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ORIGINAL ARTICLE

# Enhancement effect of *N*-methyl-*N*″-dodecylguanidine on the vacuole-targeting fungicidal activity of amphotericin B against the pathogenic fungus *Candida albicans*

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**The alkylguanidium chain attached to the polyol lactone ring of niphimycin (NM) is considered a requisite for the fungicidal activity of NM characterized by vacuole membrane fragmentation and oxidative stress induction. The addition of *N*-methyl-*N*′-dodecylguanidine to the medium can enhance the vacuole-targeting fungicidal activity of amphotericin B (AmB), in which the lactone ring has no such alkylguanidium chain, on *Saccharomyces cerevisiae* cells. In this study, the enhancement effect of *N*-methyl-*N*′-dodecylguanidine on the vacuole-targeting fungicidal activity of AmB was examined against *Candida albicans* in RPMI 1640 medium at 37 °C. *N*-methyl-*N*′-dodecylguanidine was lethal to *C. albicans* cells and additionally enhanced the vacuole disruptive activity of AmB against this pathogenic fungus. *N*-methyl-*N*′-dodecylguanidine elevated the generation of cellular reactive oxygen species when added alone in a dose-dependent manner, but its enhancement effect on AmB lethality did not accompany amplification of oxidative stress induction. The fungal vacuoles were protected against the disruptive damage even if cells were treated with $H_2O_2$ alone at a lethal concentration or treated with $H_2O_2$ at a sublethal concentration in combination with AmB. *N*-methyl-*N*′-dodecylguanidine was ineffective in enhancing AmB lethality or AmB-induced vacuole disruption when cells had been pretreated with ergosterol. Ergosterol-dependent mechanism is thus considered to be a possible target of *N*-methyl-*N*′-dodecylguanidine in enhancing the vacuole-targeting fungicidal activity of AmB in *C. albicans* cells.**
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**Keywords:** alkylguanidine; amphotericin B; *Candida albicans*; vacuole

## INTRODUCTION

Amphotericin B (AmB; Figure 1) is a polyene macrolide antibiotic widely used in chemotherapy for candidiasis, which is caused by proliferation of *Candida albicans* cells in mammalian organs.[1–4] AmB lethality is mainly explained by its ability to bind ergosterol in the fungal plasma membrane and induce plasma membrane permeability changes via the leakage of $K^+$. However, AmB-induced cell death is alternatively explained by an inhibitory effect on protein synthesis or by induction of the generation of cellular reactive oxygen species (ROS).[1,5,6] We observed vacuole membrane fragmentation as another AmB-induced lethal event in cells of *Saccharomyces cerevisiae* and *C. albicans* that was markedly enhanced in the presence of allicin, an allyl-sulfur compound from garlic (Figure 1).[7–11] A similar vacuole-targeting fungicidal activity was also detected with polymyxin B, a bactericidal antibiotic, which was also amplified by allicin or by various ionophores, such as salinomycin and monensin.[12,13] Our screening experiment additionally showed that zwiebelane A, an organosulfur compound from onion bulbs, enhances the vacuole-targeting fungicidal activity of polymyxin B.[14]

Vacuole membrane fragmentation was initially observed with the lethal action of a guanidyl polyol macrolide antibiotic niphimycin (NM; Figure 1) in *S. cerevisiae* cells.[15] NM-induced cell death was achieved at a lower concentration than AmB and was accompanied by various cytotoxic events such as disruptive plasma membrane damage and ROS production. These NM-induced events were thought to depend on the presence of the alkylguanidium chain attached to its polyol lactone ring but not to the poleyen lactone ring of AmB. The role of the alkylguanidium chain in ROS generation was later demonstrated by an experiment using *N*-dodecyl-*N*″-alkylguanidine (MC12, Figure 1) and *N*-hexadecyl-*N*″-alkylguanidine (MC16) as its synthetic analogs.[16] A comparison of AmB lethality in the absence and presence of MC12 additionally suggested the role of alkylguanidium chain in the vacuole disrupting activity of NM.[17]

