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# Effects of galanin message-associated peptide and neuropeptide Y against various non-*albicans Candida* strains

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## ABSTRACT

Antimicrobial peptides (AMPs) could represent promising therapeutic agents against fungal pathogens, especially in cases of pathogen resistance to common antifungal substances. The neuropeptides galanin message-associated peptide (GMAP) and neuropeptide Y (NPY) are both potent AMPs against certain microbes. The objective of this study was to test clinically relevant non-*albicans* *Candida* strains (*C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, *C. pelliculosa*, *C. tropicalis* and *C. utilis*) with regard to their susceptibilities to NPY and GMAP. GMAP showed a higher potency than NPY, which only inhibited growth of some isolates of *C. krusei*, *C. tropicalis* and *C. utilis*. Interestingly, the fluconazole-resistant *C. krusei* was susceptible to both AMPs. In summary, we show that these neuropeptides have *Candida* strain-dependent antifungal activity, which in some cases does not match the susceptibility of the strains to the positive controls fluconazole and magainin I. Thus, the findings demonstrate the therapeutic potential of these AMPs in cases of resistance to traditional antifungal substances. This study also confirms the research on neuropeptides as potential fungicides, which are still in the early stages. The results also suggest that testing of strain-specific susceptibility is mandatory.

## 1. Introduction

Antimicrobial peptides (AMPs) are an important arm of the innate immune system. The first epithelial AMPs, termed magainin I and II, both 23-residue peptides from the same precursor peptide, were discovered following the observation that surgical wounds in the frog *Xenopus laevis* rarely became infected even if surgery was performed under non-sterile conditions [1].

In recent years, several neuropeptides have been found to exhibit antimicrobial activities. These peptides often have multiple physiological functions and share certain features with AMPs, including being highly amphipathic and strongly conserved throughout evolution. They are produced by nerves that innervate endothelial tissues, by endothelial and epithelial cells and also by immune cells [2].

In 1996, Vouldoukis et al. showed that neuropeptide Y (NPY) exhibits antimicrobial activity against *Candida albicans* and other microbial pathogens [3]. Later, it was demonstrated that N-terminal truncation of NPY (e.g. NPY 13-36) enhanced this activity [4]. The mechanism of action was suggested to be similar to that of magainin I in damaging the plasma membrane of the fungal cells. The antimicrobial mechanism of these peptides is considered to involve initial electrostatic interactions between the positive charges of the peptides and the negative charges of the cell membrane, following penetration of the peptides into the membrane, and then formation of lipid bilayer-spanning pores that results in osmolysis [4]. However, subsequent studies using different antimicrobial activity assays and various *C. albicans* strains found either no or a 10-fold lower antifungal activity of NPY than described in the original report [5,6].

Recently, we observed that galanin message-associated peptide (GMAP), a neuropeptide with as yet no known major functions [7], exhibits antifungal activity. The full length peptide as well as the truncated peptide GMAP (16-41) inhibit growth and the yeast-to-hyphal transition of *C. albicans* [8]. The mechanism of action of GMAP is different to those of common antifungal substances such as fluconazole, which are cytotoxic to the pathogen either by inhibiting growth or direct killing. GMAP peptide appears to interfere with the signalling pathways of hyphal development, most likely via activation of a membrane receptor [8].

Yeasts of the *Candida* genus are so-called opportunistic pathogens, present as commensals in ca. 70% of healthy people. Candidiasis, a local or systemic infection with *Candida* strains (e.g. *C. albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis*), is the most common opportunistic infection, especially in persons with a weakened immune system [9,10]. *Candida* fungi can colonise and infect a wide range of microenvironments in the body, including the bloodstream, superficial sites in the mucosa as well as all of the major internal organs during systemic disease [9].

Up to 10% of bloodstream infections acquired in hospitals are due to *Candida* spp., with strain-dependent mortality rates of >50%. In recent years, several non-*albicans* *Candida* strains were found to have developed multidrug resistance, thereby increasing the need for new antifungal agents [11]. This makes new compounds with anti-*Candida* activity of special interest. Hence, neuropeptides with antifungal activity could be the basis for the development of new specific antifungal drugs. To clarify further the potential of the neuropeptides NPY and GMAP as antifungal agents, their effect on the growth of non-*albicans* *Candida* strains was analysed. These completely unrelated peptides were chosen to cover a range of different possible mechanisms of action against *Candida* strains.

