Visualization analysis of the vacuole-targeting fungicidal activity of amphotericin B against the parent strain and an ergosterol-less mutant of Saccharomyces cerevisiae

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Here, we sought to investigate the vacuole-targeting fungicidal activity of amphotericin B (AmB) in the parent strain and AmB-resistant mutant of Saccharomyces cerevisiae and elucidate the mechanisms involved in this process. Our data demonstrated that the vacuole-targeting fungicidal activity of AmB was markedly enhanced by N-methyl-N0-dodecylguanidine (MC12), a synthetic analogue of the alkyl side chain in niphimycin, as represented by the synergy in their antifungal activities against parent cells of S. cerevisiae. Indifference was observed only with Δerg3 cells, indicating that the replacement of ergosterol with episterol facilitated their resistance to the combined lethal actions of AmB and MC12. Dansyl-labelled amphotericin B (AmB-Ds) was concentrated into normal rounded vacuoles when parent cells were treated with AmB-Ds alone, even at a non-lethal concentration. The additional supplementation of MC12 resulted in a marked loss of cell viability and vacuole disruption, as judged by the fluorescence from AmB-Ds scattered throughout the cytoplasm. In Δerg3 cells, AmB-Ds was scarcely detected in the cytoplasm, even with the addition of MC12, reflecting its failure to normally incorporate across the plasma membrane into the vacuole. Thus, this study supported the hypothesis that ergosterol is involved in the mobilization of AmB into the vacuolar membrane so that AmB-dependent vacuole disruption can be fully enhanced by cotreatment with MC12.

INTRODUCTION

Amphotericin B (AmB; Fig. 1a) is a polyene macrolide antibiotic most frequently used in the medical treatment of systemic fungal infections. The mechanism of AmB lethality has been described as being related to its creation of K+-permeable channels in association with ergosterol embedded in the fungal plasma membrane (Baginski et al., 2005; Carrillo-Munoz et al., 2006). AmB lethality can also occur via the inhibition of protein synthesis through loss of intracellular K+ and ATP depletion (Alonso et al., 1979). In addition, AmB is known to cause oxidative damage in cells of the pathogenic fungus Candida albicans by generating superoxide anions (Kim et al., 2012). We previously demonstrated that lethal concentrations of AmB caused dramatic disruption of the spherical architecture of vacuoles in Saccharomyces cerevisiae and C. albicans cells, and since then, this AmB-induced intracellular event has been considered the direct cause of its lethality (Borjihan et al., 2009; Ogita et al., 2006, 2009, 2010a, 2010b, 2012).

AmB-induced vacuole disruption is substantially enhanced by allicin, an allyl-sulfur compound isolated from garlic (Allium sativum L.) (Borjihan et al., 2009; Ogita et al., 2006, 2009, 2010a, 2010b, 2012). A similar pattern of vacuole disruption is more markedly observed with the polyol macrolide antibiotic niphimycin (NM; Fig. 1b) in S. cerevisiae cells (Nakayama et al., 2002; Ogita et al., 2007). The molecular structure of NM is characterized by the presence of an alkylguanidium chain attached to its polyol lactone ring, which is absent in the polyene macrolide structure of AmB (see Fig. 1). Interestingly, AmB lethality

Abbreviations: AmB, amphotericin B; AmB-Ds, dansyl-labelled amphotericin B; FIC, fractional inhibitory concentration; FM-64, 4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide; Fmoc-AmB, 9-fluorenylmethoxycarbonyl-AMB; MC12, N-methyl-N0-dodecylguanidine; NM, niphimycin.
in *S. cerevisiae* and *C. albicans* cells can be enhanced by cotreatment with N-methyl-N′-dodecylguanidine (MC12; Fig. 1c), a synthetic analogue of the alkyl side chain in NM (Ogita *et al.*, 2007; Yutani *et al.*, 2011). Moreover, ergosterol-pretreated cells were highly resistant to the combined lethal actions of AmB and MC12, prompting us to suggest that endogenous ergosterol likely plays an important role in the vacuole-disruptive actions of AmB (Borjihan *et al.*, 2009; Ogita *et al.*, 2009).

