Effects of histatin 5 and derived peptides on *Candida albicans*

Anita L. A. RUISSSEN^{*1}, Jasper GROENINK^{*}, Eva J. HELMERHORST[†], Els WALGREEN-WETERINGS^{*}, Wim VAN'T HOF^{*}, Enno C. I. VEERMAN^{*} and Arie V. NIEUW AMERONGEN^{*}

^{*}Academic Centre for Dentistry Amsterdam (ACTA), Department of Dental Basic Sciences, Section of Oral Biochemistry, Van der Boerhorststraat 7, 1081 BT Amsterdam, The Netherlands, and [†]Department of Periodontology and Oral Biology, Goldman School of Dental Medicine, Boston University, Boston, MA 02118-2392, U.S.A.

Three anti-microbial peptides were compared with respect to their killing activity against *Candida albicans* and their ability to disturb its cellular and internal membranes. Histatin 5 is an anti-fungal peptide occurring naturally in human saliva, while dhvar4 and dhvar5 are variants of its active domain, with increased anti-microbial activity. dhvar4 has increased amphipathicity compared with histatin 5, whereas dhvar5 has amphipathicity comparable with that of histatin 5. All three peptides caused depolarization of the cytoplasmic and/or mitochondrial membrane, indicating membranolytic activity. For the variant peptides both depolarization and killing occurred at a faster rate. With FITC-labelled peptides, no association with the cytoplasmic membrane was observed, contradicting the form-

ation of permanent transmembrane multimeric peptide pores. Instead, the peptides were internalized and act on internal membranes, as demonstrated with mitochondrion- and vacuole-specific markers. In comparison with histatin 5, the variant peptides showed a more destructive effect on mitochondria. Entry of the peptides and subsequent killing were dependent on the metabolic state of the cells. Blocking of the mitochondrial activity led to complete protection against histatin 5 activity, whereas that of dhvar4 was hardly affected and that of dhvar5 was affected only intermediately.

Key words: anti-microbial, dhvar peptides, mitochondria, respiration, vacuoles.

INTRODUCTION

A large number of anti-microbial peptides, as part of the innate defence of essentially all living creatures, have been identified in the past two decades [1–4]. The common features of these peptides are a net positive charge and an amphipathic nature. These features allow their presence at lipid–water interfaces and, hence, disturbance of microbial membranes. Several models have been postulated that seek to explain the membrane-disturbing properties on a molecular level. In the ‘barrel-stave’ model, α -helical amphipathic peptides group together into barrel-like clusters that line amphipathic pores, with the hydrophobic side-chains sticking out into the lipid inside of the phospholipid bilayer and the hydrophilic side-chains pointed inward into the water-filled pore. Leakage of intracellular components through these pores subsequently leads to cell death [5,6]. In the ‘carpet’ model, massive attachment of peptides to the membrane leads to progressive destabilization. Once a certain critical concentration is exceeded, the integrity of the membrane collapses and the bilayer folds back on itself, forming worm-hole-type pores in which the space between the phospholipid head groups of the folded bilayer is filled up with individual peptide molecules [7]. Numerous studies on natural and artificial membranes, and on liposomes, indeed showed leakage of intracellular components upon exposure to anti-microbial peptides [8–14].

These models are unable to explain why some peptides permeabilize bacterial membranes at sub-lethal concentrations, whereas other peptides are very bactericidal without displaying significant membrane-permeabilizing activity [14]. Some peptides kill bacteria by inhibition of DNA synthesis [15–17], by proteinase inhibition [18], or may require some receptor-like interaction to enter the cell [19–21]. These observations have led to the

postulation of the ‘aggregate-channel’ model, in which cationic amphipathic peptides cluster together in a more or less random fashion on the membrane surface, encapsulating small negative ions or water molecules to reduce the electrostatic repulsion between the positive charged side chains. Local destabilization of the membrane allows these clusters to flip-flop through the membrane. In this manner, the peptides can migrate through the membrane without causing serious leakage. The key assumption of this model is that most, if not all, anti-microbial peptides have intracellular targets and that permeabilization of the microbial membrane may be a secondary effect rather than the primary cause of anti-microbial activity [2].

