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Synergistic Fungicidal Activities of Amphotericin B and *N*-Methyl-*N*″-dodecylguanidine: A Constituent of Polyol Macrolide Antibiotic Niphimycin

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Abstract The synergy between the alkylguanidinium chain of niphimycin (NM), a polyol macrolide antibiotic, and polyene macrolide amphotericin B (AmB) without such an alkyl side chain was examined using *N*-methyl-*N*″-alkylguanidines as its synthetic analogs. Among the analogs, *N*-methyl-*N*″-dodecylguanidine (MC12) most strongly inhibited the growth of *Saccharomyces cerevisiae* cells and those of other fungal strains in synergy with AmB. MC12 itself was not lethal but the analog could be a cause of a rapid cell death progression of yeast cells in the presence of AmB at a nonlethal concentration. Their combined actions resulted in the generation of NM-like fungicidal activity that depended on plasma membrane disability and cellular reactive oxygen species production. We also found an aberrant vacuolar morphogenesis and an associated vacuolar membrane disability in cells treated simultaneously with MC12 and AmB, as in the case of NM-treated cells. These findings support the idea that the alkylguanidinium chain plays a major role in the fungicidal activity of NM in cooperation with the polyol lactone ring as its enhancer.

Keywords niphimycin, amphotericin B, synergy, vacuole, *Saccharomyces cerevisiae*

Introduction

Niphimycin (NM, Fig. 1) was first isolated from a strain of *Streptomyces hygroscopicus*, and was designated a guanidylpolyol macrolide antibiotic because of its structure consisting of polyol lactone ring and an alkylguanidinium chain [1, 2]. Despite some structural analogy to amphotericin B (AmB, Fig. 1), a polyene macrolide antibiotic, NM shows broad antimicrobial activities against Gram-positive bacteria, which are absolutely resistant to AmB, in addition to filamentous fungi and yeasts [3]. The mode of fungicidal activity of NM is apparently distinguishable from that of AmB as observed from the disruptive effect of NM on the yeast plasma membrane by its direct interaction with phospholipids, but not with ergosterol, a molecular target of AmB [4]. We also found the involvement of cellular reactive oxygen species (ROS), which are not generated in AmB-treated cells, in the fungicidal activity of NM.

Our attention was thus focused on the role of the alkylguanidinium chain of NM, which is absent from the structure of AmB, in the fungicidal activity of NM. Among several synthetic analogs of the alkylguanidinium chain, *N*-methyl-*N*″-hexadecylguanidine (MC16, Fig. 1) was found to exert the highest fungicidal activity by directly damaging

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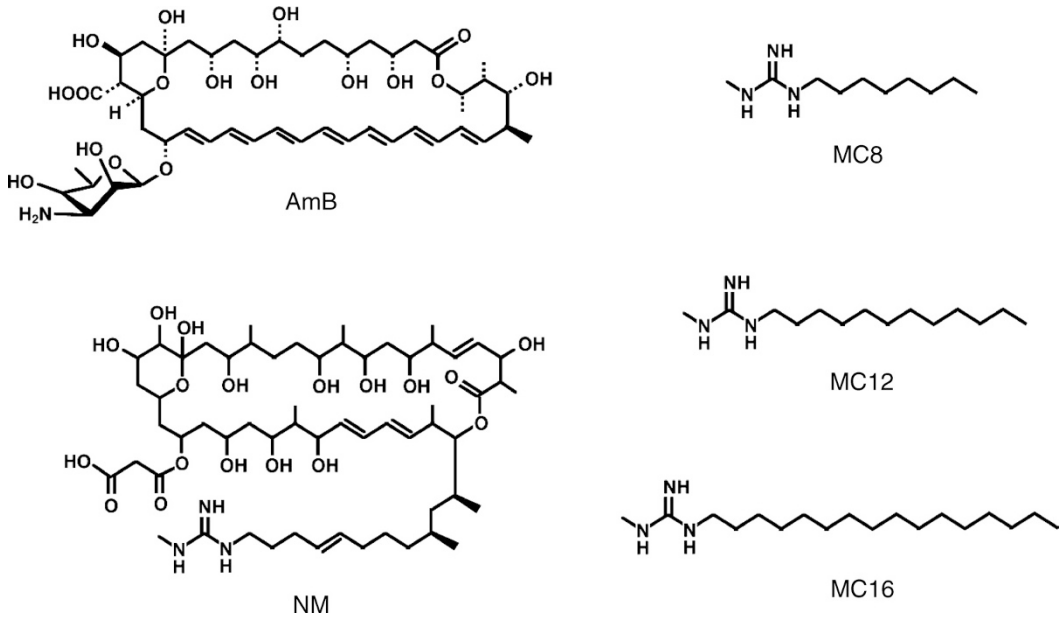