The AmB-resistant phenotype has appeared in various pathogenic fungi because of a mutation in the gene encoding one of the ergosterol biosynthetic enzymes (Martel *et al.*, 2010; Sanglard *et al.*, 2003; Young *et al.*, 2003). *S. cerevisiae* Δ*erg6* is one such mutant that harbours a deletion in the gene encoding C-24 sterol methyltransferase, characterized by its resistance to the combined lethal actions of AmB and allicin. In this mutant strain, cellular uptake of AmB into the cytoplasm was lower than that in parent cells, regardless of the presence of allicin (Ogita *et al.*, 2010b), suggesting that AmB requires ergosterol for its incorporation across the plasma membrane in addition to its ability to form transmembrane ion channels.

In this study, we evaluated the role of endogenously generated ergosterol in the vacuole-targeting fungicidal activity of AmB and the enhancement of AmB-dependent fungicidal activity by MC12 using a series of Δ*erg* mutants.

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**Fig. 1.** Structures of amphotericin B (a), niphimycin (b), N-methyl-N′-dodecylguanidine (c) and dansyl-labelled amphotericin B (d) and a part of the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae* (e).
in *S. cerevisiae*. We compared the intracellular dynamics of AmB between an AmB-sensitive parent strain and an AmB-resistant ergosterol-less mutant, with the aid of dansyl-labelled amphotericin B (AmB-Ds; Fig. 1d). Our study supported the hypothesis that ergosterol is essential for the mobilization of AmB into vacuoles and that such ergosterol-dependent trafficking is required for the MC12-dependent enhancement of vacuole disruption by AmB.

**METHODS**

**Measurement of yeast cell growth and viability.** *S. cerevisiae* strain BY4741 and its ERG6, ERG2, ERG3, ERG5 and ERG4 gene deletion mutants (Δerg6, Δerg2, Δerg3, Δerg5 and Δerg4, respectively) were obtained from the Yeast Knock Out Strain Collection (Thermo Scientific Open Biosystems) and were used in the following experiments to examine the effects of AmB, AmB-Ds and MC12 on cell growth, cell viability, K⁺ efflux and vacuole morphology. Minimum growth inhibitory concentrations (MICs) of AmB, AmB-Ds and MC12 were determined by the twofold broth dilution method, applying the chequerboard technique (Davis et al., 1994; Tanaka et al., 2000). Cells were grown overnight in YPD medium containing 1% yeast extract (Difco Laboratories), 2% bacto-peptone (Difco Laboratories), and 2% D-glucose, with vigorous shaking at 30 °C. Cells from the overnight-grown culture were then diluted with YPD medium to 10⁶ cells ml⁻¹ and incubated with each compound at various concentrations for 30°C for 24 h in a 96-well plate. An isobologram was made using the MICs obtained with either AmB or AmB-Ds and MC12. Cells from the overnight-grown culture were diluted into freshly prepared YPD medium to 10¹ cells ml⁻¹ and were then incubated with vigorous shaking at 30 °C in YPD medium containing either AmB or AmB-Ds and MC12 at various concentrations for the measurement of viable cell numbers as c.f.u. (Ogita et al., 2005).

**Leakage of potassium ions.** Cells from the overnight-grown culture were harvested by centrifugation, washed with 50 mM Tris/HCl buffer (pH 7.4) and resuspended in the same buffer to obtain a density of 10⁸ cells ml⁻¹. The cell suspensions were then shaken with 0.5 μM AmB or 7.5 μM AmB-Ds in the presence or absence of 50 μM MC12 at 30 °C for 240 min. The supernatants obtained after cell removal by centrifugation were assayed for K⁺ content using a K⁺ assay kit (HACH, Floriffoux) based on the tetraphenylborate method (Ramotowski & Szczesniak, 1967).

**Vacuole staining.** Vacuoles were visualized by staining with the fluorescent probe FM4-64 [N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide] according to previously described methods and modifications as follows (Kato & Wickner, 2001; Vida & Emr, 1995). Cells were grown in YPD medium with 200 μg genetin ml⁻¹ at 30 °C for 16 h (Kato & Wickner, 2001) and were further incubated with FM4-64 at a final concentration of 3 μM at 30 °C for 4 h. For visualization of vacuoles in ergosterol-pretreated cells, cells grown overnight were incubated with ergosterol at 240 μM with vigorous shaking at 30 °C for 60 min prior to FM4-64 staining. Cells were then collected by centrifugation, washed twice with YPD medium and suspended in the medium at a density of 1 × 10⁶ cells ml⁻¹. The cell suspensions were then incubated in the presence or absence of each compound with vigorous shaking at 30 °C for 120 min.

