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Innate immunity: involvement of new neuropeptides

M.H. Metz-Boutigue, A.E. Kieffer, Y. Goumon and D. Aunis

Unité Inserm U 575 "Physiopathologie du Système Nerveux", IFR 37, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France

Secretory granules of chromaffin cells from the adrenal medulla store catecholamines and a variety of peptides that are secreted in the extracellular medium during exocytosis. Among these fragments, several natural peptides displaying antimicrobial activities at the micromolar range have been isolated and characterized. We have shown that these peptides, derived from the natural processing of chromogranins (CGs), proenkephalin-A (PEA) and free ubiquitin (Ub), are released into the circulation and display antibacterial and antifungal activities. In this review we focus on three naturally secreted antimicrobial peptides corresponding to CGA1–76 (vasostatin-I), the bisphosphorylated form of PEA209–237 (enkelytin) and Ub. In addition, the antimicrobial properties of the synthetic active domains of vasostatin-I (CGA47–66 or chromofungin) and Ub (Ub65–76 or ubifungin) are reported.

The need for antifungal agents increases in parallel with the expanding number of immunocompromised patients at risk of invasive fungal infections resulting from, for example, chemotherapy, grafts, infection with human immunodeficiency virus type-1 (HIV-1) and prolonged antibiotherapy. A rapid and active response to challenge pathogens is essential for the survival of all living organisms. Consequently, the production of a large variety of natural non-toxic antimicrobial peptides from both plants and animals is attracting increasing attention. They are present in the hemolymph of insects, and are also stored in the secretory granules of immune cells found within mammals and birds [1–3]. The importance of these molecules is clearly established in the innate immunity of invertebrates by the microorganism-induced antibacterial activity that can be detected in the hemolymph [4]. In vertebrates, these peptides complete adaptive immunity by acting as a first line of defense against pathogens and by controlling natural flora [5]. Antimicrobial peptides are evolutionary ancient weapons, and have served a fundamental role in the successful evolution of complex multicellular organisms [5]. Furthermore, similarities have been highlighted between pathogen recognition, signaling pathways and effector mechanisms of innate immunity in *Drosophila* and mammals [6]. Adaptive immune responses are not the only mechanisms used by vertebrates to fight against microorganisms. Vertebrates also have a high array of humoral and cellular responses that are essential to health, independent of lymphocyte receptors and grouped under the heading 'innate immunity'.

Different natural antimicrobial peptides from mammals have been previously characterized in several tissues, cells and biological fluids, and some of them are presented in Table 1 [3,7–23]. The diversity in the structures of these peptides is remarkable. Among these, some have a linear structure, such as LL-37 [17]. Some are proline-rich peptides (Bac 5 [18], PR-39 [19]) and others, such as indolicidin [20] and histatin 5 [21], are tryptophane- and histidine-rich peptides. In addition, an important class of antimicrobial peptides, with intramolecular disulfide bridges, is homologous to the sequence of bactenecin 1 [22], protegrin [23], hepcidin [7] and defensins [3,8,9].

The mechanism of action of antimicrobial peptides is partially understood for bacterial targets. For example, it has been shown that LL-37 interacts with bacteria by aligning itself parallel to the bacterial membrane, initially at lower concentrations and then by reorientation at high concentrations (reviewed in [10]), whereas, defensins have been proposed to form voltage-dependent channels into the bacterial membrane [11]. Moreover, it has been reported that some antimicrobial peptides reach intracellular targets by interfering with DNA (i.e. indolicidin) [12,13] and protein synthesis (i.e. PR 39) [14]. In addition to these antibacterial molecules, several antifungal peptides (often displaying antibacterial effects) were characterized, such as protegrin 1, α-defensins and indolicidin. Antifungal peptides are classified according to their mode of action. The first group acts by lysis that occurs through several mechanisms, for example, destabilization of the membrane, formation of aqueous pores and intracellular mechanisms. Whereas, the second group interferes with cell-wall synthesis or the biosynthesis of glucan or chitin [24,25].

Secretory granules from adrenal medullary chromaffin cells contain a complex mixture of molecules including catecholamines, low-molecular mass constituents and numerous water-soluble proteins and peptides, such as chromogranins (CGs) and proenkephalin-A (PEA), which are released into the circulation in response to splanchnic nerve stimulation [16,26].

During the past decade, our laboratory has characterized the processing of chromogranins A and B (CGA and CGB; [27,28]) and PEA [29] in chromaffin granules derived from bovine adrenal medulla, and have identified other natural peptides that are secreted with catecholamines

Corresponding author: M.H. Metz-Boutigue (metz@neurochem.u-strasbg.fr).