## 2. Material and methods

### 2.1. Reagents, media and strains

The synthetic peptides magainin I (GIGKFLHSAGKFGKAFVGEIMKS) and porcine NPY 13-36 (PAEDLARYYSALRHYINLITRQRY) were purchased from Bachem Holding AG (Bubendorf, Switzerland). Human GMAP 16-41 (SIPENNIMRTIIEFLSFLHLKEAGAL) was custom-synthesised by GL Biochem (Shanghai, China). All peptides were dissolved in sterile water and were stored at  $-80^{\circ}\text{C}$ . Working stocks were diluted to  $200\ \mu\text{M}$  in water and were stored at  $-20^{\circ}\text{C}$ . Fluconazole was purchased from MP Biomedicals, Inc. (Solon, OH) and was dissolved in water and stored at  $4^{\circ}\text{C}$ . Fluconazole was chosen as the control for susceptibility because it is one of the first-class substances against *C. albicans* as well as against most of the non-*albicans Candida* strains, although there are known resistances in some strains, e.g. *C. krusei* [12] (Table 1).

Strains of *C. tropicalis*, *C. krusei*, *Candida pelliculosa*, *C. glabrata*, *Candida utilis*, *C. parapsilosis* and *Candida lusitanae* were mainly clinical isolates from a local private clinical microbiology laboratory. It was decided to use mainly clinical isolates because of greater clinical relevance. Isolates were identified by chromID<sup>TM</sup> *Candida* agar plates (bioMérieux, Vienna, Austria) and were subsequently confirmed by an automated system (Vitek2 ID-YST; bioMérieux, Vienna, Austria).

Two isolates (*C. pelliculosa* strain 70130 and *C. krusei* strain 11956) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany). Strains were maintained in stock cultures at  $-80^{\circ}\text{C}$  and were streaked on Sabouraud dextrose agar plates 2 days before the experiments.



## 2.2. Growth tests

A colony picked from the agar plates was inoculated into 5 mL of Sabouraud dextrose broth and was incubated overnight at 30 °C. The following day, cells were harvested by centrifugation at  $1000 \times g$  for 10 min, washed with sterile phosphate-buffered saline and re-suspended and diluted in 5 mL of Sabouraud dextrose broth. Then, 50  $\mu$ L of cell suspension ( $1-3 \times 10^3$  colony-forming units/mL) per well were transferred into black 96-well plates containing the different peptides and were incubated at 30 °C with shaking in a humid atmosphere. After 16 h, the amount of viable cells was assessed using the BacTiter-Glo<sup>TM</sup> Microbial Cell Viability Assay (Promega GmbH, Mannheim, Germany) according to the manufacturer's instructions. ATP production was regarded as an indicator of cell viability, providing a simple and fast method for testing the susceptibility of *Candida* spp. to an antimicrobial substance. Luminescence was read with a Zenyth 3100 microplate reader (Anthos Labtec Instruments GmbH, Eugendorf, Austria). To determine cell numbers and to exclude contamination with other microorganisms, aliquots of cell suspensions were plated onto agar plates at the beginning and end of each experiment.

## 2.3. Statistics

Each experiment was conducted independently at least three times in triplicate. The significance of changes in growth was assessed by Dunnett's multiple comparison test using GraphPad Prism 4.00 software (GraphPad Software Inc., La Jolla, CA). A *P*-value of <0.05 was considered as statistically significant.

### 3. Results

#### 3.1. Fluconazole susceptibility

Susceptibility to 65  $\mu\text{M}$  (20  $\mu\text{g/mL}$ ) fluconazole was used as a control because of its known inhibitory effect on the growth of *Candida* strains. *Candida utilis*, *C. parapsilosis* and *C. lusitanae* were highly susceptible (>95% inhibition) to this concentration, whereas the susceptibility of *C. pelliculosa*, *C. glabrata*, *C. krusei* and *C. tropicalis* was generally lower and varied between the isolates (Table 1). A pre-screen of different fluconazole concentrations on different strains revealed no augmentation of the growth inhibitory effect using fluconazole above 65  $\mu\text{M}$  (data not shown).