**Fluorescence microscopy.** For microscopy, 1.0 ml of sample was retrieved, and cells were then collected by centrifugation, washed and resuspended in 100 μl PBS [0.8% NaCl, 0.02% KCl, 0.144% Na₂HPO₄ (v/v), pH 7.4]. Cells were then observed under a phase-contrast microscope and a fluorescence microscope with excitation at 520–550 nm and emission at 580 nm for FM4-64-treated samples. Cells were also observed with excitation at 330–385 nm and emission at 515–550 nm for visualization of AmB-Ds.

**RESULTS**

**Synergy between AmB and MC12**

We first examined whether ergosterol was essential for generation of a synergistic relationship between AmB and MC12 using a series of *S. cerevisiae* Δerg mutants in which ergosterol was replaced by one of the intermediate molecules produced in its biosynthetic pathway (see Fig. 1e). As shown in Fig. 2, an AmB-resistant phenotype was generated in Δerg3 cells, in which ergosterol should be replaced by episterol as the major product in its biosynthetic pathway. The isobologram additionally suggested an indifferent relationship between the actions of AmB and MC12 in Δerg3 cells, despite the fact that the mutant cells show a slightly increased sensitivity to MC12 over other Δerg mutants (Fig. 2c). Synergistic-to-additive relationships were still observed with the other Δerg mutants, in which episterol cannot be produced at all (Δerg2) or can be metabolized further to ergostatetraenol (Δerg5) and ergostatetraenol (Δerg4). These findings are consistent with the idea that episterol accumulation in Δerg3 mutant is the most probable cause of the loss of synergistic relationship between AmB and MC12 against this mutant strain.
Effects of AmB and MC12, alone and in combination, on cell viability and K⁺ ion efflux

We next examined the effect of AmB, MC12 and a combination of AmB and MC12 on the growth of parent and Δerg3 cells. As shown in Fig. 3(a), cells of the parent strain were able to grow in medium containing AmB at 0.5 μM and were apparently viable in medium containing MC12 at 50 μM; however, addition of both 0.5 μM AmB and 50 μM MC12 caused rapid toxicity and cell death. In contrast, Δerg3 cells were completely resistant to the combined lethal actions of AmB and MC12, consistent with the pattern of indifference shown by the isobologram (Fig. 2c). These results supported the possibility that ergosterol plays an important role in the MC12-dependent enhancement of AmB lethality.

AmB has been shown to enhance plasma membrane ion permeability, especially to K⁺, by creating a transmembrane channel with ergosterol embedded in the fungal plasma membrane (Baginski et al., 2005; Carrillo-Muñoz et al., 2006). As shown in Fig. 3(c), AmB alone caused K⁺ efflux from parent cells, even at 0.5 μM, which was slightly lower than the MIC (1.0 μM). This may depend, in part, on the experimental conditions used for the K⁺ efflux assay, in which YPD medium was replaced by Tris/HCl buffer because of the high K⁺ content of this nutrient medium. In Δerg3 cells, however, the rate of K⁺ release was maintained at a level similar to that of the parent cells despite their significantly increased resistance to AmB and to the combined lethal actions of AmB and MC12 (Fig. 3d). This is consistent with the idea that AmB lethality cannot be simply elucidated by AmB-induced plasma membrane permeability to K⁺.

Enhancement of AmB-induced vacuole disruption by MC12

Thus, we next investigated the vacuole-disrupting actions of AmB as the primary cause of cellular sensitivity to the combined lethal action of AmB and MC12. MC12 alone did not cause any structural damage to vacuoles in parent or Δerg3 mutant cells, as has already been reported for a pathogenic strain of \textit{C. albicans} and another strain of \textit{S. cerevisiae} (Ogita et al., 2007; Yutani et al., 2011). As shown in Fig. 4(a), in parent cells, AmB alone did not cause vacuole disruption when added at its MIC of 1.0 μM. In contrast, vacuole disruption was provoked when parent cells were treated with 0.5 μM AmB in the presence of 50 μM MC12. Contrary to the results obtained in parent cells, Δerg3 cells were resistant to this effect, maintaining normal vacuole morphology in medium containing both AmB and MC12 (Fig. 4b).