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Table 1. Overview of antimicrobial peptides from mammals[a]

Peptides	Sequences	Tissues or fluids	Antibacterial activity	Antifungal activity	Refs
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	E, BC	+	–	[17]
Bactenecin 1	RLCRIWIRVCR	BC	+	–	[22]
Protegrin 1	RGGRLC$_1$YC$_2$RRRFC$_2$VC$_1$VGRa	BC	+	+	[23]
α-defensin (HNP3)	DC$_1$YC$_2$RIPAC$_3$IAGERRYGTC$_2$IYQGRLWAFC$_3$C$_1$	BC, E	+	+	[3]
β-defensin (TAP)	NPVSC$_1$VRNKGIC$_2$VPIRC$_3$PGSMKQIGTC$_2$VGRAVKC$_1$C$_3$RKK	E, BC	+	+	[8]
θ-defensin	GFC$_1$RC$_2$LC$_3$RRGVC$_3$RC$_2$IC$_1$TR	BC	+	+	[9]
Hepcidin	DTHFPIC$_1$IFC$_2$C$_3$GC$_4$C$_1$HRSKC$_2$GMC$_3$C$_4$KT	Liver	+	+	[7]
Bac 5	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGRPFPa	BC	+	–	[18]
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFPa	BC	+	–	[19]
Indolicidin	ILPWKWPWWPWRRa	BC	+	+	[20]
Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	Saliva	+	+	[21]

[a]Abbreviations: a, amidation of the C-terminal residue; BC, blood cell; E, epithelial tissue.

during stimulation of the chromaffin cells and possess antimicrobial activity (Figure 1) [28,30–36]. These peptides, which are derived from their protein precursors and ubiquitin (Ub), are also present in secretory granules and have been hypothesized to play a role in stress situations, acting as an immediate protective shield against pathogens [30]. They are recovered in biological fluids implicated in defense mechanisms, for example, abcess fluids, and also in secretions of stimulated polymorphonuclear neutrophils (PMNs) [31,32].

In this review we focus on three natural antimicrobial peptides derived from CGA, PEA and Ub, and their synthetic active domains. They display common structural features that are believed to be important for the molecular mechanisms implicated in their antimicrobial activities.

Vasostatin-I and chromofungin: potent inhibitors of fungi and yeast

Following separation of the soluble material present in chromaffin granules by HPLC, antibacterial activity against *Micrococcus luteus* can be detected in several fractions. One of these fractions has been identified as CGA1–76, also known as vasostatin-I, by sequencing and MALDI-TOF mass spectrometry. This peptide (Figure 1) corresponds to the N-terminal domain of bovine CGA (residues 1–76). CGA, the major member of the chromogranin family (40% of the total soluble bovine chromaffin granule proteins), has been extensively studied. This protein is the precursor of numerous peptides displaying various biological activities [37]. Vasostatin-I corresponds to a highly conserved domain, in which the disulfide bridge and the sequence 50–62 (SILRHQNLLKELQ) are strictly unchanged [32,33] (Figure 1). Furthermore, it has recently been reported that the CGA47–57-peptide contains a cell adhesion site for fibroblasts and smooth muscle cells [38], demonstrating multifunctional roles for this α-helical CGA sequence, which is highly conserved during evolution. Vasostatin-I has been immunodetected in the large and dense core vesicles that travel down sympathetic axons and are released from nerve terminals in response to stimulation [39]. In addition to the vasoinhibitory effect [40], vasostatin-I exhibits other biological activities, such as the autocrine inhibition of parathyroid hormone secretion [41], regulation of cell adhesion [42] and neurotoxic effects in neuronal–microglial cell co-cultures [43].

Natural bovine vasostatin-I is selectively active against *M. luteus* and *Bacillus megaterium*, inhibiting their growth at 1 and 0.1 micromolar, respectively (Table 2) [32]. Activity is not detectable against Gram-positive bacteria, such as *Bacillus cereus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Mycobacterium fortuitum*, *Staphylococcus aureus* and *Listeria monocytogenes*, and

