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The antifungal effect of peptides from hymenoptera venom and their analogs

Research Article

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Abstract: As the occurrence of *Candida* species infections increases, so does resistance against commonly-used antifungal agents. It is therefore necessary to look for new antifungal drugs. This study investigated the antifungal activity of recently isolated, synthesized and characterized antimicrobial α-helical amphipathic peptides (12–18 amino acids long) from the venom of hymenoptera (melectin, lasioglossins I, II, and III, halictines I and II) as well as a whole series of synthetic analogs. The minimal inhibitory concentrations (MICs) against different *Candida* species (*C. albicans, C. krusei, C. glabrata, C. tropicalis* and *C. parapsilosis*) of the natural peptides amounted to 4–20 μM (7–40 mg/l). The most active were the synthetic analog all-D-lasioglossin III and lasioglossin III analog KNWKK-Aib-LGK-Aib-IK-Aib-VK-NH_2. As shown using a) colony forming unit determination on agar plates, b) the efflux of the dye from rhodamine 6B-loaded cells, c) propidium iodide and DAPI staining, and d) fluorescently labeled antimicrobial peptide (5(6)-carboxyfluorescein lasioglossin-III), the killing of fungi by the peptides studied occurs within minutes and might be accompanied by a disturbance of all membrane barriers. The peptides represent a promising lead for the development of new, effective antifungal drugs.

Keywords: *Antifungal agents • Candida • Fluorescent microscopy • Rhodamine 6G • Fluorescein-labeled peptide*

1. Introduction

Fungal diseases are associated with various kinds of pathogens, but the *Candida* species remain a major cause of infections. Whereas in the past, *Candida albicans (C. albicans, C. a.)* was the most common, at present non-albicans species have been detected more often (*C. krusei, C. k., C. glabrata, C. g., C. tropicalis, C. t., and C. parapsilosis, C. p.*). *Candida* species are part of the normal flora of the gastrointestinal tract, mouth, vagina and skin. They cause infection when an alteration in the body environment permits their sudden proliferation (e.g. rising glucose levels from diabetes mellitus; lowered resistance due to an immunosuppressive drug, radiation, aging, or a disease such as cancer or human immunodeficiency virus (HIV) infection). *Candida* most readily colonises the skin around the nails or the soft, moist areas around body openings. Candidiasis (also called *candidosis* or *moniliasis*) is usually a mild, superficial fungal infection; more rarely *Candida* enters the bloodstream, and invades the kidneys, lungs, endocardium, brain, or other organs, causing serious infections [1-5]. In addition to the increased occurrence of *Candida* species infections, resistance against used antifungal agents is increasing. It is therefore necessary to look for new antifungal drugs.

Antimicrobial peptides (AMPs) form the first line of defense against infection [6-15] and represent a new family of antibiotics that have been extensively studied. The AMPs described in the literature not only have antibacterial activities, but some also efficiently kill fungi, viruses and cancer cells [13-15]. The significant advantage of AMPs lies in their mechanism of action, which is different from that of conventional antibiotics.

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Although the precise mechanism of AMPs action is still not known, it is generally accepted that these positively-charged peptides target the anionic bacterial membrane surface, integrate into the lipid bilayer and disrupt its structure, which leads to the leakage of cytoplasmic components and cell death [6-12,16,17]. Recently, a few papers have appeared that address the mechanism of action of AMPs (melittin, lactoferrin, dermaseptins, plant defensin) on *C. albicans* and other kinds of yeast showing that the peptides at low concentrations act *via* an apoptotic mechanism [18-21].

Very recently, we have identified new AMPs, one in the venom of the solitary cleptoparasitic bee *Melecta albifrons* which we named melectin, MEP [22], three in the venom of the eusocial bee *Lasioglossum laticeps* which we have named lasioglossins, LL-I, LL-II and LL-III [23], and two from the eusocial bee *Halictus sexcinctus* which we named halictines, HAL-1 and HAL-2 [24]. For their structures and molecular weights see Table 1.

In the papers cited, we have described the antibacterial activities of the new peptides against Gram-positive and -negative bacteria, their hemolytic and mast cell degranulating activity, as well as their potency to kill some cancer cells. We have also performed a large structure-activity relationship (SAR) study to evaluate the effect of their chemical modification on antimicrobial and hemolytic activities, as well as in an effort to obtain compounds with high antimicrobial activity and minimum hemolytic activity. In this paper, we focused our attention on antifungal activity using as an experimental model the yeast *Candida*. We studied the antifungal effect not only of the AMPs and their analogs already described [22-24] but also of some new structural analogs, whose synthesis will be described elsewhere, against the five most common *Candida* species. A preliminary communication has been published [25]. In addition, the mechanism of action was studied using a fluorescently labeled peptide, rhodamine-loaded fungi, propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) staining procedures.