In a previous study, we have found that histatin 5, belonging to a small family of histidine-rich peptides present in human parotid and submandibular saliva [22,23], is targeted to the mitochondria of *Candida albicans* [24]. Histatin-mediated killing of this yeast is accompanied by release of intracellular potassium ions [22] and of intracellular ATP [25]. The latter two observations have been explained by lytic permeabilization of the cytoplasmic membrane and by a non-lytic efflux mechanism, respectively.

In the present study we have investigated the effects of histatin 5 on the integrity of cellular membranes in greater detail. The effects of two variants of histatin 5, one with increased amphipathicity (dhvar4; where dhvar refers to a variant of dh-5, the active domain of histatin 5, amino acids 11–24) and one with amphipathicity comparable with that of histatin 5 (dhvar5), were compared with that of the parent peptide. The mean hydrophobic moment of histatin 5, calculated using the consensus hydrophobicity scale [26], is 0.091. For dhvar4 and dhvar5 this value is 0.439 and 0.061, respectively. Both variant peptides have enhanced candidacidal activity. Killing by histatin 5 as well as by

Abbreviations used: PPB, 1 mM potassium phosphate buffer; CFU, colony-forming unit; DiSC₃(5), 3,3'-dipropylthiadicarbocyanine iodide; SDA, Sabouraud Dextrose Agar; carboxy-DCFDA, 5-carboxy- and 6-carboxy-2',7'-dichlorofluorescein-diacetate; CMAc-Arg, 7-amino-4-chloromethylcoumarin-L-arginineamide; DiOC₆(3), 3,3'-dihexyloxycarbocyanine iodide.

¹ To whom correspondence should be addressed (e-mail ala.ruissen.obc.acta@med.vu.nl).

the variant peptides was always accompanied by disturbance of membrane potential. For the variant peptides, however, this occurred at a faster rate. Furthermore, using fluorescent probes with specificity for cellular organelles, we found that all peptides also affected the integrity of the vacuolar membranes. In comparison with histatin 5, the variant peptides showed a more destructive effect on mitochondria.

Previously, we described that histatin-mediated killing is dependent on the metabolic activity of *C. albicans* [24]. To investigate whether this is unique for histatins, the effect of blocking cellular respiration with sodium azide on the activity of histatin 5 and on that of the variant peptides were compared. This caused complete protection of the cells against histatin 5, while the activity of dhvar4 was hardly affected and that of dhvar5 was affected only intermediately.

EXPERIMENTAL PROCEDURES

Preparation and FITC labelling of the peptides

Histatin 5 (DSHAKRHHGYKRKFHEKHSHRGY), dhvar4 (KRLFKLLFSLRKY) and dhvar5 (LLLFLKKRKKRKY) were manufactured by solid-phase peptide synthesis using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry as described previously [27]. The peptides were labelled with FITC for FACS analysis and confocal laser microscopy. Labelling was achieved by addition of 25 μ l of FITC (1 mg/ml) in DMSO to 1 ml of a peptide solution (0.66 mM) in water, adjusted to pH 9.7 with Na_2CO_3 . After overnight incubation at 4 °C in the dark, residual FITC was inactivated by incubation with 50 μ l of 1 M NH_4Cl for 2 h. The fluorescein-peptide conjugates were stored in aliquots at -20 °C until use. As found previously [24], when used in the killing assay FITC-labelled peptides exhibited similar LC_{50} values to unlabelled peptides.

Killing assay

Killing assays were performed essentially as described previously [28]. In brief, serial dilutions of peptides (100 μ l, 0.2–131 μ M) were incubated in duplicate with an equal volume of a *C. albicans* (ATCC 10231) suspension (3.2×10^6 cells/ml) in 1 mM potassium phosphate buffer (PPB), pH 7.0. The pH of peptide solution with a concentration of 65.5 μ M was approx. pH 7. After 60 min at 37 °C, cells were diluted 200-fold in PBS, and plated on Sabouraud Dextrose Agar (SDA). After 2 days of incubation at 30 °C the number of colony-forming units (CFUs) was counted. To study the role of an active metabolism, cells were incubated for 15 min with 5 mM sodium azide before incubation with peptides. In the control experiment 5 mM NaCl was used to compensate for differences in ionic strength, since it is known that the activity of histatin 5 is sensitive to this parameter [28].