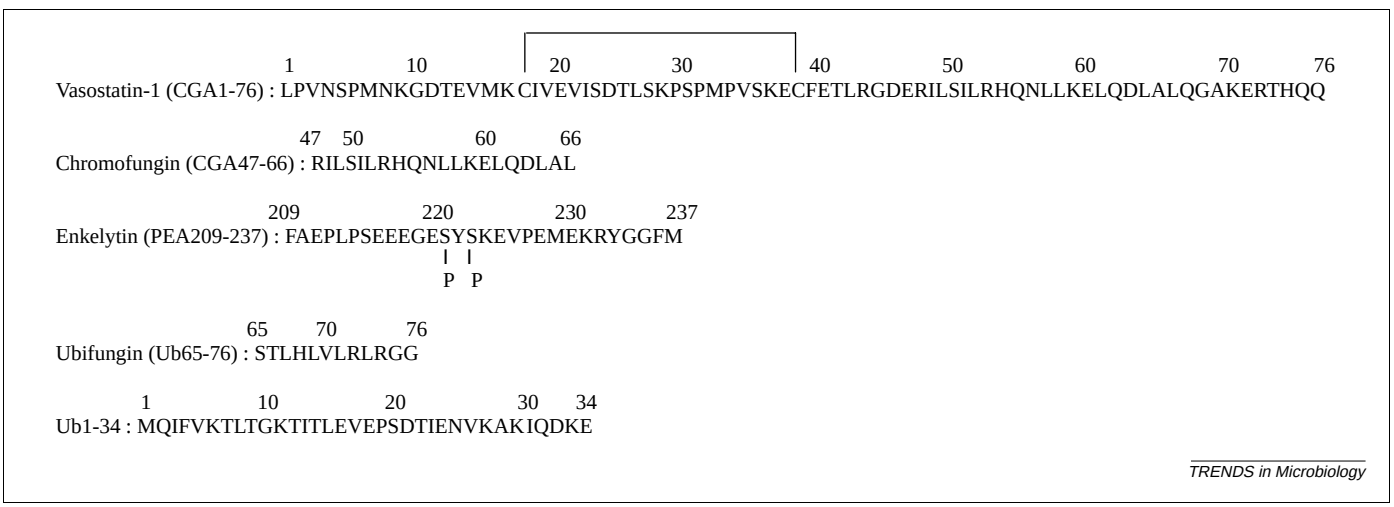

Figure 1. Antimicrobial peptides derived from chromogranin A (CGA), proenkephalin-A (PEA) and ubiquitin (Ub). The disulfide bridge and the phosphorylated serine residues are indicated. These peptides have been isolated from chromaffin secretory granules.

Table 2. Antimicrobial activity of vasostatin-I (CGA1–76) and chromofungin (CGA47–66)[a,b]

	MIC (μM)				
	CGA1–76 [32]	**CGA47–66** [33]	**PEA209–237** [31]	**Ub65–76** [54]	**Ub65–76 + 10μM, Ub1–34** [54]
Gram-positive Bacteria					
M. luteus	1	–	0.2	5	5
B. megaterium	0.1	–	0.2	4	3
S. aureus	–	–	4.5	–	–
Gram-negative Bacteria					
E. coli SBS363	–	–	–	20	20
E. coli D22	–	–	–	50	50
Filamentous fungi					
N. crassa	1	5	nd	10	4
A. fumigatus	2	15	nd	30	15
A. brassicola	1	2	nd	nd	nd
N. haematococca	0.5	2	nd	1	nd
F. culmorum	0.5	2	nd	4	nd
F. oxysporum	3	10	nd	4	nd
T. mentagrophytes	–	15	nd	20	7
T. viride	nd	nd	nd	10	nd
Yeast cells					
S. cerevisiae	2	nd	nd	nd	nd
C. albicans	2	10	nd	15	7
C. tropicalis	nd	15	nd	15	7
C. glabrata	nd	–	nd	20	10
C. neoformans	nd	15	nd	15	7

[a]Enkelytin (PEA209–237) and ubifungin (Ub65-76) in absence and presence of Ub1–34. Filamentous fungi were grown on a five-cereal medium and spores were harvested as previously reported [32]. To test the antifungal activity of the peptides, spores (final concentration, 10^4 spores/ml) were suspended in a growth medium containing potato dextrose broth (Difco; in half-strength) and yeast cells in Sabouraud medium with starting absorbance at 260 nm of 0.001. For each peptide the corresponding references are indicated.
[b]Abbreviations: MIC, minimal inhibitory concentration; nd, not determined; –, no activity.

Gram-negative bacteria such as *Escherichia coli* D31 and D22, *Enterobacter cloacae*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [32]. In addition, vasostatin-I has been tested against different fungi and yeast cells, according to the method previously reported [44]. Vasostatin-I peptide was found to be strongly active against a large variety of filamentous fungi including *Neurospora crassa*, *Alternaria brassicola*, *Nectria haematococca*, *Fusarium culmorum*, *Fusarium oxysporum* and the pathogenic *Aspergillus fumigatus*, but to be inactive against *Trichophyton mentagrophytes* (Table 2) [32]. In addition, this peptide is active against the pathogenic yeast forms of *Saccharomyces cerevisiae* and *Candida albicans* at micromolar concentrations [32]. After removal of vasostatin-I from external medium and substitution with fresh vasostatin-free medium, fungi were unable to restart growth, suggesting that this peptide possesses fungicidal activity [32]. It is important to point out the remarkable specificity of vasostatin-I towards microorganisms, because it is inactive against bovine erythrocytes and other mammalian cell-types [32]. Furthermore, these studies have been completed with structural aspects using the recombinant human vasostatin-I, corresponding to the sequence CGA1–78 and its alkylated form. The experimental data have shown that the disulfide bridge is crucial for antibacterial activity, whereas, it is not required for antifungal activity [32]. Interestingly, earlier experiments showed that chromogranin-derived peptides are present in inflammatory fluids, and later established the presence of vasostatin-I in secretions of human PMNs. These data indicated that CGA-derived fragments could be locally recovered in infectious fluids where they might exert their antimicrobial activity [32]. The concentration of circulating vasostatin-I is in the 100 nanomolar range and it can increase in infectious fluids after secretion from PMNs, therefore displaying antifungal activities.

