temporin A and temporin B [72]. In contrast, the highly active temporin L disaggregates partially when in contact with LPS and, therefore, it should be easier for this peptide to traverse the LPS leaflet into the target inner membrane [72]. Spectroscopic measurements have suggested that temporins A and B bind mostly to those portions of LPS facing the solution and not to those in proximity with the inner lipid moiety, as found for temporin L, which is able to penetrate well into the hydrophobic region of LPS [69]. The inactivity of temporins A and B on Gram-negative strains is in line with recent findings suggesting that oligomerization of AMPs causes a dramatic reduction in their anti-microbial activity because of their larger size, which makes it more difficult the diffusion of the peptides through the cell wall [79]. Besides its ability to bind LPS, temporin L has also an *in vivo* capacity to prevent lethality in rat models of *E. coli* septic shock, by means of endotoxin neutralization [69]. It is known that during or after antibiotic therapy, LPS are released from the cell envelope of Gram-negative bacteria and activate a cascade of uncontrolled systemic inflammatory responses leading to organ failure and death. According to this, it is becoming of great interest to discover new compounds able to simultaneously kill bacteria and neutralize endotoxin effects [80].

Recent analyses of the molecular mechanism underlying the synergistic action between temporin isoforms against Gram-negative bacteria have demonstrated that it differs from that already described for the magainin/PGLa pair, which is explained by the increasing perturbation of the cytoplasmic membrane [81,82]. In the case of temporins, the synergism takes place at the level of the outer membrane, and is associated with the ability of temporin L in hampering temporins A and B aggregation (Figure 1). This should promote the peptide translocation across the outer leaflet, allowing them to easily get into the inner membrane and perturb it. These data represent the first example proving how peptides synergize to overcome bacterial resistance imposed by the physical barrier of LPS.

Several reports on temporins have pointed out that although their killing mechanism involves alteration of the cytoplasmic membrane permeability in a dose-dependent manner, without destroying cell integrity, membrane permeation is not *per se* the lethal event [83]. Altogether, it cannot be excluded that these peptides act *in vivo* in a more complex way, e.g. inhibiting metabolic functions [84,85].

As indicated in Table 1, temporins A and B are mildly cationic peptides, carrying only a single basic amino acid (arginine or lysine). This finding, and the fact that electrostatic interactions between these peptides and the negatively charged components of the cell surface of *Leishmania promastigotes* (i.e. the highly anionic lipophosphoglycan (LPG) [86–88]) do not play a crucial role in their anti-parasitic action [71], might contribute to the capability of temporins to also target amastigotes, which lack in an anionic LPG layer [86]. This is in

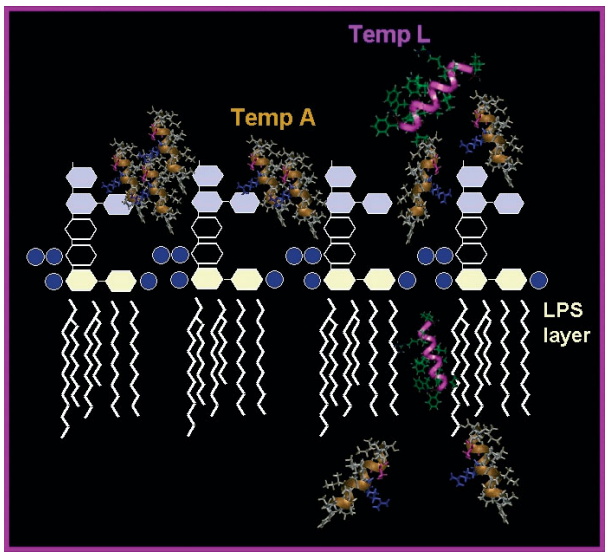

Figure 1 A possible mechanism for the synergism between temporin A and temporin L. The synergistic effect between the two peptides takes place at the level of the lipopolysaccharide layer of Gram-negative bacteria and is related to the ability of temporin L to interfere with the oligomerization process of temporin A, when in contact with LPS, and to assist it in traversing the LPS layer.