Time–kill curves were determined by incubation of 250 μ l of *C. albicans* (3.2×10^6 cells/ml) with equal volumes of the peptides at three concentrations (1.6, 3.2 or 16.6 μ M). At different time points, aliquots of 50 μ l were taken and, after dilution with PBS, plated on SDA plates to determine the number of CFUs.

To study the relationship between killing and association of peptides with *C. albicans*, a killing assay was performed as described above with FITC-labelled peptides. From this mixture 25 μ l was used to determine the number of CFUs. The remainder was used for analysis of cell-associated fluorescence with FACS analysis (Beckton Dickinson, Franklin Lakes, NJ, U.S.A.) using a 15 mV argon laser at 488 nm excitation and a 530 nm filter for detection of emitted light.

3,3'-Dipropylthiadicarbocyanine iodide [DiSC₃(5)] release

Peptide-induced release of the fluorescent probe DiSC₃(5) (Molecular Probes, Eugene, OR, U.S.A.) was monitored to study the effect of the peptides on the cytoplasmic and mitochondrial membrane potentials of *C. albicans* [29]. To a suspension of 9.6×10^6 *C. albicans* cells in 3 ml of PPB, 20 μ l of a 234 μ M DiSC₃(5) solution in PPB was added, and incubated until a constant fluorescence level was achieved (approx. 10 min). Either sodium azide (6 μ l of a 2.5 M stock solution) or NaCl (15 μ l of a 1 M stock solution) were added, to a final concentration of 5 mM, and incubated for 8 min. Peptides were added at concentrations that were 4, 8 and 16 times their LC_{50} values. Changes in fluorescence intensity upon addition of peptides were monitored with a Perkin-Elmer LS 50 B spectrofluorimeter (Perkin-Elmer, Beaconsfield, Bucks., U.K.). Simultaneously, the effect on the viability of the *C. albicans* cells was followed by taking several samples (50 μ l) over time, diluting the samples 200-fold in PBS and plating on SDA. The experiment was performed at 21 °C.

In one experiment the residual cell-associated DiSC₃(5) fluorescence after incubation with peptides was measured. A 200 μ l suspension of *C. albicans* cells (3.2×10^6 cells/ml) was preloaded with 1.5 μ M DiSC₃(5) for 10 min. Peptides were added, and after 30 min at ambient temperature samples were diluted 10-fold in PPB, and analysed by FACS analysis at 488 nm excitation and 650 nm emission.

Intracellular localization studies

Confocal laser-scanning fluorescence microscopy was used to study the intracellular target of the peptides. Double staining of *C. albicans* with FITC-labelled peptides and MitoTracker Orange (chloromethyl-H₂-tetramethyl-rosamine, Molecular Probes), a permanent mitochondrion-selective dye, was achieved as follows. A *C. albicans* cell suspension (200 μ l; 3.2×10^6 cells/ml of PPB) was incubated with 150 nM MitoTracker Orange for 15 min at 37 °C. Cells were washed with 200 μ l of PPB and treated with 17 μ M FITC-labelled peptides for an additional 15 min. Cells were collected by centrifugation (5 min at 10000 g), suspended in 20 μ l of PPB and examined by confocal microscopy with a Leica TCS NT confocal system (Leica Microsystems, Heidelberg, Germany) equipped with an Ar/Kr laser and a 100 $\times$ NA (numerical aperture) 1.4 objective lens. To study the requirement of active respiration, cells were incubated with 5 mM sodium azide for 15 min at 37 °C before the addition of MitoTracker Orange and peptides.