These experiments were extended by analysis of the recombinant-derived fragments of human vasostatin-I that are able to display antifungal activity. After digestion of human recombinant vasostatin-I with endoproteinase Glu-C, an active peptide that was identified as CGA47–60 using sequencing and MALDI-TOF mass spectrometry was found to be highly active against *N. crassa* with a minimal inhibitory concentration (MIC100), which completely inhibits fungal growth, of 7 micromolar [33]. The C-terminal domain of vasostatin-I appeared to be important for antifungal activity. Experiments testing the activities of different CGA-derived peptides (41–60, 41–70 and 47–70) located in the C-terminal domain of vasostatin-I, showed that the tripeptide RGD (43–45) hinders antifungal activity and confirmed that this region (60–70) is essential to kill fungi.

The shortest active peptide with maximum global hydrophobicity and amphipathic features corresponded to CGA47–66 and was called chromofungin [33] (Figure 1). Its global charge is the addition of positive charges +3.5 with 2R (2 × 1), 1K (1) and 1H (0.5), and the negative charges 2 with 1E (−1) and 1D (−1). The antimicrobial spectrum of this synthetic peptide is reported in Table 2. The three-dimensional structure of chromofungin has been determined in water–trifluoroethanol (50:50) using ^{1}H-NMR spectroscopy (Figure 2a). Folding in the presence of trifluoroethanol is often encountered for peptides that are able to interact with membranes, and has been widely used to study their three-dimensional structure. This analysis revealed the amphipathic helical character of the C-terminal part of the sequence 53–66, whereas

the N-terminal segments 48–51 and 53–56 were shown to present hydrophobic and hydrophilic characters, respectively [33]. The importance of the amphipathic sequence for antifungal activity was demonstrated by the loss of such activity against *N. Crassa* when two P residues were substituted for L61 and L64, disrupting the helical structure and amphipathic character of the peptide.

Lecithin monolayers are membrane models that are useful to study the molecular mechanisms implicated in the interaction of peptides with membranes [33]. The addition of chromofungin (5×19^{-9} Mres), expressed in amino acid residue per liter, induces a modest increase of the surface pressure but is indicative of its penetration into the lipid monolayer. When ergosterol, the main sterol present in yeast [45] and fungal [46] membranes, was included as a component in the lecithin monolayer, a strong increase in the surface pressure was obtained. This observation highlighted the strong specificity of chromofungin for interaction with yeast and fungal membranes [33]. Chromofungin is similar to many antimicrobial peptides as it is a cationic amphipathic molecule with a helical structure and is able to interact with inner and outer membranes to reach intracellular targets.

Enkelytin: potent inhibitor of Gram-positive bacteria

The processing of PEA has been extensively studied in chromaffin cells obtained from bovine adrenal medulla [29]. PEA degradation proceeds through an orderly series of steps. Several opioid peptides, including two C-terminally extended variants of enkephalins, the heptapeptide (M-enkephalin R-F) and the octapeptide (M-enkephalin R-F-L), are liberated by cleavage of the precursor at pairs of basic residues. The processing of PEA appears to start initially with the removal of the C-terminal domain (PEA209–239) [29]. Among the different PEA-derived peptides isolated after HPLC from the soluble intragranular material, a natural antibacterial peptide has been identified as the bisphosphorylated form of PEA209–237 (Figure 1) [34]. Using sequencing and MALDI-TOF mass spectrometry, the antibacterial activity has been correlated with the presence of two phosphate groups on serine residues S221 and S223 (Figure 2b) [34]. This bisphosphorylated peptide, known as enkelytin, is active against several Gram-positive bacteria, including *M. luteus, B. megaterium* and the pathogenic strain *S. aureus*, in the 0.2–4.5 micromolar range (Table 2). The ^{1}H-NMR spectra of PEA209–237 (Figure 2b) indicated that the residue P227 is responsible for conformational *cis–trans* isomerization changes, to which its antibacterial activity is correlated [35]. In addition, structural analysis indicates that synthetic non-modified PEA209–237 adopts an L-shape [31,35] in which the two putative phosphorylated serine residues S221 and S223 might induce linearization of the peptide by electrostatic interactions with the two residues E228 and E230 (Figure 2b). In contrast to the bisphosphorylated peptide, the non-modified fragment displays a low antibacterial activity indicating that the two phosphorylated residues S221 and S223 play an important conformational role. Furthermore, the synthetic peptide PEA209–237 that has three E residues in place of S215, S221 and S223 conserves

