The fungicidal activity of amphotericin B requires autophagy-dependent targeting to the vacuole under a nutrient-starved condition in *Saccharomyces cerevisiae*

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In this study, we demonstrated that in distilled water, a nutrient-starved condition that elicits autophagy in *Saccharomyces cerevisiae*, an array of autophagy-deficient mutants are resistant to the fungicidal effects of amphotericin B. In addition, we found that a dansyl-labelled derivative of the antibiotic colocalized with disintegrated vacuoles throughout the cytoplasm in the amphotericin B-sensitive parental strain suspended in distilled water. In contrast, the dansyl-labelled derivative was not internalized in the *Δatg18* strain, which is deficient in the formation of autophagosomes, a key early step in autophagy. However, the derivative accumulated without significant toxicity in structurally intact vacuoles in the *Δvma1* mutant, which is deficient in the degradation of autophagic bodies, the final stage in autophagy. Our data support the idea that amphotericin B can utilize autophagy-dependent trafficking into the intra-vacuolar lumen, where it interacts with the luminal leaf of the membrane to cause structurally catastrophic effects.

INTRODUCTION

Amphotericin B (AmB; Fig. 1a), a typical polyene macrolide antibiotic, is most frequently used in managing serious systemic fungal infections. The hydrophobic moiety in AmB binds ergosterol molecules embedded in the fungal plasma membrane to form K⁺-permeable ion channels (Baginski *et al.*, 2005; Carrillo-Munoz *et al.*, 2006). However, alternative modes of action have also been proposed, since ion leakage does not necessarily result in loss of cell viability (Chen *et al.*, 1978). Indeed, the antibiotic also generates superoxide anions and causes oxidative damage in the pathogenic fungus *Candida albicans* (Kim *et al.*, 2012), although this mode of action likely depends on K⁺ efflux as well.

We recently demonstrated that lethal doses of AmB dramatically alter the structure of fungal vacuoles (Borjihan *et al.*, 2009; Ogita *et al.*, 2006, 2007, 2010, 2012; Yutani *et al.*, 2011). This mode of action is markedly enhanced by allicin, an allyl sulfur compound from garlic, or by N-methyl-N'-dodecylguanidine, a synthetic analogue of the alkyl side chain in niphimycin. Notably, the effects of these compounds are not due to an increase in plasma membrane ion permeability (Borjihan *et al.*, 2009; Kang *et al.*, 2013; Yutani *et al.*, 2011). Instead, such enhancement effects on AmB lethality seem to be closely related to the machinery for its trafficking to the vacuole and/or the susceptibility of the organelle membrane to the disruptive action of the antibiotic.

Ergosterol is an essential structural and regulatory component of fungal cell membranes, and is required for both endocytosis and homotypic vacuole fusion (Kato & Wickner, 2001; Pichler & Riezman, 2004). Indeed, the most recently proposed mechanism for AmB lethality is based on its ability to extract ergosterol from the plasma membrane phospholipid layers in the form of large, extra-membranous aggregates (Anderson *et al.*, 2014). Notably, pathogenic fungi with a mutation in the ergosterol biosynthetic pathway not only accumulate a synthetic intermediate instead of ergosterol, but are also resistant to AmB (Martel *et al.*, 2010; Young *et al.*, 2003) in addition to azoles (Martel *et al.*, 2010; Pinjon *et al.*, 2003). Such a resistance to AmB may be elucidated either by the reduced affinity between the antibiotic and the synthetic...
intermediate, or by a defect in intracellular trafficking that prevents the antibiotic from reaching the vacuole (Kang et al., 2013).

Autophagy is a fundamental, well-conserved process in eukaryotic cells (Reggiori & Klionsky, 2013). The most remarkable feature of this process is the formation of autophagosomes that sequester molecules for degradation in lytic vacuoles (Nakatogawa et al., 2009). In yeast, the autophagy-competent membrane is concentrated at a phagophore assembly site, and the concerted action of the autophagy machinery at this site expands the phagophore and forms the autophagosome. Subsequent fusion between the autophagosome and the vacuole generates intravacuolar vesicles called autophagic bodies, from which cargo is released and finally broken down (Yang & Klionsky, 2010). In this study, we investigated the vacuole-dependent fungicidal activity of AmB in cells suspended in distilled water, a nutrient-starved condition that elicits autophagy in Saccharomyces cerevisiae, as a model case. As expected, the antibiotic was ineffective against autophagy-deficient mutants. Our data suggest the possibility that AmB can disrupt the yeast vacuole membrane after autophagy-dependent trafficking into the intra-vacuole luminal space and the following acidic pH-dependent degradation of the autophagic body for its release under starvation conditions.

