Reactive oxygen species play no role in the candidacidal activity of the salivary antimicrobial peptide histatin 5

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The mechanism of action of antimicrobial peptides is still a matter of debate. The formation of ROS (reactive oxygen species) has been suggested to be the crucial step in the fungicidal mechanism of a number of antimicrobial peptides, including histatin 5 and lactoferrin-derived peptides. In the present study we have investigated the effects of histatin 5 and of a more amphipathic synthetic derivative, dhvar4, on the generation of ROS in the yeast *Candida albicans*, using dihydroethidium as an indicator for ROS. With both peptides, a substantial enhancement of fluorescence was observed. However, TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl), a cell-permeant ROS scavenger, did not have an inhibitory effect on killing or on the enhancement of fluorescence.

Furthermore, antimycin and azide, which have been reported to induce ROS *in vitro*, were not able to enhance the dihydroethidium fluorescence, while chlorhexidine, a non-specific antiseptic agent, enhanced dihydroethidium fluorescence to the same extent as did the peptides. Fluorescence microscopy showed the fluorescence enhancement to be a consequence of the release of unbound preformed ethidium from the mitochondrial matrix within the cell. It is concluded that ROS do not play a role in the histatin 5-mediated killing of *C. albicans*.

Key words: amphipathic, *Candida*, dihydroethidium, histatin, membrane, reactive oxygen species (ROS).

INTRODUCTION

Antimicrobial peptides are found throughout Nature, and form a cornerstone of innate defence against microbial and viral infections. Although they display wide structural and genetic heterogeneity, these peptides share two features: they are polycationic, carrying two or more positive charges at neutral pH, and they are amphipathic, i.e. they contain a hydrophilic and a hydrophobic side [1–5]. Their mechanism of action is still a matter of debate. Originally, it was assumed that the cytolytic activity is caused by the formation of peptide pores in the cytoplasmic membrane of the target cell, resulting in leakage of essential cellular components [6]. However, alternative mechanisms have been postulated for a number of peptides, including PR-39, attacins, histatin 5, osmotin, buforin II, Bac5 and Bac7, and BMAP-28 (bovine myeloid antimicrobial peptide-28) [7–13].

For one of these peptides in particular, the human salivary peptide histatin 5, a number of studies have demonstrated that conditions resulting in decreased cellular metabolism strongly influence the sensitivity of the yeast *Candida albicans*. For instance, lowered incubation temperatures and anaerobic conditions prevented killing by histatin 5. Protective effects have also been reported with agents such as CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), sodium cyanide and sodium azide, which all interfere with ATP generation by the mitochondrial respiratory chain [9,14–18]. The finding that histatin 5-mediated killing and targeting to the mitochondria of the target cell are linked events makes it tempting to suggest a role for mitochondria in the killing process. Indeed, data have been produced suggesting that the detrimental action of histatin 5 on yeast mitochondria results in the generation of ROS (reactive oxygen species), and subsequently in cell death due to the oxidation of cellular macromolecules and a loss of cellular integrity [15]. A similar mode

of action has been suggested for other antimicrobial peptides, including lactoferrin-(1–11) and osmotin [10,19].

Based on the active domain of histatin 5, we have designed and synthesized a number of peptide variants, each of which displays increased amphipathic character, enhanced antimicrobial activity and decreased sensitivity to ionic strength [17,20,21]. Furthermore, the candidacidal activities of these peptides are insensitive to the presence of the respiratory inhibitor sodium azide, in contrast with killing by the parent peptide. One of these variants, dhvar4, was found to uncouple the mitochondrial respiration of *C. albicans*, in contrast with histatin 5, which acts as an inhibitor of electron transport [15,20]. Since uncoupling of the mitochondrial electron transport chain inhibits, rather than stimulates, ROS generation [22], it is expected that the killing activity of the variant peptide dhvar4 is not mediated by ROS. The present study was initiated to examine the effects of dhvar4, in comparison with those of histatin 5, on the formation of ROS during the killing of *C. albicans*. We found that the peptide-induced enhancement of HET (dihydroethidium), a fluorogenic indicator of ROS, was not caused by ROS formation in *C. albicans*. It is concluded that ROS do not play a role in the killing by these peptides.

