

ORIGINAL ARTICLE

Cell-penetrating peptides as antifungals towards *Malassezia sympodialis*

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Keywords

antifungal, antimicrobial peptide, atopic eczema, cell-penetrating peptide, dandruff, host defence peptide, *Malassezia sympodialis*, seborrhoeic eczema.

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Abstract

Aim: To determine whether different antimicrobial peptides (AMPs) and cell-penetrating peptides (CPPs) are able to inhibit the growth of the commensal yeast *Malassezia sympodialis*, which can act as a trigger factor in different skin disorders, such as atopic eczema (AE), seborrhoeic eczema (SE) and dandruff.

Methods and results: The antifungal activity of 21 different AMPs and CPPs was investigated by microdilution assay and plate counting to determine the number of colony forming units. Five CPPs and one AMP showed fungicidal activity at submicromolar concentrations. Importantly, no membrane damage on human keratinocytes was detected after peptide treatment.

Conclusions: Several CPPs, while being nontoxic to mammalian cells, possess growth inhibitory activity on the very stringent yeast *M. sympodialis*.

Significance and impact of study: Our findings that five CPPs and one AMP that are harmless towards mammalian cells act as antifungal agents against *M. sympodialis* opens up the possibility to use these in the treatment for AE, SE and dandruff. To our knowledge, this is the first time peptides have been identified as antifungal agents against *M. sympodialis*. Further studies to elucidate the mechanism are warranted.

Introduction

The fungus *Malassezia* belongs to the normal commensal skin microflora of humans and other warm-blooded animals. So far thirteen species of *Malassezia* have been isolated from human and animal skin (Ashbee and Scheynius 2010). The normally harmless species of *Malassezia* are associated with several human skin diseases, such as seborrhoeic eczema (SE), atopic eczema (AE), dandruff and pityriasis versicolor, as well as systemic diseases in immunodeficient humans (Ashbee and Scheynius 2010). The clinical features of SE, and the connection to pathogenic *Malassezia*, are described by Naldi and Rebora (2009). AE is an extremely itchy skin condition and is one of the most common allergic diseases in the industrial world. The occurrence of AE has increased during recent years and is now affecting 15–30% of children and 2–10% of adults (Bieber 2010). *Malassezia* is an important trigger factor in SE (Gaitanis and Mayser 2010) and AE (Scheynius and Cramer 2010), and *M. sympodialis* is

recognized as the most common *Malassezia* species recovered from both healthy individuals and patients with AE (Sandström Falk *et al.* 2005; Ashbee and Scheynius 2010).

Antimicrobial peptides (AMPs) are a component of the innate immune response and function as broad spectrum antibiotics (Boman 1995). It has been noted that the determinants for antibacterial activity include a threshold hydrophobicity, net positive charge and an ability to adopt amphipathic structures (Papo and Shai 2003). Another group of peptides called cell-penetrating peptides (CPPs) share the same determinants for activity as the AMPs. CPPs are used to transport different conjugated moieties into cells (EL Andaloussi *et al.* 2005; Heitz *et al.* 2009). Most studies of CPP drug delivery are conducted in mammalian cells; however, there are reports that some of these peptides are able to penetrate also bacteria and fungi (Nekhotiaeva *et al.* 2004; Holm *et al.* 2005). Interestingly, it has been found that a number of CPPs, such as pVEC, Tat and TP10, also function as AMPs (Nekhotiaeva *et al.* 2004; Palm *et al.* 2006; Arrighi *et al.* 2008;

Zhu and Shin 2009). Histatin 5 (His 5) is a human salivary AMP that has been demonstrated to possess potent properties against a number of medically important fungi *in vitro* (Helmerhorst *et al.* 1999). Recently, His 5 was shown to be effective also against *Leishmania* (Luque-Ortega *et al.* 2008), but it has never been tested on *Malassezia*. The role of *Malassezia* in the aetiology of different skin diseases makes this fungus an important target in the search for novel antifungal peptides. In this study, the effect of 21 different peptides (CPPs and AMPs) on *M. sympodialis* was studied. We found six peptides that inhibited the growth of *M. sympodialis* at submicromolar concentrations. None of the peptides were toxic towards human keratinocytes, making these results very promising for the future development of alternative local treatment against AE, SE and dandruff using antifungal peptides.

Materials and methods

Peptide synthesis

Peptides were synthesized using either *t*-Boc or Fmoc chemistry. The procedure for *t*-Boc chemistry and N-terminal fluorescein-labelling can be found in the studies described by Johansson *et al.* (2008). Synthesis and final cleavage from the resin using Fmoc chemistry was carried out as described by Sollenberg *et al.* (2010). Cleavage of the peptides synthesized with *t*-Boc chemistry and purification of the peptides was performed as described by Johansson *et al.* (2008).

