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Running headline: BITC boosts AmB fungicidal activity

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Significance and Impact of the Study:

Amphotericin B, a polyene macrolide antifungal agent, is widely used to treat systemic mycoses; however, a reduced dosage is preferred to avoid side effects in patients during antifungal therapy. In this study, benzyl isothiocyanate, a cruciferous plant-derived compound, considerably enhanced the fungicidal activity of amphotericin B. This combinatorial lethal effect was attributed to vacuole disruption in both the model yeast *Saccharomyces cerevisiae* and the fungal pathogen, *Candida albicans*. The fungicidal activity of benzyl isothiocyanate and amphotericin B in combination may have significant implications on the development of vacuole-targeting chemotherapy against fungal infections.

Abstract

Amphotericin B (AmB), a typical polyene macrolide antifungal agent, is widely used to treat systemic mycoses. In the present study, we show that the fungicidal activity of AmB was enhanced by benzyl isothiocyanate (BITC), a cruciferous plant-derived compound, in the budding yeast, *Saccharomyces cerevisiae*. In addition to forming a molecular complex with ergosterol present in fungal cell membranes to form K^+-permeable ion channels, AmB has been recognized to mediate vacuolar membrane disruption resulting in lethal effects. BITC showed no effect on AmB-induced plasma membrane permeability; however, it amplified AmB-induced vacuolar membrane disruption in *S. cerevisiae*. Furthermore, the BITC-enhanced fungicidal effects of AmB significantly decreased cell viability due to the disruption of vacuoles in the pathogenic fungus *Candida albicans*. The application of the combinatorial antifungal effect of AmB and BITC may aid in dose reduction of AmB in clinical antifungal therapy and consequently decrease side effects in patients. These results also have significant implications for the development of vacuole-targeting chemotherapy against fungal infections.

Keywords: benzyl isothiocyanate, amphotericin B, fungicidal activity, amplification, vacuole disruption

Introduction

Amphotericin B (AmB, Fig. 1a) is a polyene macrolide antibiotic, which has been widely employed as a potent antifungal agent in topical and systemic therapy for fungal infections. Notably, AmB also affects the integrity of cell membranes in human cells and can be toxic at higher doses (Yano *et al*. 2009; Hamill 2013). The mechanism of action of AmB involves the binding of the hydrophobic moiety of AmB to ergosterol molecules embedded in the fungal plasma membrane to form K^+-permeable ion channels (Baginski *et al*. 2005; Carrillo-Muñoz *et al*. 2006). However, alternative modes of action have also been proposed as ion leakage does not necessarily result in loss of cell viability (Chen *et al*. 1978). AmB also generates superoxide anions and causes oxidative damage in the pathogenic fungus, *Candida albicans*, although this mode of action likely depends on K^+ efflux as well (Kim *et al*. 2012). The *nss1* mutants in *Saccharomyces cerevisiae*, characterized by severe defects in vacuolar protein sorting and morphology, are strongly sensitive to polyenes such as AmB (Bhuiyan *et al*. 1999). While different modes of action are attributed to AmB, the precise mechanism of its

lethal action is poorly understood. As antifungals with novel mechanisms of action are being developed to treat fungal infectious diseases, the medical application of AmB awaits further improvement.

We recently demonstrated that lethal doses of AmB dramatically alter the structure of fungal vacuoles (Ogita *et al*. 2006; Ogita *et al*. 2007; Borjihan *et al*. 2009; Ogita *et al*. 2010; Ogita *et al*. 2012). This mode of fungicidal action of AmB is markedly enhanced by allicin, an allyl sulfur compound from garlic, and is ineffective toward vacuole-deficient cells of human promyelocytic leukemia (HL-60) cell line (Ogita *et al*. 2006). The enhancement of the vacuole-targeting activity of AmB has also been shown to be amplified in *C. albicans* by the addition of a synthetic analog of the alkyl side chain of niphimycin known as *N*-methyl-*N*"-dodecylguanidine (Yutani *et al.* 2011). These results indicate that the additive lethality of these compounds with AmB may not solely be due to an increase in plasma ion permeability (Ogita *et al*. 2006; Ogita *et al*. 2007; Ogita *et al*. 2010), and may be closely related to the vacuole trafficking machinery and/or increased susceptibility of the organelle membrane to the

disruptive action of AmB. Our recent research revealed that the fungicidal activity of AmB requires autophagy-dependent trafficking into the intra-vacuolar lumen, and AmB interacts with the luminal leaf of the vacuolar membrane to cause structurally catastrophic effects (Yoshioka *et al*. 2016). The use of synergistic compounds to enhance AmB lethality may reduce side effects by reducing the dose of AmB, thereby improving chemotherapy for fungal diseases.