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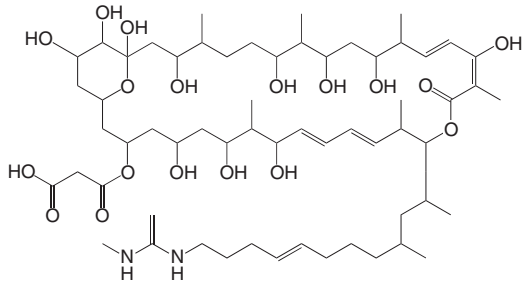
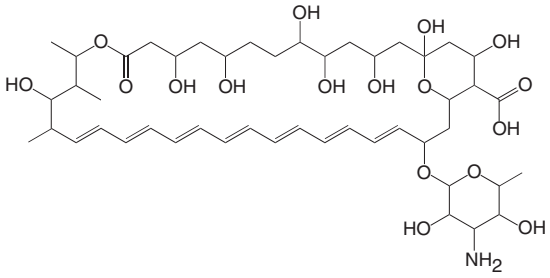
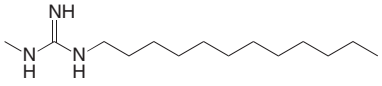


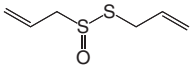
Niphimycin (NM)



Amphotericin B (AmB)



*N*-dodecyl-*N*"-methylguanidine
(MC12)



Allicin

**Figure 1** Structures of NM, AmB, MC12 and allicin.

MC12 may be more useful and valuable as an enhancer of AmB lethality than allicin because MC12 lacks odorous and volatile properties. In this study, we attempted to find the role of MC12 as an enhancer of AmB lethality against *C. albicans* cells. In contrast to *S. cerevisiae*, MC12 was lethal to *C. albicans* cells and this analog could similarly enhance the vacuole-targeting fungicidal activity of AmB against this pathogenic fungus. Our study suggests that MC12 enhances AmB lethality by inhibiting ergosterol trafficking but not by promoting ROS production.

## MATERIALS AND METHODS

### Measurement of cell growth and viability

Unless stated otherwise, *C. albicans* NBRC 1061 (formerly *C. albicans* IFO 1061) was used in the following experiments to examine the effects of AmB and MC12 on cell growth, cell viability and other physiological properties. Cells were grown in YPD medium containing 1% yeast extract (Difco Laboratories, Detroit, MI, USA), 2% Bacto-peptone (Difco Laboratories) and 2% glucose at 30 °C with vigorous shaking. Unless stated otherwise, cells from the overnight-grown culture were collected by centrifugation, washed twice with RPMI 1640 medium with L-glutamine (RPMI 1640 medium),[18] and suspended in the same medium to obtain a final cell density of $1\times10^7$ cells ml$^{-1}$. Cells were then incubated in the absence or presence of each compound with vigorous shaking at 37 °C. Viable cell number was determined by counting colony forming units after 24-h incubation on a YPD agar plate at 30 °C.[19] Methylene blue staining was used to determine cell viability.[9]

For preparation of ergosterol-enriched cells, cells from the overnight culture in YPD medium were inoculated into a freshly prepared YPD medium containing 240 μM ergosterol at a cell density of $1\times10^7$ cells ml$^{-1}$ and incubated with vigorous shaking at 30 °C for 60 min.[8,10] Ergosterol-enriched cells were washed with RPMI 1640 medium as described above and suspended in the same medium to obtain a final cell density of $1\times10^7$ cells ml$^{-1}$.

### ROS assay

ROS such as $O_2^-$ and $H_2O_2$ were measured by the method based on the intracellular deacylation and oxidation of 2′,7′-dichlorodihydrofluorescein diacetate to the corresponding fluorescent compound.[20] Cells from the overnight culture in YPD medium were collected by centrifugation, suspended in medium to obtain a cell density of $1\times10^7$ cells ml$^{-1}$ and incubated with 40 μM 2′,7′-dichlorodihydrofluorescein diacetate at 30 °C for 60 min. Cells were then collected by centrifugation and suspended in an equal volume of RPMI 1640 medium. The cell suspension (1.0 ml) was further incubated in the absence or presence of each compound at 37 °C for 120 min. Cells were collected by centrifugation, washed with phosphate-buffered saline (137 mM NaCl, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, 2.68 mM KCl, 1.47 mM $KH_2PO_4$, pH 7.4), and suspended in 100 μl of phosphate-buffered saline. The fluorescence intensities of the cell samples ($1\times10^7$ cells) were measured using a GENios Fluoreacence Detector (Tecan, Männedorf, Switzerland) at excitation and emission wavelengths of 480 and 530 nm, respectively. The arbitrary units were derived directly from the fluorescence intensity.