Culturing of *Malassezia sympodialis*

Malassezia sympodialis strain no. 42132, American Type Culture Collection (ATCC) was cultured on modified Dixon (Gueho *et al.* 1996) agar plates at 32°C. To exclude bacterial contamination and growth of other yeast, blood agar plates and Sabouraud dextrose agar plates were used in parallel. Before experiments, one colony from the culture plate was mixed with Dixon medium and incubated over night at 32°C with gentle agitation. If OD₆₀₀ was above 0.5, the next day, the culture was diluted and incubated until it reached OD₆₀₀ 0.5, which corresponds to the mid-logarithmic growth phase (experimentally determined by absorbance *vs* growth curve).

Microdilution assay

In the broth microdilution assay, absorbance of a microbial culture is measured after peptide treatment. All peptides were tested at 0.1, 1, 5 and 10 $\mu\text{mol l}^{-1}$. The concentration of TP10 was extended to 30 $\mu\text{mol l}^{-1}$ and the peptides melittin and mastoparan, which are commonly used as posi-

tive controls in antimicrobial studies, to 50 $\mu\text{mol l}^{-1}$. Antifungal activity in this work was measured as in (Yu *et al.* 2002). Briefly, a 96-well plate (Nordic Biolabs, Täby, Sweden) with 100 μl cell suspension (OD₆₀₀ 0.5), 90 μl modified Dixon growth medium and 10 μl peptide in different concentrations was incubated over night (about 17 h) at 32°C. Owing to high background absorbance of the modified Dixon growth medium, quantification of antifungal activity by absorbance measurements was not possible. However, peptides with no activity could be excluded from further studies by visual examination of the plate. Viable counts were determined from the wells showing activity to enable quantification of the effect. 50 μl (directly or diluted) was transferred from wells in the 96-well plate to Dixon agar plates and incubated at 32°C. After 3 days, colonies were counted, converted to colony forming units/millilitre and normalized against untreated *M. sympodialis*. The results are presented as mean $\pm$ standard error of the mean (SEM) from three independent experiments conducted in duplicates.

Confocal laser scanning microscopy

Microscopy was performed to show localisation of fluoresceinyl peptides and co-localisation with a mitochondrial marker (Data S1).

Results

All peptides from Table 1 were tested with the microdilution assay, as described in Materials and methods. The six most active peptides were further incubated with *M. sympodialis* on agar plates for 3 days to enable quantification of the antifungal effect. As shown in Fig. 1, all six peptides reduced the viability by more than 50% already at 0.1 $\mu\text{mol l}^{-1}$ concentrations. For Tat and pVEC, the reduction was as much as 80% at this concentration. The estimated minimal inhibitory concentrations of the peptides are shown in Table 2. Because *M. sympodialis* colonizes the skin, topologically added peptides would invariably also come into contact with the keratinocytes of the skin. It is therefore imperative that the antifungal peptides are not cytotoxic to mammalian cells. None of the peptides from Fig. 1 at concentrations up to 20 $\mu\text{mol l}^{-1}$ gave detectable levels of lactate dehydrogenase (LDH) leakage in human keratinocytes (data not shown).

To analyse intracellular uptake, fluoresceinyl peptides were incubated with *M. sympodialis* together with a mitochondrial marker. Supplementary Fig. S1 shows that the peptides were indeed internalized into *M. sympodialis*. His 5 was the most effective, while the other CPPs were taken up with similar capacity.

Table 1 Peptides tested for their antifungal activity towards *Malassezia sympodialis*

