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Review

Bombinins, antimicrobial peptides from *Bombina* species

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ABSTRACT

The skin secretions of *Bombina* species contain peptides and small proteins with interesting biological properties. These include bombesin, thyrotropin releasing hormone, BSTI and Bv8. In this review, the biosynthesis and antimicrobial activity of two groups of peptides, bombinins and bombinins H, are described. To date, these have only been found in *Bombina* skin. They are derived from common precursors containing one or two bombinin copies at the amino and a single bombinin H at the carboxyl end. Bombinins are active against Gram-positive and Gram-negative bacteria and fungi but virtually inactive in haemolysis assays. Conversely, bombinins H have lower bactericidal activities but lyse erythrocytes. In the skin secretions, bombinins H are present in two sizes with either 20 or 17 amino acids. Moreover, they occur as epimers with either an L- or a D-amino acid at position 2. An enzyme catalyzing this inversion of chirality of an amino acid in peptide linkage has been isolated from *Bombina* skin secretions. In different tests, also with different stages of the life cycle of *Leishmania* parasites, the D-forms were found to be more active. Biophysical studies have yielded some insight into the different behaviours of the epimers in model membranes.

Contents

1. Introduction

In skin secretion of amphibians, a multitude of antimicrobial peptides have been discovered [1,2]. Many of these are linear peptides that can form amphipathic helices, others have a single disulfide bridge, and a few, as discussed in some detail in this review, also contain a D-amino acid. These amphibian peptides have widely differing activities against Gram-positive or Gram-negative bacteria, fungi and a parasite. The main function of these constituents of skin secretions is the inhibition of microbial growth on the surface of frogs, many of which live in humid environments. In addition, skin secretions also contain many different peptides related to mammalian hormones and neurotransmitters [3,4]. The complete "cocktail" produced in the skin of frogs apparently also serves as a deterrent against predators.

Here we present an overview on the structure and function of antimicrobial peptides from skin secretions of *Bombina* species. These are members of an ancient group of frogs which, at least according to some herpetologists, belongs to a separate family, the Bombinatoridae of the suborder Archaeobatrachia. Besides the three main species, *Bombina bombina*, *Bombina variegata* and *Bombina orientalis*, a few others, most notably *Bombina maxima* from some regions in China, have been identified. The antimicrobial peptides produced in the skin of these frogs, the bombinins and bombinins H, have not been detected in other amphibian genera.

Secretory glands in the skin of these frogs also produce several other peptides with interesting biological activities. Two of these were already discovered more than 30 years ago. One was TRH (thyrotropin-releasing hormone), a peptide first isolated from mammalian hypothalamus. *Bombina* skin contains as much as 40 μg/g fresh tissue of this tripeptide [5], orders of magnitude more than present in the

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hypothalamus. The best known constituent of *Bombina* skin secretions is bombesin [6]. This and related peptides from other frogs are the amphibian homologues of the mammalian gastrin-releasing peptide. Bombesin is a growth factor for small cell lung cancer and other tumor cells. Numerous studies have been published describing the various pharmacological activities of bombesin and its homologues [for a recent review, see [7]]. PubMed lists more than 4000 publications where the term "bombesin" is mentioned in the title and/or the abstract.

