

## ENERGY DEPLETION PROTECTS *CANDIDA ALBICANS* AGAINST ANTIMICROBIAL PEPTIDES BY RIGIDIFYING ITS CELL MEMBRANE

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Running Title: Azide Rigidifies *Candida Albicans* Membrane

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**Inhibitors of the energy metabolism, such as sodium azide and valinomycin, render yeast cells completely resistant against the killing action of a number of cationic antimicrobial peptides, including the salivary antimicrobial peptide Histatin 5. In this study the Histatin 5-mediated killing of the opportunistic yeast *Candida albicans* was used as a model system to comprehensively investigate the molecular basis underlying this phenomenon. Using confocal and electron microscopy it was demonstrated that the energy poison azide reversibly blocked the entry of Histatin 5 at the level of the yeast's cell wall. Azide treatment hardly induced depolarisation of the yeast cell's membrane potential, excluding it as a cause of the lowered sensitivity. In contrast, the diminished sensitivity to Histatin 5 of energy-depleted *C. albicans* was restored by increasing the fluidity of the membrane using the membrane fluidizer benzyl alcohol. Furthermore, rigidification of the membrane by incubation at low temperature, or in the presence of the membrane rigidifier DMSO, increased the resistance against Histatin 5, while not affecting the cell's energy charge. In line, azide induced alterations in the physical state of the interior of the lipid bilayer. These data demonstrate that changes in the physical state of the membrane underly the increased resistance to antimicrobial peptides.**

In the last few decades an expanding number of antimicrobial peptides have been isolated from virtually all classes of organisms, where they play an important role in the innate defense against microbial and viral infections. Characterisation of these peptides has revealed a wide diversity in amino acid sequences, yet they share characteristic features: they are usually polycationic and amphipathic, containing both a hydrophilic and a hydrophobic side. This promotes their insertion into, and transmigration over the cytoplasmic membrane of the target cell, with as final consequence killing of the cell. Interestingly, cellular sensitivity to cationic proteins and

peptides such as salivary histatins and defensins, is diminished by conditions that affect the energy status of the target cell (1-6). This effect is not restricted to cationic peptides, since azoles are also sensitive to the energy status of *Candida glabrata* (7). In *Chlorella* metabolic inhibition abolishes the membrane-disruptive effects of the polyene nystatin and even of the detergent Triton X-100 (8). As explanation for the desensitizing effects of energy depletion it has been proposed that interaction of cationic peptides with the target cell would involve active transport systems, which for their activity are dependent on the cell's energy charge (1, 9-12). However, direct experimental proof corroborating this hypothesis is still lacking. The present study addresses the question how on a molecular level the energy metabolism is linked with the *Candida* cell's sensitivity to antimicrobial peptides. Thus far, many different types of mechanisms have been identified which contribute to an acquired drug-resistant phenotype in yeast cells, including the over-expression of energy-driven efflux pumps, mutations in the target enzyme, or alterations in biosynthetic pathways (13). The resistance induced by energy depletion seems fundamentally different from these ones, since it is a direct response to a physiological stress condition, which protects the yeast against a range of natural and pharmacological antifungal agents. Thus, elucidation of this molecular resistance mechanism not only will deepen our insight in the working mechanism of antimicrobial peptides but will aid in the development of (non)peptide therapeutics that are suited for fighting infections under energy-restricted conditions, such as in biofilms. The Histatin 5 (Hst5) mediated killing of *C. albicans* is used in the present study as a model system to comprehensively investigate the different aspects of the peptide-target cell interaction, including the role of the cell wall, the membrane potential, and the physical state of the membrane, in relation to the cell's energy

charge. We found that energy depletion induced a decrease in membrane fluidity, which was reversed by the membrane fluidizer benzyl alcohol. In line, the increased resistance of energy-depleted *C. albicans* against Hst5 was reversed by the membrane fluidizer benzyl alcohol, without restoration of the cell's energy charge. On the other hand, inducing increased membrane rigidity by lowering the temperature, or by treatment with a membrane-rigidifying agent, led to an increased resistance against Hst5. It is hypothesised that the actin cytoskeleton, which is highly sensitive to the cell's energy charge mediates the effect, since the cytoskeleton inhibitor jasplakinolide induced also resistance to Hst5.

## EXPERIMENTAL PROCEDURES

**Preparation and FITC labelling of the peptides** - Peptides (Table 1) were manufactured by solid phase peptide synthesis using Fmoc-chemistry with a MilliGen 9050 peptide synthesizer (Milligen-Bioscience, Bedford, MA) according to the manufacturer's procedures. N- $\alpha$ -Fmoc protected amino acids and preloaded PEG-PS supports were obtained from Applied Biosystems (Foster City, CA). Peptides were purified by RP-HPLC (Jasco Corporation, Tokyo, Japan) to a purity of at least 90%, and the authenticity of the peptides was confirmed by ion trap mass spectrometry with a LCQ Deca XP (Thermo Finnigan, San Jose, CA). FITC labelling of Hst5 and Dhvar5 was performed as described previously (3). The labeled peptides were designated F-Hst5 and F-Dhvar5, respectively. Both in the viability assay and in the propidium iodide assay, fluorescein-labeled peptides exhibited comparable activities against *C. albicans* as the parent peptides.

**Growth conditions** - *C. albicans* (ATTC 10231), cultured aerobically at 30 °C on Sabouraud dextrose agar plates (SDA, Oxoid, Hampshire, UK) was suspended in 25 ml of Sabouraud dextrose broth in a 100 ml erlenmeyer flask. After 20 h of incubation at 30 °C, 1 ml from this suspension was subcultured for 1-2 h in 20 ml of Sabouraud dextrose broth, to obtain a mid-log phase culture. Cells were washed twice in 1 mM potassium phosphate (PPB), and resuspended to a cell density of 2 McFarland (approximately  $10^7$  cells/ml).

**Spheroplast preparation** - One gram (wet weight) of *C. albicans* of a mid-log phase culture, was suspended in 100 mM Tris buffer (pH 7.4), supplemented with 100 mM EDTA (TE buffer) and incubated for 45 min with  $\beta$ -mercaptoethanol. Cells were washed and suspended in 4 ml TE buffer, supplemented with 1 M sorbitol, and incubated with 100  $\mu$ l zymolase (50 units). After treatment, more than 90 % of the cells were converted to spheroplasts, as determined by counting cells after lysis in distilled water.

Spheroplasts were resuspended in PPB, supplemented with 0.5 M sorbitol as osmoprotectant.

**Determination of the membrane-disruptive activity of peptides (PI assay)** - Membrane-disruptive activity of peptides was determined by monitoring the fluorescence enhancement of propidium iodide (Invitrogen, Breda, The Netherlands) in peptide-treated cells, as described previously (14). The membrane impermeant propidium iodide (PI) only enters membrane-compromised cells, after which the fluorescence of this probe is enhanced by 20–30-fold due to its binding to nucleic acids. Cell suspensions were mixed with the indicated agents and incubated at 30 °C for 30 min (unless indicated otherwise) with gentle shaking. NaN<sub>3</sub> was dissolved in PPB, CCCP (Sigma-Aldrich, The Netherlands) was dissolved in methanol, rapamycin (Sigma-Aldrich), valinomycin (Sigma-Aldrich), latrunculin B (MP Biomedicals, The Netherlands) and jasplakinolide (Invitrogen) were dissolved in DMSO and diluted prior to use. The final concentrations of methanol and DMSO did not exceed 0.1%. Controls were incubated with PPB, supplemented with appropriate volumes of the corresponding vehicle (DMSO or methanol) alone. Cell suspensions were supplemented with PI (final concentration 10  $\mu$ M) and subsequently added to two-fold serial dilutions of peptides. PI fluorescence was measured at 5 min intervals for 1 h, at excitation and emission wavelengths of 544 and 620 nm, respectively, in a Fluostar Galaxy microplate fluorimeter (BMFG Labtechnologies, Offenburg, Germany). Afterwards, the numbers of surviving cells were determined by plating aliquots on SDA plates and counting colony forming units (CFUs), as described below.

**Candidacidal activity of peptides** - The effects of the various agents and treatments on the viability of *C. albicans* were determined in a microdilution viability assay, essentially as described previously (3). In brief, 50  $\mu$ l aliquots from a mid-log phase culture of *C. albicans* were incubated with equal volumes of peptide solutions (0.3 to 100  $\mu$ M) and incubated at 30 °C for 60 min. The incubation mixtures were appropriately diluted (200- to 500-fold) in PBS and 25  $\mu$ l aliquots were plated on SDA. After 48 h incubation at 30 °C the numbers of CFUs were counted.

**DiSC<sub>3</sub>(5) fluorescence to monitor membrane potential** - Induction of release of the fluorescent probe DiSC<sub>3</sub>(5) (Invitrogen) was monitored to study the effect of azide on the membrane potential of *C. albicans* (15). *C. albicans* (approximately  $10^7$  cells/ml)

in PPB were incubated with a final concentration of 1.6  $\mu\text{M}$  DiSC<sub>3</sub>(5) at 30 °C until a constant fluorescence level was achieved (after approx. 10 min). Next either 5 mM NaN<sub>3</sub> or 5 mM NaCl (control experiment) was added, followed by excess of Hst5. An excess of Dhvar4 (8 times its LC<sub>50</sub>-value) was added to achieve complete dissipation of the cytoplasmic membrane potential. Changes in peptide mediated fluorescence intensity were monitored with a Perkin-Elmer LS 50 B spectrofluorimeter (Perkin-Elmer Ltd., Buckinghamshire, UK).

**FACS analysis** - FACS-analysis was performed essentially as described previously (3). *C. albicans* (approximately 10<sup>7</sup> cells/ml) were pre-incubated in PPB+5 mM NaN<sub>3</sub> at 30 °C for 15 min. Subsequently, equipotent concentrations of FITC-labeled peptides were added (65.5  $\mu\text{M}$  F-Hst5 or 17  $\mu\text{M}$  F-Dhvar5) and incubation was continued for another 15 min. Cells were washed twice with PPB+NaN<sub>3</sub>, suspended to a density of approximately 10<sup>7</sup> cells/ml and diluted 10-fold in the same buffer before use. Cell-associated fluorescence was measured with a FACS apparatus (Beckton Dickinson, Franklin Lakes, NJ) using a 15 mW Argon laser at 488 nm for excitation and a 530 nm filter for detection of emitted light. In the control experiment, cells were treated the same way, except that NaN<sub>3</sub> was replaced by NaCl.