**METHODS**

**Strains.** S. cerevisiae strain BY4741, as well as deletion mutants listed in Table 1, were obtained from the Yeast Knock Out Strain Collection (Thermo Scientific Open Biosystems, Waltham, MA, USA). The mutant strains Δatg1 to Δatg18 are deficient in autophagosome formation, maturation or vacuole fusion. In mutants Δvph1 and Δvma1 to Δvma16, autophagosomes are incorporated into vacuoles, but are not efficiently degraded, because of defects in H\(^+\) transport by v-ATPase. On the other hand, the mutant strains Δldb17, Δart5, Δend3 and Δmon2 are deficient in endocytosis of extracellular molecules, as well as of molecules embedded in the plasma membrane. These strains were tested for sensitivity to AmB by measuring the minimum fungicidal concentration in distilled water. Further, strains Δatg18 and Δvma1 were used to examine the effects of unlabelled or dansyl-labelled AmB (Fig. 1b) on cell viability, K\(^+\) efflux and vacuole morphology.

**Fungicidal activity.** Unless otherwise stated, cells were grown overnight at 30 °C with vigorous shaking in YPD medium consisting of 1 % yeast extract (Difco Laboratories), 2 % bacto-peptone (Difco Laboratories) and 2 % D-glucose. Cells were then harvested, washed with distilled water, and diluted to 1 × 10\(^8\) cells ml\(^{-1}\) in distilled water containing AmB at various concentrations. Cell suspensions were then incubated at 30 °C for 24 h to measure minimum fungicidal concentration, as previously described (Ogita et al., 2006). In addition, cells were also diluted to 1 × 10\(^7\) cells ml\(^{-1}\) in distilled water containing unlabelled or dansyl-labelled AmB (Kang et al., 2013) at various concentrations. Cell suspensions were then incubated with vigorous shaking at 30 °C, and plated on YPD medium containing 1.8 % (w/v) agar. Viability was determined as the number of colonies formed after 48 h at 30 °C.

**Leakage of K\(^+\) ions.** Overnight cultures were harvested by centrifugation, washed with distilled water and resuspended in distilled water at 1 × 10\(^8\) cells ml\(^{-1}\). Cell suspensions were then shaken at 30 °C with or without 0.63 μM AmB. K\(^+\) was measured in culture supernatants at the time points indicated using a K\(^+\) assay kit (HACH) based on the tetraphenylborate method (Ramotowski & Szczesniak, 1967).

**Table 1. Minimum fungicidal concentrations of AmB against various gene deletion mutants of S. cerevisiae**

Cells (1 × 10\(^8\) cells ml\(^{-1}\)) were incubated with or without various concentrations of AmB in distilled water at 30 °C for 24 h. Data are means of triplicate experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MFC* (μM)</th>
<th>Strain</th>
<th>MFC* (μM)</th>
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<tbody>
<tr>
<td>Parental</td>
<td>0.313</td>
<td>Δvma4</td>
<td>2.5</td>
</tr>
<tr>
<td>Δatg1</td>
<td>5.0</td>
<td>Δvma5</td>
<td>1.25</td>
</tr>
<tr>
<td>Δatg2</td>
<td>5.0</td>
<td>Δvma6</td>
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<td>Δatg7</td>
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<td>Δatg8</td>
<td>2.5</td>
<td>Δvma8</td>
<td>1.25</td>
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<tr>
<td>Δatg9</td>
<td>5.0</td>
<td>Δvma10</td>
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</tr>
<tr>
<td>Δatg12</td>
<td>5.0</td>
<td>Δvma11</td>
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<td>Δvma16</td>
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</tr>
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<td>2.5</td>
</tr>
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<td>5.0</td>
<td>Δldb17</td>
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</tr>
<tr>
<td>Δvma1</td>
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<td>Δart5</td>
<td>0.625</td>
</tr>
<tr>
<td>Δvma2</td>
<td>2.5</td>
<td>Δend3</td>
<td>0.625</td>
</tr>
<tr>
<td>Δvma3</td>
<td>1.25</td>
<td>Δmon2</td>
<td>0.625</td>
</tr>
</tbody>
</table>