Staining with yeast vacuole markers

Yeast vacuole markers 5-carboxy- and 6-carboxy-2',7'-dichloro-fluorescein-diacetate (carboxy-DCFDA, Molecular Probes; both isomers are present in the same mixture and both have the same effect) and 7-amino-4-chloromethylcoumarin-L-arginineamide (CMAC-Arg, Molecular Probes) were used to study the effect on the vacuole. A *C. albicans* suspension (200 μ l; 3.2×10^6 cells/ml of PPB) was incubated with the peptides for 15 min at 37 °C. Final peptide concentrations were 17 μ M for histatin 5, 27 μ M for dhvar4 and 27 μ M for dhvar5. Cells were collected by centrifugation and resuspended, either in 50 mM sodium citrate buffer, pH 5.0, containing 2% glucose, or in 10 mM Hepes buffer, pH 7.4, containing 5% glucose. To the suspension in citrate buffer, carboxy-DCFDA was added to a final concentration of 10 μ M (10 μ l from a 1 mM stock solution in citrate buffer). To the suspension in Hepes buffer, CMAC-Arg was added to a final concentration of 100 μ M (10 μ l from a 10 mM

stock solution in DMSO). Cells were incubated subsequently for 30 min at ambient temperature and examined by confocal laser microscopy. In addition, 50 μ l samples were taken, diluted in 50 mM sodium citrate, and analysed on a FACScan at 488 nm excitation and 530 nm emission.

RESULTS

Killing activity

Previously it has been demonstrated that cellular respiration is necessary for the candidicidal action of histatin 5. To investigate whether this was also true for more potent histatin derivatives, the anti-microbial activities of two synthetic variants (dhvar4 and dhvar5) were compared with that of histatin 5, in the presence and absence of 5 mM sodium azide (Figure 1). In the presence of sodium azide, the fungicidal activity of histatin 5 was completely lost. At 70 μ M no significant killing was observed (the LC_{50} value in the absence of azide was 3.8 μ M). The activity of dhvar5 was intermediately sensitive to azide, with an LC_{50} value increasing from 2 to 10 μ M. Activity of dhvar4 was the least sensitive to azide. Its LC_{50} value increased 2-fold from 0.1 μ M to approx. 0.2 μ M. Time-kill experiments indicated differences in killing rate between histatin 5 and the synthetic variants (Figure 2). At a concentration of 8.3 μ M, 15 min of incubation with histatin 5 was needed to reach complete killing, whereas dhvar4 and dhvar5 killed virtually all cells within 2 min at this concentration. Essentially the same results were obtained when killing was measured by monitoring the time-dependence of the uptake of the viability marker propidium iodide (results not shown).

Effects of histatin 5, dhvar4 and dhvar5 on membrane depolarization

We next examined the effect of histatin 5, dhvar4 and dhvar5 on the cytoplasmic and mitochondrial membrane potentials of *C. albicans* cells, by monitoring the release of DiSC₃(5) from preloaded *C. albicans* cells (Figure 3). The peptides were tested at concentrations with corresponding activities to compensate for the higher activity at equal molar concentrations of the dhvars compared with histatin 5. Upon addition of DiSC₃(5) to the *C.*

Figure 1 Effect of sodium azide on peptide-mediated *C. albicans* killing

C. albicans cells (100 μ l, 3.2×10^6 cells/ml PPB) were incubated in the presence of either 5 mM NaCl ($\blacktriangle$) or 5 mM NaN_3 ($\blacksquare$) for 15 min at 37 $^{\circ}C$. Subsequently, equal volumes of serial dilutions of the three peptides in PPB containing the same additives were added (final peptide concentration, 0.1–65 μ M). After 1 h of incubation, cell viability was determined by plating on SDA plates. Sodium azide protected *C. albicans* completely against the killing activity of histatin 5, partially against that of dhvar5 and non-significantly against that of dhvar4.