#### 3.2. Peptide susceptibility

A pre-screen of different concentrations (0.5–16  $\mu\text{M}$ ) of magainin I, NPY and GMAP revealed that at <4  $\mu\text{M}$  the effects were substantially lower and at >8  $\mu\text{M}$  they were not further increased (data not shown). Therefore, the positive control peptide magainin I, NPY 13-36 and GMAP 16-41 (hereafter termed NPY and GMAP) were tested at concentrations of 4  $\mu\text{M}$  and 8  $\mu\text{M}$  against two isolates of each *Candida* strain (Fig. 1).

##### 3.2.1. *Candida utilis*

Growth of isolate 1 of *C. utilis* was significantly reduced by all peptides tested. Isolate 2 was less susceptible than isolate 1 and was significantly inhibited only by GMAP (4  $\mu\text{M}$  and 8  $\mu\text{M}$ ) and magainin (8  $\mu\text{M}$ ). Regarding the effect of peptide concentration on growth, application of 8  $\mu\text{M}$  instead of 4  $\mu\text{M}$  led to further growth inhibition only of isolate 1 with NPY, and of both isolates with magainin, whereas the higher concentration of GMAP was not able to inhibit growth of *C. utilis* further.

### 3.2.2. *Candida parapsilosis*

Growth of *C. parapsilosis* isolate 1 was inhibited by only ca. 20% with 4  $\mu$ M and 8  $\mu$ M magainin, NPY and GMAP. Isolate 2 even showed significantly increased growth upon application of 4  $\mu$ M and 8  $\mu$ M NPY (121% and 122% growth compared with the untreated controls). A tendency toward increased growth in this isolate could also be observed with magainin (8  $\mu$ M) and GMAP (8  $\mu$ M).

### 3.2.3. *Candida lusitanae*

Magainin inhibited the growth of both *C. lusitanae* isolates by up to 90% at 4  $\mu$ M. The effect of GMAP on *C. lusitanae* was highly dependent on the isolate; 4  $\mu$ M GMAP resulted in 44% inhibition and 8  $\mu$ M resulted in 58% inhibition of isolate 1, but neither concentration had a significant effect on isolate 2, a trend that was also observed with NPY.

### 3.2.4. *Candida pelliculosa*

Isolate 1 was significantly inhibited by 8  $\mu$ M magainin (35%) as well as by 4  $\mu$ M (48%) and 8  $\mu$ M (59%) GMAP, but not by NPY. Only 4  $\mu$ M GMAP showed a significant effect on isolate 2 (44%).

### 3.2.5. *Candida glabrata*

None of the peptides tested at 4  $\mu$ M had a significant effect on the growth of *C. glabrata*. The only significant growth inhibition was observed with 8  $\mu$ M magainin on isolate 1 (41%).

### 3.2.6. *Candida krusei*

Growth of both isolates of *C. krusei* was inhibited by 4  $\mu$ M magainin (36% and 97%) and 8  $\mu$ M magainin (81% and 99%). GMAP also inhibited the growth of both isolates (47% and 55% at 4  $\mu$ M and 36% and 65% at 8  $\mu$ M). For NPY, isolate 1 was more susceptible than isolate 2 (57% and 31% at 4  $\mu$ M and 62% and 42% at 8  $\mu$ M).

### 3.2.7. *Candida tropicalis*

*Candida tropicalis* isolate 1 was significantly inhibited by GMAP, NPY (both at 4  $\mu$ M) and magainin (8  $\mu$ M). Significance was lost with 8  $\mu$ M NPY. Growth was significantly increased at 4  $\mu$ M magainin (150% growth compared with the untreated control). Isolate 2 was less influenced by GMAP and was not significantly inhibited by NPY, but was strongly inhibited by magainin (77% at 4  $\mu$ M and 91% at 8  $\mu$ M).

## 4. Discussion

Here we report the effect of the neuropeptides GMAP and NPY as well as the frog skin-derived AMP magainin I on the growth of non-albicans *Candida* strains. Susceptibility of the *Candida* isolates to fluconazole determined in the present study is in agreement with data from the literature [12–14], indicating that measurement of ATP production is a reliable system for measuring *Candida* growth.