EXPERIMENTAL

Fluorescence microscopy

C. albicans in the exponential growth phase were preloaded with 20 µg/ml HET and suspended in PPB (1 mM potassium phosphate, pH 7.0) to a D_{620} of approx. 2.0. Histatin 5 was added, and the mixture was examined on a Leica DM-RA microscope equipped with a Leica DC-200 digital camera, using Leica Qwin

Abbreviations used: HET, dihydroethidium; PI, propidium iodide; PPB, 1 mM potassium phosphate, pH 7.0; ROS, reactive oxygen species; TEMPO, 2,2,6,6-tetramethylpiperidine-N-oxyl; TEMPO-9-AC, 4-[(9-acridinecarbonyl)amino]-2,2,6,6-tetramethylpiperidin-1-oxyl.

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imaging software. Excitation and emission filters were 515–560 nm and 590 nm respectively.

Measurement of ROS

The formation of ROS in *C. albicans* was determined using HET (Molecular Probes, Eugene, OR, U.S.A.) as a ROS indicator. *C. albicans* cells in the exponential growth phase were suspended in PPB to a D_{620} of approx. 2.0. Then HET was added from a 10 mM stock solution in DMSO to a concentration of 20 μ M. After incubation (15 min at 30 °C) the cells were collected by centrifugation and resuspended in PPB to a D_{620} value of approx. 2.0. Then 100 μ l of this mixture was added to dilution series of histatin 5 and dhvar4 in PPB in a black microtitre plate (Greiner, Recklinghausen, Germany). To assess the involvement of ROS in the HET-derived fluorescence, cells preloaded with HET were preincubated in PPB supplemented with 3 mM of the membrane-permeant superoxide dismutase mimetic TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl; Molecular Probes), which acts as a scavenger of ROS. The development of fluorescence was monitored at 2.5 min intervals for 1 h at 30 °C at $\lambda_{\text{excitation}} = 485$ nm and $\lambda_{\text{emission}} = 620$ nm for each concentration of the peptide. In addition to peptides, the following agents or conditions were tested for their effects on the HET-derived fluorescence: 1 μ M antimycin A, a complex III inhibitor of respiration; 5 mM sodium azide, a complex IV inhibitor of respiration; and chlorhexidine (1–20 μ M), a general disinfectant.

ROS formation was also determined using the ROS indicator TEMPO-AC {4-[(9-acridinecarbonyl)amino]-2,2,6,6-tetramethylpiperidin-1-oxyl; Molecular Probes}, a fluorogenic probe for detecting hydroxyl and superoxide radicals. TEMPO-AC was added from a stock solution of 10 mg/ml in DMSO to *C. albicans* cells grown in the exponential phase and suspended in PPB (D_{620} value approx. 2.0) to a concentration of 20 μ M. After 10 min of incubation, 100 μ l of this mixture was added to dilution series of histatin 5 and dhvar4 in PPB in a black microtitre plate, as described above. Fluorescence was monitored at 5 min intervals for 1 h at 30 °C at $\lambda_{\text{ex}} = 358$ nm and $\lambda_{\text{em}} = 424$ nm for each concentration of the peptides.

Another probe that has been used to monitor ROS is H_2 -DCFDA (dichlorodihydrofluorescein diacetate). However, in the presence of histatin 5 and dhvar4, a steady increase in the background fluorescence level of this probe was measured (even in the absence of *C. albicans*), making it unsuitable as a ROS detector under these conditions.

Growth conditions

In brief, cells were grown on Sabouraud dextrose agar (Oxoid, Basingstoke, Hants., U.K.) and transferred to 100 ml of Sabouraud dextrose broth in a 250 ml Erlenmeyer flask. After 20 h of incubation at 30 °C, 300 μ l of this suspension was subcultured for 1–2 h in 100 ml of Sabouraud dextrose broth, to obtain an exponential-phase culture.

Killing assays

The fungicidal activity of the peptides was measured by monitoring the fluorescence enhancement of PI (propidium iodide; Molecular Probes) by killed cells. PI was added from a 10 mM stock solution in DMSO to *C. albicans* cells grown in the exponential phase and suspended in 1 mM PPB ($D_{620} = 2.0$) to a final concentration of 2 μ M. After incubation (15 min at 30 °C), 100 μ l of this mixture was added to dilution series of histatin 5 and dhvar4 in PPB in a black microtitre plate. Fluorescence was

monitored at 5 min intervals for 1 h at 30 °C at $\lambda_{\text{ex}} = 544$ nm and $\lambda_{\text{em}} = 620$ nm for each concentration of each peptide. To examine whether ROS are involved in the fungicidal process, incubations were conducted in parallel in the presence of 3 mM of the membrane-permeant ROS scavenger TEMPO.