Figure 2 Time kinetics of peptide-mediated *C. albicans* killing

C. albicans cells (0.8×10^6 cells in 250 μ l) were incubated with 250 μ l of peptide dilutions, 1.6 ($\blacksquare$), 3.2 ($\blacktriangle$) and 16.6 ($\bullet$) μ M, in PPB at 37 $^{\circ}C$. At indicated points in time, 50 μ l samples were taken and the cell viability was determined by plating on SDA. Histatin 5 killed *C. albicans* at a much slower rate than dhvar4 and dhvar5. At the highest concentrations dhvar4 and dhvar5 needed only 2 min to achieve complete killing, whereas with histatin 5 this was only accomplished after 20 min.

albicans cell suspension, a decrease in fluorescence intensity was observed, indicating uptake of the dye by the yeast cells. Addition of dhvar4 or dhvar5 at concentrations exceeding their LC_{50} values by 4- and 8-fold respectively resulted in a sharp rise in fluorescence intensity, highlighting a rapid release of the probe from the cells (Figures 3B and 3C). After approx. 4 min the fluorescence signal reached a constant level. Addition of excess histatin 5 (60 μ M) resulted also in a rise in fluorescence, but equilibrium was reached after more than 20 min (Figure 3A). Incubation with lower concentrations of histatin 5 and dhvar peptides (below their LC_{50} values) resulted in a proportionally smaller release of DiSC₃(5) (results not shown).

To investigate the effect of metabolic activity on these processes, DiSC₃(5)-loaded cells were incubated with 5 mM sodium azide before peptides were added. Addition of azide resulted in a small increase in fluorescence (Figures 3D–3F), probably due to minor depolarization of the cytoplasmic and mitochondrial membranes. Subsequent addition of histatin 5 or dhvar5 in concentrations exceeding their LC_{50} values by 16- and 8-fold, respectively, did not result in an increase in the fluorescence signal (Figures 3D and 3F). In contrast, addition of dhvar4 at a concentration exceeding its LC_{50} value by 4-fold resulted in release of DiSC₃(5), but to a somewhat lower final level (Figure 3E). Simultaneous determination of viability showed that in all of these experiments the kinetics of DiSC₃(5) release corresponded with that of killing (results not shown), indicating a correlation between the effect on membrane potential and the lethal effect. It can be noted that the release caused by histatin 5 in the absence of azide (Figure 3A) occurs at a lower rate than the killing by histatin 5, as presented in Figure 2. However, the release experiment was performed at 21 $^{\circ}C$, whereas the time-kill experiment of Figure 2 was performed at 37 $^{\circ}C$. Following the killing activity of the peptides at the concentrations used in the release experiment, at 37 and 21 $^{\circ}C$ concurrently, showed that at 21 $^{\circ}C$ this occurred at a slower rate than at 37 $^{\circ}C$ (results not shown). This probably explains the discrepancy between the rate of killing in Figure 2 and the release rate in Figure 3(A).

To measure residual cell-associated fluorescence, DiSC₃(5)-loaded cells were incubated with peptides and analysed by FACScan analyses. Figure 4 shows that *C. albicans* cells that had been incubated for 30 min with either 17 μ M histatin 5 or 27 μ M of the dhvars still displayed DiSC₃(5) fluorescence that was

Figure 3 Peptide-mediated membrane depolarization of *C. albicans*

To a suspension of 9.6×10^6 *C. albicans* cells in 3 ml of PPB, 20 μ l of a 234 μ M DiSC₃(5) solution in DMSO was added. After a constant fluorescence level had been achieved (about 10 min), sodium azide or NaCl was added, to a final concentration of 5 mM (left-hand arrows), and incubation was continued for approx. 8 min. Peptides were added in concentrations varying between 4 and 16 times their previously determined LC₅₀ values, as shown (right-hand arrows). In line with the killing kinetics, histatin 5 (A) induced a much slower release of the membrane-potential-dependent probe than dhvar4 (B) and dhvar5 (C). In the presence of sodium azide (D–F), the membrane depolarization by histatin 5 and dhvar5 was almost completely abolished.