2. Experimental Procedures

2.1 Materials

Fmoc-protected L-amino acids and Rink Amide MBHA resin were purchased from IRIS Biotech GmbH, Marktredwitz, Germany. Tetracycline, Clotrimazole, Fluconazole, Amphotericine B, p-nitrophenyl-N-acetyl-b-D-glucosaminidine, Rhodamine 6G (R6G), Triton X-100 (Trit), fluorescent stains propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI), Luria-Bertani (LB) and Yeast extract-Peptone-Glucose (YPG) broth and agar were obtained from Sigma-Aldrich, Octyl-beta-D-glucopyranoside (OBG) from Fluka and 5(6)-carboxyfluorescein (Fluo) from NovaBiochem. The RPMI 1640 medium was from the PAA laboratories, Austria. All of the other reagents were of the highest purity available from commercial sources.

2.2 Peptides

The peptides were prepared by the solid-phase method as previously described [22-24,26]. They were purified by preparative RP-HPLC using a Vydac C-18 column (250 x 10 mm) at a 3.0 ml/min flow rate. Their identity was checked by MS. Their purity determined by analytical HPLC was higher than 97%. Fluorescein-labeled peptide Fluo-LL-III (see Table 2) was prepared by the acylation of α-amino terminal group of resin-bound LL-III peptide by 5(6)-carboxyfluorescein using N,N'-diisopropylcarbodiimide/1-hydroxy-benzotriazole (DIPC/HOBT) activation in dimethylformamide as described in [27].

peptide	Sequence	*M.w.*	Ref.
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18		
MEP	H-Gly-Phe-Leu-Ser-Ile-Leu-Lys-Lys-Val-Leu-Pro-Lys-Val-Met-Ala-His-Met-Lys-NH_2	2 038.23	[22]
LL-I	H-Val-Asn-Trp-Lys-Lys-Val-Leu-Gly-Lys-Ile-Ile-Lys-Val-Ala-Lys-NH_2	1 722.14	[23]
LL-II	H-Val-Asn-Trp-Lys-Lys-Ile-Leu-Gly-Lys-Ile-Ile-Lys-Val-Ala-Lys-NH_2	1 736.16	[23]
LL-III	H-Val-Asn-Trp-Lys-Lys-Ile-Leu-Gly-Lys-Ile-Ile-Lys-Val-Val-Lys-NH_2	1 764.19	[23]
HAL-1	H-Gly-Met-Trp-Ser-Lys-Ile-Leu-Gly-His-Leu-Ile-Arg-NH_2	1 408.8	[24]
HAL-2	H-Gly-Lys-Trp-Met-Ser-Leu-Leu-Lys-His-Ile-Leu-Lys-NH_2	1 451.9	[24]

Table 1. Abbreviation, sequence and molecular mass of peptides isolated from the Hymenoptera species.

2.3 Antibacterial activity

The antibacterial activity of the new analogs was determined quantitatively as previously described [22-24,26]. As test organisms we used *Bacillus subtilis* (*B. s.*) 168, kindly provided by Prof. H. Yoshikawa (Princeton University, Princeton, NJ, USA); *Escherichia coli* (*E. c.*) B No. CCM 7372 from the Czech Collection of Microorganisms, Brno; *Staphylococcus aureus* (*S. a.*) and *Pseudomonas aeruginosa* (*P. a.*) were obtained as multi-resistant clinical isolates, No. 4231 and 8567, respectively, from Liberec Hospital, Czech Republic.

2.4 Antifungal activity

The minimal inhibitory concentrations, *i.e.* lowest concentrations of peptides completely inhibiting the yeast growth, (MICs), were established by observing yeast growth in multi-well plates. In brief, 0.1 ml of appropriately diluted *Candida* in mid-exponential phase in YPG broth or RPMI (Roswell Park Memorial Institute) medium was added to the individual wells that contained solutions (0.1 ml) of different concentrations of the tested peptides (two fold dilutions) in the LB broth or RPMI medium (final volume 0.2 ml, final peptide concentration in the range of 0.25–100 μM). The plates were incubated at 37°C for 20 h while being continuously shaken in a Bioscreen C instrument (Oy Growth Curves AB Ltd, Helsinki, Finland) and the absorbance was measured at 540 nm every 15 min. Routinely, 1.2×10^3–7.5×10^3 CFU of *Candida* cells per well were used. For the preparation of *Candida* species cultures, the cells were transferred from agar to 2.5–5 ml of YPG liquid medium and shaken at 37°C for the time necessary to acquire a suspension having an absorption at 600 nm of 0.400–0.600 (exponential growth). Each peptide was tested at least 3 times in duplicates. For MIC assessment, 1.2×10^3 – 7.5×10^3 CFU of *Candida* per well was routinely used, but 10, 100 and 1000 times more CFU was used for the activity determination in order to establish the dependence of the MICs on the CFU quantity. The incubation time for the MIC assessment was generally 20 h; for some compounds (randomly selected, LL-III, HAL-1/5, HAL-1/18 and HAL-2/2) a 48 h-long incubation was also used. Tetracycline, Fluconazole, Clotrimazole and Amphotericin B were tested as standards in the concentration range of 0.25–200 μM. The quantity of CFU was determined by counting colonies on agar plates.