The effects of the various agents and treatments on the viability of *C. albicans*, determined in the PI assay, were verified in a viability assay [9]. In short, exponential-phase *C. albicans* cells were washed three times in PPB and suspended to a D_{620} of 2.0. Aliquots from this suspension were added to a dilution series of histatin 5 and dhvar4 in PPB, resulting in final cell densities of approx. 1×10^6 cells/ml. The incubation mixtures were diluted 200-fold in PBS, and 25 μ l aliquots were plated on Sabouraud dextrose agar. After 48 h of incubation at 30 °C, the numbers of colony-forming units were determined.

Peptides

Histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGY) and dhvar4 (KRLFKKLLFSLRKY) were synthesized on a Milligen 9050 (Milligen Biosearch) peptide synthesizer using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry. They were purified by reverse-phase HPLC on a Jasco HPLC System (Jasco, Tokyo, Japan), as follows. Peptides were dissolved in 0.1 % (v/v) trifluoroacetic acid and applied on a Vydac C_{18} column (218TP, 1.0 cm $\times$ 25 cm, 10 μ m particles; Vydac, Hesperia, CA, U.S.A.) equilibrated in 0.1 % trifluoroacetic acid. Elution was performed with a linear gradient from 30 to 45 % (v/v) acetonitrile containing 0.1 % trifluoroacetic acid in 20 min at a flow rate of 4 ml/min. The absorbance of the column effluent was monitored at 214 nm, and peak fractions were pooled, lyophilized and re-analysed by reverse-phase HPLC and by capillary electrophoresis on a Biofocus 2000 apparatus (Bio-Rad, Hercules, CA, U.S.A.). The authenticity of the peptides was confirmed by quadrupole-time of flight MS on a tandem mass spectrometer (Micromass Inc., Manchester, U.K.), as described previously [23].

ATP determination in *C. albicans* supernatants

Exponentially grown *C. albicans* cells were suspended to a density of 10^7 cells/ml of PPB. To this suspension was added 25 μ M histatin 5 or dhvar4, and incubation was conducted at 37 °C. After 5, 15 and 60 min, aliquots were taken and centrifuged at 10 000 *g* for 2 min. Supernatants were analysed for the presence of nucleotides (ATP) by capillary zone electrophoresis on a Biofocus 2000 instrument, as described previously [24]. ATP concentrations were determined from ATP standard curves, using integration software provided by the manufacturer (Bio-Rad). Control samples, obtained from cell suspensions that were incubated in parallel in the absence of peptides, were completely negative.

RESULTS

Effects of TEMPO on the antifungal properties of histatin 5 and dhvar4

In the present study we compared histatin 5, which reportedly kills through the induction of ROS [15], with its more potent variant dhvar4, which is expected to kill via another mechanism. We tested the effects of the ROS scavenger TEMPO on the candidacidal activities of both peptides using the vital stain PI (Figure 1). When the membrane becomes permeable to PI, fluorescence of this probe is enhanced by 20–30-fold due to its binding to nucleic acids. A close correlation between PI

Figure 1 Effects of TEMPO on the killing of *C. albicans* by histatin 5 or dhvar4

PI was added at a concentration of 2 µM to a *C. albicans* suspension in PPB ($D_{620} = 2.0$). After a 15 min incubation at 30 °C, 100 µl aliquots of this mixture were added to dilution series of histatin 5 (●, ○) or dhvar4 (■, □) containing PPB with (●, ■) or without (○, □) 3 mM of the ROS scavenger TEMPO. Equipotent starting concentrations of histatin 5 and dhvar4 were used. Fluorescence (expressed in arbitrary units; AU) was monitored after 60 min at $\lambda_{ex} = 544$ nm and $\lambda_{em} = 620$ nm. Curves are representative of three independent experiments, carried out in duplicate. Values represent means $\pm$ S.D. of the means of duplicate measurements.

fluorescence and killing as determined in the viability assay has been observed [9]. Both histatin 5 and dhvar4 induced an enhancement in the fluorescence signal of PI (Figure 1). To examine whether ROS are involved in the fungicidal process, parallel incubations were conducted in the presence of 3 mM of the membrane-permeant ROS scavenger TEMPO. However, TEMPO did not protect *C. albicans* against the fungicidal effects of either histatin 5 or dhvar4, as determined with both the PI assay (Figure 1) and the viability assay (results not shown). Also, when using TEMPO-AC, a fluorogenic ROS indicator, no ROS induction by either of these peptides was detected (results not shown).