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Table 2 Sequences of bombinins H and bombinin H-related peptides

Peptide	Frog species	Sequence	Reference
Bombinin H1	*B. variegata*	IIGPVLGMVGSALGGLLKKI-NH_2	[36]
Bombinin H2	*B. bombina, B. variegata*	IIGPVLGLVGSALGGLLKKI-NH_2	[34, 36, 89]
Bombinin H3	*B. variegata*	I*I*GPVLGMVGSALGGLLKKI-NH_2	[36]
Bombinin H4	*B. bombina, B. variegata*	I*I*GPVLGLVGSALGGLLKKI-NH_2	[34, 36, 89]
Bombinin H6	*B. bombina, B. orientalis*	ILGPILGLVSNALGGLL----NH_2	[24, 89]
Bombinin H7	″	I*L*GPILGLVSNALGGLL----NH_2	[24, 89]
[a]GH-1	*B. orientalis*	IIGPVLGLVGKPLESLLE	[27]
[a]GH-2	″	ILGPVLDLVGRALRGLLKKI-NH_2	[90]
Maximin H1	*B. maxima*	ILGPVISTIGGVLGGLLKNL-NH_2	[91]
Maximin H2	″	ILGPVLSMVGSALGGLIKKI-NH_2	[91]
Maximin H3	″	ILGPVLGLVGNALGGLIKKI-NH_2	[91]
Maximin H4	″	ILGPVISKIGGVLGGLLKNL-NH_2	[91]

Basic and acidic residues are indicated by red and blue letters, respectively. Italicised letters within green boxes indicate D-amino acids. Gaps (-) are inserted to maximize identities.
[a], Peptide sequence deduced from gene.

contrast with what found for the majority of highly cationic peptides.

BOMBININS H

Discovery and Structural Features

The term bombinins H refers to a family of 17–20-residue hydrophobic AMPs, isolated from the skin secretions of frogs belonging to the *Bombina* genus such as *B. bombina*, *B. orientalis* and *B. variegata* (Table 2). All bombinins H are amidated at the *C*-terminus and adopt an amphipathic α-helical structure in membrane-mimicking environments [35]. Their existence was predicted from the cDNA sequences encoding the precursors of longer anti-microbial peptides, named bombinins (25–27 amino acids), previously detected in the skin secretions of *B. orientalis* and *B. variegata* [33,34]. Different from the precursors of other AMPs, these preproproteins include the information for one or two identical copies of a bombinin-like peptide (BLP), and a single copy of a bombinin H-like peptide, separated by acidic intervening sequences. Noteworthy, BLP mRNAs have also been identified in *Bombina* brain and stomach [33], suggesting their function in the central nervous system and gastrointestinal tract.

Bombinins H1–H4 (20-residues long) are weakly cationic peptides (net charge +3 at neutral pH, because of two lysines at the carboxyl end) and differ by one or two amino acids (Table 2). The most surprising outcome is the presence of a D-alloisoleucine at the second *N*-terminal position of some of them, as a consequence of post-translational modification of the respective gene-encoded L-isoleucine (there is no codon for a D-amino acid [92,93]), where the chirality of the α-carbon is changed (Table 2). The enzyme responsible for this L- to D-isomerization has been purified and characterized from skin secretions of *B. variegata* [94]. Two shorter (17-residues), more hydrophobic and less cationic forms of bombinins H (Table 2, H6 and H7, net charge +1) were isolated from *B. orientalis*. They lack the lysine-lysine-isoleucine motif at the *C*-terminus and differ one from each other by only the configuration of the second residue (an L- or D-leucine for H6 and H7, respectively).

On the basis of the sequences of two bombinin genes from *B. orientalis*, two additional bombinin H-like peptides were predicted, GH1 and GH2, but they were never found directly in the skin secretion [27,90]. Moreover, series of bombinin H-related peptides, termed maximins H, have been isolated from skin glands of the chinese red belly toad *Bombina maxima*. However, the presence of a D-amino acid has not been mentioned [91]. Their sequences are also shown in Table 2.