**Confocal microscopy** - *C. albicans* (approximately 10<sup>7</sup> cells/ml) were pre-incubated with PPB+NaN<sub>3</sub> at 37 °C for 15 min. FITC-labeled peptides were added (final F-Hst5 concentration of 65  $\mu\text{M}$ , final F-Dhvar5 concentration 17  $\mu\text{M}$ ) and incubation was continued for another 15 min. To remove unbound peptide, cells were washed with PPB+NaN<sub>3</sub>, centrifuged (5 min at 10,000 g) and resuspended in the same buffer. The resulting suspensions were divided into two equal volumes, which were centrifuged (5 min at 10,000 g) and of which one pellet was resuspended in PPB+NaN<sub>3</sub>, the other in PPB+NaCl. Cells treated with Hst5 or Dhvar5 in PPB+NaCl served as positive controls. Cells were examined with a Leica TCS NT confocal system (Leica Microsystems, Heidelberg, Germany) equipped with an Ar/Kr laser and a 100x NA 1.4 object lens.

**Electron microscopy** - Ultrastructural localisation was examined using immunogold-labelling and transmission electron microscopy as described previously (6). *C. albicans* (approximately 10<sup>7</sup> cells/ml) were pre-incubated with either PPB+NaN<sub>3</sub> or PPB+NaCl and incubated with 65.5  $\mu\text{M}$  F-Hst5, or 17  $\mu\text{M}$  F-Dhvar5 at 37 °C for 15 min. Fixation, preparation of cryo-sections and incubation with gold-labeled mouse anti-FITC antibodies (Aurion, Wageningen, The Netherlands) were performed as described previously (6). Cells were examined using a Philips EM-420

transmission electron microscope (Philips, Eindhoven, The Netherlands).

**Determination of the adenine nucleotide content of *C. albicans*** - Cellular content of adenine nucleotides was determined as previously described (14). In short, *C. albicans* (approximately 10<sup>7</sup> cells/ml) were suspended in PPB, supplemented with 5  $\mu\text{g/ml}$  guanosine bromide as internal standard. After treatment with various agents or incubation under various conditions, 400  $\mu\text{l}$  of the cell suspension (in duplicate) was transferred to 80% boiling ethanol, buffered with 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.8 (16) to lyse the cells, and boiled for 3 min. After freeze-drying, the resulting pellet was resuspended in 100  $\mu\text{l}$  distilled water, and centrifuged (10 min at 10,000 g). Aliquots of the supernatants were analyzed by capillary zone electrophoresis with a BioFocus 2000 capillary electrophoresis system (Bio-Rad, Hercules, CA), equipped with an uncoated fused-silica capillary (internal diameter, 50  $\mu\text{m}$ ; length, 50 cm).

Kinetics of azide-induced effects on the adenine nucleotide composition were determined in the same way using NaN<sub>3</sub> (final concentration 5 mM) in the incubation buffer. At different time intervals 400  $\mu\text{l}$  aliquots were taken and transferred into buffered boiling ethanol and processed as described above. Control incubations were carried out in the same way, except that NaCl was added instead of NaN<sub>3</sub>.

To monitor the reversibility of the effect of azide on the intracellular adenine nucleotide composition, cells were incubated with 5 mM NaN<sub>3</sub> for 30 min. Then 400  $\mu\text{l}$  aliquots (in duplicate) were taken and processed as described above. Subsequently, NaN<sub>3</sub> was removed by washing twice in 9 ml PPB. From the resulting suspension 400  $\mu\text{l}$  aliquots (in duplicate) were taken at different time intervals and processed as described above for measurement of the adenine nucleotide content in comparison with the initial content.

**Fluorescence anisotropy of DPH** - The effect of energy poisons on the physical state of the yeast plasma membrane was measured in whole cells using the membrane fluidity probe DPH as described previously (17, 18). In short, a mid-log phase culture of *C. albicans* was washed twice and then suspended in PPB to a density of 8 mg/ml wet weight. The cells were incubated at 20 °C for 5 min and the DPH was added to a final concentration of 2  $\mu\text{M}$ . The same volume of the solvent (DMSO) was added to the control cells. Incubation was continued for 20 min at 20 °C, after which the cells were washed twice with

PPB. Subsequently the DPH-treated cells were suspended in the same volume of PPB supplemented with 5 mM NaN<sub>3</sub>, 5 mM NaN<sub>3</sub>+50 mM benzyl alcohol, PPB+50 mM benzyl alcohol and PPB + 5% (v/v) DMSO respectively. Immediately thereafter, fluorescence emission anisotropy measurements were performed in a 1 cm quartz cuvette on a Fluoromax-3 fluorimeter (Horiba Jobin Yvon, Longjumeau, France) in the right angle conformation. The optical densities of the samples were kept low to avoid inner filter effects. The samples were stirred continuously throughout the measurements. Upon 365 nm excitation, emission wavelengths were scanned from 422 to 432 nm using emission and excitation slits of 4.5 nm. In the 422-432 nm wavelength range, the value for fluorescence anisotropy of DPH is constant (19). The temperature of the samples was maintained at 31°C or 4°C using a thermostated water bath. All four combinations of vertically and horizontally polarized excitation and emission light were measured and the fluorescence anisotropy (*r*) was automatically calculated. Anisotropy values of three repeated measurements of various conditions were statistically analysed by one-way ANOVA, followed by post-hoc multicomparison with Tukey test. Data were analysed with SPSS.

## RESULTS

*Effect of energy metabolism on candidacidal activities of antimicrobial peptides* - In previous studies we found that Hst5 accumulates in the mitochondria of *C. albicans* (3, 20). Since treatment with mitochondrial poisons such as sodium azide renders *C. albicans* cells insensitive to Hst5 (3, 20), it was tempting to speculate about a causative relationship between the cellular target of the peptide and the desensitizing effects of azide. Therefore, we addressed the issue if the effects of azide and other energy poisons were specific for Hst5, and tested a number of structurally different peptides for their sensitivity to azide: (i) Hst5 and Hst5-derived peptides, including dh5 (residues 11-24), Dhvar4 (a multi-substituted variant of dh5), P-113 (encompassing residues 4-15 of Hst5) and its D-enantiomer P-113D; (ii) the bovine lactoferrin-derived peptides (21) LFcin 17-30 and LFampin 265-284; (iii) drosocin and a drosocin variant (22), and (iv) the artificial peptide Dhvar5. Peptides were tested in a viability assay as well as in a microtiterplate assay using PI to monitor their membranolytic effects. The membrane impermeant propidium iodide (PI) only enters membrane-compromised cells, after which the fluorescence of this probe is enhanced by 20–30-fold due to its binding to nucleic acids. The candidacidal activities (Table 1) and the membranolytic activities of all peptides tested were inhibited in the presence of azide. Differences in sensitivity to azide, however, were

noted: Hst5, dh5, both P-113 and its enantiomer P-113D, LFcin 17-30 and the drosocin peptides were completely inactive in the presence of azide. On the other hand, Dhvar5 and LFampin 265-284 were intermediately active, whereas the activity of Dhvar4 was marginally diminished (Table 1). These results were mirrored in the membranolytic assays with PI (not shown). This was in line with previous results which showed that peptide-mediated PI-uptake is accompanied by leakage of vital cell constituents, including ATP and other adenine nucleotides, resulting in cell death (14).

We decided to use the extremely azide-sensitive Hst5 as a model peptide to explore the molecular basis underlying the apparent correlation between the energy metabolism of the yeast cell and its sensitivity for antimicrobial peptides. In parallel, the effect on the intracellular adenine nucleotide content was systematically monitored. Addition of azide virtually instantaneously inhibited Hst5-mediated influx of PI (Fig 1A), an concomitantly depleted the energy charge of the cell (Fig 2A). These effects were readily reversed after washing out of the energy poison (Fig 1B and 2B). This illustrated that the cell's sensitivity to Hst5 was closely linked with its energy charge. Other energy poisons such as the potassium ionophore valinomycin and the protonophore CCCP likewise depleted the cell's energy charge and blocked the Hst5-mediated PI-influx (Figs. 1C and 2C). The inhibitory effects of these energy poisons were also confirmed in viability assays (not shown)

*Azide reversibly inhibits the internalisation of Hst5 in C. albicans* - Several stages can be distinguished in the candidacidal process. It has been proposed that in the first stage, Hst5 associates transiently with a putative receptor at the cell wall (23). This is followed by transmigration over the membrane, and accumulation in the cell. To identify the step in the killing process that is sensitive to the energy charge of the yeast cell, we examined the effect of azide on the association between F-Hst5 and *C. albicans* by FACScan analysis (Fig 3). Introduction of the fluorescein group marginally influences the candidacidal and membrane-disrupting activity of the peptides (Table 1). To compensate for ionic strength effects due to the presence of sodium and azide ions, the incubations without sodium azide were carried out in PPB supplemented with an equimolar concentration of sodium chloride. After incubation with F-Hst5 in the absence of azide the cell-associated fluorescence

increased to a value which was thousand times higher than that of control cells, which had been incubated with either a fluorescein-tagged irrelevant peptide or without any peptide. Cells treated with F-Hst5 in PPB+NaN<sub>3</sub> exhibited a 100-fold lower fluorescence, but this was still approximately 10-fold higher than that of the negative control. This fluorescence remained after repeated washing of the cells with PPB+NaN<sub>3</sub>. For comparison we examined the effect of energy depletion on the association between Dhvar5, which was moderately sensitive to azide, and *C. albicans*. In this case the F-Dhvar5 fluorescence was decreased in about 50% of cells, while the rest of the cells remained intensely labeled (Fig 3, lower frame).