Peptide	Sequence	H	MW [g mol ⁻¹]	pI
Peptides with antifungal activity				
Arg 9	RRRRRRRR	-1.76	1442	13.3
Tat (47-57)	YGRKKRRQRRR	-1.20	1560	12.7
Histatin5	DSHAKRHHGYKRKFHEKHSHRGY	-0.54	3034	10.7
Penetratin	RQIKIWQNRRMKWKK	-0.53	2250	12.7
pVEC	LLILRRRIRKQAHASK	-0.37	2210	12.9
Scrambled pVEC	IAARIKLSRQHIKLRHL	-0.37	2210	12.9
Peptides with no antifungal activity				
eCATH-1	KRFGR LAKSFLRRILLPRRKILLAS	-0.31	3007	13.1
Fragment eCATH-1	KSFLRRILLPRRKILLAS	-0.26	2178	12.9
Flatfish Antimicrobial	GWRTLLKKA EVKTVGKLALKHYL	-0.16	2651	10.8
Camel 102	GWKLGGKILRV LKVL	-0.10	1749	11.8
Camel 46	KWKLFKGIRAV LKVL	-0.09	1797	11.8
BMAP28	GGLRSLGRKILRAWKKYGPIIPIIRIG	-0.09	3129	12.4
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	-0.08	2850	12.4
Mastoparan B	LKLSIVSWAKKVL	-0.06	1611	11.0
Piscidin 1	FFKKIFRGIVHVGKTIHRLVTG	-0.06	2552	12.4
MAP	KLALKLALKALKAALKLA	-0.01	1880	11.1
Piscidin-3	FIHHIFRGIVHAGRSIGRFLTG	0.00	2489	12.7
Ponericin W5	FWGALIKGA AKLIPSVVGLFKKKQ	0.01	2598	11.1
Piscidin-2	FFHHIFRGIVHVGKTIHKLVTG	0.03	2542	11.6
Mastoparan	INLKALAALAKKIL	0.04	1480	10.8
TP10	AGYLLGKINLKALAALAKKIL	0.05	2180	10.6

H, hydrophobicity; Mw, molecular weight; pI, isoelectric point.

All peptides are C-terminally amidated. The mean hydrophobicity was calculated with algorithm described by Eisenberg *et al.* (1984) using HydroMCalc peptide sequence analysis tool developed by Alex Tossi and Luca Sandri (<http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html>).

Discussion

Bacterial resistance towards antibiotics is an increasing problem that has earned much attention and many AMPs have been investigated for their capacity to kill bacteria (Boman 1995). The increased use of antifungal agents for prophylaxis and therapy has also led to the development of antifungal drug resistance (Pitman *et al.* 2011). A recent study showed that a novel AMP was toxic to the yeast *Malassezia furfur*, with no severe toxicity towards mammalian cells (Ryu *et al.* 2011). In the present study, we tested a battery of peptides, both antimicrobial and those known as CPPs, in the search for new agents to be used against the yeast *M. sympodialis*. From the 21 peptides tested with the microdilution assay, six were shown to have antifungal activity against *M. sympodialis* (Table 1).

Histatin 5 is a human salivary peptide with antifungal properties against *Candida albicans* (Xu *et al.* 1991), but its effect on *Malassezia* has, to our knowledge, never been tested. It was one of the most active peptides and a concentration of 0.1 $\mu\text{mol l}^{-1}$ reduced *M. sympodialis* viability to 35% of the control (Fig. 1), but showed no toxicity

towards human keratinocytes up to 20 $\mu\text{mol l}^{-1}$ (data not shown). For a peptide to possibly be used therapeutically as an antifungal agent on human skin, this is a crucial characteristic.

Penetratin, Arg9, Tat and pVEC are all well-known CPPs that have been used as transporting vehicles for different cargos in mammalian cell cultures without causing toxicity (EL Andaloussi *et al.* 2005). However, in *M. sympodialis*, they caused more than 50% reduction in viability already at 0.1 $\mu\text{mol l}^{-1}$. Penetratin is reported to be harmless to *Escherichia coli* (Palm *et al.* 2006), *Saccharomyces cerevisiae* and *C. albicans* (Holm *et al.* 2005). Therefore, we were surprised to find that penetratin was very efficient against *M. sympodialis* (Fig. 1). Arg9 has, to our knowledge, never been tested for its antimicrobial activity. There are a few reports of Tat acting as an AMP (Jung *et al.* 2006, 2008; Zhu and Shin 2009; Wang *et al.* 2010) and pVEC has been shown to possess antibacterial activity against *Mycobacterium smegmatis* (Nekhotiaeva *et al.* 2004), but to our knowledge, this is the first time pVEC is reported to have antifungal effect (Fig. 1). Also, the scrambled version of pVEC was tested to investigate whether the amino acid order is crucial for antifungal effect. Indeed,