As test organisms, we used: *Candida albicans* (F7-39/IDE99), *Candida glabrata* (CG 196/98 IDE), *Candida parapsilosis* (Z1 50/119/IDE99), *Candida krusei* (CK 802/97) and *Candida tropicalis* (CT 14/HK), which were kindly provided by Olga Hrušková from the Institute of Organic Chemistry and Biochemistry. These yeasts were clinical strains from a collection of fungi at the Institute of Microbiology, Faculty of Medicine and Dentistry, Palacký University Olomouc, Czech Republic. The *Candida albicans* and *Candida parapsilosis* have been studied previously [28].

2.5 Determination of hemolytic activity

Hemolytic activity was tested using rat or human (healthy volunteer) red blood cells as described previously [22-24].

2.6 Rhodamine efflux studies

C. albicans cells (5–20 ml, $1–2\times10^8$ CFU/ml) were incubated for 20 min at 37°C in a rhodamine solution (R6G, 10 μM) in PBS according to [29]. Subsequently, the rhodamine-loaded fungi were centrifuged (for 10 min at 2000 x g), the supernatant was removed and the pellet washed twice with PBS. After the last wash, the rhodamine-loaded *Candida* cells were tested for viability and were found to form colonies on agar plates as the starting *C. albicans* suspension. The rhodamine-loaded fungi suspension (10^5–10^9 cells/ml) in fresh PBS was added to various concentrations of peptides (2–50 μM in PBS) and incubated for 0.5–60 min. Then the suspension was centrifuged for 1–5 min at 2000 x g and the fluorescence of the aliquots of the supernatants was determined at 530/580 nm (using a Tekan iControl spectrophotometer). Some of the pellets were suspended in PBS or PBS containing DAPI (5 μg/ml), and an aliquot (0.5 μl) was prepared for fluorescent microscopy. Similar experiments were conducted using the non-ionic detergents Triton X-100 (Trit) or Octyl-beta-D-glucopyranoside (OBG) in a concentration range of 0.01–10% in PBS. The aliquots of the final suspension were also used for the viability test, *i.e.* the counting of colonies grown on agar plates.

2.7 Uptake of the fluorescein-labeled analog Fluo-LL-III

C. albicans cells (10^7–10^9 CFU/ml) in PBS were incubated with Fluo-LL-III (1–2 μM) for different time intervals in the presence or absence of an unlabeled peptide LL-III (5–100 μM). After the incubation, the suspension was diluted 10 times with PBS and centrifuged for 5 min at 2000 x g. The pellet was resuspended in PBS and prepared for fluorescent microscopy or the viability test.

2.8 Propidium iodide staining

C. albicans cells (10^7–10^9 cells/ml) were incubated for 10 min in the presence or absence of an AMP or detergent, centrifuged, washed by PBS, suspended in 100 μl PI solution (5 μg/ml) and incubated for a further 5 min. After that, the suspension was diluted, centrifuged, the pellet washed with PBS and finally

suspended in 10 µl of PBS and prepared for fluorescent microscopy. An aliquot of the suspension was used for the viability test.

2.9 DAPI staining

C. albicans cells (10^7–10^9 cells/ml) were incubated for 10 min in the presence or absence of an AMP or detergent, centrifuged, washed with PBS, suspended in 10 µl DAPI solution (5 µg/ml) and prepared for fluorescent microscopy.

2.10 Fluorescent Microscopy

Fluorescence was studied with the Olympus IX81 microscope (Olympus C&S s.r.o., Prague, Czech Republic). In the case of fluorescein, rhodamine and PI, and DAPI, the excitation/emission filters 460-490/505/510 nm (U-MWIB2), 510-550/570/590 nm (U-MWG2), and 330-385/400/420 (U-MWU2), respectively, were used.

3. Results

3.1 Antifungal Activity

The AMPs melectin, lasioglossins and halictines and all their analogs previously described [22-24] were screened for anticandidal activity (*C. a.*). Table 2 provides the primary structures and MIC values of the parent peptides and those analogs which have shown high antifungal activity (a MIC lower than 10 µM). The MIC values of the fluorescently labeled analog synthesized for this study and those of two new highly active analogs which had not yet been described, namely all-D-LL-III (LL-III/12) and [Lys^1, Aib^{6,10,13}]LL-III (LL-III/A), are also shown in Table 2. The three last mentioned new compounds were also tested for their antibacterial and hemolytic activity. The MIC values for Fluo-LL-III, LL-III/12 and LL-III/A are as follows: for *B. s.* 2.2 µM, 0.4 µM, and 0.5 µM, respectively, for *S. a.* 25 µM, 2.5 µM, and 10 µM, respectively, for *E. c.* 3.5 µM, 0.7 µM, and