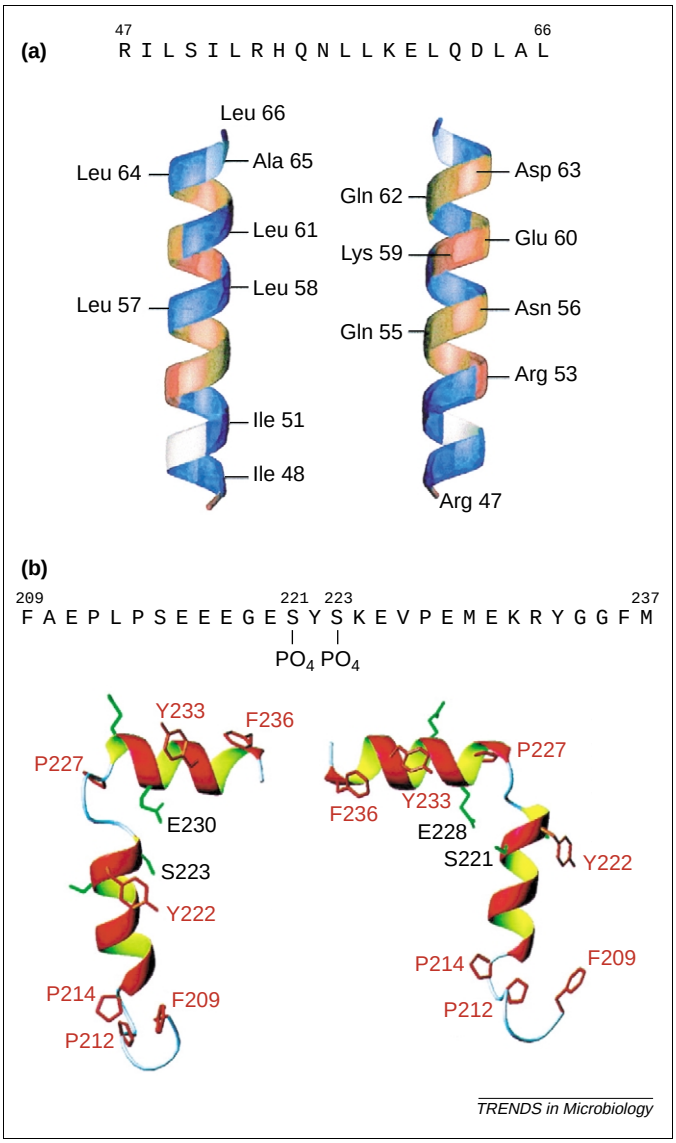

Figure 2. **(a)** Sequence of chromofungin and three-dimensional ^{1}H-NMR structure of CGA47–66. Reproduced, with permission, from [33]. **(b)** Sequence of enkelytin and three dimensional ^{1}H-NMR structure of non-phosphorylated PEA209–237 (left, *cis*-isomer; right, *trans*-isomer). Reproduced, with permission, from [35]. The structures have been determined by ^{1}H-NMR in a 50% trifluoroethanol–water solution using the X-PLOR program [35].

antibacterial activity suggesting the importance of negative charges in the expression of such activity. Antibacterial peptides have to be positively charged to bind bacterial surfaces, which are usually negatively charged. Curiously, enkelytin possess a negative charge (-7) and because of this might be compared with polyaspartic acid peptides identified in secretions from the lung [47].

To characterize the biological function of enkelytin, several fluids and PMN secretions from injured animals with infection have been examined [31]. Different biological fluids have been analysed: bovine knee periarthritis abscess fluid, and also bovine caesarean abscess and rabbit abscess fluid induced by subcutaneous injection of complete Freund's adjuvant. These experiments have revealed the presence of several peptides with antibacterial activities: defensins, bactenecins, dodecapeptide as expected, and also enkelytin [31]. Following isolation at the inflammatory area, enkelytin was quantified by

sequencing, and its concentration (in bovine periarthritis abscess fluid) was estimated from 0.5 to 1 micromolar. Therefore, enkelytin locally exerts genuine antibacterial activity in specific fluids. As a continuation of these studies, we have shown its presence in human PMNs [31]. PEA has been reported to be significantly expressed in the immune system and might provide a basis for neuroimmune interactions [48]. The local inflammatory response initiates the synthesis and secretion of opioid peptides by immune cells. Therefore, enkelytin degradation by neuropeptide-degrading endopeptidase (NEP) and angiotensin converting enzyme (ACE) present in granulocytes generate Met-enkephalin and its derived peptides [31]. Met-enkephalin enhances the immune reaction in patients with cancer or AIDS [49]; moreover, this pentapeptide can bind opioid receptors present in peripheral inflamed tissues to mediate an analgesic effect [50]. Taken together the major bisphosphorylated form of PEA209–237 and Met-enkephalin would provide a highly beneficial survival strategy for the proinflammatory process.