Figure 4 *C. albicans* cell-associated fluorescence of DiSC₃(5)

After a 15 min incubation to obtain a constant fluorescence level, suspensions of 3.2×10^6 *C. albicans* cells/ml in PPB containing 1.5 μ M DiSC₃(5) were treated with an excess of peptides (17, 27 and 27 μ M histatin 5, dhvar4 and dhvar5, respectively). After 30 min of incubation the residual cell-associated fluorescence was determined. FACS analysis shows for all three peptides a fluorescence comparable with cells that had been incubated with DiSC₃(5) without further peptide treatment (C+), indicating that DiSC₃(5), to a certain extent, also bind to cells in a non-membrane-potential-dependent manner. As a reference, fluorescence of untreated cells (C–) is shown.

comparable with or even higher than that displayed by the untreated control cells loaded with DiSC₃(5). This suggests that not all of the preloaded DiSC₃(5) molecules were released by subsequent treatment with these peptides.

Cellular localization of FITC-labelled peptides

The characteristics of binding of FITC-labelled peptides to *C. albicans* were examined further by confocal microscopy, in

combination with FACS analysis (Figures 5 and 6). With FITC–histatin 5, a granular intracellular staining pattern was observed in *C. albicans* cells, indicating binding to distinct cell organelles. Double-staining with MitoTracker Orange and FITC-labelled histatin 5 showed overlapping patterns (Figure 5), suggesting association of histatin 5 with the mitochondria. Double-staining with MitoTracker Orange and FITC-labelled dhvar peptides showed more heterogeneous labelling patterns. In some cells the FITC signal appeared to be more uniformly distributed over the cell, suggesting that the mitochondrial

Figure 5 Double staining of *C. albicans* cells with FITC-labelled peptides and MitoTracker Orange

C. albicans cells (3.2×10^6 cells/ml) were incubated at 37 °C with 150 nM MitoTracker Orange in PPB for 15 min. After washing, the cells were incubated with 17 µM FITC-labelled peptides in the same buffer. Confocal laser microscopy showed that incubation with FITC-labelled histatin 5 resulted in a granular fluorescein signal (green), that co-localized (yellow) with the MitoTracker Orange signal (red). Treatment with dhvar4 and dhvar5 resulted in more diffuse distribution of the labels, suggesting a collapse of the mitochondrial structure.

Figure 6 Effect of sodium azide on the uptake of FITC-labelled peptides by *C. albicans* cells

C. albicans cells (1.6×10^6 cells/ml) that had been pre-incubated at 37 °C with 5 mM NaCl or NaN₃ in PPB for 15 min were treated with peptides at a concentration of 8.2 µM. FACS analysis shows that sodium azide almost completely blocked the uptake of FITC-labelled histatin 5. The uptake of FITC-labelled dhvar5 was partially blocked, whereas the uptake of FITC-labelled dhvar4 was virtually unaffected. As a reference, untreated *C. albicans* cells were used (C).

organization was disturbed. In a few cells the granular staining of mitochondria was observed. Occasionally, labelled dhvar4 and dhvar5 were found associated with the cytoplasmic membrane. MitoTracker Orange labelling patterns overlapped partly with the FITC signal in some cells and completely in others. In a control experiment, no association of inactivated FITC or a

FITC-labelled negative control peptide with *C. albicans* was found. Confocal fluorescence microscopy (results not shown) and FACS analysis showed that pre-treatment with azide almost completely abolished the uptake of FITC-labelled histatin 5, whereas under these conditions the majority of cells were labelled with FITC-dhvar4 and FITC-dhvar5 (Figure 6).

Figure 7 Effect of peptides on *C. albicans* vacuoles

C. albicans cells (3.2×10^6 cells/ml) were incubated in PPB at 37 °C with final concentrations of 17 μ M histatin 5, 27 μ M dhvar4 and 27 μ M dhvar5 for 15 min. Cells were collected by centrifugation and suspended in 50 mM sodium citrate buffer, pH 5.0. Carboxy-DCFDA was added to a final concentration of 10 μ M, followed by an incubation of 30 min at ambient temperature. Confocal laser microscopy (upper panels, transmission; lower panels, fluorescence) showed that, upon incubation with carboxy-DCFDA, the probe accumulated in the vacuoles of control cells (not treated with any of the peptides), visible as clustered spherical structures. In cells treated with histatin 5 or dhvar5, the fluorescence was distributed diffusely over the entire cell, suggesting that the integrity of the vacuolar membrane was lost. In cells treated with dhvar4 only very weak fluorescence was observed.