More recently, two other constituents of *Bombina* skin secretions have been discovered. One is the trypsin inhibitor BSTI (*B*ombina *s*kin *t*rypsin/*t*hrombin *i*nhibitor) which surprisingly is related to protease inhibitors from nematodes [8]. In addition, a small protein termed Bv8 (*B. variegata*, molecular mass 8 kDa) was found [9]. This had originally only a relative in the venom of the black mamba [10], but in recent years, two mammalian homologues have been characterized. These are EG-VEGF/prokineticin 1 and mammalian Bv8/prokineticin 2 [11–13]. They all belong to a small group of proteins containing around 80 amino acids including 10 cysteines, for which, derived from the conserved amino-terminal sequence, the name AVIT family has been proposed [14]. In mammals, members of this family bind to two G-protein-coupled receptors [15,16] and thereby elicit an astounding variety of biological effects [see ref. [17] for a recent review]. For example, they induce hyperalgesia [8,18], stimulate endothelial cell vascular growth [19], and play a role in circadian rhythm [20]. Moreover, mammalian Bv8 produced by myeloid cells was shown to promote tumor growth [21], a finding with promising therapeutic implications. Mutations in human prokineticin 2/Bv8 and/or its receptor are one of the causes for the Kallmann syndrome, a genetic disease characterized by an impaired development of the olfactory bulb and severe defects in the gonads [22]. This is another example how research on the constituents of skin secretions from amphibians can lead to important discoveries relevant for mammalian physiology.

2. Bombinins: structure and biosynthesis

Studies on the skin secretions of the two European species *B. bombina* and *B. variegata* were already started in the early sixties in the group of H. Michl in Vienna [23]. At that time, the pentapeptide Glu-His-Phe-Ala-Asn-amide could be characterized from this source. About 10 years later it was shown that this was the carboxy-terminal part of a larger peptide termed bombinin which was reported to have antibacterial and haemolytic activities [24].

After a hiatus of about 20 years, the constituents of skin secretions of *Bombina* species were studied again by groups in Rome, Salzburg and San Francisco using modern separation and amino acid sequencing techniques as well as cDNA cloning. In case of *B. variegata* it could be shown that a family of peptides related to the original bombinin existed. They all contain 27 residues which differ at some positions in the amino-terminal half, while the rest is identical [25]. Similar results were obtained with bombinins from another species, *B. orientalis* [26]. Amino acid sequences of bombinins, obtained either by direct peptide sequencing or by cDNA cloning, are reported in Fig. 1.

From the sequence of the bombinin precursors obtained via cDNA cloning one could predict the existence of another peptide [25,26]. Indeed, several variants of this peptide could be detected in skin secretions of *B. variegata* and *B. orientalis*. These were named bombinins H to indicate that, as opposed to bombinins, these are more *h*ydrophobic and have *h*aemolytic activity [27]. Some bombinins H isolated from *B. orientalis* contained 17 instead of 20 amino acid residues [28]. More recently, homologues of both groups of peptides were also characterized from skin of *B. maxima*; these were named maximins and maximins H, respectively [29].

Interestingly, in some of these bombinins H the second amino acid was found to be D-alloisoleucine [27]. As reviewed elsewhere, peptides containing a D-amino acid have been found in skin of other frogs [30], in the venom of the male platypus [31], and in a variety of invertebrate species [reviewed in [28,30,32]].

The function of D-amino acids in secreted peptides varies. In case of the bombinins, the antimicrobial activity of the all-L-versus the D-2-variant is relatively small (see below). Conversely, in case of the dermorphins and deltorphins only those with a D-amino acid at position two bind to opiate receptors. If the amino-terminal sequence has a beta-strand structure, the side chain of the D-residue would be "sandwiched" between the ones of the L-amino acids in position one and three, thereby yielding a new structural element.

An enzyme converting L-isoleucine to D-alloisoleucine at position 2 of the mature peptide has been characterized from *Bombina* skin secretions [33]. This inversion of chirality of an amino acid in peptide linkage occurs via de-protonation/protonation at the α-carbon, apparently in the absence of any cofactor. Such a reaction mechanism has also been demonstrated for an isomerase from the venom of a spider [34]which converts an L-Ser close to the carboxyl end of a 48-residue toxin to the D-isomer. However, the frog and the spider enzyme have different amino acid sequences.

Interestingly, DNA sequences potentially coding for proteins related to the *Bombina* isomerase are present in the genome of different vertebrates. The closest homologue was found in an EST library from the frog *Xenopus tropicalis* (65% sequence identity in a fragment of close to 300 amino acids). More distant relatives have been found in the genomes of e.g. fish, chicken and man [33]. It is, however, currently not known whether any of these genes codes for an active isomerase.