Ub and its N- and C-terminal fragments

Ub is a peptide of 76 residues found in all eukaryotic cells that displays well-conserved sequences, from protozoa to vertebrates [51,52]. It conjugates to intracellular target proteins in a process that appears to be the initiation step for selective protein degradation by the 26S proteasome and serves as a signal for the endocytosis of plasma membrane proteins [53]. Recently, the subcellular localization of free Ub in bovine adrenal chromaffin cells has been reported [54]. Ub is present in secretory granules and is secreted with catecholamines during chromaffin cell stimulation. In addition, natural Ub displays *in vitro* antimicrobial activities [54] and inhibits the growth of *M. luteus* and *B. megaterium* at a MIC of 60 micromolar [54]. At a concentration of 100 micromolar, Ub completely inhibits the growth of *N. Crassa* [54]. Subsequently, the question regarding the shortest active peptide that has been derived from Ub was addressed. Following proteolytic digestion with endoproteinase Glu-C and separation on reverse phase HPLC, a potent active fraction was identified. The C-terminal fragment with the sequence STLHLVLRLRGG (Ub65–76), which results from enzymatic cleavage at E64, is a highly active fragment. As reported in Table 2, Ub65–76 is active against Gram-positive bacteria *M. luteus* and *B. megaterium* with a MIC estimated at 5 and 4 micromolar, respectively. Also, complete inhibition of their growth occurs at a concentration of 10 micromolar [54]. It is also active against *E. coli* (SBS 363 and D22) with a MIC of 20 and 50 micromolar, respectively [54]. However, activity is not detectable at concentrations up to 100 micromolar against other bacteria that have been tested so far [54]. The spectrum of the antimicrobial activity of Ub65–76 is remarkable because it also strongly affects the growth of filamentous fungi and yeast cells. This active domain of Ub, known as ubifungin, completely inhibits the growth of various filamentous fungi, including pathogenic strains (*A. fumigatus, N. haematococca, F. culmorum, F. oxysporum, T. mentagrophytes and T. viride*) with MIC values in the micromolar range (Table 2) [54]. In addition, Ub65–76 is active against the pathogenic yeast forms of *C. albicans, C. tropicalis, C. neoformans and C. glabrata* with a MIC varying from 15 to 20 micromolar [54]. The antibacterial and antifungal activity of Ub65–76 at 41°C was tested to examine the putative role of this peptide in humans with severe infectious diseases that induce a fever increase. In this condition ubifungin is active against Gram-positive bacteria (5 micromolar with *B. megaterium*), Gram-negative bacteria (75 micromolar with *E. coli* D22) and filamentous fungi (35 micromolar with *A. fumigatus*) [54]. Because microorganism growth had not resumed after 48 hours, this peptide is believed to exert a lytic mechanism. Importantly, ubifungin is inactive against erythrocytes [54]. In contrast to chromofungin, ubifungin is described as a cationic non-helical peptide that exhibits a global charge of 2.5.

When looking at the three-dimensional structure of Ub [55], the residue E64 appears to be located close to the N-terminal sequence surrounding the surface residue F4. Furthermore, the N-terminal fragment (Ub1–34) adopts a β-hairpin (residues 1–17), followed by an α helix (residues 23–34), the latter being important for membrane interactions. Antimicrobial assays of Ub1–34 have indicated that this peptide displays weak activity against *N. crassa* at a concentration of 100 micromolar. Interestingly, when Ub1–34 was added to Ub65–76 the two peptides acted synergistically to inhibit the growth of several filamentous fungi (Table 2). These effects have been reported for the pathogenic strains of *T. mentagrophytes* and *A. fumigatus* [54]. In normal serum the cellular concentration of Ub from erythrocytes and immune cells (neutrophils) is estimated at 6 micromolar and 10 nanomolar, respectively. Furthermore, immune cells in infectious fluids release higher concentrations of free Ub, which might be degraded by endoprotease V8 from *S. aureus,* inducing the release of Ub65–76 at concentrations corresponding to the antifungal activity.

Interaction of chromofungin, ubifungin and Ub1–34 with microorganisms

Confocal microscopy has allowed analysis of the interaction of chromofungin, ubifungin and Ub1–34 with fungal membranes of pathogenic strains of *A. fumigatus* and *C. albicans* as illustrated in Figure 3 [54]. By comparison with the control experiment (no peptide), rhodaminated chromofungin was visible at the level of the cell wall and in the inner part of fungus and yeast, where it appeared to accumulate after 15 minutes incubation (Figure 3a). By contrast, the labeled inactive peptide CGB602–626 was undetectable within microorganisms (Figure 3a). Experiments performed using ubifungin and Ub1–34 produced similar data. Labeled ubifungin was visible at the level of the cell wall and in the inner part of the fungi after two minutes incubation (Figure 3b). Furthermore, Figure 3b shows the interaction of rhodaminated Ub1–34 with *N. crassa*, indicating that the peptide was visible at the level of cell wall but not within cells. A similar observation was obtained with *A. fumigatus* [54]. However, when *A. fumigatus* was treated with unlabeled ubifungin before incubation with rhodaminated Ub1–34, an intense fluorescence was observed (Figure 3b)

indicating that ubifungin destabilizes the cell wall, allowing the peptide Ub1–34 to actively penetrate the fungi [54].

Chromofungin and ubifungin: inhibition of the phosphatase activity of calcineurin

In addition to destabilizing the cell wall, chromofungin and ubifungin might also exert activity on intracellular targets. It has previously been shown that vasostatin-I is responsible for the interaction of CGA with calmodulin in the presence of calcium [56]. Similarly, it has been reported that calmodulin is mono-ubiquitinylated in a Ca^{2+}-dependent manner with a Ub–calmodulin synthetase [57], a modification that induces a strong negative influence on the biological function of calmodulin. Furthermore, it has been established that calcineurin, the calmodulin-activated phosphatase B, plays a crucial role in hyphal growth, morphology and maintenance of the apical Ca^{2+} gradient in filamentous fungi [58]. When chromofungin and ubifungin were tested *in vitro* on the phosphatase activity of calcineurin, 80% of the activity was inhibited [33,54]. The ability of these two antifungal peptides to inhibit calcineurin activity at the same concentration range as that inhibiting fungal growth, suggests the implication of a calcineurin-involved molecular mechanism. Moreover, in addition to calcineurin, other calmodulin-dependent enzymes might be the target for these active peptides, therefore affecting intracellular signalling pathways and provoking intracellular damages.