The existence of a D-amino acid in ribosomally made peptides of animal origin was first detected in dermorphin and delthorphins, opioid peptides from the skin of South American frogs *Phyllomedusa sauvagei* and *Phyllomedusa bicolor* [95,96], and later in other neuropeptides and toxins from invertebrates [97–100]. Although such a modification has already been described for bacterial and fungal AMPs [93], bombinins H represent the first example of natural AMPs bearing a single D-amino acid, deriving from a post-translational isomerization, and coexisting with the corresponding, but less abundant, all-L counterparts [36]. Actually, in contrast with opiates, where the all L-isoform is apparently not detectable as a mature peptide and is

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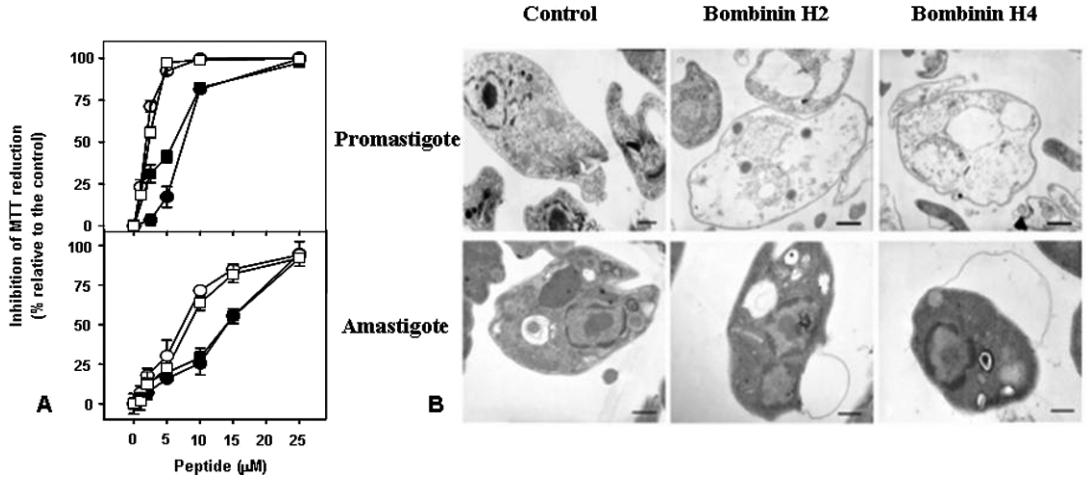

Figure 2 Effect of bombinins H on the viability and morphology of *Leishmania* parasites. Panel A: Anti-parasitic activity. The inhibition of MTT reduction in *L. donovani* promastigotes (upper graph) and *L. pifanoi* amastigotes (lower graph), caused by H2 (closed symbols) or H4 (empty symbols) was measured either immediately after peptide incubation (squares), or after allowing the surviving parasites to proliferate (circles), as explained in Mangoni *et al.* [103]. MTT reduction to insoluble formazan by mitochondrial reductases is used as a viability parameter of the parasites. Data are expressed as means ± standard deviations. Panel B: Transmission Electron Microscopy images of *Leishmania* protozoa after peptide treatment. *Leishmania donovani* promastigotes and *Leishmania pifanoi* amastigotes were incubated for 1 h under standard conditions with bombinins H2 and H4 at the equipotent concentrations causing approximately 80% or 30% killing of promastigotes or amastigotes, respectively. The values of the peptide concentrations were determined according to the results reported in panel A. Parasites were then fixed for 1 h with 5% (w/v) glutaraldehyde in phosphate buffered saline, containing 2.5% (w/v) osmium tetraoxide, gradually dehydrated in ethanol and propylene oxide. Membrane disruption, membrane blebbing and breakages as well as depletion of electron-dense cytoplasmic material can be observed for both peptides (Bar = 0.5 μm). Taken from Ref. [103].

completely inactive (the occurrence of D-amino acids is essential for the interaction with opioid receptors), the all L-bombinins H are considerably active, although with a different potency and cell selectivity from that of the corresponding D-amino acid-containing isoforms (see below).