More recently, isomerase activity has been detected in the venom of the male platypus. This venom contains two peptides, one related to beta-defensins and the other to C-type natriuretic peptides [31], which both contain a D-amino acid at position 2. Using a partially

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Bombinin   (B. variegata)   GIGGALLSAAKVGLKGLAKGLAEHFAN-NH2
BLP-1      (B. orientalis)  GIGASILSAGKSALKGLAKGLAEHFAN-NH2
BLP-3      (B. orientalis)  GIGAAILSAGKSALKGLAKGLAEHF---NH2
Maximin 1  (B. maxima)      GIGTKILGGVKTALKGALKELASTYAN-NH2
                            ***   *   *  ***  * **

Bombinin H2   (B. variegata)     IIGPVLGLVGSALGGLLKKI-NH2
          H4   (B. variegata)     IIGPVLGLVGSALGGLLKKI-NH2
          H6   (B. orientalis)    IIGPILGLVSNALGGLL----NH2
          H7   (B. orientalis)    ILGPILGLVSNALGGLL----NH2
Maximin   H1   (B. maxima)        ILGPVISTIGGVLGGLLKNL-NH2
                                  * **         *****
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Fig. 1. Some representative amino acid sequences of bombinins, bombinins H and maximins H. D-amino acids in position 2 are italicized. Identical residues are marked (*).

purified enzyme preparation, the substrate specificity of the platypus isomerase has been determined [35].

Genes encoding the precursors of bombinins were cloned from *B. orientalis* [36,37] (see Fig. 2). These are comprised of two exons separated by large introns (1–2 kb). Exon 1 codes for the signal peptide; exon 2 includes the remaining coding region comprising the sequences of the mature peptides as well as acidic and spacer peptides. The genetic information differs in the two genes: the first gene (*BLP-3*) codes for two copies of a bombinin-like peptide (BLP-3) and one copy of a bombinin H-related peptide, whereas the second gene (*BLP-7*) codes for a single copy of each peptide. The same organization was present in the various cDNA clones isolated from *B. variegata* [1,25]. The nucleotide sequences of exon 1 and the acidic and spacer peptides in the two genes are very similar. Canonical Lys-Arg sequences are flanking the putative peptides for the correct processing, plus a Gly residue necessary for the C-terminal amidation. In the promoter regions of these genes, binding sites for NF-κB and NF-IL6 are present (Fig. 2). A common regulation of the expression of antimicrobial peptide genes occurs in amphibians involving the cooperation of a multitude of transcription factors, including those acting in the activation of the mammalian acute-phase response.

3. Biological activity of bombinins

The antimicrobial activity of either bombinin-like peptides or bombinins H can be distinguished on the basis of their cytolytic properties. Bombinins were found to be active against Gram-positive (*Bacillus megaterium* Bm11, *Staphylococcus aureus* Cowan1) and Gram-negative (*Escherichia coli* D21, *E. coli* D22, *Yersinia pseudotuberculosis*, *Pseudomonas aeruginosa* ATCC 15692) bacteria as well as against *Candida albicans*; however, they have no appreciable haemolytic capacity (Table 1) [38]. Conversely, bombinins H generally have a lower antibacterial but a higher haemolytic activity [1,25,27].

Subsequently, the interest of researchers has focused on the functional significance of the D-amino acid present in the sequences of some bombinins H containing either 20 or 17-residues. These