From Figure 3 no distinction can be made between surface-bound and internalised peptides. To obtain information on the location of the peptides, fluorescence microscopy was conducted on cells treated with F-Hst5 in the absence and presence of azide (Fig 4). In the absence of azide F-Hst5 accumulated inside the cells, producing a granular labelling pattern, while the cell boundaries were negative (Fig 4A). Upon depletion of the ATP-charge by azide, in virtually all cells the cytoplasmic labelling was absent. In 5% - 10% of the cells a faint peripheral labelling could be observed (Fig 4B). After subsequent removal of azide, which restored the energy charge of the cells, the intracellular labelling pattern returned (Fig 4C). This suggests that the peripheral labelling in the presence of azide represented a transient stage that under normal conditions is not detected.

*The cell wall is not involved in the desensitizing effects of energy depletion.*

To identify the site at which F-Hst5 accumulated in azide-treated *C. albicans* cells, we first attempted to study its localisation by immunogold-electron microscopy. In the presence of sodium azide, however, more than 90% of the cells were unlabeled, and in the remaining cells only few gold-particles were found with a seemingly preferred labelling of the cell wall (not shown). This scarce labelling, however, did not allow us to draw firm conclusions about the ultrastructural localisation of F-Hst5. Therefore we examined the association between *C. albicans* and the more potent Dhvar5, which is moderately sensitive to azide (Table 1). Confocal fluorescence microscopy revealed that in the absence of azide F-Dhvar5 labeled the interior of the cells (Fig 5A). Some cells exhibited a granular staining pattern plus some surface labelling, while in other cells the label was distributed more uniformly and intensely throughout the interior. In the presence of azide, a shift in localisation of F-Dhvar5 occurred, with now approximately 70% of the cells exhibiting also labelling at their boundaries (Fig 5B). These cells were faintly intracellularly labelled with strong

fluorescent spots observed in about 50% of the cells. The remaining 30% of the cells showed cytoplasmic labelling, similar to that observed in the absence of azide. Immunogold-electron microscopy of F-Dhvar5-treated *C. albicans* (Fig 5C) confirmed the predominant intracellular labelling patterns found by confocal microscopy. In addition, gold particles were occasionally found at the cell wall, but not in the plasma membrane. In the presence of sodium azide, however, the number of gold particles associated with the cell wall was substantially increased (Fig 5D). Taken together these experiments revealed that in the presence of azide the interaction of peptides with the cell was blocked at the cell wall. This was reminiscent to a previous report that in *Saccharomyces cerevisiae* stress proteins, expressed at the cell wall, conveyed resistance to osmotin, an antimicrobial peptide from the tobacco plant (24). This prompted us to examine if removal of the cell wall would abolish the effect of azide. This, however, appeared not to be the case: spheroplasts were as sensitive to azide as whole cells, both in the PI assay (Fig 6), and in the viability assay (not shown), excluding a role of the cell wall in conferring resistance to Hst5.

*The plasma membrane potential does not play a role in the azide-induced decreased sensitivity of C. albicans to Hst5* - It has been reported that an inside negative membrane potential can enhance the activity of membrane-directed cationic antimicrobial peptides against living bacteria cells and against model membrane vesicles (25, 26). To establish whether reduction of the membrane potential plays a role in the azide-induced decreased sensitivity, we examined the effect of energy depletion on the membrane potential of *C. albicans*, using the potential-dependent distributional fluorescent dye DiSC<sub>3</sub>(5) (Fig 7). Upon addition of DiSC<sub>3</sub>(5) to a *C. albicans* suspension, a fast decrease in DiSC<sub>3</sub>(5) fluorescence occurred, reflecting the potential-driven uptake of the probe by the yeast cells, accompanied by self-quenching of the fluorescence. Subsequent addition of Hst5 led to a steady rise in fluorescence, highlighting the dissipation of the membrane potential and concomitant leaking of the probe (Fig 7A). Addition of azide to *C. albicans* cells preloaded with DiSC<sub>3</sub>(5) led to a small increase in DiSC<sub>3</sub>(5) fluorescence (Fig 7B), likely due to the dissipation of only the mitochondrial membrane potential (3). This is in line with a previous study which revealed that azide has little if any effect on the membrane potential of yeast cells (28). Subsequent

addition of excess Hst5 to these azide-pretreated *Candida* cells did not result in further dissipation of the membrane potential (Fig 7B). In contrast, the strongly membrane-disruptive peptide Dhvar4 (6, 27) induced a rapid dissipation of the membrane potential, both in the control cells and in the azide-pretreated cells (Figs 7C and D). These findings were in line with the results of Table 1, which showed similar differences between Hst5 and Dhvar4 with regard to their fungicidal activity to azide-treated *C. albicans*. Altogether, these experiments, demonstrated that in the presence of azide the membrane potential was maintained for the larger part.

*Energy poisons affect the fluidity of the membrane probe DPH* - Because of the intrinsic membrane-active features of cationic antimicrobial peptides, we next focused on the role of the membrane of the yeast cell and investigated if energy depletion affected the physical properties, in particular the fluidity, of the phospholipid bilayer. To address this point, we treated *C. albicans* cells with the fluorescent membrane probe DPH, which is used to monitor the physical environment in different regions of the lipid bilayer. DPH is a rod-like molecule and partitions into the interior (hydrophobic core) of the bilayer. This partitioning is accompanied by an increase in fluorescence, since the quantum yield of DPH in the membrane is much higher than that in water (29). It was observed that upon addition of azide or valinomycin to DPH-pretreated *C. albicans*, an immediate rise in DPH fluorescence occurred (not shown), indicating a change in the direct molecular environment of DPH. However, a number of conditions, including the fluidity, the water content and the dielectric constant in the environment of the probe, influence the fluorescence properties of DPH, and this hampers an unambiguous interpretation of fluorescence intensity data. We therefore determined the effect of azide treatment on the steady state anisotropy of DPH. This parameter is inversely related to the membrane fluidity (30). For comparison, we tested the effect of agents known to influence the membrane fluidity, including the membrane-fluidizer benzyl alcohol (31) and the membrane-rigidifier DMSO (Fig 8). It was found that azide treatment induced an increase in the DPH anisotropy. Treatment with DMSO induced an increase in the DPH anisotropy, in line with the anticipated rigidifying effect on the membrane. On the other hand benzyl alcohol caused a decrease in DPH anisotropy, reflecting an increase in membrane fluidity. Furthermore, benzyl alcohol reversed the effect of azide on the DPH anisotropy (Fig 8). Hst5-treatment had no effect on the anisotropy value of DPH (not shown).

*The membrane fluidizer benzyl alcohol abolishes the azide-induced resistance to Hst5* - We hypothesized that the membrane-rigidifying effect of azide was the underlying cause of the resistance against Hst 5, and therefore tested if the fluidizer benzyl alcohol might reverse the protective effect of azide (Fig 9). These experiments revealed that 75 mM benzyl alcohol completely restored the sensitivity of azide-treated *C. albicans* cells to Hst5, both in the PI-assay (Fig 9A), and in the viability assay (not shown). Furthermore, benzyl alcohol restored the cytoplasmic localization of Hst5 in azide-treated *C. albicans* cells (not shown). Benzyl alcohol did not restore the energy charge of the yeast cell ruling this out as an explanation for the increased sensitivity (Fig 9D). The inhibitory effects of high ionic strength, however, could not be abolished by benzyl alcohol, even at a 100 mM concentration (not shown). This indicates that the effect of azide occurs downstream from the initial, ionic-strength sensitive, interaction between the positively charged Hst5 and the negatively charged surface of the yeast cell. At low temperature (4 °C) the activity of Hst5 was substantially diminished (Fig 9B), while the energy content of the cells remained unchanged. This inhibition was reversed by 25 mM benzyl alcohol, underlining that the decreased fluidity of the membrane was responsible for the increased resistance against Hst5 at low temperature (Fig 9B). The membrane rigidifying agent DMSO increased the resistance to Hst5 at 30 °C, thus mimicking the effect of low temperature. In PPB supplemented with 10% (v/v) DMSO the Hst5 induced PI-influx was inhibited for approximately 70% (Fig 9C). Also in the viability assay was confirmed that DMSO and low temperature protected against the fungicidal activity of Hst5 (not shown). Despite the relatively high DMSO concentration, in the absence of peptides no enhancement in PI-fluorescence occurred (Fig 9C), nor a decrease in the energy charge (Fig 9D) indicating that the integrity of the yeast's plasma membrane was maintained. Culturing confirmed that treatment for 60 minutes with 10% DMSO had no adverse effects on the viability of the yeast cells (not shown) in line with other reports (*e.g.* 32).

*The cytoskeleton inhibitor jasplakinolide inhibits the membranolytic action of Hst5* - The previous experiments revealed that low energy conditions induced changes in the cell membrane, rendering it less sensitive to membrane-active peptides such as Hst 5. The molecular events, however, linking the cell's

energy charge with the membrane fluidity remain unclear. In a first attempt to identify the structures involved we focused on major energy consuming systems in the cell. Because the very short timescale of the effects (<1 min, Fig 1), we deemed the involvement of transcriptional or translational processes unlikely, and focused on major energy-consuming systems in the cell. One major energy drain in eukaryotic cells is the maintenance of the actin cytoskeleton, which in nerve cells and platelets is responsible for about 50% of the energy consumption (33). The turnover of the cytoskeleton is a continuously ongoing, energy-demanding process, which renders it directly sensitive to changes in the energy charge of the cell. Moreover, the cytoskeleton is connected at multiple points with the cell membrane, making direct communication with the membrane physically possible. We investigated therefore the effect of two membrane-permeating marine natural products, latrunculin B and jasplakinolide, which influence the turnover of the actin cytoskeleton (Fig 10). Latrunculin B sequesters actin monomers as they are released from filaments and prevents their reassembly, inducing depolymerisation of the actin cytoskeleton. In contrast, jasplakinolide, by inhibiting the ATP-driven release of actin monomers, induces actin polymerisation (34), thus mimicking energy depletion (35). At 100  $\mu\text{M}$  concentration, Latrunculin B had no effect on the Hst5-*C. albicans* interaction, neither in the PI-assay (Fig 10A) nor in the viability assay (not shown). On the other hand, jasplakinolide inhibited in a dose-dependent way the Hst5-mediated influx of propidium iodide (Fig 10B). At 10  $\mu\text{M}$  jasplakinolide, a 50% reduction in PI influx, which is a measure for membrane disintegration, was observed. By measuring the adenine nucleotide content of the *C. albicans* cells it was verified that jasplakinolide did not decrease the ATP content of the cell (not shown), excluding that the observed inhibition was indirectly caused by energy depletion.