### Vacuole staining

Vacuoles were visualized by staining with the fluorescent probe FM4-64.[21] Cells from the overnight culture in YPD medium were suspended in a freshly prepared medium to obtain a cell density of $1\times10^7$ cells ml$^{-1}$. After incubation with 5 μM FM4-64 at 30 °C for 30 min, the cells were collected by centrifugation, washed twice with RPMI 1640 medium and suspended in the same medium at a final cell density of $1\times10^7$ cells ml$^{-1}$. The cells were then incubated in the absence or presence of each compound with vigorous shaking at 37 °C for 60 or 120 min and were observed under a phase-contrast microscope and a fluorescence microscope with excitation at 520–550 nm and emission at 580 nm.

### Chemicals

AmB was purchased from Sigma Aldrich (St Louis, MO, USA) and FM4-64 was purchased from Molecular Probes (Eugene, OR, USA). MC12 was synthesized as described previously.[16]

## RESULTS

### Effects of AmB and MC12 alone and in combination on cell viability

AmB inhibited the growth of *C. albicans* cells at 0.5 μM and exhibited an apparent lethal effect when its concentration was increased up to 2 μM (Figure 2a). MC12 exhibited limited lethality to this pathogenic fungus at 20 μM and was fully effective at promoting cell death at 40 μM (Figure 2b). The lethal action of MC12 in *C. albicans* cells was distinguishable from its static growth inhibition pattern observed in *S. cerevisiae* cells.[17] This difference may depend on different functional mechanisms for protection against MC12-induced oxidative stress in *C. albicans* and *S. cerevisiae*, as discussed below. *C. albicans* cells were extremely sensitive to the lethal action of AmB in the presence of MC12, suggesting a synergistic relationship in the mechanism of cell death progression between this polyene macrolide antibiotic and an analog of alkylguanidium chain in NM (Figure 2c).

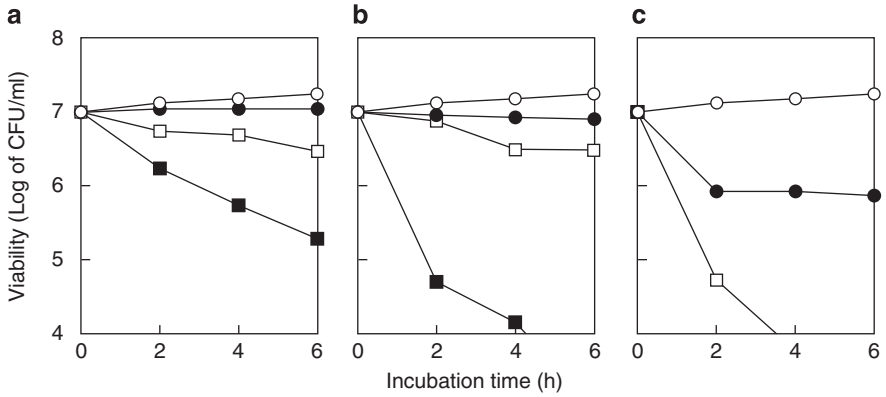



**Figure 2** Effects of AmB (**a**), MC12 (**b**), and AmB and MC12 in combination (**c**) on the viability of *C. albicans* cells. For **a**, cells ($1\times10^7$ cells ml$^{-1}$) were incubated in RMPI 1640 medium containing AmB at 0 μM (○), 0.5 μM (●), 1.0 μM (□) or 2 μM (■) at 37 °C. For **b**, cells ($1\times10^7$ cells ml$^{-1}$) were incubated in RPMI 1640 medium containing MC12 at 0 μM (○), 10 μM (●), 20 μM (□) or 40 μM (■) at 37 °C. For **c**, cells ($1\times10^7$ cells ml$^{-1}$) were incubated in RPMI 1640 medium containing none (○), 0.5 μM AmB+10 μM MC12 (●) or 0.5 μM AmB+20 μM MC12 (□) at 37 °C.