Biological Activity

The anti-microbial activity of bombinins H and their mechanisms of action were investigated mainly for the two pairs of diastereomers, bombinins H2/H4 and bombinins H6/H7. Each pair includes an all L-isomer (H2 and H6) and the corresponding D-amino acid-containing peptide, H4 and H7 (Table 2). The anti-bacterial and anti-yeast activities were tested using the inhibition zone assay on agarose plates, and expressed as lethal concentration (LC), the lowest peptide concentration inhibiting microbial growth [101]. As shown in Table 3, in which temporin L is included as reference, only the more cationic H2 and H4 display anti-microbial activity against the selected microorganisms, with the exception of *A. hydrophila* Bo-3N, a member of *B. orientalis* natural flora [35]. In all cases, H2 is less potent than H4, whose LC values are approximately 2–3-fold higher and 2-fold lower than those of temporin L against bacterial and *Candida* species, respectively (Table 3). The anti-fungal activity toward *Phytophthora nicotianae* spores was also carried out, and a minimal fungistatic concentration corresponding to 18, 10 and 16 μM, was found for H2, H4 and temporin L, respectively [102]. Rate of killing experiments, which were performed at high ionic strength such as phosphate buffered saline, have revealed that the pair H2/H4 expresses higher activity on Gram-positive bacteria, in agreement with their LC values, and with a faster killing kinetic for the D-amino acid-containing peptide [35]. Surprisingly, H7 appears to be the best bactericidal isoform against *A. hydrophila* Bo-3N, which is thought to be its natural target [35]. Since a dansylated derivative of this peptide (net charge 0 at neutral pH) maintains the same trend of activity, the single positive charge of H7 is not an essential element for its anti-microbial action.

Bombinins H are also lethal to promastigotes and amastigotes of *Leishmania* parasites with LC_{50} values significantly lower for H4 (Figure 2) [103]. The effectiveness of H4, which is better than that of H2, mirrors the disparity previously described for their bactericidal action [35].

Regarding the hemolytic activity of bombinins H against human erythrocytes, the all-L H6 is the most active one [35]. On the contrary, within the pair H2/H4, the D-amino acid-containing H4 is more hemolytic at concentrations above 15 μM (Table 3). Note, a remarkable hemolysis is manifested by maximins H [91].

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Table 3 Anti-bacterial and haemolytic activities of bombinins H and temporin L

Microorganism	H2	H4	H6	H7	Temp L
	Lethal concentration (μM)				
Gram-negative bacteria					
Escherichia coli D21	21.4	4.7	NA	NA	1.5
Escherichia coli D22	4.4	3.1	NA	NA	0.7
Yersinia pseudotuberculosis YPIII	7.3	2.0	NA	NA	0.7
Pseudomonas syringae pv *tabaci*	32.0	8.2	NA	NA	3.0
Aeromonas hydrophila Bo-3N	NA	NA	NA	NA	NA
Enterobacter agglomerans Bo-1S	30.0	11.3	NA	NA	NA
Gram-positive bacteria					
Bacillus megaterium Bm11	1.4	0.8	NA	25	0.3
Staphylococcus aureus Cowan I	4.7	3.0	NA	NA	0.5
Staphylococcus lentus	2.0	0.6	NA	NA	0.2
Micrococcus luteus	2.0	0.2	NA	NA	0.3
Yeasts					
Candida albicans ATCC 10231	3.1	1.6	NA	NA	2.7
Candida guiller-mondii	1.3	0.7	NA	NA	1.8
Candida tropicalis	1.1	0.6	NA	NA	1.0
% Haemolysis at 15 μM	11.0	28.0	78.0	20.0	90.0

NA, not active.
Data taken from Refs. 35,102.