## DISCUSSION

Lowering the energy content of *C. albicans* affects the activity of a wide variety of peptides (Table 1), indicating that this effect is not restricted to the Hst5-*C. albicans* interaction. Similarly, various processes taking place at the membrane of eukaryotic cells, including the entry of polyarginine and polylysine into fibroblasts (36, 37), the endocytosis of TAT peptides into HeLa cells (38) and influenza virus budding from the plasma membrane of infected kidney cells (39), are inhibited by energy depletion. On the other hand, the sensitivity of *E. coli* to Hst5 is not changed by azide (ECI. Veerman, unpublished), despite the fact that this bacterium, like *C. albicans*, does contain an

azide-sensitive respiratory chain. The common intuitive explanation of how energy poisons decrease the activity of these peptides is that peptide entry into the cell requires the presence of active transport systems or a membrane potential. This implies that the energy charge of the cell, directly or indirectly, drives translocation of peptides across the cell membrane with the concomitant toxic consequences. Support for the involvement of the membrane potential has come from model studies showing that the induction of a membrane potential led to enhanced peptide translocation into protein-free lipid vesicles (26, 40). However, in agreement with other studies (28), we found that blocking the mitochondrial respiration did not diminish the plasma membrane potential of *C. albicans* cells, while still the entry of Hst5 into the cell was abolished. In line, the potassium ionophore valinomycin and the protonophore CCCP, which neither affect the plasma membrane potential in yeast (41-43), almost completely abolished the sensitivity to Hst5. In this study we found that energy depletion affected the physical state of the plasma membrane, as reflected by an increased value of the DPH anisotropy. Since increasing the fluidity by benzyl alcohol reversed both the effect of azide on the Hst5 mediated membrane-disruption, and on the membrane-fluidity (Figs 8 and 9A), it is tempting to conclude that not energy depletion itself but rather its effect on the physical state of the membrane causes the protective effect of energy poisons. Rigidifying the membrane by DMSO or low temperature, likewise decreased the membranolytic activity of Hst5, albeit to a smaller extent. Membrane rigidifying effects of energy depletion have previously been reported by Haidekker *et al.* (44) using a laser-based scatter system, and Florine-Casteel *et al.* (45) using video fluorescence polarisation microscopy. The latter authors reported that the plasma membrane of cultured rat hepatocytes becomes uniformly rigid within a few minutes after the addition of metabolic inhibitors, when ATP levels have fallen by >95%. These authors speculated that increased phospholipase activity, leading to an altered cholesterol/phospholipid ratio, or lipid peroxidation might account for the increased lipid order. However, in our opinion the fast kinetics (<5 min, Fig 1) combined with the ready reversibility of these effects (Figs 2 and 4), make it less likely that an increase in activity of phospholipid-modifying enzymes would be the primary mechanism underlying the observed phenomena.

Although the molecular principles linking the energy status with the cell's membrane properties and its sensitivity to antimicrobial peptides, are not completely understood, a number of observations point to a role for the actin cytoskeleton. The cytoskeleton inhibitor jasplakinolide, which promotes actin polymerisation, concurrently renders *Candida* less sensitive to membrane disruption by Hst5, thus having the same overall effect as energy poisons, but without lowering the ATP levels. Maintenance of the cytoskeleton is, also in yeast (46), an ongoing process that requires a continuous and substantial supply of ATP. In human platelets and nerve cells this may require as much as 50% of the cell's energy sources (33). This metastable situation is inherently sensitive to energy depletion, as illustrated by the observation that decreased intracellular ATP levels are associated with an immediate increase in the fractions of polymerized actin observed *in vivo* and *in vitro* (34), an effect that partly can be mimicked by jasplakinolide. In line with the present findings, Sheikh *et al.* (47) found that jasplakinolide caused a marked increase in cell rigidity in neutrophils. Because of the abundant connections between the actin cytoskeleton and the plasma membrane, cytoskeletal rearrangements can induce direct changes in cell and membrane morphology (48-51). In *Dictyostelium* cells, for example, depletion of cellular ATP by azide causes a 'rigor' contraction of the cytoskeleton, which renders the cells stiffened and spherical in appearance (52). Such cytoskeletal rearrangements occur in minutes (53), a time scale on which energy poisons depleted the cell's energy charge (Fig 2A), induced resistance against Hst5 (Fig 1B) and affected the physical state of the membrane.

In the present study the molecular mechanism was investigated underlying the long-known phenomenon that energy depletion in yeast induces resistance against cationic peptides and proteins (1). At variance with the generally accepted explanation which implies a role for ATP-driven transport systems, we found that energy depletion induces membrane rigidification, possibly mediated by the cytoskeleton, which desensitises *C. albicans* to antimicrobial peptides. Because of the similar responses of yeast and mammalian cells towards energy depletion, the peptide-yeast model may provide a new model system to study the early cellular events triggered by ATP depletion, e.g. in hypoxia, or in ischemic attacks.



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## FIGURE LEGENDS

### Figure 1. The effect of energy poisons on Histatin 5 mediated influx of propidium iodide in *C. albicans*.

**A.** *C. albicans* was incubated with PPB+5 mM NaCl, and directly transferred to equal volumes of 20  $\mu$ M Hst5 in PPB+NaCl (■), or PPB+NaN<sub>3</sub> (◆), supplemented with PI. After mixing, the development of fluorescence was monitored at 100 s intervals at 30 °C at  $\lambda_{exc}$  485 nm and  $\lambda_{em}$  620 nm.

**B.** Suspensions of *C. albicans* (10<sup>7</sup> cells/ml PPB) were mixed with either NaN<sub>3</sub> or NaCl (final concentration 5 mM) at 30 °C for 30 min. To remove azide, cells were washed twice in PPB+NaCl. As control served cells that were washed in PPB+NaN<sub>3</sub>. Immediately after washing, cells were transferred to equal volumes of 20  $\mu$ M Hst5 in the indicated buffer, supplemented with PI. The development of fluorescence was monitored at 30 s intervals for 1 h at 30 °C at  $\lambda_{exc}$  485 nm and  $\lambda_{em}$  620 nm. ■: Cells preincubated in PPB+NaCl, then resuspended in PPB+NaCl. ◆: Cells preincubated in PPB+NaN<sub>3</sub>, then resuspended in PPB+NaCl. ▲: Cells preincubated in PPB+NaN<sub>3</sub>, then resuspended in PPB+NaN<sub>3</sub>. Curves are representative of two independent experiments, carried out in duplicate.

**C.** Suspensions of *C. albicans* (2 $\times$ 10<sup>7</sup> cells/ml) were incubated with PPB supplemented with 5 mM NaCl (◆), 5 mM NaN<sub>3</sub> (■), 10  $\mu$ M valinomycin (▲) or 2.5  $\mu$ M CCCP (●) for 30 min. PI was added and cells were transferred to wells containing equal volumes of serially diluted Hst5 in PPB, supplemented with the corresponding agent in the same concentration. Development of fluorescence was followed for each concentration of the peptides at 5 min intervals for 1 h at  $\lambda_{exc}$  485 nm and  $\lambda_{em}$  620 nm. The figure shows the fluorescence, expressed in arbitrary units (AU), after 1 h of incubation. Curves are representative of three independent experiments, carried out in duplicate.

### Figure 2. The effect of energy poisons on the energy charge of *C. albicans*.

**A.** *C. albicans* (2 $\times$ 10<sup>7</sup> cells/ml PPB) was mixed with 5 mM NaN<sub>3</sub>. At the indicated time points, aliquots were taken and transferred into boiling ethanol to lyse the cells. Adenine nucleotide content was determined by CZE. ◆: ATP; ▲: AMP.

In control cells treated with NaCl, no measurable decrease in ATP occurred during the time-course of the experiment (not shown).

**B.** 10 ml suspension of *C. albicans* ( $2 \times 10^7$  cells/ml) was incubated in PPB supplemented with 5 mM  $\text{NaN}_3$  at 30 °C. After 5 min a 400  $\mu\text{l}$  aliquot was taken and boiled in buffered ethanol. The remaining cells were washed two times in PPB to remove  $\text{NaN}_3$ , and resuspended in 9 ml PPB. At the indicated time points, 400  $\mu\text{l}$  aliquots were taken and processed for determination of the intracellular adenine nucleotide content. ◆: ATP; ▲: AMP. Curves are representative of two independent experiments, carried out in duplicate.

**C.** *C. albicans* was incubated in PPB supplemented with 5 mM NaCl, 10  $\mu\text{M}$  valinomycin, 2.5  $\mu\text{M}$  CCCP, and 5 mM  $\text{NaN}_3$ , respectively. After 30 min incubation, cells were lysed by transfer into boiling ethanol. Adenine nucleotide content was determined by CZE: ATP ■; ADP ▤; AMP □. No measurable amounts of adenine nucleotide were released in the supernatant of intact untreated cells in the time course of these experiments. Data are representative of at least two independent experiments, carried out in duplicate. Values represent the mean of duplicate measurements.

### Figure 3. The effect of energy depletion on the binding of Histatin 5 and Dhvar5 to *C. albicans*, analyzed with

**FACScan.** Suspensions of *C. albicans* ( $3.2 \times 10^6$  cells/ml) were pre-incubated with PPB (at 37 °C for 15 min) supplemented either with 5 mM NaCl or with  $\text{NaN}_3$ . Subsequently, F-Hst5 (A) or F-Dhvar5 (B) was added and incubation was continued for an additional 15 min.

**A.** In the presence of  $\text{NaN}_3$ , binding of F-Hst5 to *C. albicans* was approximately 100-fold lower than in the presence of NaCl. The fluorescence signal of cells incubated with F-Hst5 in the presence of  $\text{NaN}_3$  was still approximately ten-fold higher than that of cells which had been with an irrelevant fluoresceinated peptide, F-CystS, (control).

**B.** Binding of Dhvar5 to *C. albicans* was approximately 100-fold diminished in approximately 50 % of the cells. Also in this case the residual fluorescence was approximately 10-fold higher than that of the negative control.