This prompted us to study more closely the formation of ROS using HET, which has been used as a ROS indicator in *C. albicans* [15]. ROS detection with HET is based on the oxidation of the non-fluorescent HET to the fluorescent ethidium by ROS. Similar to that of propidium, the fluorescence of ethidium is strongly enhanced when this dye intercalates with the bases of nucleic acids. First we optimized HET-based ROS detection in *C. albicans*. *C. albicans* was preloaded with a dilution series of HET, in a concentration range from 5 to 25 µM, and mixed with dilution series of histatin 5. After 60 min the fluorescence was quantified in a microtitre fluorimeter. With all HET concentrations tested, a histatin-dependent increase in HET fluorescence was found, but the best signal was obtained when cells had been preloaded with 20 µM HET (results not shown). It was therefore decided to use this concentration in further experiments.

Effects of histatin 5 and dhvar4 on HET-derived fluorescence in *C. albicans*

The generation of ROS in *C. albicans* by histatin 5 and dhvar4 was monitored by the oxidation of HET to the fluorescent ethidium, in the presence or absence of TEMPO (Figure 2). After 35 min of incubation, both histatin 5 and dhvar4 had induced a dose-dependent increase in the fluorescence intensity of

Figure 2 Effects of TEMPO on histatin 5- and dhvar4-induced HET fluorescence in *C. albicans*

Exponential-phase *C. albicans* cells, loaded with HET, were preincubated in PPB in the absence (○, □) or presence (●, ■) of 3 mM TEMPO, a ROS scavenger. Aliquots of 100 µl of the mixture were added to dilution series of histatin 5 or dhvar4 in PPB, and development of fluorescence was followed for each concentration of each peptide at 5 min intervals for 1 h at 30 °C at $\lambda_{ex} = 485$ nm and $\lambda_{em} = 620$ nm. Shown is the fluorescence, expressed in arbitrary units (AU), after 35 min of incubation with dilution series of histatin 5 (●, ○) or dhvar4 (■, □), in the presence (●, ■) or absence (○, □) of 3 mM TEMPO. TEMPO had no effect on the development of HET fluorescence induced by either peptide. Curves are representative of four independent experiments, carried out in duplicate. Values represent means $\pm$ S.D. of the means of duplicate measurements.

C. albicans cells preloaded with HET. However, the ROS scavenger TEMPO did not inhibit the development of the fluorescence signal. Monitoring the fluorescence signal over time revealed that the histatin-induced increase in fluorescence was maximal after approx. 15 min, and remained stable for the rest of the incubation period (Figure 3). In contrast, dhvar4, at the higher concentrations, induced a transient increase in HET-derived fluorescence: the fluorescence reached a maximum after approx. 15 min and then decayed. Notably, at the highest dhvar4 concentration tested, the fluorescence signal had returned to its baseline value after 60 min. When cells were preloaded with lower HET concentrations, the decay was even faster (results not shown). This anomalous behaviour may explain why, after a 1 h incubation with the more potent antimicrobial peptide PGLa, in an end-point reading no enhancement of HET fluorescence was found [15]. Altogether, these experiments confirmed that histatin 5 enhanced the fluorescence of cells preloaded with HET. However, in contrast with what was expected, dhvar4 also induced a (transient) increase in fluorescence. TEMPO did not inhibit the fluorescence enhancement induced by either peptide, suggesting that ROS were not responsible for the observed increase in HET fluorescence.