Benzyl isothiocyanate (BITC, Fig. 1b), a cruciferous plant-derived compound, has been shown to inhibit chemically-induced cancer in animal models (Srivastava and Singh 2004) and growth suppression of pancreatic cancer cells due to NF-κB inactivation *in vitro* and *in vivo* (Batra *et al.* 2010). Methylsulfinylhexyl isothiocyanate, a compound similar to BITC, is known to have important medical benefits such as ameliorating diabetic nephropathy in mice (Fukuchi *et al.* 2004). BITC is a potential inducer of glutathione *S*-transferase (GST), decreasing intracellular glutathione (GSH) levels and inducing oxidative stress in rat liver epithelial RL34 cells (Nakamura *et al*. 2000). Also, BITC exhibits rapid and strong

bactericidal effect against oral pathogens involved in periodontal disease and other gram-negative bacteria, whereas gram-positive bacteria mainly display growth inhibition or remain unaffected (Sofrata *et al*. 2011). Furthermore, the antifungal activities of BITC against *Sclerotinia sclerotiorum* (Lib.) de Bary and *Gibberella moniliformis* Wineland [anamorph *Fusarium verticillioides* (Sacc.) Nirenberg] have also been reported (Kurt *et al*. 2011; Azaiez *et al*. 2013). Although BITC is a promising health-promoting, anti-cancer, and antifungal compound, its application is limited due to its ability to cause morphological and functional aberrations to certain cells/tissues *in vitro* and *in vivo* (Bruggeman *et al*. 1986; Temmink *et al*. 1986).

In this study, we focused on the enhanced fungicidal effects exhibited by BITC in combination with AmB in *S. cerevisiae* and the pathogenic fungus, *C. albicans*. We investigated whether BITC could increase AmB efficacy at low concentrations, with the potential to minimize the drug-induced side effects of AmB during antifungal therapy.

Results and discussion

Effect of BITC on the fungicidal activity of AmB in S. cerevisiae

Enhancement of the effects of polyenes such as AmB (see Fig. 1a) is essential in clinical therapy as a minimum dose is required to reduce side effects (Laniado-Laborín and Cabrales-Vargas 2009). We first examined the effect of BITC on AmB lethality by measuring changes in the colony-forming capacity of *S. cerevisiae* cells. As shown in Fig. 2a, while cells are mostly resistant to the action of AmB at 0.15 μmol l^{-1}, *S. cerevisiae* cells were subjected to considerable lethal damage when AmB was added at 1 μmol l^{-1} in a dose-dependent manner. BITC (see Fig. 1b) exhibited only a weak lethal effect against *S. cerevisiae* cells in S-buffer and slightly reduced the cell viability at 50 μmol l^{-1}. The number of viable cells was reduced by approximately 50% after treatment with 100 μmol l^{-1} BITC for 2 h (Fig. 2b). Interestingly, AmB exhibited a drastically lethal effect on yeast cells even at 0.15 μmol l^{-1}, a non-lethal concentration when used alone, in the presence of 50 μmol l^{-1} BITC (Fig. 2c). These results indicated that the simultaneous addition of BITC and a sub-inhibitory (non-lethal)

concentration of AmB was sufficient to reduce cell viability, suggesting that BITC enhanced the lethal effects of AmB.

Effects of BITC and AmB on plasma membrane permeability

Despite some models proposing alternate modes of action, it has been widely accepted that the fungicidal activity of AmB is a result of channel formation across the plasma membrane after it selectively binds to ergosterol, thereby enhancing the leakage of intracellular K^+ ions and other ionic substances (Brajtburg *et al*. 1990; Ellis 2002; Baginski *et al*. 2005). Therefore, the effects of K^+ ion leakage caused by BITC, AmB, and combinations from intact cells were investigated as an indicator of cell membrane damage to determine whether the lethal effects are associated with the cell membrane damage. AmB is usually used as a positive control for cell membrane integrity assays because it specifically leaks K^+ ions from intact cells. However, this experiment focused on the action of AmB; the membrane-disruptive action should be compared with compounds other apart from AmB. On the other hand, ethanol has