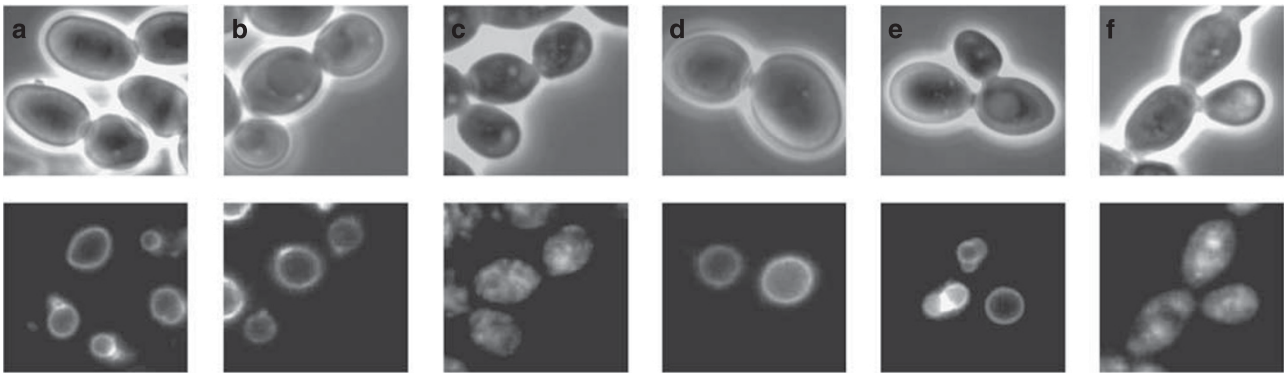



**Figure 3** Effects of AmB and MC12 alone and in combination on vacuole membrane fragmentation in *C. albicans* cells. After treatment with the fluorescent dye FM4-64, cells ($1\times10^7$ cells ml$^{-1}$) were incubated in RPMI 1640 medium containing none (**a**), 0.5 μM AmB (**b**), 2 μM AmB (**c**), 20 μM MC12 (**d**), 40 μM MC12 (**e**) or a combination of 0.5 μM AmB and 20 μM MC12 (**f**) at 37 °C for 60 min. Cells were observed under a bright-field microscope (top) and a fluorescence microscope (bottom).

### Effects of AmB and MC12 alone and in combination on vacuole morphology

Electron microscopic observation clearly revealed fragmentation of a membrane-enclosed rounded architecture of the vacuole in NM-treated cells of *S. cerevisiae*.[17] This drastic morphological change of the organelle could be also visualized under a microscope with the aid of a fluorescent probe. The vacuole was therefore evaluated as a target of NM lethality against *S. cerevisiae*, although it remains unknown whether or not vacuole disruption occurs as a result of NM-induced ROS production. We examined the contribution of MC12 to AmB lethality against *C. albicans* cells in RPMI medium at 37 °C. Vacuole membrane fragmentation was observed when *C. albicans* cells were treated with AmB alone at the lethal concentration (Figure 3c), whereas MC12 did not cause any disruptive damage to the organelle at the lethal concentration (Figure 3e). The organelle was also observed as fragmented particles or patches in the cytoplasm when cell death was achieved by the combined actions of AmB and MC12 added at each of their non-lethal concentrations (Figure 3f). This indicates that MC12 effectively enhances AmB lethality by enhancing the vacuole disruptive activity of AmB in *C. albicans* cells.

### Effects of AmB and MC12 alone and in combination on ROS production

ROS can induce cell death when they are produced at an increased level or when the resulting toxic effects cannot be suitably eliminated.[19,22,23] In our previous study, MC12 induced ROS production in *S. cerevisiae* cells during incubation in YPD medium at 30 °C; however, the cells were still viable, presumably because of a functional mechanism of protection against oxidative stress.[19] We therefore examined the effect of MC12 on cellular ROS production in *C. albicans* cells under conditions with or without AmB. MC12 enhanced cellular ROS production at 20 μM and significantly elevated the level of ROS production as the concentration was increased to 40 μM (Figure 4), suggesting that a relationship exists between ROS production and MC12-induced cell death. In the medium containing both 20 μM MC12 and 0.5 μM AmB, however, the level of ROS production was not significantly increased compared with the level in the medium containing 20 μM MC12 alone ($P>0.01$). This result supports the idea that the combined lethal actions of AmB and MC12 can be attributed to an event other than the amplification of ROS-dependent cytotoxicity.