Effect of histatin 5 and dhvar peptides on the distribution of vacuole markers carboxy-DCFDA and CMAC-Arg

The effect of histatin 5, dhvar4 and dhvar5 at the subcellular level was examined further using the fluorescent probes carboxy-DCFDA and CMAC-Arg. The mechanism of carboxy-DCFDA labelling depends on removal of the acetate ester group by non-specific esterase, followed by accumulation of the anionic carboxyfluorescein in the vacuole, driven by the low vacuolar pH. Upon incubation with carboxy-DCFDA in control untreated cells, the probe accumulated in the vacuoles, visible as spherical structures clustered together (Figure 7). In cells treated with histatin 5 or dhvar5, the fluorescence became distributed over the entire cell, suggesting that the integrity of the vacuolar membrane was lost. After incubation with dhvar4, fluorescence was very weak, suggesting the probe was not retained in the cell. FACSscan analysis confirmed these results, showing an even increase in carboxy-DCFDA fluorescence in cells treated with histatin 5 and dhvar5. Using CMAC-Arg, a thiol-reactive probe that is converted into a membrane-impermeant glutathione-fluorescent dye adduct, all peptides, including dhvar4, produced similar fluorescence patterns, with the probe more uniformly distributed over the entire cell with respect to cells not treated with peptides (results not shown). Reversal of the incubation order, i.e. pre-incubation of the cells with the vacuole markers first followed by treatment with the peptides, gave essentially the same results. After incubation of cells with histatin 5 in the presence of azide, the signals were comparable with those of the controls (results not shown), indicating that under these conditions the vacuolar membranes remained intact.

DISCUSSION

In the present study three cationic peptides with different amphipathic moments were compared with respect to their effects on *C. albicans*. Histatin 5, a natural peptide of human saliva, is moderately amphipathic. dhvar4, derived from the

active domain of histatin 5, is strongly amphipathic, while the amphipathicity of dhvar5 is comparable with that of histatin 5. This study demonstrated that all three peptides became internalized by *C. albicans* and that internalization was closely associated with their killing activity. However, cellular localization of these peptides was clearly different. Confirming our previous results [24], we found that histatin 5 was targeted to the mitochondria and was most sensitive to inhibition of the mitochondrial respiratory chain by sodium azide. On the other hand, both dhvar4 and dhvar5 gave more diffuse labelling of the cells. Strikingly, the MitoTracker Orange signal was also affected by treatment of *C. albicans* with the variant peptides. MitoTracker Orange is a fixable cell-permeant mitochondrion-selective dye that contains a mildly thiol-reactive chloromethyl moiety, which keeps the dye covalently associated with mitochondrial components. Once their mitochondria are labelled, cells can be permeabilized without disturbance of the MitoTracker Orange staining pattern. The present finding that the staining pattern is completely disturbed upon incubation with dhvar4 and dhvar5 suggest that, after incubation with the variant peptides, the entire mitochondrial organization collapsed.

All peptides caused membrane depolarization, as determined by the release of the membrane-potential-dependent probe DiSC₃(5). However, histatin 5 induced the release at a much slower rate than dhvar4 and dhvar5. This occurred at approximately the same rate as the killing, measured in the killing assay and using the viability probe propidium iodide, suggesting that these processes are correlated. This finding is different from that of Koshlukova et al. [25], who suggested that histatin 5 had no effect on the membrane potential on the basis of FACSscan analysis monitored by 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)]. The disparity between these conclusions is probably due to the different methods of membrane-potential measurement. In the study of Koshlukova et al. [25], the presence of cell-associated probe was considered as evidence for the presence of membrane potential. In the present study, dissipation of the