been known to exhibit remarkable cell membrane damage on a wide range of microbial strains, causing the non-specific leakage of various substances, including intracellular ions, such as K^+ and H^+ ions, in yeast cells (Lam *et al*. 2014). Since almost all the intracellular K^+ ions leak due to cell membrane damage from ethanol treatment, ethanol was used as an index to confirm the relative intracellular ion leakage scale, which is a measure of membrane damage effect. In line with the study by Lam *et al*. (2014), we observed that K^+ ion leakage from intact cells of *S. cerevisiae* was drastically increased by the addition of 70% ethanol (v/v) in the S-buffer for 3 h (Fig. 3). The membrane disruption, as represented by K^+ ion leakage, upon addition of AmB at a non-lethal concentration (0.15 μmol l^{-1}) was approximately 50% of that observed by the addition of 70% ethanol. The addition of 50 μmol l^{-1} BITC resulted in a disrupted membrane integrity effect comparable to that from the addition of AmB alone at a non-lethal concentration. Although amplifying fungicidal activity (see Fig. 2c), the membrane-disruptive action of AmB was not altered in the presence of BITC at 50 μmol l^{-1} (Fig. 3). These results

indicate that the enhanced lethal action of BITC and AmB in combination cannot be attributed to membrane disruption due to an enhanced efflux of K^+ ion from intact cells.

Effects of BITC and AmB on vacuole morphology

Vacuoles are organelles involved in osmoregulation, ion homeostasis, and cell volume regulation in fungal cells (Wickner 2002). Various hydrolytic enzymes, including proteases and nucleases, are thought to accumulate in vacuoles; hence, damage to these organelles is considered a critical step in the induction of cell death (Obara *et al*. 2001). We recently reported a stimulatory effect of allicin, an allyl sulfur compound from garlic, on the fungicidal activity of AmB, in which the vacuole was determined to be the target of their combined actions (Ogita *et al*. 2012). As the yeast cells were sensitive to BITC and AmB in combination, we examined the mechanism of BITC-dependent fungicidal activity of AmB as regards yeast vacuolar morphology using the vacuolar-staining fluorescent dye FM4-64. Vacuoles of *S. cerevisiae* cells were observed to possess extremely spherical morphology in

cells treated with no drug, and such typical spherical morphology was also observed for vacuoles of most cells treated with either 50 μmol l^{-1} BITC or 0.15 μmol l^{-1} AmB, which are both non-lethal concentrations (Fig. 4a). In contrast, scattering of the fluorescent dye, FM4-64, into the cytoplasm was observed in cells treated with AmB at a lethal concentration of 1 μmol l^{-1} for 3 h, reflecting a defective vacuolar morphology (78.6 ± 12.6%). Furthermore, AmB-treatment, even at a non-lethal concentration of 0.15 μmol l^{-1}, was able to disrupt the vacuolar morphology in yeast cells (80.3 ± 4.6%) (Fig. 4a and b) in the presence of 50 μmol l^{-1} BITC. Since the ratio of these vacuole-disrupted cells appeared to reflect a decrease in cell viability (see Fig. 2), the combined fungicidal action of BITC and AmB was attributed to the disruption of vacuolar morphology rather than alteration of plasma membrane permeability.

Effects of BITC and AmB in the pathogenic fungus, C. albicans

C. albicans, a pathogenic fungus, is known to be the main causative agent of invasive candidiasis and poses a serious healthcare problem with a high mortality rate in patients with

immune diseases (Thompson *et al*. 2019). Despite its toxicity at particular doses, AmB has been a mainstay of antifungal therapy and remained the 'gold standard' for treating disseminated life-threatening fungal infections primarily caused by *C. albicans* (Ellis 2002). We examined whether BITC enhances the fungicidal activity of AmB against *C. albicans* and observed changes in the vacuole morphology owing to the combined action of BITC and AmB. The cell viability of *C. albicans* NBRC 1061 was reduced to 11.0 ± 1.0% after a 3-h treatment with 50 μmol l^{-1} BITC (data not shown). Thus, *C. albicans* was more sensitive to BITC than *S. cerevisiae* considering the results in Fig. 2b, while 40 μmol l^{-1} BITC did not show any fungicidal effects against *C. albicans* (Fig. 5a). The concentration of BITC at 40 μmol l^{-1}, which is the highest concentration showing no lethal effect, was applied to confirm the amplification effect of the combination with AmB against *C. albicans* cells. In this experiment, 0.15 μmol l^{-1} AmB, which has no lethal effect against *S. cerevisiae* cells (see Fig. 2a), slightly reduced the viability of *C. albicans* cells to 76.0 ± 4.1% (Fig. 5a). The observation that *C. albicans* cells were more sensitive to AmB than *S. cerevisiae* cells