### Figure 4. Confocal fluorescence microscopy of *C. albicans* after incubation with F-Histatin 5.

**Left row:** Suspensions of *C. albicans* ( $3.2 \times 10^6$  cells/ml) were pre-incubated at 37 °C for 15 min in PPB+NaCl or PPB+ $\text{NaN}_3$ . Then F-Hst5 was added and incubation was continued for 15 min.

**A.** *C. albicans* treated with F-Hst5 in PPB+NaCl.

**B.** *C. albicans* treated with F-Hst5 in PPB+ $\text{NaN}_3$ .

**C.** *C. albicans* treated with F-Hst5 in PPB+ $\text{NaN}_3$ , subsequently washed in PPB+ $\text{NaN}_3$  to remove unbound Hst5, and then washed and resuspended in PPB+NaCl to wash-out  $\text{NaN}_3$ . Controls incubated with F-CystS peptide were completely negative.

**Right row.** Adenine nucleotide content of *C. albicans* cells treated the same way, in the absence of Hst5.

**Figure 5. Localisation of F-dhvar5 in *C. albicans*** Suspensions of *C. albicans* ( $3.2 \times 10^6$  cells/ml) were pre-incubated at 37 °C for 15 min in PPB+NaCl or PPB+ $\text{NaN}_3$ . F-Dhvar5 (17  $\mu\text{M}$ ) was added and incubation was continued for 15 min.

**A.** *C. albicans* treated with F-Dhvar5 in PPB+NaCl.

**B.** *C. albicans* treated with F-Dhvar5 in PPB+ $\text{NaN}_3$ . Controls incubated with F-CystS peptide were completely negative.

**C.** Immuno-electronmicroscopy of cells treated with F-Dhvar5 in PPB+NaCl. Cells treated with F-Dhvar5 in PPB+NaCl exhibit predominantly an intracellular labelling pattern (arrows).

**D.** Immuno-electronmicroscopy of cells treated with F-Dhvar5 in PPB+ $\text{NaN}_3$ , showing labelling of the cell wall by Dhvar5. w: cell wall, m: cell membrane.

### Figure 6. Effect of azide on Histatin 5 mediated PI influx in *C. albicans* spheroplasts.

Suspensions of whole cells (c) or spheroplasts (s) of *C. albicans* ( $2 \times 10^7$  cells/ml) were incubated with PPB supplemented with 0.5 M sorbitol as osmoprotectant and NaCl or  $\text{NaN}_3$ . After 30 min PI was added and cells were transferred to wells containing equal volumes of serially diluted Hst5 in the corresponding buffer. ◆: spheroplasts in PPB+NaCl; ▲: whole

cells in PPB+NaCl X: spheroplasts in PPB+ $\text{NaN}_3$ ; ■: whole cells in PPB+ $\text{NaN}_3$ . Development of fluorescence was

followed for each concentration of the peptides at 5 min intervals for 1 h at  $\lambda_{\text{exc}}$  485 nm and  $\lambda_{\text{em}}$  620 nm. The figure shows the fluorescence, expressed in arbitrary units (AU), after 1h of incubation. Curves are representative of three independent experiments, carried out in duplicate.

**Figure 7. Effect of azide on the plasma membrane potential of *C. albicans*.** To a suspension of *C. albicans* ( $3.2 \times 10^6$  cells/ml) in PPB DiSC<sub>3</sub>(5) was added to a final concentration of 1.5  $\mu$ M.

**A.** After a constant fluorescence level had been achieved (about 10 min), excess Hst5 was added, resulting in a steady dissipation of the membrane potential.

**B.** Addition of 5 mM NaN<sub>3</sub> resulted in a small increase in fluorescence. Subsequent addition of excess Hst5 did not depolarise the membrane further.

**C.** Addition of excess Dhvar 4 resulted in a virtually complete depolarisation of the membrane in the control cells

**D.** Addition of Dhvar4 induced a virtual complete depolarisation of the membrane in the presence of azide.

**Figure 8. Change in DPH fluorescence anisotropy after treatment of *C. albicans* with azide, benzyl alcohol and DMSO.**

*C. albicans* were incubated with the membrane probe DPH for 20 min. Cells were washed in PPB to remove unbound probe, and suspended in PPB alone (control), and in PPB supplemented with 5 mM NaN<sub>3</sub> (azide); 5 mM NaN<sub>3</sub> +50 mM benzyl alcohol (azide+BA); 50 mM benzylalcohol (BA) and 5% DMSO (DMSO). Relative changes in anisotropy value relative to PPB alone are plotted. \*:  $p < 0.01$  (relative to control). Increase in anisotropy reflects a decrease in membrane fluidity

**Figure 9. Effect of membrane-modulating agents and -conditions on the sensitivity of *C. albicans* to Histatin 5.**

**A.** *C. albicans* cells were preincubated either in PPB+NaN<sub>3</sub> or in PPB+NaCl. After 30 min, benzyl alcohol was added. After 15 min two-fold serially diluted Hst5 was added and PI-influx was determined. Development of fluorescence was followed for each concentration of the peptide at 5 min intervals for 1 h at  $\lambda_{exc}$  485 nm and  $\lambda_{em}$  620 nm. The figure shows the fluorescence, expressed as arbitrary units, after 1 h. ●: PPB+NaN<sub>3</sub>; ◆: PPB+5 mM NaN<sub>3</sub>+100 mM benzyl alcohol; ▲: PPB+5 mM NaCl; ■: PPB+5 mM NaCl + 100 mM benzyl alcohol

**B.** *C. albicans* cells were incubated with serial dilutions of Hst5 in PPB supplemented with various concentrations of benzyl alcohol, at 4 °C (no azide). PI was added, and after 1 h the fluorescence was measured at  $\lambda_{exc}$  485 nm and  $\lambda_{em}$  620 nm. \*: PPB; ●: PPB+12.5 mM benzyl alcohol; ▲: PPB+ 25 mM benzyl alcohol; ◆: PPB+50 mM benzyl alcohol.

**C.** *C. albicans* cells were suspended in PPB supplemented with varying DMSO concentrations (no azide). PI was added and cells were transferred to wells containing equal volumes of serially diluted Hst5 dissolved in the corresponding buffers. The figure shows the fluorescence, expressed as arbitrary units, after 1h. ■: PPB; ▲: PPB+1% DMSO; ◆: PPB+2.5% DMSO; \* : PPB+5% DMSO; ●: PPB+10% DMSO. Curves are representative of two independent experiments, carried out in duplicate.

**D.** *C. albicans* was incubated in PPB at 4 °C and 30 °C for 30 min. In addition, *C. albicans* was incubated in PPB supplemented with 10% DMSO, 5 mM NaN<sub>3</sub>, and 5 mM NaN<sub>3</sub>+100 mM benzyl alcohol at 30 °C. After 30 min, cells were lysed by transfer into boiling ethanol. Adenine nucleotide content was determined by CZE. ■: ATP; ▨: ADP; □: AMP. No measurable amounts of adenine nucleotide were released in the supernatant of intact untreated cells in the time course of these experiments. Data are representative of at least two independent experiments, carried out in duplicate. Values represent the mean of duplicate measurements.

**Figure 10. Effect of the cytoskeleton inhibitors latrunculin B and jasplakinolide on the sensitivity of *C. albicans* for Histatin 5.**

**A.** *C. albicans* were preincubated for 30 min in PPB supplemented with 100  $\mu$ M concentrations of latrunculin B. PI was added and cells were transferred to wells containing equal volumes of serially diluted Hst5 in PPB, supplemented with latrunculin B in the corresponding concentration. Development of fluorescence was monitored for each concentration of the peptides at 5 min intervals for 1 h at  $\lambda_{exc}$  485 nm and  $\lambda_{em}$  620 nm. The figure shows the fluorescence, expressed in arbitrary units, after 1h of incubation. Data are representative of three independent experiments, carried out in duplicate.

**B.** *C. albicans* were preincubated for 30 min in PPB supplemented with various concentrations of jasplakinolide. PI was added and cells were transferred to wells containing equal volumes of serially diluted Hst5 in PPB, supplemented with jasplakinolide in the corresponding concentration. Development of fluorescence was followed for each concentration of the peptides at 5 min intervals for 1 h at  $\lambda_{exc}$  485 nm and  $\lambda_{em}$  620 nm. The figure shows the fluorescence, expressed in arbitrary units, after 1h of incubation. Data are representative of three independent experiments, carried out in duplicate.

**Table 1. The effect of energy depletion on the activity of various antimicrobial peptides**

Peptide	Sequence	LC <sub>50</sub> (μM)	
		NaCl	NaN <sub>3</sub>
Histatin 5	DSHAKRHHGYKRKFHEKHHSRGRY	1.1	>100
F-Histatin 5		3.0	> 100
Dh5 (11-24)	KRKFHEKHHSRGRY	2.5	> 100
P-113 (4-15)	AKRHHGYKRKFH	2.7	>100
P-113D	a k r h h g y k r k f h	2.4	>100
Dhvar4	KRLFKKLLKFSLRKY	0.1	0.2
Dhvar5	LLLFLLKKRKKRKY	2	10
F-Dhvar5		2.5	6.5
LFampin 265-284	DLIWKLLSKAQEKFGKNKSR	0.8	2.0
LFcin 17-30	FKCRRWQWRMKKLG	0.5	>40
Drosocin-1	GKPRPYSPRPTSHRPIRV	3.5	>100
Drosocin-4	GKPRPYTPRPTSHRPIRV	2.3	>100

*C. albicans* were preincubated with PPB+NaCl or PBB+NaN<sub>3</sub> for 15 min and then incubated with two-fold serially diluted peptides in the same buffers for 1h. After appropriate dilution, aliquots (in duplicate) were plated on SDA plates. After 2 days the numbers of CFUs were determined. LC<sub>50</sub>: concentration at which 50% of the cells were killed.