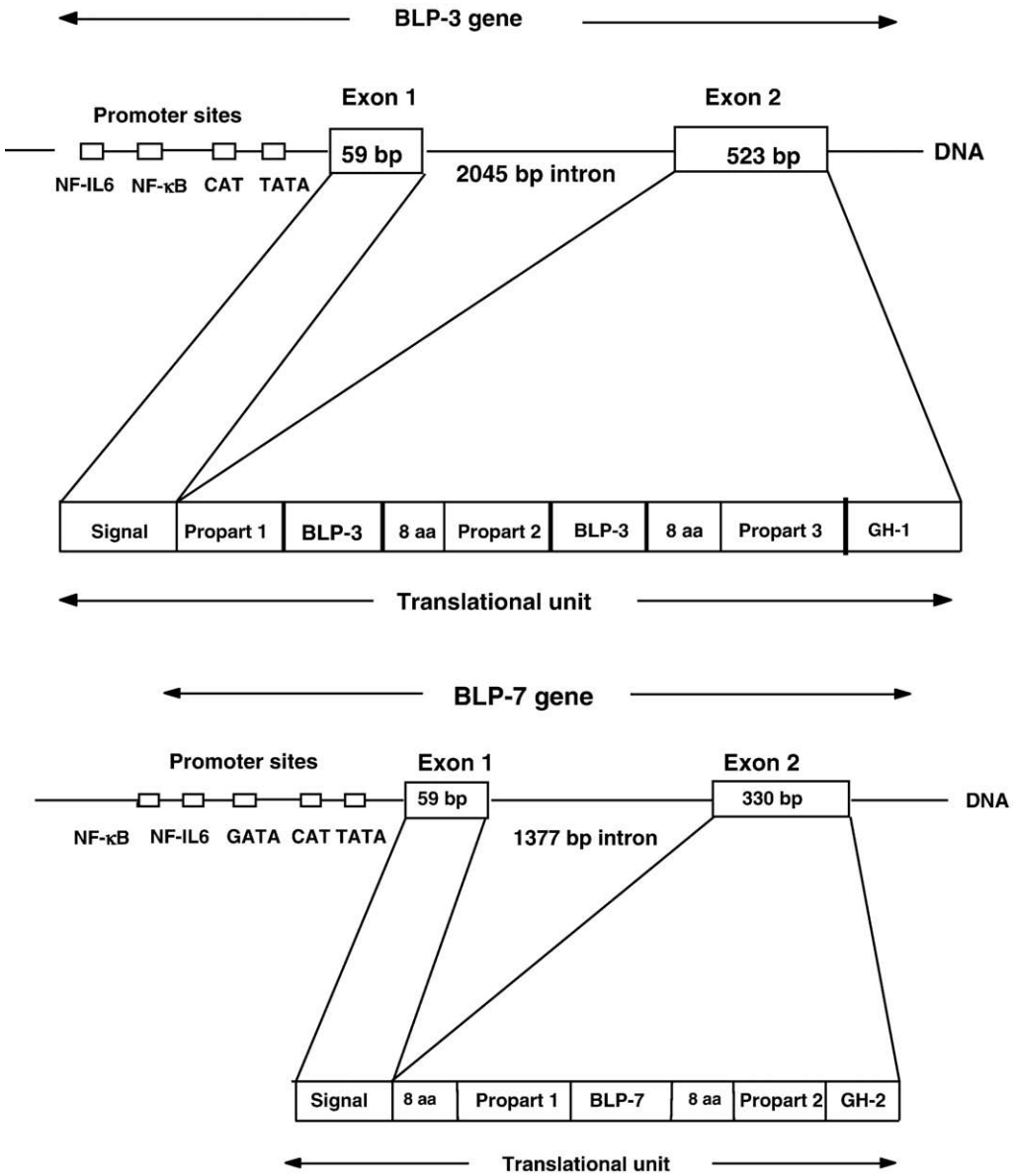

Fig. 2. Schematic representation of the *BLP-3* and *BLP-7* genes from *B. orientalis* with the indication of promoter sites. Exon 1 contains the predicted signal sequence. Exon 2 contains pro-parts, spacer peptides, one or two copies of a bombinin-like peptide (BLP) and one copy of a bombinin H peptide (GH, gene-derived bombinin H) [modified from ref. [32 and 33]].