Synergistic effect of ubifungin on the antifungal activity of chromofungin

At local infectious sites, the V8 protease of *S. aureus* might generate neuropeptides following proteolysis of the precursors, such as CGA, vasostatin-I or Ub. When chromofungin and ubifungin were combined and tested against the growth of *A. fumigatus*, *T. mentagrophytes*, *N. crassa* and *C. albicans*, their individual activities were maximized (Figure 4).

Chromofungin and ubifungin display complex mechanisms of antimicrobial action. They destabilize the cell wall of fungi by forming pores and exert intracellular effects by inhibiting calcineurin and perhaps other calmodulin-dependent enzymes. Therefore, these peptides are different from defensins that form a voltage-dependent channel into membranes [11]. They are also different from other peptides, such as indolicidin [13] and protein PR 39 [14], which reach intracellular targets and interfere with DNA and protein synthesis. Therefore, chromofungin and

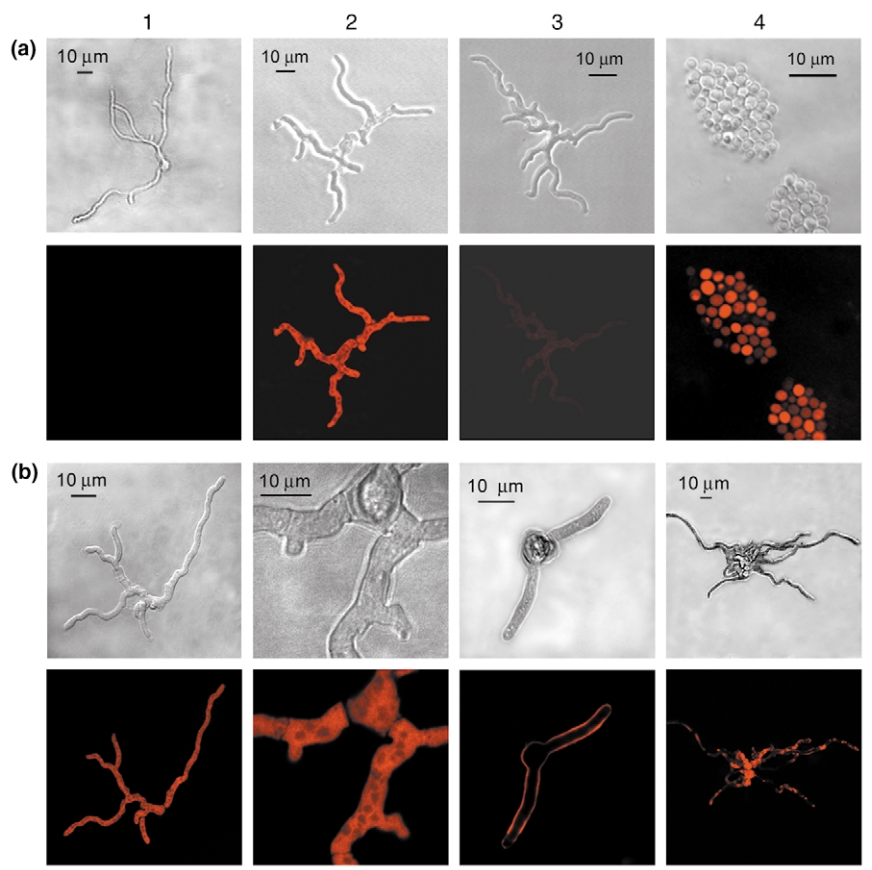

Figure 3. Phase contrast and fluorescence confocal laser micrographs of *Aspergillus fumigatus, Neurospora crassa* or *Candida albicans* with rhodamine-labeled synthetic peptides chromofungin, ubifungin, CGB602–626 and Ub1–34. **(a)** 1, *A. fumigatus* in the absence of peptide; 2, *A. fumigatus* after 60 minutes incubation with chromofungin (10 micromolar); 3, *A. fumigatus* after 60 minutes incubation with rhodaminated CGB602–626 (10 micromolar); 4, *C. albicans* after 60 minutes incubation with chromofungin (10 micromolar). **(b)** 1, *A. fumigatus* after 2 minutes incubation with ubifungin (2 micromolar); 2, at higher magnification, the core of fungi and unlabeled vacuoles and septum are clearly visible; 3, *N. crassa* was incubated with 5 micromolar Ub1–34 for 15 minutes: note the labeling along the cell membrane; 4, *A. fumigatus* was first incubated with 20 micromolar unlabeled ubifungin for 45 minutes before a final incubation with 5 micromolar rhodaminated Ub1–34 for 15 minutes. Note the fluorescence within cells revealing Ub1–34 penetration due to membrane destabilization evoked by ubifungin.

ubifungin should be considered as a new specific class of antimicrobial peptides.