membrane potential was monitored as release of the probe from preloaded cells into the medium. The use of carbocyanine dyes like DiOC₆(3) and DiSC₃(5) as potential-sensitive probes is based on the fact that these molecules accumulate on hyperpolarized membranes and that they are translocated into the lipid bilayer, which results in decreased fluorescence. It is assumed that release of the probe into the medium, which is accompanied by an increase in fluorescence, highlights membrane depolarization. Confirming the results of Koshlukova et al. [25], we found that upon prolonged incubation of the variant peptides or histatin 5 with cells preloaded with DiSC₃(5), the cell-associated fluorescence measured by FACScan hardly changed, or, in the case of dhvar4, even increased. Yet, upon addition of dhvar4 and dhvar5 we found a direct release from the probe into the medium (Figure 3), indicating dissipation of the membrane potential. Though commonly used as membrane-potential sensitive probes, carbocyanine dyes such as DiOC₆(3) and DiSC₃(5) also bind to intracellular membranes, including the endoplasmic reticulum, in both live and formaldehyde-fixed cells [30].

The molecular mechanism of how histatin 5 kills *C. albicans* is not completely understood. Several studies demonstrated that, upon incubation with histatin 5, the cytoplasmic membrane of *C. albicans* becomes permeable to small molecules, including K⁺ and ATP [22,25]. Depletion of ATP (and probably also of ADP) from the cells is a lethal event, but it is not clear how histatin 5 causes leakage of the cytoplasmic membrane. Originally, it was assumed that because of their amphipathic properties, peptides such as histatin 5 form pores in the cytoplasmic membrane through which small molecules can diffuse. In the present study, as well as in our previous study, we could not demonstrate association of histatin 5 with the cell surface, suggesting that histatin does not simply act by forming permanent pores in the cytoplasmic membrane. Xu et al. [31] also concluded that binding of histatin 3 to the cell surface alone (without subsequent internalization) was not sufficient for killing by histatin 3. Similarly we found that when histatin 5 was not internalized, e.g. by lowering the state of energy of the cell, neither the cellular membranes, the mitochondrial membranes [24] or the vacuolar membranes were depolarized.

Another issue pertaining to the mechanism of action is the decrease in candidacidal activity when agents affecting mitochondrial activity lower the cell metabolism. This effect has been demonstrated for a number of cationic peptides, including histatins, HNP-1 (human neutrophil peptide-1) and protamine [24,25,32–34]. In a previous study this effect was explained by assuming that depolarization of the mitochondrial membrane potential would decrease the affinity of histatin 5 for the mitochondrial membrane. In the present study we found that azide afforded generic protection against the detrimental effects of histatin 5 on the plasma membrane, as well as on the mitochondrial and vacuolar membranes. Thus the minimal hypothesis is that lowering the metabolic state hampers the permeabilization through the plasma membrane of histatin 5 molecules (and other peptides), which subsequently cannot reach their intracellular receptors. There seems to be a gradual difference in sensitivity among the peptides. Whereas the activity and entrance of histatin 5 is completely blocked, dhvar5 was affected only intermediately and dhvar4 was hardly affected at all. This might be due to differences in amphipathicity: dhvar4 is strongly amphipathic, whereas histatin 5 and dhvar5 have comparable low amphipathicity. It is conceivable that a more amphipathic peptide migrates more easily through lipid bilayers. The inhibition of peptide uptake by *C. albicans* under conditions in which cellular metabolism is lowered might be attributed to a decreased activity of ATP-driven transport systems. However, a

crucial role of such systems in the candidacidal action of amphipathic peptides is questionable. It is hard to envisage how transport of peptides via such aspecific peptide transporters will disturb membrane integrity, leading to the release of small molecules [22,25] and dissipation of the membrane potential. Besides, if the passage of peptides through the cell membrane involved active transport, all peptides would be equally sensitive to metabolic inhibitors, e.g. sodium azide. In contrast, in the present study we found gradual differences in sensitivity to azide, suggesting that this is more likely to be an intrinsic property of the peptide. One can hypothesize that, secondary to the decrease in metabolic activity, physicochemical or structural changes might occur at the perimeter of the cell that influence the peptide–membrane interaction.

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