Table 1
Antibacterial and haemolytic activities of bombinins

Peptide	BLP-1	BLP-3	H2	H4	H6	H7
	Lethal concentration (μM)					
Gram-negative bacteria						
Escherichia coli D21	1.7	3.0	21.4	4.7	NA	NA
Escherichia coli D22	1.5	0.9	4.4	3.1	NA	NA
Yersinia pseudotuberculosis YPIII	0.8	0.5	7.3	2.0	NA	NA
Pseudomonas aeruginosa ATCC 15692	6.3	9.2	NA	NA	NA	NA
Pseudomonas syringae pv *tabaci*	ND	ND	32.0	8.2	NA	NA
Aeromonas hydrophila Bo-3N	ND	25.7	NA	NA	NA	NA
Enterobacter agglomerans Bo-1S	ND	1.9	30.0	11.3	NA	NA
Gram-positive bacteria						
Bacillus megaterium Bm11	0.3	0.8	1.4	0.8	NA	25
Staphylococcus aureus Cowan I	3.4	1.7	4.7	3.0	NA	NA
Staphylococcus lentus	ND	ND	2.0	0.6	NA	NA
Micrococcus luteus	ND	ND	2.0	0.2	NA	NA
Yeasts						
Candida albicans ATCC 10231	0.4	0.4	3.1	1.6	NA	NA
Candida guiller-mondii	ND	ND	1.3	0.7	NA	NA
Candida tropicalis	ND	ND	1.1	0.6	NA	NA
% haemolysis at 15 μM	<10	<10	11.0	28.0	78.0	20.0

Lethal concentration values were measured with the inhibition zone assay.
NA, not active; ND, not determined.
Data taken from refs. [35,36].

comparative studies on the activity of two corresponding isomers led to a deeper knowledge of their biological properties [38,39]. In particular, the pairs bombinin H2/H4 and H6/H7 were analysed. Of these, the cationic H2/H4 peptides containing 20 amino acids were more active against Gram-positive bacteria. Between the two, peptide H4 containing the D-amino acid had a stronger antibiotic activity and a higher rate of killing against the microorganisms tested. The only exception was *Aeromonas hydrophila* Bo-3N, a bacterial species belonging to the natural flora on the skin of *B. orientalis* [40], where the all-L H2-peptide was more active. Both H2 and H4 were also found to be active against spores of the fungus *Phytophthora nicotianae*, with a minimal fungistatic concentration of 10 and 18 μM, respectively [41].

The more hydrophobic bombinins H6 and H7, when assayed in agar medium, were devoid of any antibacterial activity, possibly due to their poor solubility and reduced diffusion rate. In solution, only the D-amino acid-containing H7 had a bactericidal effect against some bacterial strains. In particular, H7 was found to be specifically active against *A. hydrophila*, thus representing a possible modulator of the natural flora. Additional experiments indicated that the increase in hydrophobicity does not increase the antimicrobial activity and that the single positive charge of H7 is not essential [38,42].

The membrane permeabilization experiments indicated that bombinins H2 and H4 cause large damage at the cell membranes which leads to protein leakage. Apparently, H7 damages the membrane to a lesser extent so that it becomes leaky only to small molecules. The biological activity of H6 is mainly directed against eukaryotic cells, such as erythrocytes, containing membranes composed of zwitterionic phospholipids. Indeed, H6 caused 60% haemolysis at a very low peptide concentration (4 μM) [38].

Bombinins H2/H4 were also assayed on *Leishmania*, a protozoan pathogen endemic in many parts of the world. The peptide H4 was found to be lethal to promastigotes and amastigotes of *Leishmania* parasites with LC_{50} values significantly lower than H2 [43]. However, the final effect on the parasite morphology was similar for both epimers and characterized by a marked damage at the membrane (Fig. 3).