Evaluation of AmB-Ds as a tool for visualization of AmB

AmB was detected for the first time in the cytoplasmic fraction of AmB-treated \textit{C. albicans} cells by means of HPLC-dependent analysis (Borjihan et al., 2009). This apparent cytoplasmic localization of AmB is consistent with its direct vacuole-disruptive actions, suggesting that the vacuole-targeting ability of AmB could be more precisely analysed with the aid of a fluorescence-labelled AmB derivative. As shown in Fig. 5(a), the MIC of AmB-Ds in parent cells was calculated as 15 μM, reflecting the significant decrease in the growth inhibitory activity of this structurally modified AmB derivative. However, similar to AmB, AmB-Ds could fully synergistically inhibit the growth of parent cells in the presence of MC12, and the combined actions of these two compounds resulted in a significant loss in cell viability, as shown in Fig. 5(a,b). As is the case with AmB, AmB-Ds also increased plasma membrane permeability to K⁺, regardless of the presence of MC12 (Fig. 5c). These findings clearly supported the usefulness of AmB-Ds as a...
Next, we examined the relationship between AmB-Ds-dependent vacuole disruption and the intracellular dynamics of AmB-Ds in parent and Δerg3 mutant cells. In parent cells, AmB-Ds alone did not cause any disruptive damage to the vacuole at the MIC of 15 μM, although its fluorescence was clearly detected in the vacuole membrane (Fig. 6a’). However, a combination of mutually nonlethal concentrations of AmB-Ds (7.5 μM) and MC12 (50 μM) resulted in serious structural damage to the vacuoles, and the fluorescence of AmB-Ds was observed to be colocalized with the fluorescence of FM4-64 in the fragmented vacuole membranes scattered throughout the cytoplasm (Fig. 6a’’). On the other hand, in Δerg3 cells, AmB-Ds alone at the MIC of 15 μM and a combination of 7.5 μM AmB-Ds and 50 μM MC12 did not cause any apparent vacuole disruption. The fluorescence of AmB-Ds was scarcely detected in the cytoplasm when added alone at 15 μM and added at 7.5 μM in combination with MC12, and no clear image reflecting its incorporation and concentration into the vacuole was found in this ergosterol-less mutant (Fig. 6b’’, ‘’). Thus, the failure of MC12 to enhance vacuole disruption induced by AmB should also arise from the failure of AmB to mobilize to the vacuole.

**Effects of AmB-Ds and MC12, alone and in combination, on vacuole morphology in ergosterol-pretreated cells**

Our previous study indicated that MC12 is ineffective in enhancing the vacuole-disruptive action and lethality of AmB in ergosterol-pretreated *C. albicans* cells, suggesting the possibility that MC12 inhibits cellular ergosterol trafficking from the plasma membrane to the vacuole membrane, which is evaluated as a cellular response provoked to protect against the disruptive action of AmB (Yutani et al., 2011). Therefore, we examined the intracellular dynamics of AmB-Ds in ergosterol-pretreated *S. cerevisiae* cells in order to evaluate the role of ergosterol in the vacuole-targeting lethal action of AmB and the enhancement of this effect by MC12. As shown in Fig. 7, AmB-Ds was detected throughout the cytoplasm, unable to penetrate into the vacuolar membrane of ergosterol-pretreated cells, suggesting that exogenously added ergosterol contributed to the cytoplasmic localization of AmB-Ds, but not to its penetration into the vacuolar membrane. Thus, our study ultimately supported the idea that MC12 stimulates AmB-induced vacuole disruption only when AmB is mobilized into the vacuolar membrane, possibly via its interaction with the vacuolar membrane-embedded ergosterol (see Fig. 6).

**DISCUSSION**

The vacuole is one of the intracellular organelles unique to plants and eukaryotic microbes. Vacuoles function in storage and decomposition of waste and cytotoxic compounds as well as the regulation of intracellular osmotic balance and pH for the maintenance of cell viability. Thus, the function of vacuoles must be precisely regulated in order to protect against cell death. Allicin was found to enhance AmB lethality in *S. cerevisiae* cells in distilled water, but not in YPD medium, and in *C. albicans* cells in a serum-containing synthetic medium by means of
enhancing its vacuole-disruption activity (Borjihan et al., 2009; Ogita et al., 2006). Allicin can also enhance the vacuole-targeting fungicidal activity of polymyxin B, a bactericidal antibiotic that is effective against Gram-negative bacteria (Ogita et al., 2012). In contrast, in the current study, we demonstrated that MC12 was quite effective at enhancing the vacuole-disruption activity of AmB in S. cerevisiae cells, even in YPD medium, suggesting that allicin and MC12 have different roles.