Peptide acronym	Structure	MW	MIC [μM]
MEP	GFLSILKKVLPKVMAHMK-NH_2	2038.23	8.3±1.8
LL-I	VNWKKVLGKIIKVAK-NH_2	1722.14	9.1±3.9
LL-II	VNWKKILGKIIKVAK-NH_2	1736.16	10.0±1.6
LL-III	VNWKKILGKIIKVVK-NH_2	1764.19	11.7±2.4
Fluo-LL-III	**Fluo**-VNWKKILGKIIKVVK-NH_2	2694	40±10
LL-III/12[a]	*VNWKKILGKIIKVVK-NH_2*	1764.19	5±1
LL-III/A[b]	**K**NWKK-**Aib**-LGK-**Aib**-IK-**Aib**-VK-NH_2[c]	1695.2	3.7±1.3
HAL-1	GMWSKILGHLIR-NH_2	1408.8	6.2±2.2
HAL-1/5	GMW**K**KILGHLIR-NH_2	1449.8	4.5±0.5
HAL-1/10	GMW**K**KILG**K**LIR-NH_2	1440.1	6.0±2.3
HAL-1/12	G**K**WSKILGHLIR-NH_2	1406.0	7.4±1.8
HAL-1/15	GMWSK**L**LGHL**L**R-NH_2	1408.8	5.0±0.5
HAL-1/18	GMWSKIL**K**HLIR-NH_2	1480.0	3.7±0.5
HAL-1/19	G**K**W**K**KILGHLIR-NH_2	1446.9	5.3±0.5
HAL-1/22[a]	*GMWSKILGHLIR-NH_2*	1408.8	6.0±1.6
HAL-2	GKWMSLLKHILK-NH_2	1451.9	6.6±2.0
HAL-2/2	GKWM**K**LLKHILK-NH_2	1492.9	4.5±0.5
HAL-2/6	GKWMS**F**LKHILK-NH_2	1485.9	6.8±0.9
HAL-2/10	GKW**W**SLLKHILK-NH_2	1506.9	5.3±0.5
HAL-2/11	GKW**L**SLLKHILK-NH_2	1433.9	8.7±1.9
HAL-2/13	GKWM**T**LLKHILK-NH_2	1465.9	7±2.2
HAL-2/25	GK**Na**lMSLLKHILK-NH_2[d]	1463.1	6.0
Tetracycline		444.43	>200
Fluconazole		306.27	>200
Clotrimazole		344.84	20-60
Amphotericin B		924.08	0.6

Table 2. The structure, molecular weight, and minimal inhibitory concentrations (MICs) against *C. albicans* of melectin, lasioglossins, halictines and some of their analogs.

[a] all D-peptides; [b] Aib – aminoisobutyric acid; [c] a bold letter means a primary structure modification (attachment of a fluorescein group or replacement of an amino acid in the given position) in comparison to the parent peptide; [d] Nal means L-3-(1-naphthyl)alanine.

1.1 μM, respectively, for *P. a.* >100 μM, 24.2 μM and 20 μM, respectively. All the three compounds had low hemolytic activity, LC_{50} >100 μM.

The best fungicidal compounds tested are able to stop the growth of the *Candida* species in a concentration of 3.7–10 μM (5.5–20 mg/l) when a standard (low) quantity of CFU of *C. albicans* is used for the testing. The dependence of the MIC value on the titer of inoculum (number of CFU/well) has been established for 4 different peptides, the LL-III, LL-III/12, HAL-1/5 and HAL-1/10. The results are summarized in Table 3. From the Table, it can be seen that even if the cell number in the inoculum is three orders of magnitude higher, only about 4–10 times higher concentrations of the tested peptides are necessary to kill the yeast cells.

From the set of standard compounds, tetracycline – a broad spectrum antibiotic – was, as expected, not effective in the antifungal test (MIC>200 μM) against all of the tested *Candida* species. It was quite active against bacteria such as *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* or *Pseudomonas aeruginosa*). Amphotericin B, an antifungal drug used for the treatment of *Candida* infections for more than 40 years, was more active (MIC ~0.6 μM, *i.e.* ~0.5 mg/l) in our tests. Two other antifungal drugs, Clotrimazole and Fluconazole, tested for comparison, were much less active (MIC 20-60 μM and >200 μM, *i.e.* 6-18 mg/l or >60 mg/l, respectively) under our testing conditions.

Figure 1 shows the results of testing the effect of selected peptides against other *Candida* species. The higher the column, the lower the anticandidal potency. As can be seen, the most resistant strains proved to be *C. parapsilosis* and *C. glabrata*. Two- to five-fold higher concentrations of peptides were necessary to stop the growth of these species than were needed against *C. albicans*.

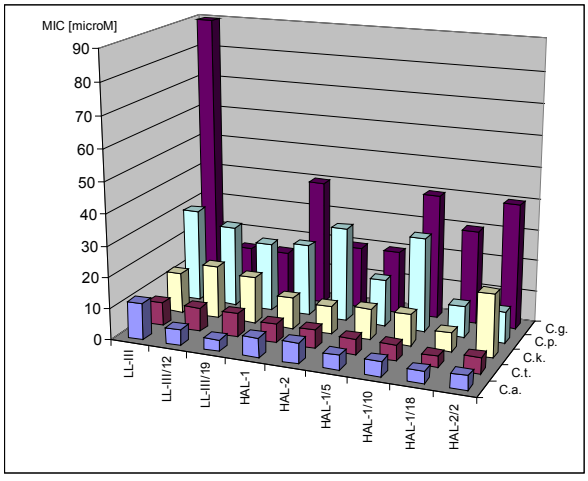

Figure 1. An illustration of the potency of selected antimicrobial peptide analogs against the different *Candida* species (*C. a., Candida albicans, C. k., Candida krusei, C. g., Candida glabrata, C. t., Candida tropicalis* and *C. p., Candida parapsilosis*). The higher the column, the lower the anticandidal potency.