Fig. 1

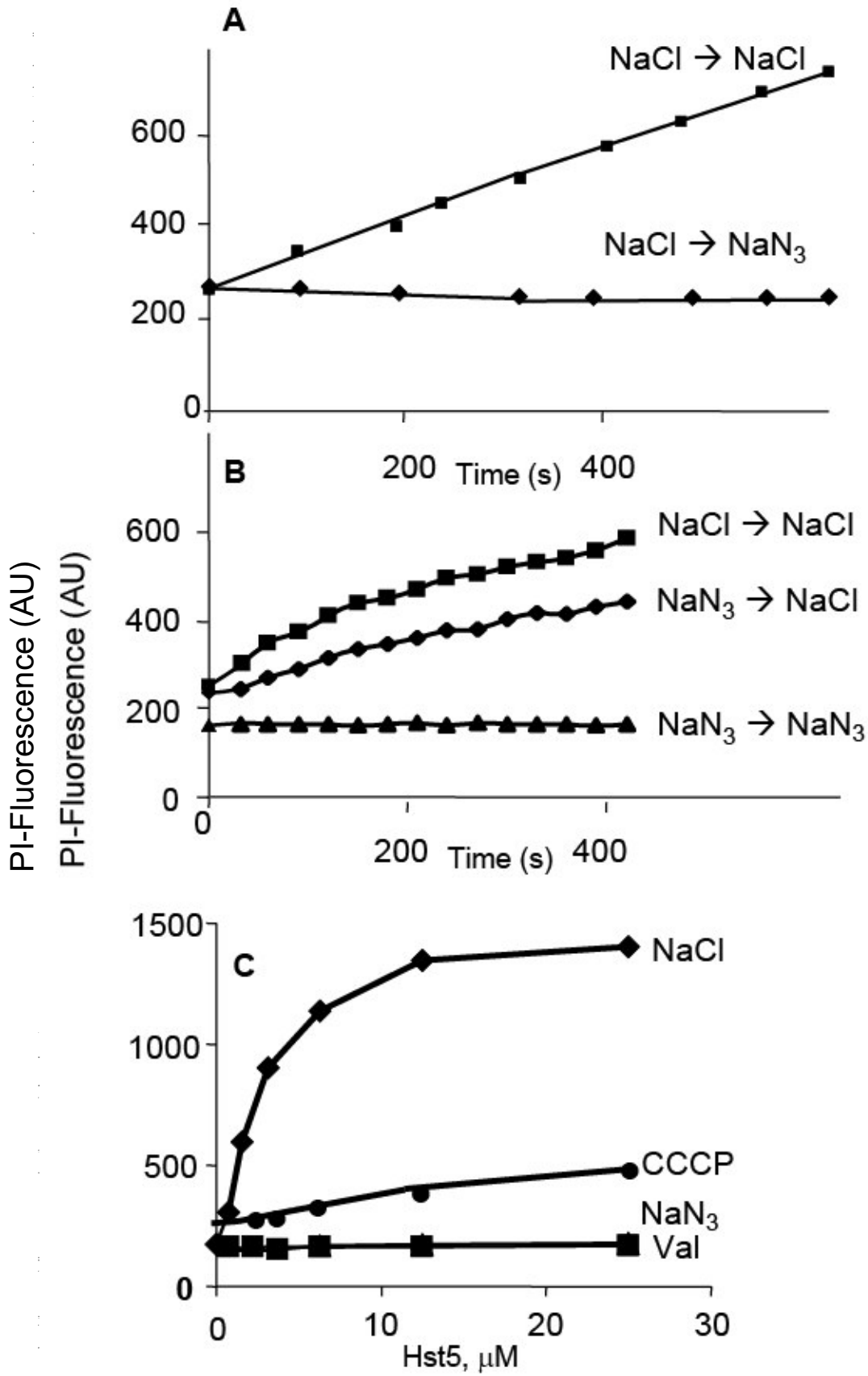


Fig. 2

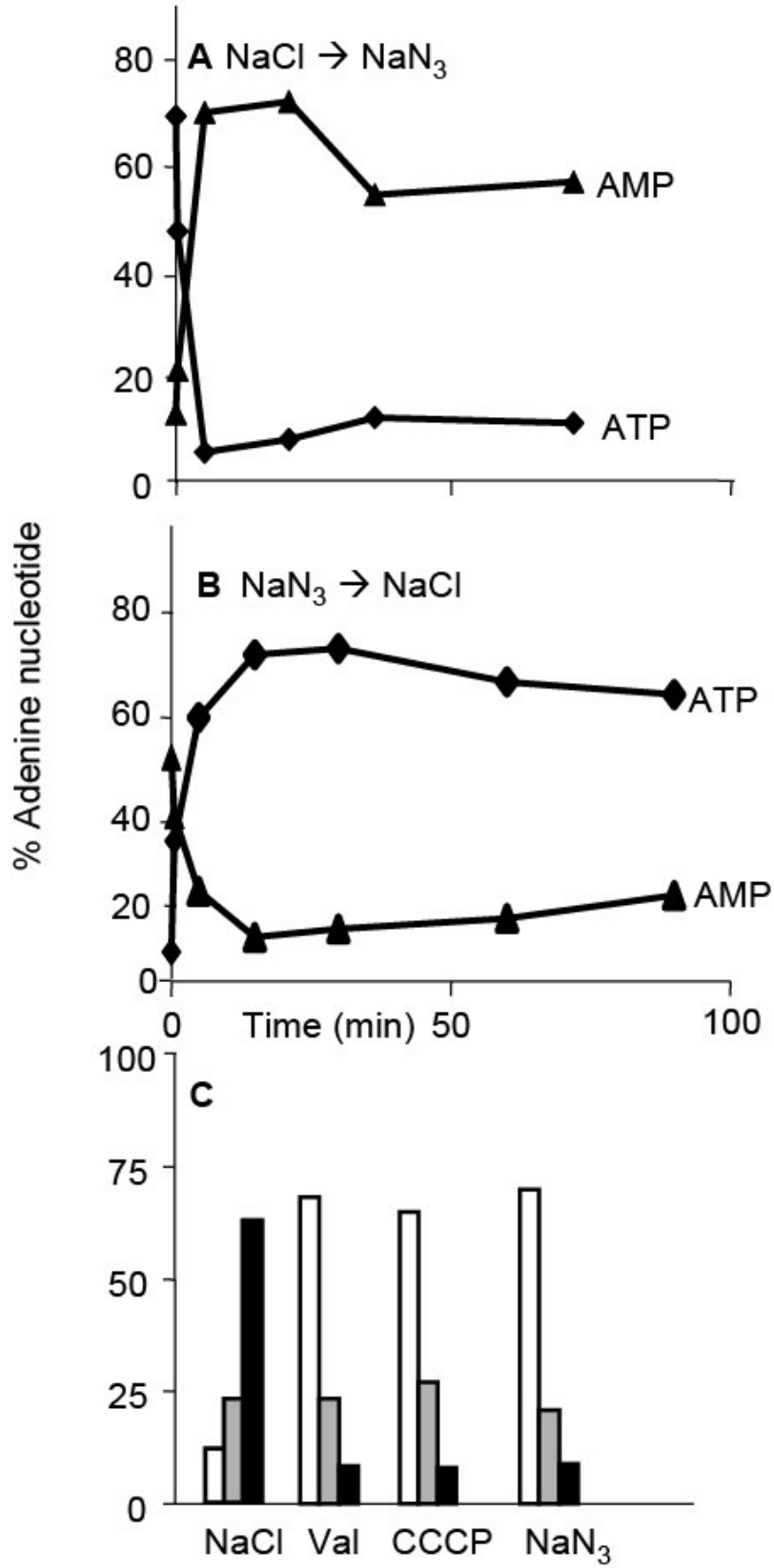
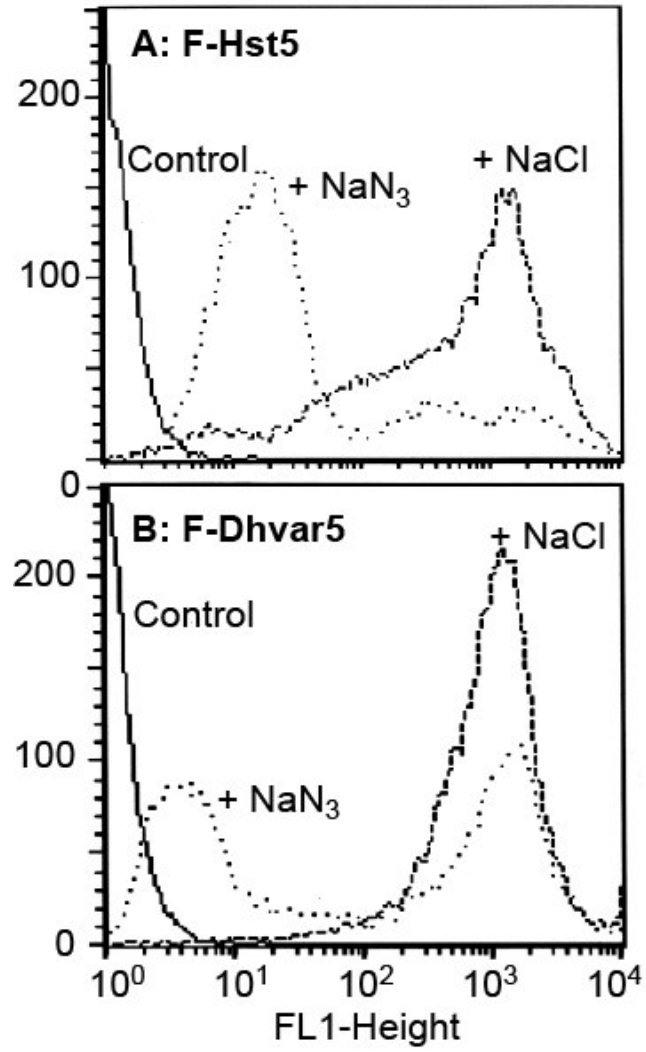