Interestingly, in addition to the observed varying AMP susceptibilities among species and even between isolates of the same species, the susceptibility of individual isolates to the different peptides was non-uniform. Magainin, for example, completely inhibited both *C. lusitaniae* isolates, which were not susceptible to NPY and only partly to GMAP. In

contrast, GMAP was the only peptide that significantly inhibited *C. pelliculosa*, doing so at 4  $\mu$ M. The phenomenon of strain-specific susceptibility has also been observed with human  $\beta$ -defensins on oral *Candida* isolates [15]. Using determination of ATP production as an indicator of microbial viability, it is not possible distinguish between a static and fungicidal effect, but in this study this differentiation is not of clinical relevance.

The most surprising result of the present study was the observation of increased growth of *C. tropicalis* (4  $\mu$ M magainin on isolate 1) and *C. parapsilosis* (4  $\mu$ M and 8  $\mu$ M NPY on isolate 2) upon exposure to certain concentrations of AMPs.

With NPY, significantly increased growth in one isolate of *C. parapsilosis* was observed, a trend that could also be seen with GMAP and magainin. In experiments with magainin and *C. tropicalis* isolate 1, it was observed that the peptide had no effect on growth at lower doses (<4  $\mu$ M; data not shown), a growth-increasing effect at intermediate doses (4  $\mu$ M) and a growth-inhibiting effect at high doses (>8  $\mu$ M). The growth-increasing effect was not seen with isolate 2. A possible explanation for the apparent growth increase could be a faster release or leakage of ATP through an already perforated cell membrane at an early stage of apoptosis of the lysed *Candida* cells.

Increases in extracellular ATP concentration after treatment with cationic peptides, for example magainin, histatin-5, lactoferricin, human neutrophil defensins, battenecin and cathelicidin, may serve to potentiate cell killing and facilitate further peptide uptake.

NPY has been suggested to exhibit a similar mode of action as the magainins, namely penetration of the pathogen's lipid bilayer and subsequent formation of pores [4]. For GMAP, no mechanism of action has been proposed so far, although pore formation is

rather unlikely as this peptide is not capable of killing even at high doses and its effect on the yeast-to-hyphal transition of *C. albicans* implies an influence on intracellular signalling [8].

In conclusion, these data indicate that, depending on the *Candida* spp. and isolates, AMPs may have therapeutic potential, especially in cases of resistance to common antifungal substances. The peptides themselves might not have enough stability for a therapeutic application and therefore biologically more stable analogues have to be developed, which are ideally non-peptidergic.

One of the most remarkable finding of this study is that the fluconazole-resistant *C. krusei* is susceptible to all three peptides tested. However, the data also indicate that strain-specific testing is necessary to determine susceptibility to antifungal agents.

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### **Competing interests**

None declared.

**Ethical approval**

Not required.

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**Fig. 1.** Effect of (a) magainin I, (b) galanin message-associated peptide (GMAP) 16-41 and (c) neuropeptide Y (NPY) 13-36 on the growth of *Candida* spp. Change in ATP production (%) is in relation to the respective untreated culture. Asterisks indicate significant effect on growth (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ). Error bars indicate standard error of the mean.

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**Table 1**

Susceptibility of *Candida* isolates to fluconazole

<i>Candida</i> strain	Isolate	% growth inhibition <sup>a</sup>
<i>C. utilis</i>	1	98 ± 0.3
	2	95 ± 0.7
<i>C. parapsilosis</i>	1	97 ± 0.8
	2	98 ± 0.1
<i>C. lusitaniae</i>	1	96 ± 0.3
	2	99 ± 0.2
<i>C. pelliculosa</i>	1	52 ± 3.4
	2	92 ± 1.7
<i>C. glabrata</i>	1	46 ± 5.2
	2	84 ± 1.5
<i>C. krusei</i>	1	89 ± 3.4
	2	43 ± 6.4
<i>C. tropicalis</i>	1	83 ± 2.3
	2	74 ± 2.8

<sup>a</sup> Assessed in the presence of 65 µM fluconazole (± standard error of the mean).