To study the biophysical basis for the different behaviours of the two epimers, the structure of the peptides was analysed with ATR-FTIR and CD spectroscopy in membranes mimicking those of mammals, bacteria and *Leishmania* promastigotes, respectively [43]. 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) only H2 forms β-sheet aggregates in the model promastigote's membrane. In addition, using surface plasmon resonance measurements, the binding affinity of these peptides to the *Leishmania* model membrane was determined. Probably due to its reduced hydrophobicity, bombinin H2 was found to have a lower binding affinity than H4 [43]. Further studies using high-resolution ^{1}H NMR spectroscopy and a paramagnetic probe indicated that in a membrane mimetic environment the randomly ordered N-terminal segment was exposed to solvent [44].

Folding propensities of H2 and H4 were also investigated by means of circular dichroism measurements and molecular dynamics simulations [45]. It could be shown that in solution H2 has a markedly higher tendency to fold, whereas the stereochemical isomerization of isoleucine to D-alloisoleucine in position 2 drastically decreases the ability of bombinin H4 to form intrapeptide contacts [45].

The insertion of a D-amino acid in the peptide sequence makes it more resistant to enzymatic degradation and slows down serum clearance. However, the reason for the different activities of the two isomers against *Leishmania* seems to be more complex. The isomerization of an L- to a D-amino acid can lead to new structural elements which can not be formed by all-L sequences, like e.g. the side-chain of the D-residue being "sandwiched" between the side chains of the two adjacent L-amino acids [46]. This can modulate not only stability and bioavailability but also, as in case of the bombinins, the biophysical properties by preventing the formation of oligomers thereby increasing its antimicrobial activity.

The structural and the related functional differences of these peptides suggest that they act through more than one mechanism, and this may represent a possible way to prevent the development of bacterial resistance. For these reasons, several amphibian peptides and their analogues have been assayed against multidrug-resistant

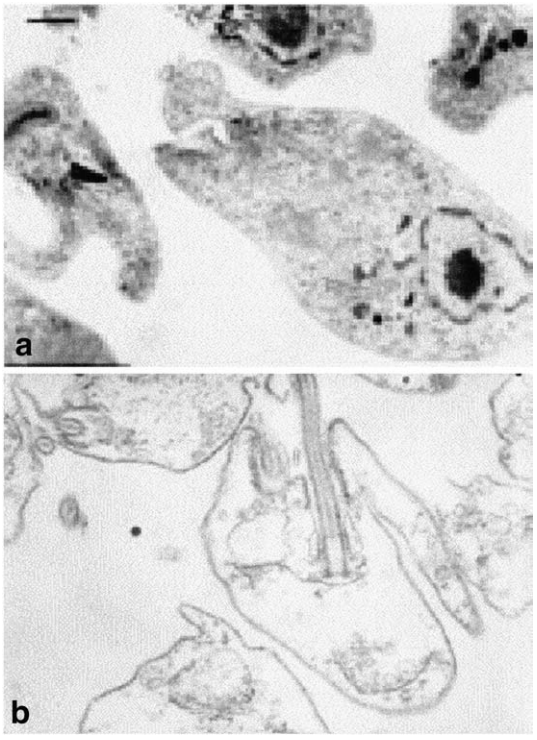

Fig. 3. Transmission Electron Microscopy images of *Leishmania donovani* promastigotes. a, control; b, parasites after treatment with bombinin H2 (9 μM). Parasites were fixed for 1 h with 5% (w/v) glutaraldehyde in phosphate buffered saline, containing 2.5% (w/v) osmium tetraoxide, gradually dehydrated in ethanol and propylene oxide. Membrane disruption, as well as depletion of electron-dense cytoplasmic material can be observed in peptide-treated promastigotes (Bar = 0.5 μm). (Photo by J.M. Saugar).

nosocomial bacterial strains [47] as promising candidates for therapeutic use.

Moreover, as recently demonstrated for the amphibian peptides temporins [48], but not yet addressed in the studies carried out on bombinins, synergistic effects in the activity of the different combinations of bombinin and bombinin H peptides may in fact determine the overall antimicrobial potency of the skin secretion of these frogs.

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