Ergosterol is a fungal plasma membrane component essential for stabilizing phospholipid bilayers. Allicin-dependent enhancement of the vacuole-disruption activity of AmB depends on the presence of ergosterol in the fungal plasma membrane, as demonstrated by the resistance of an ergosterol-less S. cerevisiae mutant to the combined lethal effects of AmB and allicin (Ogita et al., 2010b). Additionally, S. cerevisiae and C. albicans cells were similarly protected against AmB-mediated lethality when

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**Fig. 4.** Effects of amphotericin B (AmB), N-methyl-N'-dodecylguanidine (MC12) and a combination of AmB and MC12 on vacuole morphology in parent (a) and Δerg3 (b) cells. After treatment with the fluorescent dye FM4-64, cells (1×10⁷ cells ml⁻¹) were incubated in YPD medium containing no drug, 1.0 μM AmB, or a combination of 0.5 μM AmB and 50 μM MC12 at 30 °C for 120 min. Cells were observed with a bright-field microscope (top) and a fluorescence microscope (bottom).

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**Fig. 5.** Effects of dansyl-labelled amphotericin B (AmB-Ds), N-methyl-N’-dodecylguanidine (MC12) and a combination of AmB-Ds and MC12 on the growth (a), cell viability (b) and leakage of K⁺ (c) in parent cells. For (a), cells (1×10⁶ cells ml⁻¹) were incubated in YPD medium containing AmB-Ds and MC12 at the varying concentrations at 30 °C for 24 h. In the isobologram, each point represents the combination of the two agents that is needed for minimum growth inhibitory activity. For (b), cells (1×10⁷ cells ml⁻¹) were incubated in YPD medium containing no drug (○), 7.5 μM AmB-Ds (●), 50 μM MC12 (□), or a combination of 7.5 μM AmB-Ds and 50 μM MC12 (■) in YPD medium at 30 °C. For (c), cells (1×10⁷ cells ml⁻¹) were incubated in 50 mM Tris/HCl buffer (pH 7.4) containing no drug (○), 7.5 μM AmB-Ds (●), 50 μM MC12 (□), or a combination of 7.5 μM AmB-Ds and 50 μM MC12 (■) at 30 °C.
the cells had been pretreated with exogenous ergosterol (Borjihan et al., 2009; Ogita et al., 2009). These findings suggest that cellular sensitivity to AmB depends on the subcellular localization of ergosterol and its expression in each of the cytoplasmic compartments. Here, we examined the mechanisms through which ergosterol was related to the fungal sensitivity of AmB and MC12 (and the combination of the two compounds) using the following ergosterol-less mutants: Δerg6, Δerg2, Δerg3, Δerg5 and Δerg4 (see Fig. 1e). We found that ergosterol was a necessary sterol component for the synergistic relationship between AmB and MC12. Among the ergosterol-less mutants, Δerg3 appeared to give us the most valuable information on the role of ergosterol in the vacuole-targeting action of AmB and its dependence on MC12. In fact, Δerg3 cells were as sensitive as the parent cells to AmB with regard to AmB-induced plasma membrane permeability changes, as reflected by K⁺ efflux (see Fig. 3), indicating that the ability of episterol to bind to AmB was similar to that of ergosterol when these molecules were embedded in the plasma membrane phospholipid bilayers.

In the current study, we developed AmB-Ds, which we expected to be valuable for visualizing the intracellular dynamics and action of AmB since this fluorescent derivative exhibited the same lethality as AmB, except with a higher MIC. Moreover, AmB-Ds was not clearly visualized in the plasma membrane of both parent and Δerg3 cells, although this fluorescent derivative was expected to be embedded within the plasma membrane as a molecular component of K⁺ channels (see Fig. 5c). The fluorescence intensity of AmB-Ds may have been weakened when this molecule was embedded within the plasma membrane phospholipids via the formation of a complex with either ergosterol or episterol. The most surprising feature of AmB-Ds was its failure in vacuole disruption, despite the fact that this molecule could be efficiently incorporated into the vacuoles of parent cells at the non-lethal concentration.