Species	MIC, HAL-1/5 [μM]				MIC, HAL-1/10 [μM]			
	A	10A	100A	1000A	A	10A	100A	1000A
Candida albicans	5	5	7.5 ± 2.5	20 ± 14	5	7 ± 2	9 ± 1	24 ±12
Candida glabrata	20	25 ± 5	30 ± 10	40 ± 16	40	40	70 ± 10	87 ± 9
Candida krusei	10	15 ± 5	20	30 ± 10	10	13 ± 4	14 ± 3	28 ± 8
Candida parapsilosis	15 ± 5	20	20	30 ± 10	30 ± 14	20	40	45 ± 5
Candida tropicalis	5	10	13 ± 8	39 ± 25	5	7.5 ± 2.5	20	50 ± 31
	MIC, LL-III/12 [μM]				MIC, LL-III/A [μM]			
	A	10A	100A	1000A	A	10A	100A	1000A
Candida albicans	5	5	7.5 ± 2.5	32 ± 20	4.5 ± 0.5	6 ± 1	9 ± 4	28 ± 22
Candida glabrata	15 ± 5	10	10	30 ± 10	15 ± 4	15 ± 4	15	30 ± 8
Candida krusei	11 ± 2	10	10	47 ± 25	15 ± 8	15 ± 4	22.5 ± 7.5	47 ± 25
Candida parapsilosis	9 ± 6	15 ± 5	20	53 ± 34	14 ± 11	25 ± 5	45 ± 15	68 ± 23
Candida tropicalis	7.5 ± 2.5	5	5	36 ± 20	7 ± 2	6 ± 1	9 ± 1	26 ± 14

Table 3. The MIC values for peptides HAL-1/5, HAL-1/10, LL-III/12 and LL-III/A, for different *Candida* species and for different numbers of cells in suspension (a different quantity of CFU); A is equal to 1–5 x 10^3 CFU/well; the values are averages of two to four experiments performed in duplicates (mean ± SD); in the case when the same value was obtained in two or three independent experiments, the SD would be 0 and is not explicitly expressed in the Table.

3.2 Release of dye from rhodamine loaded fungi

Rhodamine 6G is often used as a tracer dye within water to determine the rate and direction of flow and transport. The dye has a remarkably high photostability, its absorption maximum is approximately 530 nm, emission maximum at 566 nm. It is taken up by cells and binds to the mitochondrial membranes. At higher concentrations it inhibits transport processes, especially the electron transport chain. It is the substrate for multidrug resistant pump [30]. Below 10 μM rhodamine 6G, no mitochondrial swelling, loss of matrix protein, or endogenous K^+, Ca^{2+}, or Mg^{2+}, was observed; the dye was bound very tightly to mitochondria [30].

The release of the stain from rhodamine 6G-loaded *Candida* was monitored in several ways. First, the time course of the release was tested. The experiments showed that the efflux of the dye from the cells after incubation with AMP (LL-III, LL-III/12 and HAL-1/10) is very quick – more than 80% of rhodamine was released from the cells within 1 min after AMP addition (Figure 2A). The viability tests showed that more than 95% of the cells were already dead. Next, the release of rhodamine dye was monitored as a function of the concentration of three different AMPs after 10 min of incubation with the peptides (Figure 2B). The concentration at which the release reaches its maximum corresponds to the MIC determined by the dilution technique (the highest number of CFU in inoculum). Triton X-100 (10% solution) released rhodamine to a much lower extent than the peptides and the viability test showed that the cells were not dead. The second detergent used, OBG, on the other hand released a higher quantity of fluorescence from the cells than the peptides (about 20-40% more) but at rather high concentrations – 2.5–10% solution. However, only the highest concentrations of OBG caused cell death.

The rhodamine-loaded fungi were also studied by means of fluorescent microscopy. The AMP-untreated cells showed high fluorescence, which was unevenly distributed in the cells. After incubation with AMPs (LL-III, LL-III/12 and HAL-1/10 were tested), the cells shrank a little and the fluorescence was distributed evenly. After centrifugation and wash, the cells lost