supported a previous study describing the antifungal efficacy of AmB (Ogita *et al*. 2006). As expected, the combined action of BITC-AmB considerably reduced the colony-forming units of *C. albicans* cells after a 3-h treatment (Fig. 5a). Similar to cells treated with no drug, the vacuoles of *C. albicans* cells displayed spherical morphology in most cells treated individually with either 40 μmol l^{-1} BITC or 0.15 μmol l^{-1} AmB. In contrast, the vacuoles of BITC-AmB-treated cells were mostly disrupted, reflected by the diffused FM4-64 staining observed in the cytoplasm of *C. albicans* cells (73.5 ± 4.6%) (Figs 5b and c). These results indicate that BITC enhances fungicidal effects mediated by AmB-induced vacuolar membrane disruption not only in *S. cerevisiae* but also in the pathogenic fungus, *C. albicans*.

Numerous reports indicate that BITC prevents chemically-induced cancer in laboratory animals, and it has been postulated that BITC might also be chemoprotective in humans. On the other hand, there is accumulating evidence that this compound is a potent genotoxin in mammalian cells (Kassie et al. 1999). Although it may be necessary to eliminate the toxicity of BITC in patients, the combined fungicidal activities of AmB and BITC may

have significant implications for the development of vacuole-targeting chemotherapy with minimized antibiotic-induced side effects in clinical therapy against fungal infections.

Materials and methods

Chemicals

Amphotericin B and benzyl isothiocyanate were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). FM4-64 and K^+ ion assay kit were purchased from Thermo Fisher Scientific (Kanagawa, Japan) and HACH (Loveland, CO, U.S.A.), respectively. All other reagents were of analytical grade.

Measurement of cell growth and viability

S. cerevisiae W303-1A and *C. albicans* NBRC 1061 (formerly IFO 1061) cells were used in this experiment. Cells were grown overnight at 30ºC with vigorous shaking in yeast-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% D-glucose). Overnight

cultured cells were harvested, washed with S-buffer (50 mmol l^{-1} succinate, pH 6.0), and diluted into fresh S-buffer to a concentration of 5×10^7 cells ml^{-1}. Cells were then incubated with vigorous shaking at 30°C in S-buffer with or without the addition of each compound at various concentrations and plated on YPD medium plates containing 1.8% (w/v) agar. The cell viability and lethality upon AmB treatment with or without BITC were characterized by assessing colony-forming units (CFU) after 48 h at 30°C.

Leakage of K^+ ions

Cells cultured overnight in YPD medium were harvested by centrifugation, washed twice, and resuspended in S-buffer at a cell density of 5×10^7 cells ml^{-1}. The cell suspensions were supplemented with or without each compound and incubated with vigorous shaking at 30°C for 3 h. The supernatants obtained after removal of the cells by centrifugation were used for the quantification of K^+ ions released from the cells. K^+ ions from the cells were reacted with sodium tetraphenylborate to form potassium tetraphenylborate, an insoluble white solid. The

amount of turbidity produced is proportional to the K^+ concentration. The measurement wavelength is 650 nm for spectrophotometers. This quantification was performed with a K^+ ion assay kit (Loveland, CO, U.S.A.) based on the tetraphenylborate method (Ramotowski and Szczesniak 1967).

Vacuole staining

Vacuoles were stained with the fluorescent probe FM4-64 (*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)-phenyl)hexatrienyl)pyridinium dibromide) according to previously described methods (Vida and Emr 1995; Kato and Wickner 2001) with some modification. Cells from an overnight culture were inoculated into a freshly prepared YPD medium to obtain a density of approximately 5×10^7 cells ml^{-1}. After incubation in YPD medium containing 5 μmol l^{-1} FM4-64 at 30°C for 1 h, cells were collected by centrifugation, suspended into S-buffer, and incubated again in the presence or absence of each compound at 30°C for 3 h. After washing the cells, they were resuspended in distilled water. The cells were observed

under a phase-contrast microscope and a fluorescence microscope at an excitation of 520-550 nm and emission of 580 nm to visualize vacuoles. The percentage of cells with disrupted vacuoles was determined by counting 10 cells in 10 different microscopic fields.