In Δerg3 cells, AmB-Ds was scarcely visible in the cytoplasm even after treatment with MC12. This failure to penetrate into the organelle was thought to be a cause of the indifferent relationship between the actions of AmB and MC12. As mentioned above, ergosterol can be fully replaced by episterol for K⁺ channel formation with AmB, whereas this replacement was inhibitory to the penetration of AmB (AmB-Ds) across the plasma membrane. It is also apparent that MC12 cannot enhance the vacuole-disruptive action of AmB unless AmB is suitably localized into the vacuole. In this sense, ergosterol can be considered as a prerequisite for the MC12-dependent enhancement of AmB lethality, and thus, its replacement with episterol may be the cause of the indifferent relationship between AmB and MC12. On the basis of these findings, a model has been proposed for the roles of ergosterol and MC12 in the vacuole-targeting fungicidal activity of AmB (Fig. 8). In

**Fig. 6.** Effects of dansyl-labelled amphotericin B (AmB-Ds) and a combination of AmB-Ds and N-methyl-N'-dodecylguanidine (MC12) on vacuole morphology and intracellular AmB-Ds localization in parent (a, a’, a’’) and Δerg3 (b, b’, b’’) cells. After treatment with the fluorescent dye FM4-64, cells (1×10⁷ cells ml⁻¹) were incubated in YPD medium containing no drug (a, b), 15 μM AmB-Ds (a’, b’), or a combination of 7.5 μM AmB-Ds and 50 μM MC12 (a’’, b’’) at 30 °C for 120 min. Cells were observed with a bright-field microscope (top) and with a fluorescence microscope for visualization of vacuole morphology (middle) and intracellular AmB-Ds localization (bottom).
parent cells, MC12 enhanced the vacuole-disruptive action of AmB when this antibiotic had been incorporated into the vacuolar membrane, possibly via its molecular interaction with ergosterol. In \( \text{D} \text{erg3} \) cells, however, MC12 was unable to enhance the vacuole-disruptive action of AmB because of the failure of AmB to mobilize across the plasma membrane into the vacuolar membrane. MC12 was also ineffective at enhancing the vacuole-disruptive action of AmB in ergosterol-pretreated cells of the parent strain, in which AmB-Ds was apparently detected in the cytoplasm, but not in the vacuolar membrane. Under aerobic conditions, exogenously added ergosterol is not suitably incorporated into cells of a \( \text{S. cerevisiae} \) ergosterol-less mutant unless the \( \text{SUT1} \) gene is overexpressed (Bourot & Karst, 1995). However, it has been shown that as in the case with the parent strain, ergosterol can be incorporated into cells of a \( \text{D} \text{sut1} \) mutant to a limited extent, suggesting its dependence on a mechanism functional for ergosterol uptake under aerobic conditions. Thus, our study ultimately suggests the possibility that AmB-dependent antifungal therapy may be improved, even against AmB-resistant ergosterol-less mutants, with the aid of an agent that enhances the vacuole-targeting mobilization of AmB.

Fig. 7. Effects of dansyl-labelled amphotericin B (AmB-Ds) and a combination of AmB-Ds and \( \text{N}-\text{methyl-N}^\prime-\text{dodecyl} \)guanidine (MC12) on vacuole morphology and intracellular AmB-Ds localization in ergosterol-pretreated parent cells. After treatment with the fluorescent dye FM4-64, ergosterol-pretreated cells (1 \( \times \) 10\(^7\) cells ml\(^{-1}\)) were incubated in YPD medium containing no drug (a), 15 \( \mu \text{M} \) AmB-Ds (b), or a combination of 7.5 \( \mu \text{M} \) AmB-Ds and 50 \( \mu \text{M} \) MC12 (c) at 30 °C for 120 min. Cells were observed with a bright-field microscope (top) and a fluorescence microscope for visualization of vacuole morphology (middle) and intracellular AmB-Ds localization (bottom).

![Fig. 7](image)

Fig. 8. A proposed model for the role of \( \text{N}-\text{methyl-N}^\prime-\text{dodecyl} \)guanidine (MC12) and ergosterol in the vacuole-targeting fungicidal activity of amphotericin B (AmB) against cells of the parent strain (a), \( \text{D} \text{erg3} \) mutant (b) and ergosterol-pretreated cells of the parent strain (c).

![Fig. 8](image)

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Vacuole-targeting fungicidal activity of amphotericin B


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