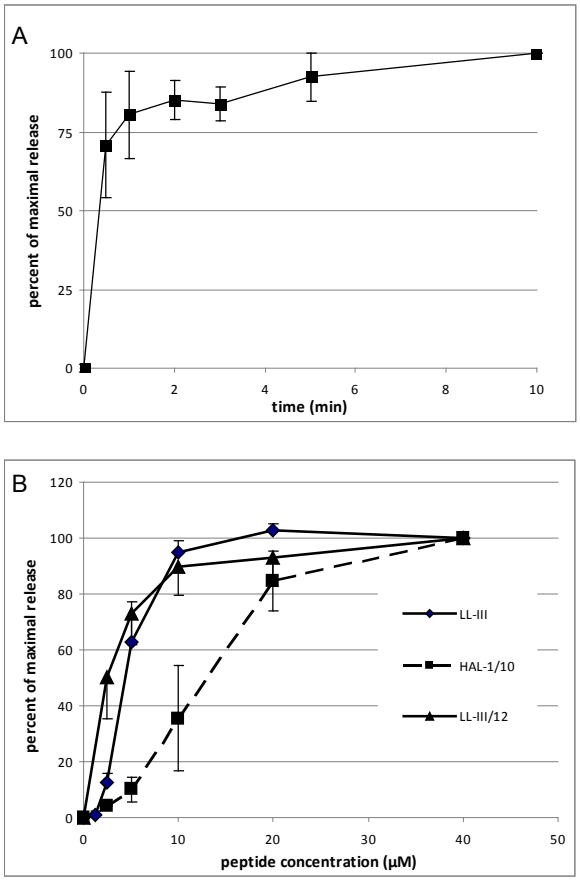

Figure 2. Rhodamine 6G dye efflux from the rhodamine-loaded *C. albicans*. A. The time dependence of the release from rhodamine-loaded *C. a.* cells (1–5 x 10^7 CFU/ml) in the presence of LL-III with the final concentration being 50 μM. Similar curves were obtained using LL-III/12 and HAL-1/10. B. The release of the dye *C. a.* cells (1–5 x 10^7 CFU/ml) in the presence of various peptide concentrations after a 10-min incubation with the peptides. ♦ LL-III; ■ HAL-1/10; ▲ LL-III/12

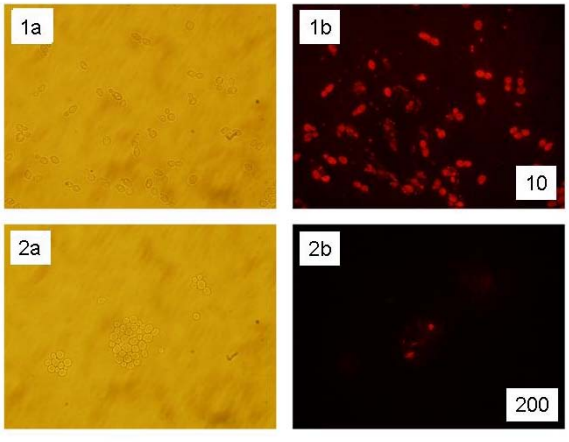

Figure 3. Images of rhodamine 6G-loaded *C. albicans* cells after a 10-min incubation without (1) and with (2) LL-III (40 μM), centrifugation and wash. 1a, 2a = pictures in visible light; 1b, 2b = pictures in green light (rhodamin 6G) using an exposure time of 10 and 200 ms, respectively. In the absence of peptide, the cells are large and unevenly colored; after incubation with an AMP, the cells are smaller and much less intensively colored.

their fluorescence. This is illustrated in Figure 3, which refers to the particular case of peptide LL-III.

3.3 Propidium iodide (PI) and DAPI staining

When *C. a.* or *C. k.* cells were incubated in the absence of an AMP, then centrifuged and suspended in DAPI solution, only a low percentage of cells (about 3.7–6.3%) have stained the nucleus (blue color inside the cell), whereas after 10 min incubation with an AMP (HAL-1/10), then centrifugation and suspension in DAPI solution, more than 90% of cells have stained the nucleus (compare Figure 4, panels 1b and 2b). The same is true when rhodamine-loaded *Candida* cells are treated with AMPs and DAPI (data not shown). Figure 5 illustrates the uptake of PI into *C. a.* cells after 10 minutes' incubation with an AMP (using the example of the peptide HAL-1/10) followed by incubation with PI. After incubation with the peptide, the cells were intensively colored by PI (2b), and only a few uncolored cells (no PI entry) can be seen when comparing panels 2a and 2b in Figure 5. Incubation without a peptide followed by incubation with PI led to negligible entrance of the stain into the cells (Figure 5, panel 1b). It appears that Triton did not damage the membrane as the PI was not entering the cells even at the highest Triton concentration (10%). On the other hand, the OBG caused damage of the membrane as judged by the staining of the cells by PI at OBG concentrations higher than 1% (not shown). Similar results were obtained using DAPI stain.

3.4 Carboxyfluorescein-labeled peptide interaction with *Candida* cells

Carboxyfluorescein is a green-emitting fluorescent dye with excitation and emission maxima at 495 and 517 nm, respectively. It is commonly used as a tracer agent which does not permeate membranes. The carboxyfluorescein was attached to the N-terminal of the LL-III molecule to get carboxyfluorescein-labeled peptide Fluo-LL-III. The MIC of Fluo-LL-III to *C. albicans* amounted to 40 μM, that is, it was about 4 times less antifungal than the unmodified compound LL-III. Experiments performed using the carboxyfluorescein-labeled peptide Fluo-LL-III at a concentration of 1–2 μM showed that there was very scarce penetration of the fluorescent peptide into the cells, only single cells were intensively colored. However, after the incubation of the *Candida* cells with a mixture of Fluo-LL-III (2 μM) and an unlabelled peptide (LL-III or HAL-1/10) in MICs and higher concentrations (10–100 μM), all of the cells were immediately uniformly green (compare Figure 6, panels 2b and 1b) and their size was smaller.