Mode of action studies and the effect of L-to D-amino acid conversion on the biophysical properties of bombinins H

Mode of action studies on intact bacteria have indicated that bombinins H alter the permeability of the target cell membrane, triggering leakage of large cytosolic components (e.g. proteins in the case of H2/H4) or diffusion of smaller molecules through local membrane disruptions (H7) [35]. Despite of growing efforts to gain an insight into the anti-bacterial activity and mechanism of action of AMPs against bacteria, only a few of them have been tested so far against *Leishmania,* and very little has been determined about the parameters contributing to this activity. Yet, similar to temporins A and B, bombinins H2 and H4 rapidly perturb the parasite's membrane with a pronounced blebbing and loss of intracellular material (Figure 2). The extent of membrane permeation correlates with the peptide concentration used and with the inhibition of parasite proliferation [103]. To shed light into the biophysical basis accounting for the quantitative discrepancies between the two diastereomers, the peptide's structure in membranes mimicking those of mammals, bacteria and *Leishmania* promastigotes was analysed using ATR-FITR and CD spectroscopies. These studies revealed that: (i) a D-amino acid in the second position does not destabilize the α-helical content of the peptide and (ii) H2 adopts β-sheet aggregates specifically in the promastigote's mimicking membranes. In addition, surface plasmon resonance measurements have shown a lower binding affinity to the *Leishmania* model membrane for H2 than H4, according to its lower hydrophobicity, as estimated using reverse-phase HPLC [36]. Although the reason is not yet clear, the formation of aggregated strands by H2 and its weaker membrane-binding affinity could contribute to the reduction of the anti-parasitic activity of this peptide compared to H4.

It is well known that inclusion of D-amino acids in the sequence of AMPs makes them more resistant to enzymatic degradation and serum clearance. However, as highlighted by Mangoni *et al.* [103], a difference in the susceptibility to proteolysis is not the reason for the different activities of the two isomers against *Leishmania.*

In summary, a single natural L-to D-amino acid substitution stands for a new approach developed by Nature to modulate not only the peptide bioavailability (e.g. higher solubility) and biostability (i.e. protection from proteolytic degradation) but also the peptide's biophysical properties (e.g. hindering its oligomeric state) to make it as a more potent weapon against microbes.

CONCLUDING REMARKS

Temporins and bombinins H are among the shortest and mild cationic (net charge ranging from 0 to +3) amphipathic α-helical AMPs found in Amphibia to date. Beside their anti-microbial activity, other functions, such as the ability to mediate protection against endotoxic shock [69] and to promote chemotaxis and prophylaxis against infections, have been delineated for temporins in animal models [104,105]. Furthermore, the control mechanism governing the expression of gene-encoded AMPs in *Rana* and *Bombina* is similar to that involved in the regulation of mammalian inflammatory genes, and has a common signal transduction cascade. Therefore, these two families of AMPs represent fascinating molecules to expand our knowledge on the immunomodulatory role of skin AMPs in the innate defence system of vertebrates, their regulation, and further physiological functions. In addition, both temporins and bombinins H are provided with fundamental features to assist the design of new antibiotics: (i) they are short-length peptides allowing a readily cost-efficient chemical production, without compromising the peptide's pharmacological value by means of a reduction in size; (ii) they are fast membrane-active AMPs against a broad range of pathogens (bacteria, yeasts, filamentous fungi, viruses and protozoa) at

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concentrations not lytic towards normal mammalian cells and (iii) they retain good activity in 33% human serum and in physiological salt concentration. Yet, their *in vivo* efficacy and therapeutic index have not been examined in depth and studies along this line are in progress.

Finally, the synergistic effect between short and homologue AMPs to overcome resistance due to the LPS protective layer of Gram-negative strains and the stronger anti-microbial activity of D-amino acid-containing peptides, made by Nature, might suggest innovative and viable strategies for the development and manufacture of new peptide-based anti-infective medical preparations.

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