Fig. 3





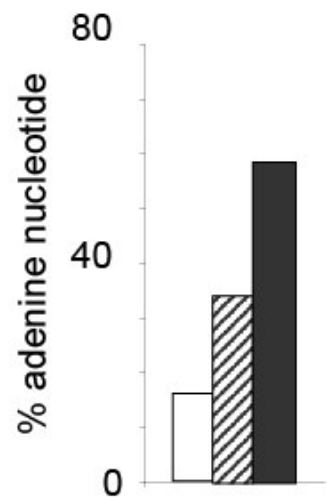
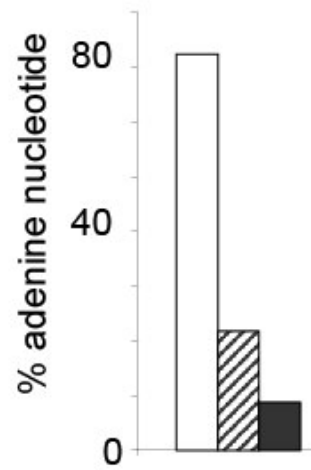
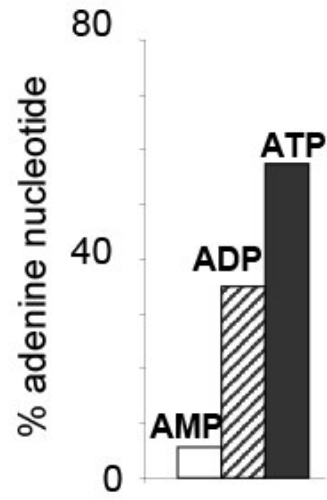
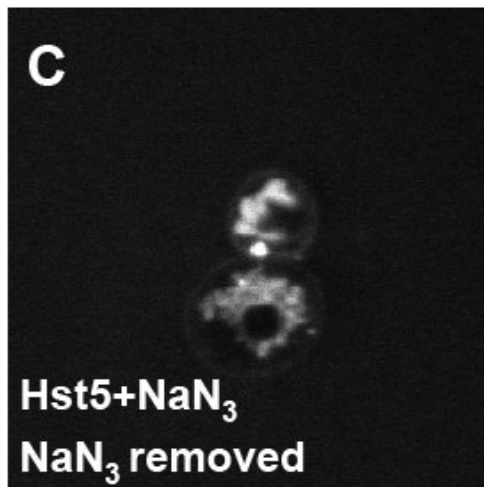
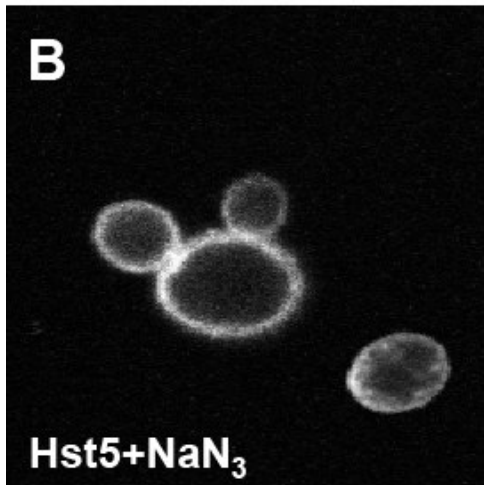
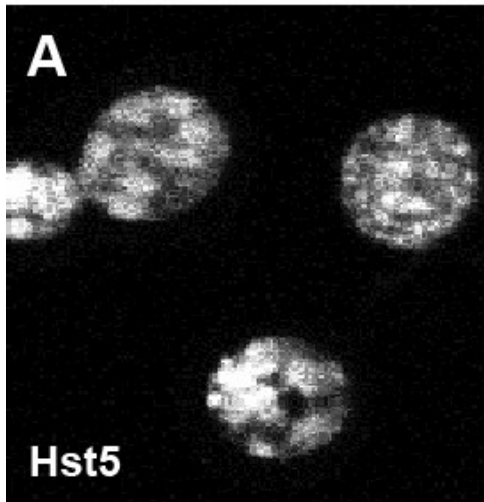


Fig. 5

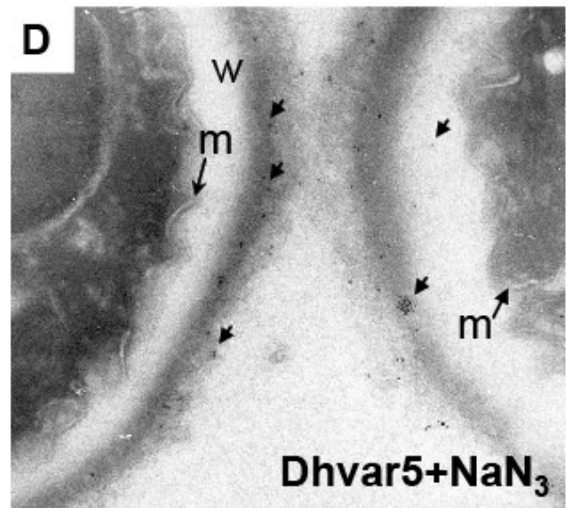
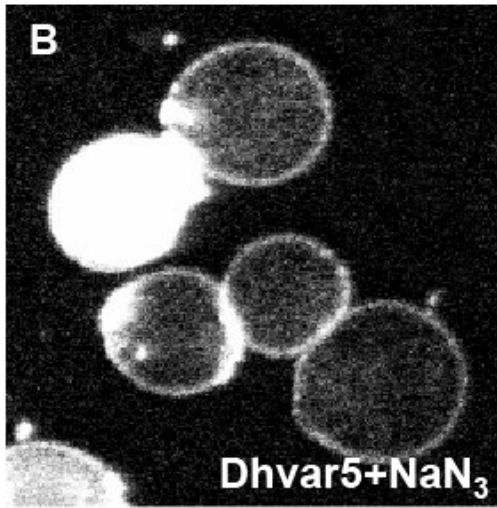
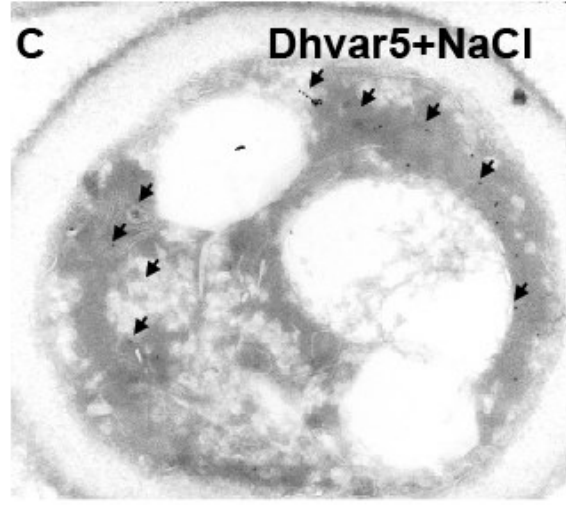
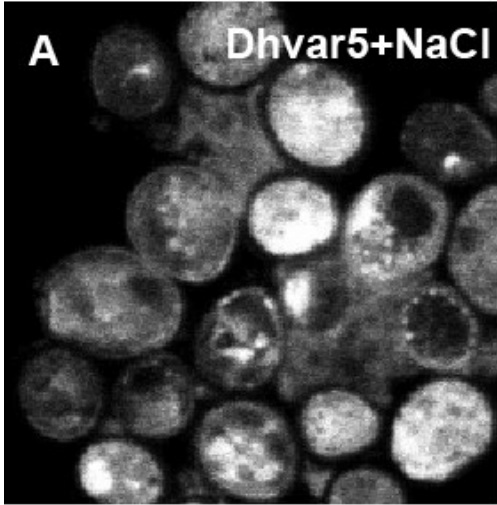