Statistical methods

Statistical evaluation was performed using Student's *t*-test; $p < 0.05$ was considered to represent statistical significance.

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Conflict of Interest

The authors of this manuscript declare no conflict of interest.

Author contributions

All authors contributed to the conception and design, and analysis of data. N.Y., W.M., and A.O. carried out the experiments. All authors contributed to drafting or critically revising the manuscript and approval of the final submitted version.

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Legend to figures

Figure 1. Structure of amphotericin B (a) and benzyl isothiocyanate (b).

Figure 2. Effect of amphotericin B (AmB) on cell viability in the absence or presence of benzyl isothiocyanate (BITC) in *S. cerevisiae*. Cells (5×10^7 cells ml^{-1}) were incubated with AmB alone at the following concentrations; 0 (○), 0.15 (●), 0.5 (□), or 1 μmol l^{-1} (■) (a). Cells were incubated with BITC alone at 0 (○), 50 (●), or 100 μmol l^{-1} (□) (b). Cells were incubated with AmB at 0 (○), 0.15 (●), or 0.5 μmol l^{-1} (□) in the presence of 50 μmol l^{-1} BITC (c). Each data point represents the mean ± standard deviation of triplicate assays.

Figure 3. Effect of amphotericin B (AmB), benzyl isothiocyanate (BITC), and a combination of AmB and BITC on K^+ leakage from intact cells of *S. cerevisiae*. Cells (5×10^7 cells ml^{-1}) were incubated in S-buffer at 30°C for 3 h with each compound at indicated concentrations. Each data point represents the mean ± standard deviation of triplicate assays, wherein n.s. indicates no significant differences between AmB and

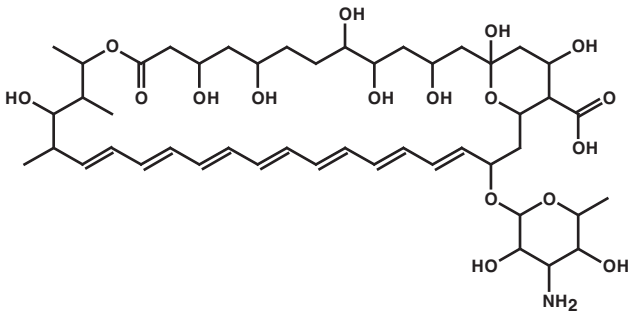
BITC, AmB and BITC-AmB, or BITC and BITC-AmB-treated cells, and asterisk indicates significant differences between ethanol and AmB, BITC, or BITC-AmB-treated cells ($p < 0.05$).

Figure 4. Effect of amphotericin B (AmB), benzyl isothiocyanate (BITC), and a combination of AmB and BITC on the vacuole morphology. Cells (5×10^7 cells ml^{-1}) were preincubated with FM4-64 followed by incubation at 30°C for 3 h in S-buffer in the absence or presence of each compound at the indicated concentrations and were imaged by bright-field (top) and fluorescence microscopy (bottom). The most representative photographic images of bright-field (top) and fluorescence microscopy (bottom) are shown (a); scale bar, 2 μm. The percentage of cells with disrupted vacuoles was determined by counting 10 cells in 10 different microscopic fields (b). The data are expressed as means ± standard deviation, and asterisk indicates significant differences compared to the cells treated with no drug ($p < 0.05$).

Figure 5. Effect of amphotericin B (AmB), benzyl isothiocyanate (BITC), and a combination of AmB and BITC on cell viability (a), the vacuole morphology (b), and the percentage of cells with disrupted vacuoles (c) in *C. albicans*. Cells (5×10^7 cells ml^{-1}) were incubated at 30°C for 3 h with S-buffer in the absence or presence of each compound at the indicated concentrations (a). Cell viability is indicated by the percentage of CFU per ml after 3 h incubation, in which the initial inoculum size corresponding to 5×10^7 cells ml^{-1} was considered to be 100%. Each data point represents the mean ± standard deviation of triplicate assays. Asterisk indicates significant differences between AmB alone and BITC-AmB-treated cells ($p < 0.05$). Cells (5×10^7 cells ml^{-1}) were preincubated with FM4-64 followed by incubation at 30°C for 3 h in S-buffer in the absence or presence of each compound at the indicated concentrations and the most representative photographic images of bright-field (top) and fluorescence microscopy (bottom) are shown (b); Scale bar, 2 μm. The percentage of cells with disrupted vacuoles was determined by counting 10 cells

in 10 different microscopic fields (c). The data are expressed as means ± standard deviation. Asterisk and n.s. indicate significant differences and no significant differences compared to the cells treated with no drug, respectively ($p < 0.05$).