*M*Minimum fungicidal concentration.
Vacuole staining. Vacuoles were stained with the fluorescent probe FM4-64 (N-(3-triethylammoniumpropyl)-4-[6-[4-(diethylamino)phenyl]hexatrienyl]pyridinium dibromide) according to published methods (Kato & Wickner, 2001; Vida & Emr, 1995), with some modification. Briefly, overnight cultures were incubated for another 4 h at 30 °C in YPD with 2 μM FM4-64. Cells were then collected by centrifugation and suspended in distilled water at 1 × 10⁷ cells ml⁻¹. Cell suspensions were then incubated with vigorous shaking at 30 °C for 2 h to investigate the effects of unlabelled or dansyl-labelled AmB on vacuole morphology.

Fluorescent microscopy. After treatment with unlabelled or dansyl-labelled AmB, FM4-64-stained cells (1 × 10⁷ cells ml⁻¹) were collected by centrifugation, washed, resuspended in 100 μl PBS, and imaged by bright-field and fluorescence microscopy. Cells labelled with FM4-64 were imaged with excitation at 520–550 nm and emission at 580 nm. Dansyl-labelled AmB was visualized with excitation at 330–385 nm and emission at 515–550 nm (Kang et al., 2013).

Chemicals. AmB and FM4-64 were purchased from Sigma Aldrich Chemicals. AmB and Molecular Probes, respectively. All other reagents were analytical grade. The antibiotic was labelled with dansyl as previously reported (Kang et al., 2013).

Statistical methods. Statistical evaluation was done by using Student’s t-test, in which P < 0.05 was considered statistically significant.

RESULTS

AmB resistance in autophagy-deficient yeast

Autophagy is a highly conserved pathway that degrades and recycles cytoplasmic contents in response to stressors such as starvation. In S. cerevisiae, autophagy can be uniformly induced in almost all cells using nutrient-deficient media (Takeshige et al., 1992) such as distilled water, a stressor that imposes nitrogen starvation. As summarized in Table 1, the minimum fungicidal concentration for AmB against S. cerevisiae suspended in distilled water was 4- to 16-fold higher in autophagy-deficient derivatives than in the WT parental strain, for which the minimum fungicidal concentration was 0.31 μM. In contrast, the minimum fungicidal concentration was only slightly increased to 0.63 μM in the endocytosis-deficient strains Δart5, Δend3 and Δamon2. Notably, the endocytosis-deficient mutant Δldb17 was more sensitive to AmB than the parental strain, and had a minimum fungicidal concentration of 0.078 μM.

AmB fungicidal activity against Δatg18 and Δvma1

AmB has been shown to cross the plasma membrane and accumulate in the cytoplasm despite its hydrophobicity, and its vacuole-targeting fungicidal activity is likely to require intracellular trafficking machinery (Borjihan et al., 2009) in addition to or independent of plasma membrane permeabilization. Thus, we examined the effects of unlabelled or dansyl-labelled AmB on cell viability, K⁺ efflux and vacuole morphology in the autophagy-deficient mutants Δatg18 and Δvma1, which have large swollen vacuoles. Cell viability was measured in c.f.u., and, as shown in Fig. 2(a), AmB was fungicidal against the parental strain in distilled water at 0.63 μM. In contrast, autophagy-deficient mutants Δatg18 and Δvma1 were significantly resistant to the lethal action of AmB at this concentration (Fig. 2b, c), in line with measured minimum fungicidal concentrations (Table 1).

Plasma membrane permeabilization in autophagy-deficient mutants

The fungicidal activity of AmB does not simply depend on increased K⁺ efflux (Chen et al., 1978). We previously found that the fungicidal activity of AmB depends more directly on vacuole disintegration than on altered plasma membrane permeability, although osmotic imbalance from the latter may enhance the former (Ogita et al., 2010).

![Fig. 2. Effect of AmB on the viability of parental (a), Δatg18 (b) and Δvma1 (c) strains of S. cerevisiae. Cells (1 × 10⁷ cells ml⁻¹) were incubated in distilled water at 30 °C without antibiotics (○) or with 0.31 μM (■), 0.63 μM (□) or 1.25 μM (△) AmB. Data are means ± SD of triplicate experiments.](image-url)
Nevertheless, we investigated the possibility that AmB resistance in Δatg18 and Δvma1 may be due to a decrease in antibiotic-induced permeabilization of the plasma membrane, even though autophagy genes may not directly affect the structural robustness of the plasma membrane. Therefore, we compared antibiotic-induced K⁺ leakage in parental and autophagy-deficient mutants. As shown in Fig. 3(a), AmB dramatically enhanced K⁺ efflux from parental cells at 0.63 μM, at which the antibiotic is reliably lethal in distilled water. However, the antibiotic also induced a similar level of K⁺ efflux from Δatg18 and Δvma1 cells (Fig. 3b, c), despite significantly increased resistance (Fig. 2).