Fig. 6

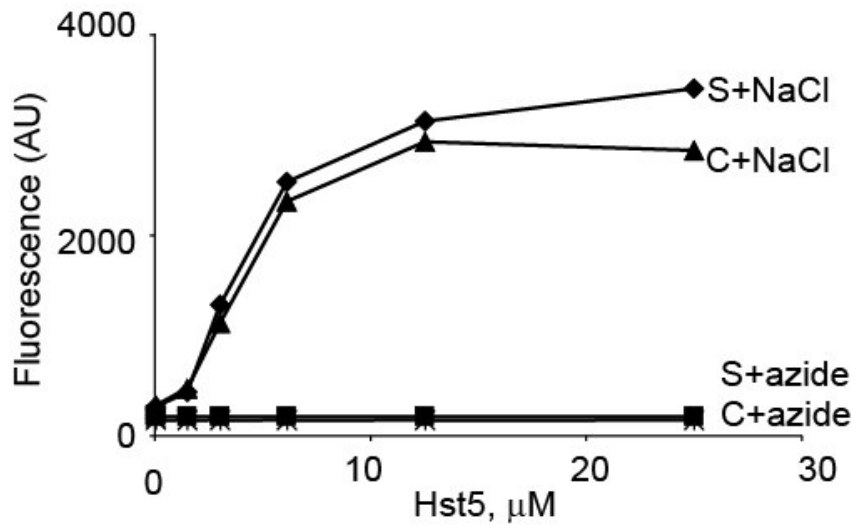


Fig. 7

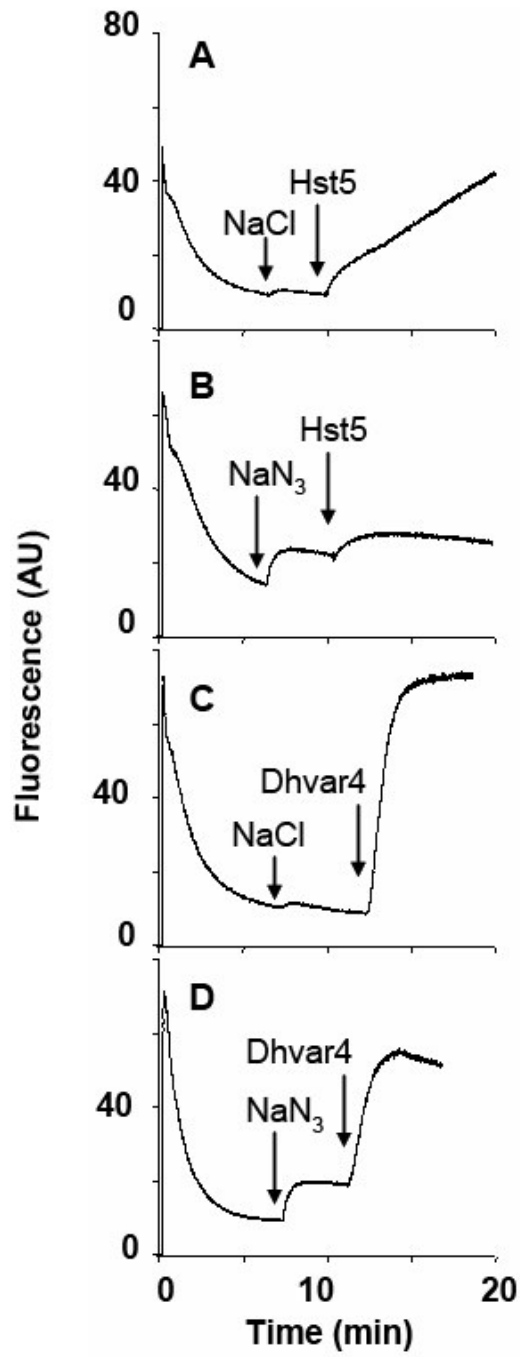
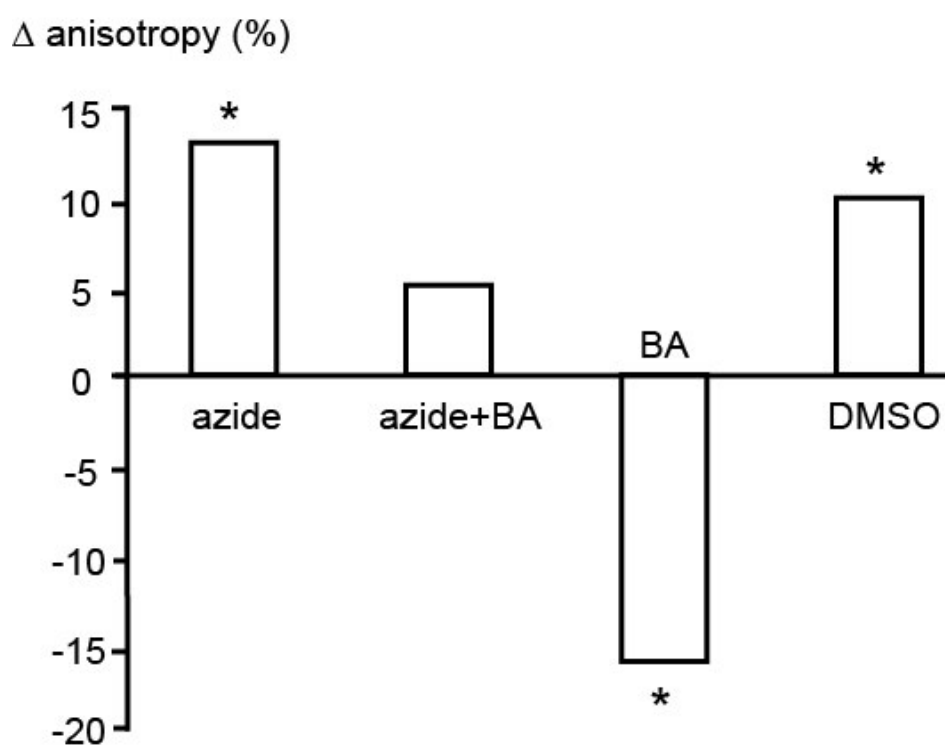


Fig. 8



**Fig. 9**

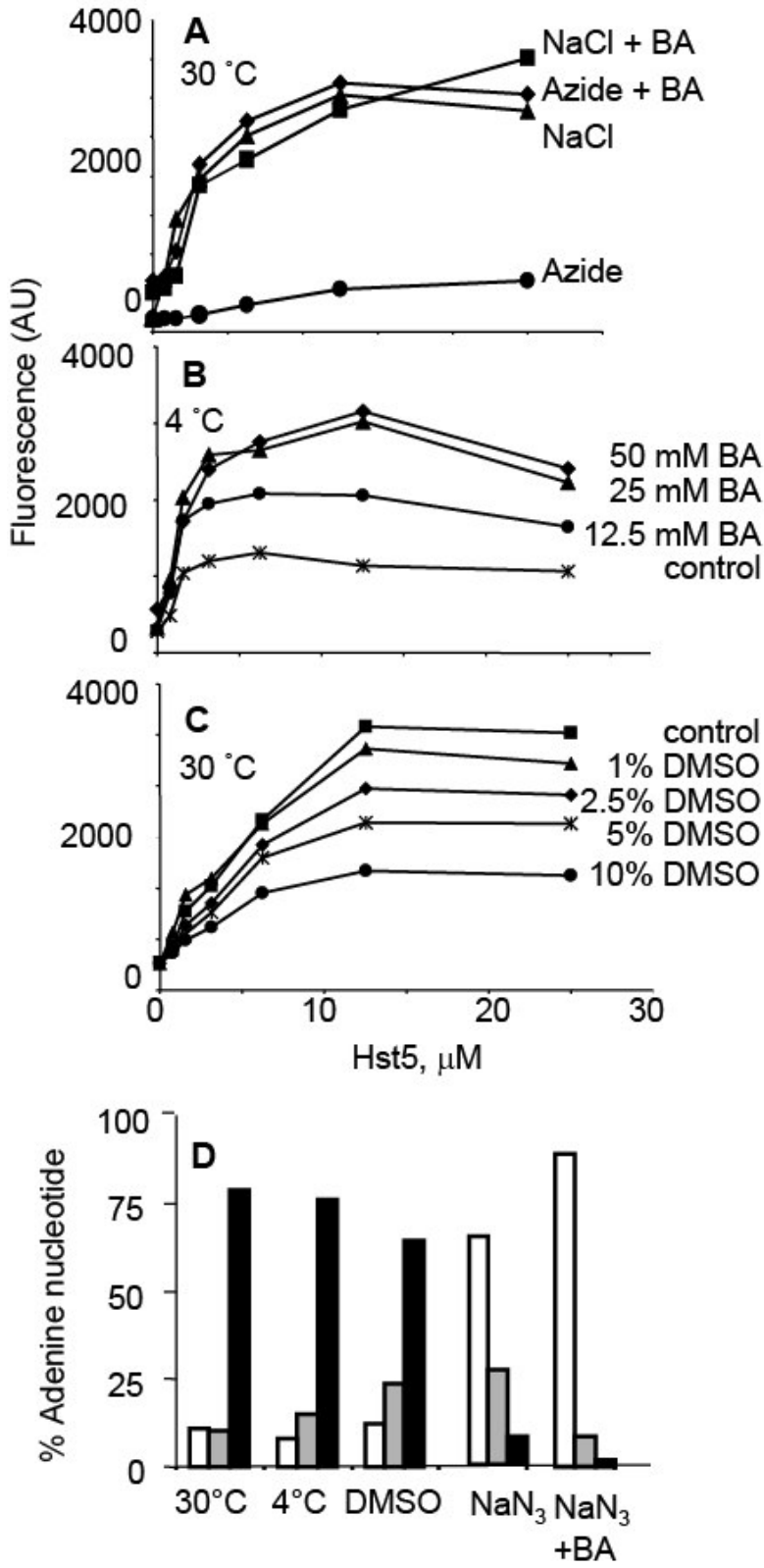
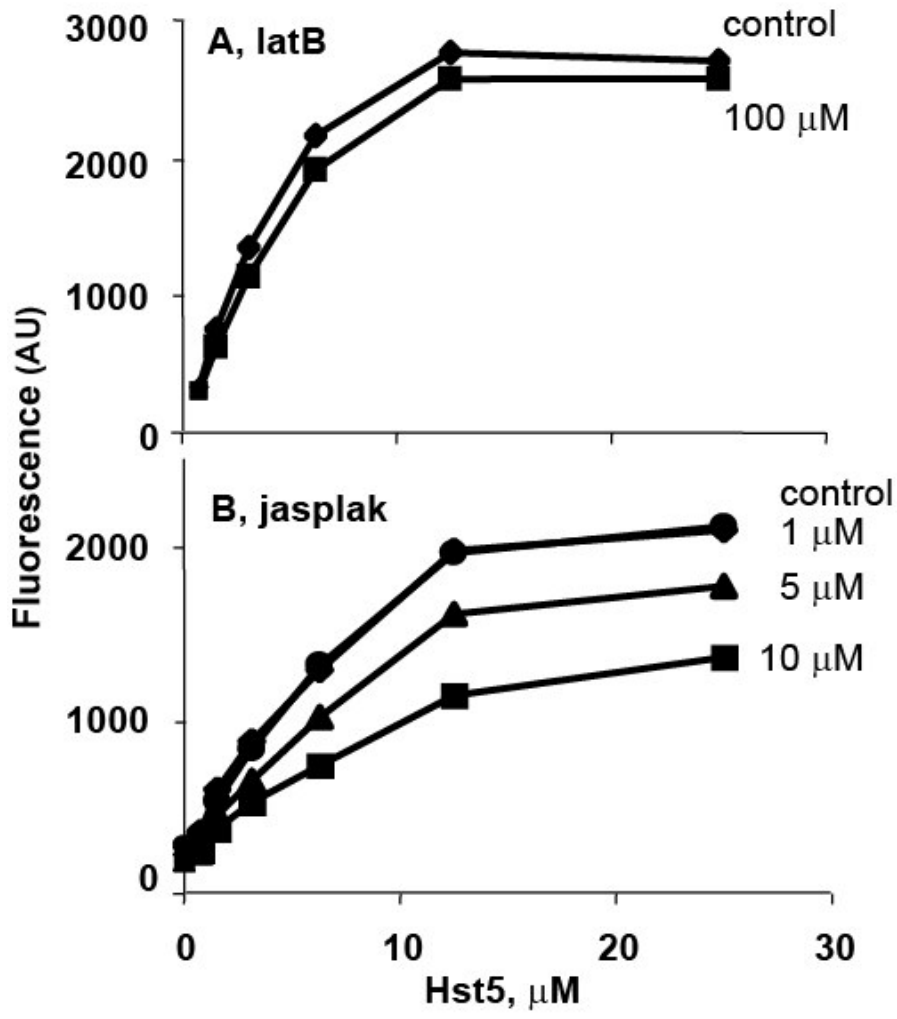


Fig. 10



**Energy depletion protects candida albicans against antimicrobial peptides by rigidifying its cell membrane**

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