We further evaluated the use of HET as an indicator of ROS in *C. albicans* by examining the effects of antimycin and sodium azide on HET-derived fluorescence. Both agents act as inhibitors of the mitochondrial respiratory chain, and induce formation of ROS *in vitro* [22,25,26]. However, these agents did not exhibit any antifungal activity, either in the PI assay or in the viability assay (results not shown), in line with previous reports [9,15]. Furthermore, we found that neither antimycin A nor sodium azide increased HET-derived fluorescence, even after a 1 h incubation period. On the other hand, when HET-preloaded *C. albicans* cells were incubated with the aseptic agent chlorhexidine, a time- and

Figure 3 Time course of HET fluorescence induced by various concentrations of histatin 5 (A) and dhvar4 (B)

Exponential-growth-phase *C. albicans* cells, loaded with HET, were incubated with various concentrations of histatin 5 (A) or dhvar4 (B). The development of fluorescence was monitored at 5 min intervals for 1 h at 30 °C at λ_{ex} = 485 nm and λ_{em} = 620 nm. Whereas the fluorescence increase induced by histatin 5 remained stable, dhvar4-induced fluorescence decayed over time, particularly at higher peptide concentrations. Curves are representative of three independent experiments, carried out in duplicate.

concentration-dependent increase in the HET-derived fluorescence signal was found (results not shown). This indicates that HET-derived fluorescence is enhanced in killed yeast cells, independent of the killing agent.

HET-derived fluorescence in *C. albicans*

The histatin 5-dependent rise in HET-derived fluorescence (Figures 2 and 3) has been interpreted as evidence for ROS formation, caused by blocking of the mitochondrial respiratory chain by histatin 5 [15]. However, HET is oxygen-sensitive, and is readily oxidized by viable cells to ethidium [26]. Once formed inside the cell, the cationic ethidium will accumulate in mitochondria in response to the mitochondrial membrane potential [25,26]. Since both histatin 5 and dhvar4 dissipate this potential [9], we examined the possibility that the fluorescence enhancement by peptides is a secondary effect caused by release of ethidium from its mitochondrial stores, and its subsequent binding to nucleic acids. We therefore studied the effects of histatin 5 on HET-preloaded *C. albicans* cells by fluorescence microscopy (Figure 4). In the absence of histatin 5, a weak intracellular red fluorescent labelling was observed, mainly at the perimeter of the yeast cell. This confirmed that, during the preincubation step, internalized HET became oxidized to ethidium and accumulated in cellular stores, presumably mitochondria.

After approx. 15 min of incubation with histatin 5, the fluorescence became brighter and spread over the whole interior of the cell, except for a central spot, probably the vacuole.

Figure 4 Fluorescence microscopy of *C. albicans* cells loaded with HET, upon exposure to histatin 5

C. albicans cells in the exponential growth phase were loaded with HET and suspended in PPB to a D_{620} of approx. 2.0. Histatin 5 was added at a concentration of 20 µg/ml and the suspension was examined by fluorescence microscopy. Top panel: fluorescent image of cells preloaded with 20 µM HET, in the absence of histatin 5 (His5). Weak fluorescence is noted mostly at the perimeter of the cell. Middle panel: fluorescent image of the cells 5 min after addition of 20 µg/ml histatin 5. The fluorescence has a granular appearance, and is located mostly at the perimeter of the cytoplasm. The central vacuole is negative. Bottom panel: fluorescent image of cells 15 min after addition of histatin 5. The fluorescence intensity is increased, and the whole cell, except for the vacuole, is brightly fluorescent.

This suggests that most of the increase in histatin 5-induced fluorescence in the HET experiment is caused by a redistribution of pre-existing ethidium over the whole cell, rather than by the generation of ROS in mitochondria.

Histatin 5 and dhvar4 induce the release of ATP

Both histatin 5 and dhvar4 translocate over the cellular membrane of *C. albicans* [9,17]. To investigate whether this compromises the barrier function of the membrane, we analysed the supernatant after incubation of *C. albicans* with either peptide. Within 5 min of the start of the incubation, substantial amounts of essential nucleotides were detected in the incubation buffer, and after 15 min the process was virtually complete. Figure 5 illustrates the effects of these peptides on the release of ATP. However, simultaneously leakage of other nucleotides, including NAD⁺, AMP and ADP, also occurred (A. L. den Hertog, unpublished work). Strikingly, the leakage induced by either peptide was comparable with that induced by the antiseptic agent chlorhexidine, which adversely affects cytoplasmic membranes by binding specifically to negatively charged components, such

Figure 5 Time-dependent ATP release from *C. albicans* induced by histatin 5 and dhvar4

Exponential-growth-phase *C. albicans* cells were suspended to a density of 10^7 cells/ml of PPB, and incubated with 25 μ M histatin 5 or dhvar4. At the indicated times, an aliquot was taken from the supernatant, briefly centrifuged (15 000 *g*, 2 min) to remove cells and analysed by capillary zone electrophoresis. Curves are representative of two independent experiments. The supernatants of cells that had been incubated in the absence of any agent were completely negative.

as phospholipids (results not shown). This indicates that both histatin 5 and dhvar4 induced the leakage of vital compounds from the yeast cell, probably concomitant with their transmigration over the cell membrane.