Fig. 1 Structures of amphotericin B (AmB), niphimycin (NM), *N*-methyl-*N″*-octylguanidine (MC8), *N*-methyl-*N″*-dodecylguanidine (MC12), and *N*-methyl-*N″*-hexadecylguanidine(MC16).

the plasma membrane and inducing ROS generation in a NM-like manner [5]. However, MIC of MC16 was much higher than that of NM, suggesting the role of the polyol lactone ring as a constituent that enhances the interaction of the alkylguanidinium chain with a corresponding target molecule. The polyol lactone ring of NM may alternatively exhibit its own fungicidal activity as expected from the polyene lactone ring of AmB without such an alkyl side chain. This constituent has never been prepared by the chemical degradation of NM or by any other methods and thus its physiological function remains unclear.

We therefore examined the effects of the simultaneous application of the alkylguanidine and AmB as a substitute for the polyol lactone ring of NM. It was also valuable to confirm whether AmB can enhance the antifungal activity other than that dependent on the plasma membrane permeability change. *N*-Methyl-*N″*-dodecylguanidine (MC12) was not lethal but this analog could strongly inhibit the growth of *Saccharomyces cerevisiae* and some other fungal strains when used in combination with AmB. Their combined actions resulted in the generation of NM-like fungicidal activity that depended on plasma membrane disability and cellular ROS production in *S. cerevisiae* cells. In addition, we found an aberrant vacuole morphogenesis in cells treated with MC12 and AmB each at the nonlethal concentration, as in the case with NM-treated cells.

Materials and Methods

Measurement of Cell Growth and Viability

MICs of *N*-methyl-*N″*-alkylguanidines, fatty acids, and alkylamines were determined for *S. cerevisiae* X 2180- 1A (a) in the presence or absence of AmB by the serial 2-fold broth dilution method as follows [6]. After diluting an overnight culture with YPD medium (yeast extract 1%, peptone 2%, and glucose 2%) to 10^6 cells/ml, the cell suspension was incubated in a freshly prepared medium in a 96-well microplate at 30°C for 48 hours. MICs of MC12 and AmB were also determined for *Candida albicans* IFO1061, *Aspergillus niger* ATCC 6275, *Rhizopus oryzae* IFO 4766 and *Bacillus subtilis* IFO 3007 in addition to *S. cerevisiae* X2180-1A (a) by applying the checkerboard technique [7]. The filamentous fungi were precultivated in 2.5% malt extract medium (Oriental Yeast Co., Tokyo, Japan) at 30°C for 24 hours and the overnight cultures were diluted 100-fold with the same medium for MIC determination at 30°C for 48 hours.

S. cerevisiae X2180-1A (a) was used in the following experiments to examine the effects of MC12, AmB and their combination on the growth and other properties of the yeast cells. Cells from an overnight culture were incubated in YPD medium at an initial cell density of approximately 10^7 cells/ml in the presence or absence of each compound at 30°C for the indicated times. The cell viability was

determined by counting viable cell number as colony forming units [8].

Assay of Plasma Membrane Permeability Change

The plasma membrane permeability change was assayed by measuring the efflux of cytoplasmic K^+ as follows. Overnight cultured cells were harvested by centrifugation, washed with 50 mM succinate buffer (pH 6.0) and suspended in the buffer to obtain a density of 10^8 cells/ml. The cell suspension with or without the addition of each compound was shaken at 30°C and the supernatant obtained after removing cells by centrifugation was assayed for its content of K^+ according to our previously described method [4].