4. Discussion

In this paper, we have studied the antifungal activity of newly discovered antibacterial peptides from wild-bee venom [22-24] and their analogs. We have screened all the analogs previously described [22-24] as well as some additional ones. We have compared

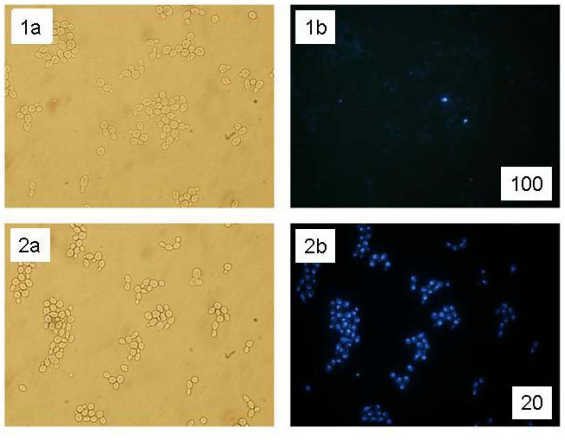

Figure 4. Images of *C. a.* cells after a 10-min incubation without (1) and with (2) the AMP (HAL-1/10, 50 μM) resuspended after centrifugation and wash in a DAPI solution (5 μg/ml). In the absence of the peptide, only a few cells are stained, whereas after 10-min incubation with the peptide almost all of the cells are stained (cf. 1b and 2b). 1a, 2a = pictures in visible light, 1b, 2b = pictures in UV light, using exposure times of 100 and 20 ms, respectively.

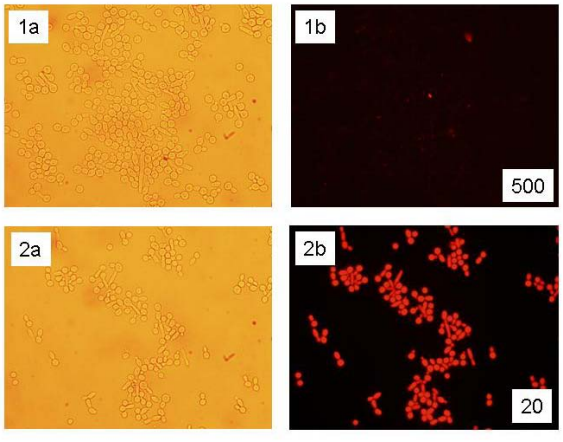

Figure 5. Images of *C. a.* after a 10-min incubation without (1) and with (2) peptide HAL-1/10 (40 μM) followed by a 10-min incubation with propidium iodide (5 μg/ml), centrifugation and wash. 1a, 2a = pictures of cells in visible light, 1b = picture in green light (PI, exposition 500 ms) – no staining – all cells are alive, 2b = picture in green light – the dye enters the damaged cells and stains them, exposure 20 ms.

their antifungal activity with their antibacterial activity as previously described [22-24] and found that the analogs which showed high antibacterial activity also showed high antifungal activity. Peptides with low or no antibacterial activity displayed low (MIC>100 μM) or no antifungal activity (not shown). We have found that, under our experimental conditions using the yeast cells in suspension (planktonic form), the compounds are fairly active, with MICs in tens of μM, against all the *Candida* species tested, with *C. albicans* being the most sensitive to the peptides, followed by *C. tropicalis.* For the other three species, two to ten times higher concentrations of the peptides were needed to achieve the same response, *i.e.* no growth of the fungi for 20 h at 37°C and continuous shaking. The peptides were equally active if the determination was performed in different media, either LB or YPG broth or an RPMI medium. However, in the RPMI medium, the *C. albicans* cells grew very slowly and did not reach the same maximal density as in the case of the other two media. In the case of the selected peptides (LL-III, LL-III/18, HAL-1/5, HAL-1/10 and HAL-1/19), the MIC value was also assessed after a 48-h incubation at 37°C and continuous shaking in the LB/YPG broth, and it was found that it either had not changed or slightly decreased (max. 2 times) in comparison to the 20 h incubation value.

The best antifungal potency was demonstrated by three previously-described analogs HAL-1/5, HAL-1/18 and HAL-2/2 (all three having enhanced charge for 1) as well as two new analogs, namely the all D analog of the natural peptide lasioglossin III LL-III/12 (*VNWKKILGKIIKVVK-NH*$_2$) and the analog that has an amino acid Aib in peptide chain positions 6, 10, and 13 and enhanced charge LL-III/A (KNWKK-Aib-LGK-Aib-IK-Aib-VK-NH$_2$). The two new analogs (LL-III/12 and LL-III/A) also had low hemolytic activity (LC_{50} >100 μM) towards both rat and human erythrocytes. The hemolytic activity of the other three most potent antifungal peptides amounted to 45-100 μM (rat erythrocytes) [24].

The Wang database (http://aps.unmc.edu/AP/database/antiF.php) currently lists 484 AMPs with antifungal activity. They include peptides with higher antifungal activity than our peptides (e.g. peptide Gomesin), comparably active peptides (e.g. PW2, tachyplessins, maculatin, histatins *etc.*) or peptides with antifungal activity lower by orders of magnitude. A comparison is however difficult, as the MIC values depend on the testing conditions [31]. Altogether, we can classify the peptides studied among those with high potency.