**Vacuole morphology in cells exposed to AmB**

In a previous study, we demonstrated that a lethal concentration of AmB dramatically disrupts the spherical architecture of vacuoles in *S. cerevisiae* and *C. albicans* (Borjihan et al., 2009; Ogita et al., 2006, 2012). Accordingly, 0.63 μM AmB was fully effective for vacuole disruption in parental cells suspended in distilled water (Fig. 4a), but its vacuole-disruptive action was only partly effective against AmB-resistant strains Δatg18 and Δvma1 (Fig. 4b, c).

**Visualization of dansyl-labelled AmB**

AmB has been shown by HPLC to accumulate in the cytoplasm of *C. albicans* cells (Borjihan et al., 2009). This result indicated that the antibiotic is transported across the plasma membrane through an undefined cellular process. Thus, we tracked the distribution of dansyl-labelled AmB by fluorescence microscopy. Like unlabelled AmB, 0.63 μM of the dansyl-labelled antibiotic reduced the viability of the parental strain in distilled water to less than 1.0 % over 2 h. Strains Δatg18 and Δvma1 were resistant to the dansyl-labelled antibiotic as well, and remained at least 95 % viable (data not shown). As shown in Fig. 5(a), dansyl-labelled AmB seriously damaged vacuoles in parental cells, and colocalized with FM4-64 fluorescence in fragmented vacuole membranes scattered throughout the cytoplasm. However, dansyl fluorescence was observed only at the extracellular surface of Δatg18 cells, but not in structurally intact vacuoles (Fig. 5b). On the other hand, dansyl fluorescence was observed at both the extracellular surface and intravacuolar space of Δvma1 cells (Fig. 5c).

**DISCUSSION**

We found that AmB targets the vacuole in nutrient-starved *S. cerevisiae*, via a mechanism that depends on autophagic genes such as ATG18 and VMA1. ATG proteins, especially Atg18p, are essential for autophagosome formation, a key structure early in autophagy (Obara et al., 2008). On the other hand, mutants lacking VMA, a gene that encodes a subunit of v-ATPase, accumulate but do not degrade autophagic bodies in the vacuole in response to nitrogen starvation (Nakamura et al., 1997). Notably, AmB resistance was higher in Δatg18 than in Δvma1. The difference in resistance is likely due to differences in the subcellular localization of the antibiotic, and is consistent with the idea that fungicidal activity of AmB requires autophagy-dependent mobilization to the vacuole. This result implies that accumulation in the vacuole is a key determinant of fungicidal activity, as AmB is trafficked to the vacuole in Δvma1, but not in Δatg18 (Fig. 2). A small release of AmB from autophagic bodies in Δvma1 may thus explain the fungicidal activity observed in this mutant.

Ergosterol, a neutral lipid and a structural component of the fungal plasma membrane, is essential for endocytosis and homotypic vacuole fusion (Kato & Wickner, 2001; Pichler & Riezman, 2004). Thus, ergosterol may help traffic AmB to the vacuole, presumably at the first step of the process. Δerg3 *S. cerevisiae*, which has a defect in ergosterol
In particular, the mutant was found resistant to AmB-induced vacuole disintegration, although the antibiotic permeabilized K\(^+\) ions across the plasma membrane to a similar extent as in the parental strain. Indeed, the most striking feature of this mutant was the inability of AmB to cross the plasma membrane, suggesting that the antibiotic is taken up by ergosterol-dependent endocytosis of the plasma membrane. In this study, we revealed that AmB dramatically enhanced K\(^+\) efflux from parental cells in which the antibiotic is reliably lethal in distilled water. However, the antibiotic also induced a similar level of K\(^+\) efflux from \(\Delta\)atg18 and \(\Delta\)vma1 cells (Fig. 3b, c), despite significantly increased resistance. These results reinforce the model that AmB fungicidal activity is mostly due to vacuole disintegration rather than K\(^+\) leakage across the plasma membrane. Nonetheless, permeabilization of the plasma membrane cannot be excluded entirely, as the antibiotic also induced a slight decrease in the viability of \(\Delta\)atg18 and \(\Delta\)vma1 cells (Fig. 3b, c). Furthermore, we demonstrated that fluorescence from dansyl-labelled AmB accumulated in the plasma membrane of parental, \(\Delta\)atg18 and \(\Delta\)vma1 cells, indicating the formation of K\(^+\) ion channels in complex with ergosterol (Fig. 5). Fluorescence from dansyl-labelled AmB was also found to be spread slightly into the cytoplasm in any of the antibiotic-sensitive as well as the antibiotic-resistant strains, suggesting its endocytic incorporation via internalization of a section of the plasma membrane (Fig. 5).