### Effects of AmB and $H_2O_2$ alone and in combination on cell viability and vacuole morphology

Assuming that a non-lethal range of AmB causes vacuole membrane fragmentation more rapidly under conditions where ROS are produced, it is possible that MC12-induced ROS production could enhance AmB lethality. We therefore examined the effect of exogenously added $H_2O_2$ on the ability of AmB to induce lethality and disrupt vacuoles. We observed vacuoles with normal rounded architecture in medium containing 100 mM $H_2O_2$ (Figure 5), in which the cell viability was markedly reduced to 20% of the original level after a 60-min incubation. In medium containing 50 mM $H_2O_2$, the cell viability was reduced only to 80% of the original level, and it was completely lost in medium containing 50 mM $H_2O_2$ and 0.5 μM AmB (data not shown). Nevertheless, the organelles were still observed without any disruption, suggesting the possibility that the vacuole-targeting lethal action of AmB is not accelerated under the condition of ROS production (Figure 5). The effect of MC12 thus could be distinguished from any cytotoxic events arising from ROS production, as this alkylguanidium compound can indeed enhance AmB-mediated vacuole membrane fragmentation.

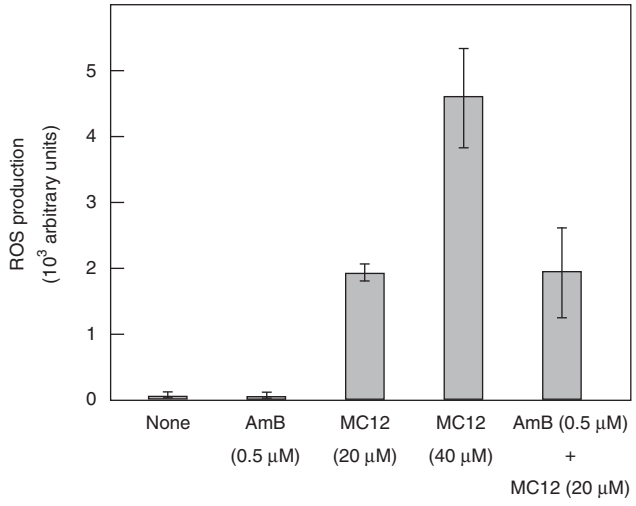



**Figure 4** Effects of AmB and MC12 alone and in combination on ROS production in *C. albicans* cells. Cells ($1\times10^7$ cells ml$^{-1}$) were incubated in RPMI 1640 medium containing AmB and MC12 at the indicated concentrations at 37 °C for 120 min. Data are expressed as the mean (s.d.) of the arbitrary units measured in triplicate assays. Data were analyzed by Student's *t*-test and $P<0.05$ was considered statistically significant.

### AmB lethality in ergosterol-enriched cells

The enhancement effect of allicin on the vacuole-targeting fungicidal activity of AmB has been explained by its inhibitory effect on ergosterol trafficking from the plasma membrane to the vacuole membrane.[8,10] Ergosterol enrichment in the vacuole membrane is proposed to be a cellular response to increase the vacuole membrane stability, thereby protecting the organelles against the disruptive action of AmB. In agreement with this finding, AmB did not induce lethality in ergosterol-enriched *C. albicans* cells when added alone at the lethal concentration (Figure 6a). Ergosterol-enriched cells were also much more resistant to the combined lethal actions of AmB and MC12 than untreated cells. However, such a marked difference was not observed on ergosterol pretreatment when cells were simply incubated with MC12 alone. In ergosterol-enriched cells, the organelles were indeed protected against the disruptive action of AmB or a combination of AmB and MC12 (Figure 6b). These results may suggest a possibility that MC12 has an allicin-like inhibitory effect on the process of ergosterol trafficking from the plasma membrane to the vacuole membrane.[8]