As far as activity is concerned, the synthetic alpha helical beta-peptides of ten amino acid residues described by Karlsson *et al.* [32] are the most similar to our peptides. Their most active peptides showed MIC values of 8–16 μg/ml, *i.e.* 4–9 μM, and are described to be active also against the biofilm form of *C. a.* In preliminary experiments (using the same experimental conditions [32] for biofilm formation and incubation with a peptide), our peptides were similarly active (MIC of ~50 μM).

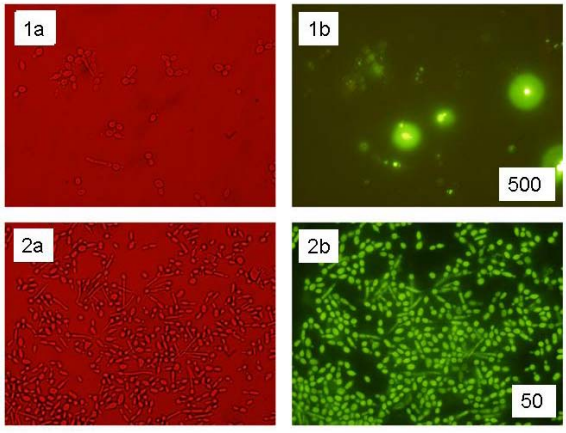

Figure 6. Images of *C. a.* after a 10-min incubation with 1 μM Fluo-LL-III peptide and without (1) and with (2) unlabelled peptide LL-III (50 μM). 1a, 2a = pictures in visible light, 1b and 2b = pictures in blue light (fluorescein) using an exposure time of 500 and 50 ms, respectively. In the absence of unlabeled peptide, only a few cells have been labeled; in the presence of the AMP, all of the cells are colored, which means that the fluorescent-labeled peptide entered the cells.

The killing process owing to the addition of AMPs melectin, lasioglossins, halictines and their analogs is very quick and the cells are dead within minutes. This is in agreement with the previously published results (see *e.g.* review [7]) for short cationic amphipathic α-helical antimicrobial peptides. It is difficult to draw any justification concerning the mechanism as we have not measured any factor typical *e.g.* for apoptosis as was the case of other authors [18-21]. In contrast to others, we have used a short interval for the detection of dead and live cells. We have found that 10 min are sufficient for the maximal release of rhodamine 6G from rhodamine-loaded cells and for obtaining the maximal fluorescence signal within the cells using PI and DAPI fluorescence marks or carboxyfluorescein-labeled peptides. The two compounds PI and DAPI, which bind to DNA, do not enter *Candida* cells that have an unimpaired cell membrane. In the case of DAPI, this differs from mammalian cells. To stain *Candida* cells with DAPI, the cells have to be permeabilized *e.g.* by ethanol treatment. This means that the 10 min incubation of *Candida* cells with the AMP studied leads to such a degree of cell membrane damage that there in an uptake of both, PI and DAPI, and outflow of rhodamine 6G.

The efflux of the R6G dye from the cells is highly likely to be connected with the release of all of the cell content. The OBG (non-ionic, dialyzable detergent for the solubilization and isolation of membrane proteins) strongly disturbs the *Candida* cells and its effect can be seen within a minute in comparison with Triton X-100 (also a nonionic surfactant which has however a longer hydrophilic polyethylene oxide group and a hydrocarbon hydrophobic group), which seems to remove the rhodamine dye only from the surface of the cells. Altogether, our experiments indicate a necrotic mechanism rather than apoptosis. We therefore agree with the hypothesis that the action mechanism may differ if high or low concentrations of AMPs are used. Programmed cell death may be induced at low concentrations of peptides, but only a small percentage of the cells is affected. This mechanism seems unlikely with our peptides as they do not enter the cells easily if in low concentrations as was seen in the experiments with a fluorescein-labeled peptide. More peptide molecules must thus cooperate to overcome the cell membrane barriers (see the models of pore forming [7,8]).

Helmerhorst *et al*. [33] performed similar experiments using *C. albicans* cells and AMP histatin 5, a 24-amino-acid-long salivary peptide. Although the killing and permeabilisation of *C. a.* cells showed some similarities, the mechanism of action of histatin 5 and that of our peptides seem to be different. According to our results, peptides studied in this paper destroy not only the cell membrane but also other intracellular barriers. After the peptide reaches the MIC concentration, the fluorescence of R6G, which was unevenly distributed before, colors the whole cell evenly. Below the MIC concentration, negligible amounts of the fluorescein-labeled peptide enter the cells. The incubation of cells with NaN_3 or NaCN does not influence the effectiveness of our peptides (data not shown).

To conclude, the analogs of the newly discovered peptides melectin, lasioglossins and halictines represent a promising lead for the development of new, effective antifungal drugs. They are fungicidal, have low hemolytic activity, and kill the yeast cells immediately. The concomitant high antibacterial activity might be an advantage as *Candida* infections are usually accompanied by the presence of other microbes.

Acknowledgments

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