In nutrient-rich YPD medium, the fungistatic concentration of AmB was higher than the treatment in distilled water, a nutrient-starved condition (Kang et al., 2013; Nakayama et al., 2002). Under this condition, AmB synthesis, is resistant to AmB lethality (Kang et al., 2013).

Fig. 4. Microscopic observation of cells of parental (a), \(\Delta\)atg18 (b) and \(\Delta\)vma1 (c) strains of \(S.\) cerevisiae exposed to AmB. Cells \((1 \times 10^7 \text{ cells ml}^{-1})\) were labelled with the vacuole-specific fluorescent dye FM4-64, incubated at 30 °C for 120 min in distilled water spiked with or without 0.63 μM AmB, and imaged by bright-field (top) and fluorescence microscopy (bottom). The most representative photographic images are shown in (a), (b) and (c). Bars, 2 μm. Cells with non-disrupted vacuole images were observed at a ratio of less than 3 % of total cells in (a), and were observed at significantly higher ratios of 75 ± 9 % in (b) and 63 ± 9 % in (c), respectively. The data are means ± SD of the ratio of vacuole-disrupted cells upon observation of 10 cells at each of 10 different stages.

Fig. 5. Vacuole morphology and subcellular localization of dansyl-labelled AmB in parental (a), \(\Delta\)atg18 (b) and \(\Delta\)vma1 (c) \(S.\) cerevisiae. Cells \((1 \times 10^7 \text{ cells ml}^{-1})\) were incubated at 30 °C for 120 min in distilled water supplemented with 0.63 μM dansyl-labelled AmB, and imaged by bright-field (top) and fluorescence microscopy for FM4-64-labelled vacuoles (middle) and dansyl-labelled AmB (bottom). The most representative photographic images are shown in (a), (b) and (c). Bars, 2 μm. Cells with non-disrupted vacuole images were observed at a ratio of less than 5 % of total cells in (a), and were observed at significantly higher ratios of 71 ± 9 % in (b) and 64 ± 8 % in (c), respectively. The data are means ± SD of the ratio of vacuole-disrupted cells upon microscopic observation of 10 cells at each of 10 different stages.
should only increase K⁺ ion efflux across the plasma membrane, but it should not reach the vacuole because of minimal autophagy. Therefore, in the condition such as human serum, the antibiotic may be lethal to the pathogenic fungi to a lesser extent than expected from its dose in patients with serious systemic fungal infections. Thus, in a nutrient-rich condition, other compounds are required to enhance the activity of AmB against vacuoles, which, in comparison with plasma membrane permeabilization, is fungicidal rather than fungistatic. One such compound is N-methyl-N’-dodecylguanidine, a synthetic compound that enhances the fungicidal activity of AmB against S. cerevisiae and C. albicans in nutrient-rich media even at lower doses (Kang et al., 2013; Yutani et al., 2011).

Our study indicated the possibility that AmB is trafficked to the vacuolar lumen via autophagy, and that the antibiotic interacts directly with the luminal leaf of the vacuolar membrane. A recent study showed a sterol sponge model in which extramembranous aggregates of AmB extract ergosterol from phospholipid bilayers and thereby kill yeast (Anderson et al., 2014). In fungi, trafficking of AmB to the vacuole may be an attempt to eliminate such a fungicidal compound that increases plasma membrane permeability or causes ergosterol extraction on the cell surface. However, this response is instead likely to result in fungicidal toxicity due to the consequent disintegration of the vacuole. Thus, autophagy-dependent transport of AmB to the vacuole is considered to be a suicide-like response, not a protective response against toxicity. At present, it is unclear whether AmB newly acquires the vacuole-disruptive activity due to structural modification by a certain vacuolar enzyme.

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