## DISCUSSION

MC12 was lethal to *C. albicans* cells grown at 37 °C under the *in vitro* conditions generally used as a substitute for *C. albicans* invasive growth conditions. *C. albicans* cells are expected to be more sensitive to oxidative stress at a higher growth temperature. We therefore suspected that MC12 enhances the vacuole-targeting

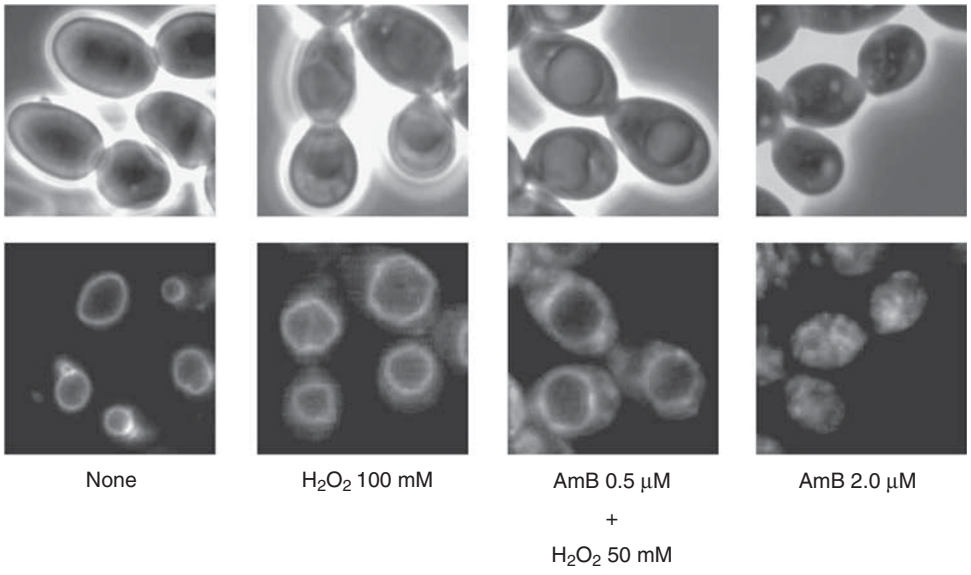



**Figure 5** Effects of AmB and $H_2O_2$ alone and in combination on vacuole membrane fragmentation in *C. albicans* cells. After treatment with the fluorescent dye FM4-64, cells ($1\times10^7$ cells ml$^{-1}$) were incubated in RPMI 1640 medium containing $H_2O_2$ and AmB at the indicated concentrations at 37 °C for 60 min. Cells were observed under a bright-field microscope (top) and a fluorescence microscope (bottom).