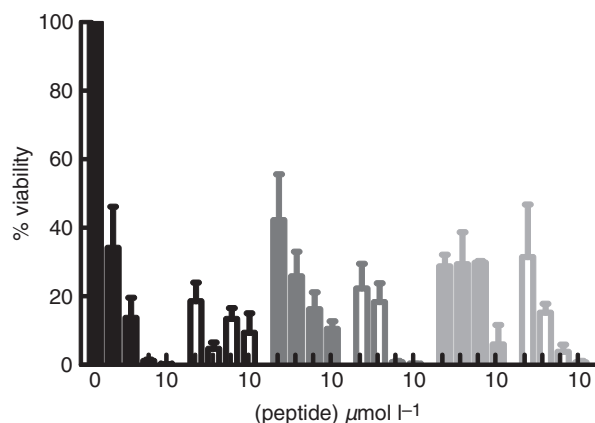


Figure 1 Growth inhibition of *Malassezia sympodialis* treated with peptides at concentrations 0.1, 1, 5 and 10 $\mu\text{mol l}^{-1}$. *M. sympodialis* was incubated with Histatin 5 (■), Tat (□), penetratin (▒), pVEC (□), scrambled pVEC (▒) and Arg9 (□) for 3 days. Colony forming units (CFU) was calculated and normalized against untreated *M. sympodialis* in each experiment. In the graph, untreated is set to 100% (0 $\mu\text{mol l}^{-1}$). The results are given as mean $\pm$ standard error of the mean from three independent experiments conducted in duplicates.

scrambled pVEC did act as an antifungal agent, although at higher concentrations than pVEC (Fig. 1).

There are reports showing that the CPP TP10 is toxic to both bacteria, fungi, parasites and mammalian cells (Nekhotiaeva *et al.* 2004). Therefore, we were surprised to find that TP10 had no effect at all on *M. sympodialis* up to 30 $\mu\text{mol l}^{-1}$ (the highest concentration tested). Furthermore, melittin, which is derived from bee venom and commonly used as positive control for AMPs (Asthana *et al.* 2004), and mastoparan, a peptide toxin from wasp venom, did not either have an effect on the viability of *M. sympodialis*. Thus, *M. sympodialis* seems to be very stringent and difficult to kill, and still His 5 and the other five CPPs were able to reduce the viability at low concentrations. Albeit inhibiting the growth of *M. sympodialis* at very low concentrations, none of these peptides induced elevated leakage of LDH compared with untreated human

keratinocytes and can thus be defined as nontoxic. The action of AMPs has historically been attributed to their membrane disrupting properties; however, more recently, intracellular effects have gained increasing attention (Marcos and Gandía 2009). Some of these intracellular effects are the result from peptide activity in mitochondria (Luque-Ortega *et al.* 2008; Komatsu *et al.* 2011). Therefore, we added the mitochondrial probe Mitotracker, which accumulates in active mitochondria, to enable the detection of colocalization with fluoresceinyl peptides. Unfortunately, from these images, it is difficult to draw conclusions regarding colocalization with mitochondria (Fig. S1).

It is generally accepted that critical determinants for the efficacy of AMPs is overall charge, amphipathicity and hydrophobicity (Takahashi *et al.* 2010). The same is true for CPPs (EL Andaloussi *et al.* 2005). Therefore, it came as a great surprise to us that the opposite seems to be true regarding *M. sympodialis* (Table 1). From Table 1, it can be concluded that the molecular weight (i.e. length of the peptide) and isoelectric point are less important factors regarding their antifungal activities against *M. sympodialis*, because no pattern can be discerned between the active peptides and the ones without effect. However, the six peptides having activity towards *M. sympodialis* are also the least hydrophobic ones. Thus, hydrophobicity does not seem to be a beneficial factor in this case.

In summary, we have found six peptides that possess antifungal activity towards *M. sympodialis* at submicromolar concentrations. Importantly, none of these peptides are toxic towards human keratinocytes up to 20 $\mu\text{mol l}^{-1}$. Therefore, these peptides emerge as promising leads for future alternative treatment for skin disorders such as AE, SE and dandruff.

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Peptide	Sequence	MIC [$\mu\text{mol l}^{-1}$]	MIC [$\mu\text{g ml}^{-1}$]
Arg9	RRRRRRRRR	10	14.0
Histatin5	DSHAKRRHHGYKRFHEKHSHRGY	5	15.2
Penetratin	RQIKIWFQNRRMKWKK	>10	>22.5
pVEC	LLILRRRIKQAHAHSK	5	11.0
Scr pVEC	IAARIKLRSRQHIKLRHL	>10	>22.1
Tat	YGRKKRRQRRR	>10	>17.2
Mastoparan	INLKALAALAKKIL	>50	>44.3
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	>50	>85.3

All peptides are C-terminally amidated. Mastoparan and melittin are two widely used cytolytic peptides included as controls. No growth inhibition of *Malassezia sympodialis* was induced in the concentration range tested (up to 50 $\mu\text{mol l}^{-1}$).

Table 2 Minimal inhibitory concentrations of the antifungal peptides

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and methods.

Figure S1. Peptide internalization into *M. sympodialis*.

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