DISCUSSION

The fact that a wide variety of antimicrobial peptides require actively respiring cells has sparked interest in a possible role for free radicals, especially ROS, in the initiation of cell death by antimicrobial peptides [10,15,19]. A major source of ROS in cells is the mitochondrial respiratory chain, where transient quinone radicals are intermediates in the chemiosmotic Q-cycle. When electron transfer is blocked, the half-life of the formed semiquinone radical increases, enhancing non-enzymic transfer to molecular oxygen with the generation of O_2^- . Accordingly, electron transport inhibitors (e.g. antimycin and sodium azide) stimulate ROS formation, while uncouplers, which enhance electron flow through the respiratory chain, abolish ROS formation [22,25,26]. The present study was initiated in order to compare the ROS-inducing properties of two antimicrobial peptides: histatin 5, an inhibitor of mitochondrial respiration [15], and dhvar4, an uncoupler of respiration [20]. The results obtained, however, are inconsistent with a killing mechanism that involves a pivotal role for ROS.

First, TEMPO, a validated membrane-permeant ROS scavenger, did not inhibit killing by histatin 5 or dhvar4. Furthermore, in line with previous studies [9,16,17], we found no killing by inhibitors of electron transport, e.g. sodium azide and antimycin, which have been reported to induce ROS.

The conclusion that histatin 5 induces ROS in *C. albicans* has been based on the enhancement of HET-derived fluorescence by this peptide. We have reproduced this observation of fluorescence enhancement, but did not find any inhibitory effect of TEMPO, suggesting that the increase was not caused by ROS. In addition, when using TEMPO-AC as a ROS indicator, no ROS formation could be detected. Neither did we find enhancement of HET-derived fluorescence by antimycin or sodium azide. This is understandable, because incubations in peptide-mediated killing assays typically are carried out in 1 mM phosphate buffers that are devoid of substrates needed to fuel the electron transport chain. However, dhvar4 and chlorhexidine, a surface-active antiseptic,

enhanced the HET-derived fluorescence, indicating that HET-derived fluorescence is not linked exclusively to ROS formation. While HET may be an authentic detection system for ROS in cell-free systems, we conclude that, under the conditions used in the killing assays, it rather behaves as a vital stain, because it is partly oxidized to ethidium (Figure 4; [27]). As first reported by Rottenberg [28], ethidium distributes across the mitochondrial membrane in response to the membrane potential. Intracellular mitochondrial depolarization, such as that induced by histatin 5 [9,20], will result in rapid efflux from the matrix, allowing ethidium to bind to nuclear DNA with extensive enhancement of fluorescence. Thus the enhanced fluorescence seen when HET-loaded cells are treated with fungicidal agents is likely to reflect an enhanced fluorescence yield of preformed ethidium rather than an increase in ROS generation. In conclusion, there is no evidence supporting a role for ROS in the histatin 5-mediated killing of *C. albicans*.

The observation that some antimicrobial peptides are directed against the mitochondria has resulted in the hypothesis that their killing activity may involve the induction of apoptotic pathways. Indeed, it has been shown that, in mononuclear cells, the bovine cathelicidin BMAP-28 may act as an inducer of the mitochondrial permeability transition pore, causing release of cytochrome *c* from the mitochondria, accompanied by the initiation of a cell-death programme [29]. However, for histatin 5, no clear disintegration of intracellular organelles [30] or effects on macromolecules such as DNA or cytochrome *c* could be demonstrated [31]. On the other hand, the present study shows that treatment of *C. albicans* cells with dhvar4 or histatin 5 results in instantaneous leakage of vital compounds such as ATP and NADH, which directly shuts down the cellular energy supply. Apparently, these peptides compromise the barrier function of the membrane without forming permanent pores (Figure 5; [14,16]). This is most likely to happen during their passage over the membrane. This was corroborated by a model study, revealing that the passive diffusion of dhvar4 over a lipid bilayer was accompanied by the induction of defects that allowed the passage of molecules with sizes comparable with that of ATP [32].

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