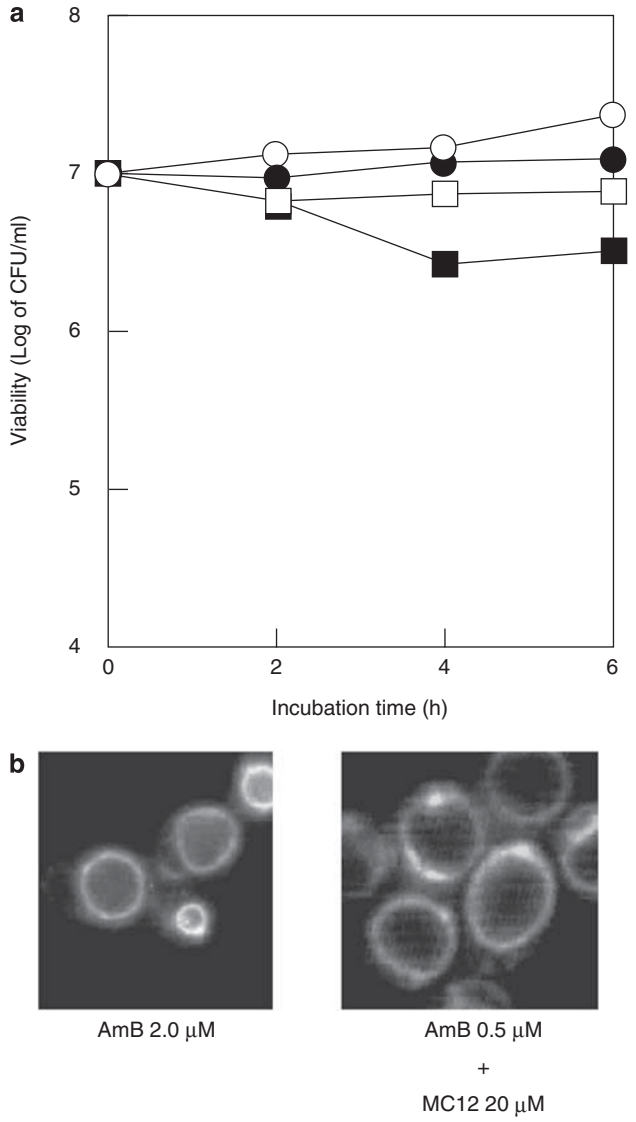



**Figure 6** Effects of AmB and MC12 alone and in combination on cell viability (**a**) and vacuole morphology in ergosterol-enriched *C. albicans* cells (**b**). For **a**, ergosterol-enriched cells ($1\times10^7$ cells ml$^{-1}$) were incubated in RPMI 1640 medium containing none (○), 2 μM AmB (●), 20 μM MC12 (□) or 0.5 μM AmB + 20 μM MC12 (■). For **b**, ergosterol-enriched cells ($1\times10^7$ cells ml$^{-1}$) were incubated in RPMI 1640 medium containing AmB and MC12 at the indicated concentrations at 37 °C for 60 min after treatment with the fluorescent dye FM4-64. Cells were observed under a bright-field microscope (top) and a fluorescence microscope (bottom).

fungicidal activity of AmB against *C. albicans* because of its enhancement of ROS production at 37 °C. In this study, oxidative stress induced in *C. albicans* cells by the exogenous addition of $H_2O_2$ scarcely affected the vacuole morphology even when cell death was achieved with $H_2O_2$ alone or in combination with AmB (Figure 5). Nevertheless, a synergistic relationship was observed between the lethality of $H_2O_2$ and AmB, as judged by the absolute cell death in medium containing 50 mM $H_2O_2$ and 0.5 μM AmB. This is consistent with the previously reported enhancement of AmB lethality against *C. albicans* cells by superoxide radical.[6]

Ergosterol is a fungal plasma membrane component that increases the stability of plasma membrane phospholipid bilayers. AmB creates a transmembrane ion channel by aggregate formation with ergosterol, thereby accelerating the leakage of intracellular ionic substances, such as $K^+$.[1–4] The leakage of $K^+$ seems essential for AmB lethality even if AmB-induced cell death inevitably depends on vacuole disruption, as the hypertonic condition achieved by the addition of $K^+$ and $Mg^{2+}$ into medium resulted in simultaneous protection from vacuole disruption and cell death.[11] AmB is less active on ergosterol-less mutants (*erg6*Δ) of *C. albicans* and *Candida lusitaniae* than wild-type strains.[24] This lower activity is thus explained by the loss of ergosterol, which is essential for the ion channel formation with AmB in the fungal plasma membrane, but may be also explained by the failure of AmB to penetrate through the plasma membrane to exert its direct disruptive action on vacuoles.[9,10] In the case, plasma membrane ergosterol should have an important role in the cellular uptake of AmB into the cytoplasm.

In the previous study, we visually confirmed the intracellular trafficking of plasma membrane ergosterol into the vacuole membrane in response to AmB-treatment of *S. cerevisiae* cells.[8] Incubation of yeast cells in medium supplemented with ergosterol results in ergosterol accumulation in the vacuole membrane, producing ergosterol-enriched cells that are highly resistant to the vacuole disruptive actions of AmB alone or even in combination with allicin.[8,10] Ergosterol-enriched vacuoles isolated from *C. albicans* cells were indeed resistant to the direct disruptive action of AmB.[10] However, viability was partly lost in medium in which ergosterol-enriched *C. albicans* cells were incubated with 20 μM MC12 and 0.5 μM AmB (Figure 6). The loss of cell viability coincided with that of *C. albicans* cells during incubation with 20 μM MC12 without ergosterol pretreatment (Figure 2b). The lethality detected with MC12 alone may instead depend on MC12-induced ROS production as well as an increased sensitivity to oxidative stress of *C. albicans* cells.[25]

## ACKNOWLEDGEMENTS

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