Discussion

The past decade has witnessed a dramatic increase in the knowledge of natural antimicrobial peptides, most of them concerning antibacterial molecules. The emergence of fungal pathogens that are resistant to current therapies requires new non-toxic active molecules. The secretory granules of chromaffin cells from the adrenal medulla synthesize and store catecholamines and a large number of neuropeptides, and subsequently release them into circulation to activate target organs during cell-stimulation that occurs as a result of stress. Furthermore, chromofungin and enkelytin are released from PMNs, suggesting that they might act locally in infectious sites as defensive molecules [31,32]. Our previous study on the natural processing of CGA has indicated that vasostatin-I is predominantly generated in the matrix of chromaffin granules and co-released with catecholamines in the extracellular medium during chromaffin cell stimulation [27]. In addition, vasostatin-I is also present in numerous neuroendocrine tissues, and its release from sympathetic nerves suggests an important biological function. Vasostatin-I displays *in vitro* antimicrobial activities at micromolar concentrations. The concentration of circulating vasostatin-I is in the 100 nanomolar range and its half-life in circulation remains to be determined [33].

Enkelytin, which displays a potent antibacterial activity against Gram-positive bacteria including *S. aureus*, has been well conserved during evolution [34], and proteolytic processing of PEA in the adrenal medulla appears to start at the C-terminal region [59]. In addition, immunoreactive forms of this peptide can be found in circulating bovine plasma [60] and in infectious fluids [31].

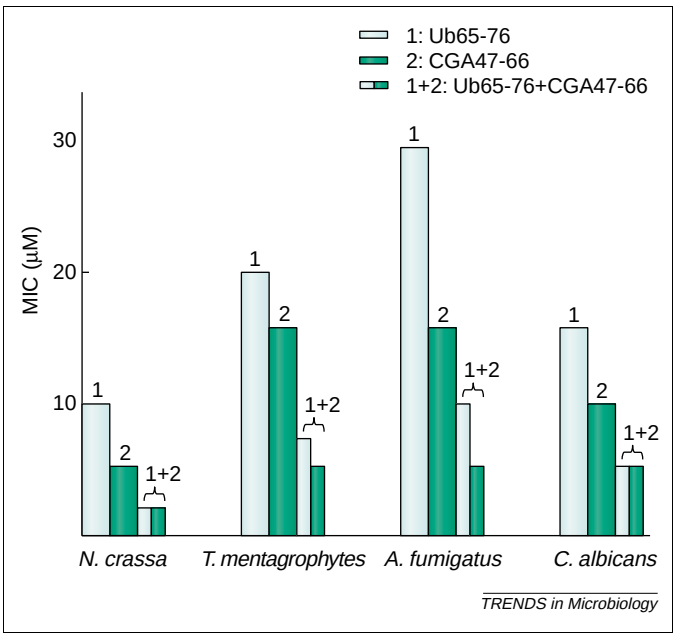

Figure 4. Synergistic effect of ubifungin (Ub65–76) and chromofungin (CGA47–66) on filamentous fungi (*Neurospora crassa, Trichophyton mentagrophytes, Aspergillus fumigatus*) and yeast (*Candida albicans*). For each strain the MIC (minimal inhibitory concentration, micromolar) is indicated for peptide 1 (Ub65–76), peptide 2 (CGA47–66) and the mixture of the two peptides.

Interestingly, the concentration of active enkelytin (0.5–1 μM) in these fluids is in accordance with the antibacterial activity.

In normal serum the cellular concentration of free Ub from erythrocytes and immune cells (neutrophils) [61] is evaluated at 6 micromolar and 10 nanomolar, respectively. Furthermore, in infectious fluids immune cells release higher concentrations of free Ub, which might be degraded by endoproteinase Glu-C from *S. aureus*, inducing the release of Ub65–76 locally at concentrations corresponding to the antifungal activity. Ub65–76-derived peptide has not been isolated from the intragranular matrix and secretions but it represents, with the N-terminal sequence Ub1–34, the Ub domains that are important for interactions with the membranes of microorganisms and antimicrobial properties.

As reported, vasostatin-I, enkelytin and Ub correspond to highly conserved peptides and their antimicrobial activities probably occurred early in evolution. Furthermore, they are widely distributed not only in endocrine, neuroendocrine and nerve cells but also in immune cells. Their liberation from cells indicates that these peptides probably play a role in inflammatory processes. Therefore, we suggest that in stress situations these peptides might provide a highly beneficial strategy against pathogenic invasion.

Finally, chromofungin, enkelytin and ubifungin represent new non-toxic antimicrobial agents. Chromofungin and ubifungin are able to cross cell-wall and plasma membranes, and probably interact with enzymes crucial for the growth of fungi. They might be used together or in combination with classical antifungal molecules to increase their efficiency.

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