(a)



(b)

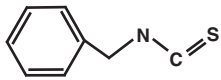


Fig. 1. Yamada N, et al.

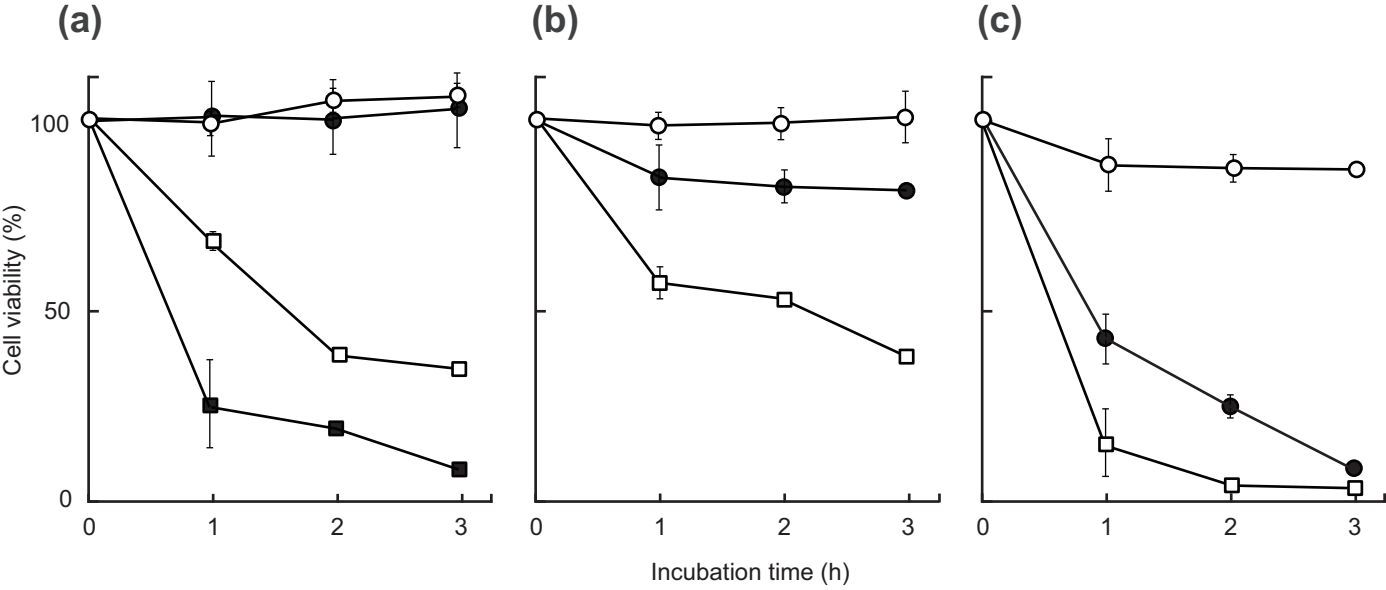
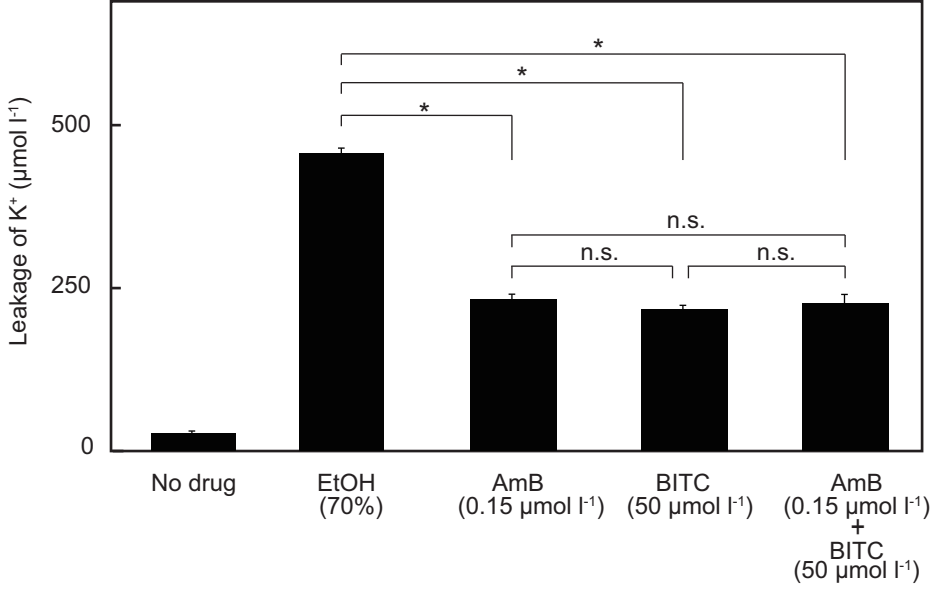


Fig. 2. Yamada N, et al.



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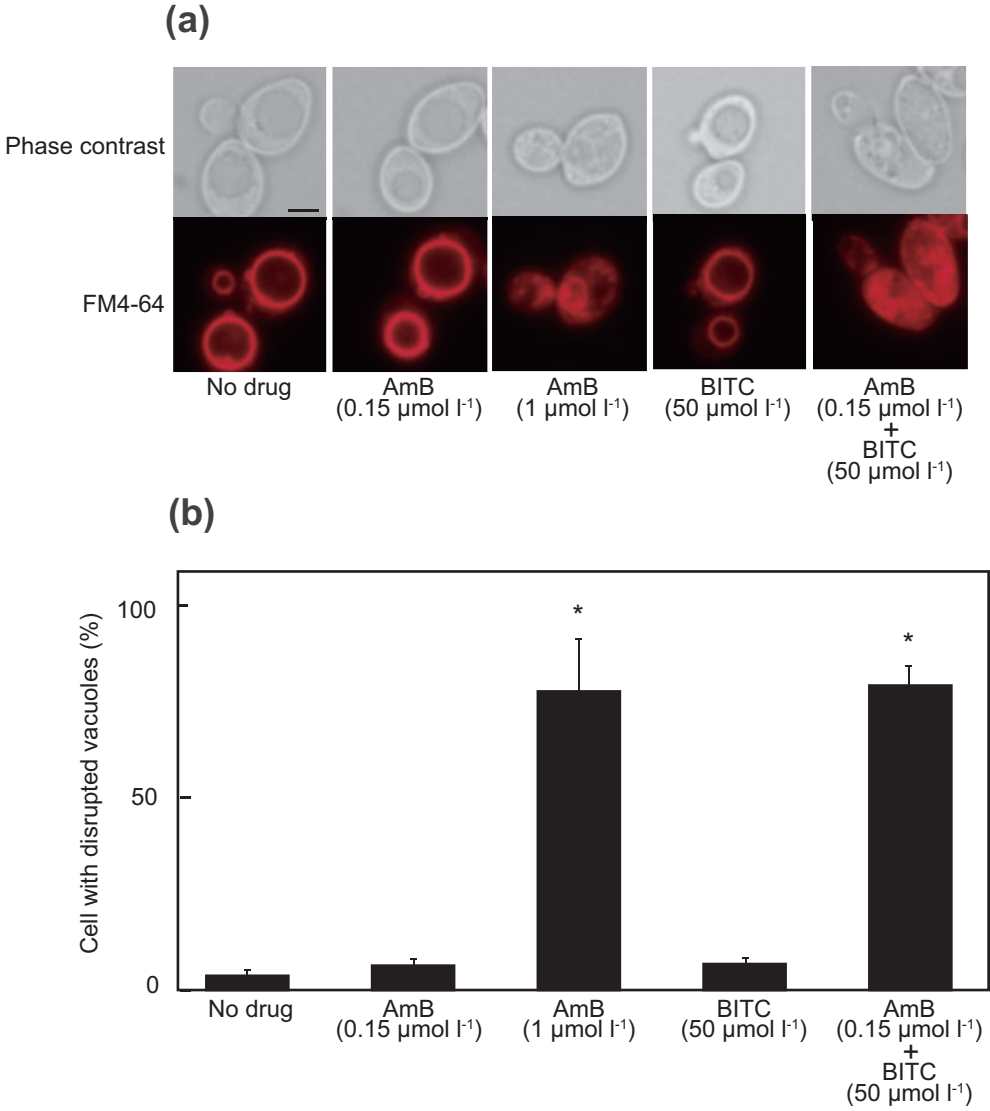

Fig. 4. Yamada N, et al.

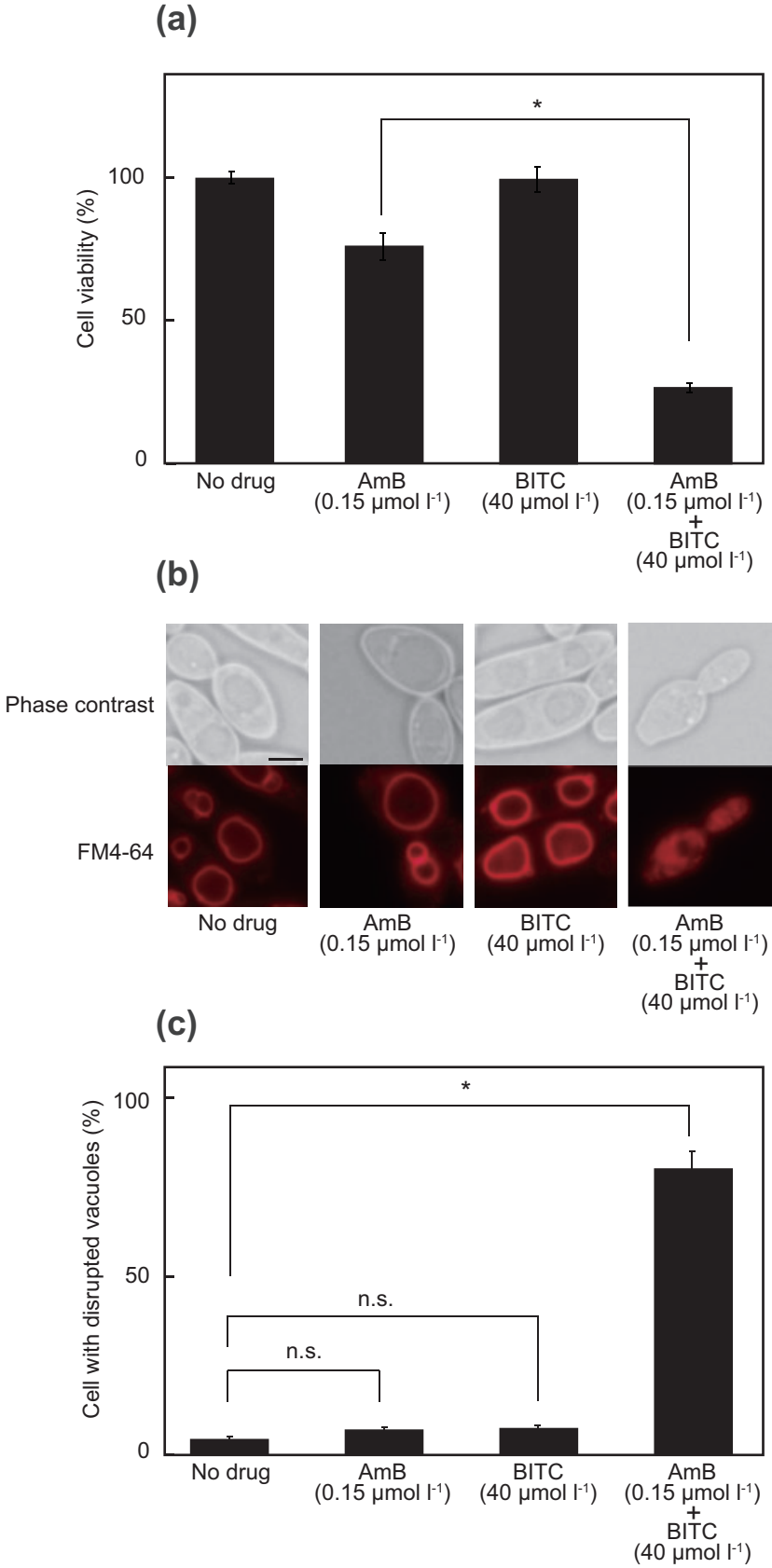

Fig. 5. Yamada N, et al.