Assay of Cellular ROS Production

The effects of MC12, AmB and their combination on cellular ROS productions were assayed by following a previously described method [9]. Cells from an overnight culture were inoculated into a freshly prepared medium to obtain a density of approximately 10^7 cells/ml. Cells were then incubated with 40 μM 2′,7′-dichlorodihydrofluorescein diacetate at 30°C for 60 minutes, and then collected by centrifugation and suspended in an equal volume of the medium. The cell suspensions (1.0 ml) were further treated with or without each compound for 60 minutes, and then washed and resuspended in 100 μl of phosphate-buffered saline. Fluorescence from cells was then measured with excitation at 480 nm and emission at 530 nm.

Vacuole Staining

Vacuoles were stained with a fluorescent probe, FM4-64 (*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)-phenyl)hexatrienyl)pyridinium dibromide) as follows [10]. Cells from an overnight culture were inoculated into a freshly prepared medium to obtain a density of approximately 10^7 cells/ml. After incubating with 5 μM FM4-64 at 30°C for 30 minutes, cells were collected by centrifugation, suspended in a fresh medium, and incubated again in the presence or absence of each compound at 30°C for 30 minutes. Cells were then observed under a phase-contrast microscope and a fluorescence microscope with excitation at 480 nm and emission at 530 nm.

Transmission Electron Microscopy

Cells were treated for transmission electron microscopy as follows. An overnight grown culture was inoculated into freshly prepared medium to obtain an initial cell density of approximately 10^7 cells/ml. Cells were then grown in the presence or absence of each compound 30°C for 60 minutes with vigorous shaking. After fixing with 2.5% (v/v) glutaraldehyde at room temperature for 3 hours, cells were washed three times in 0.1 M sodium potassium phosphate buffer (pH 7.0) and postfixed with 2% (v/v) osmium tetroxide for 60 minutes. Cells were then dehydrated with 10-minutes serial ethanol washes (50, 70, 90, 95, and 4 times in 100%) and infiltrated with 25% quetol 651 in 75% ethanol on a rotator overnight at room temperature. The ratio of quetol was increased gradually to 100% for 3 days. Cells were pelleted, transferred to capsules, overlaid with fresh quetol for polymerization at 65°C for 24∼48 hours. Thin sections were cut, stained with uranyl acetate and lead acetate, and observed under a transmission electron microscope.

Chemicals

N-Methyl-*N*″-octylguanidine (MC8), MC12, and MC16 were synthesized as described previously [5]. FM4-64 was a product of Molecular Probes (Eugene, OR, USA). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Results and Discussion

Synergy between MC12 and AmB

We first measured MICs of MC8, MC12 and MC16 (Fig. 1) against *S. cerevisiae* in the presence or absence of 0.5 μM AmB in comparison with the compounds having long-chain alkyl moieties such as free fatty acids and alkylamines. AmB did not inhibit yeast cell growth at this concentration of 0.5 μM as its MIC was determined to be 1 μM. As summarized in Table 1, free fatty acids tested did not

Table 1 MICs of fatty acids, alkylamines, and *N*-methyl-*N*″-alkyl guanidine derivates in the presence or absence of AmB against *Saccharomyces cerevisiae* cells

Test compounds	MIC (μM) with	
	None	AmB[a]
Lauric acid	>400	>400
Myristic acid	>400	>400
Palmitic acid	>400	>400
Octylamine	>400	>400
Laurylamine	50	25
Hexadecylamine	50	25
MC8	>400	>400
MC12	100	6.25
MC16	100	12.5

[a] Amphotericin B (AmB) was added at 0.5 μM.

inhibit the growth of *S. cerevisiae* cells at 400 μM even in the presence of AmB regardless of the length of their alkyl or acyl chains. Alkylamine with the lauryl or hexadecyl chain, but not with the octyl chain, inhibited the yeast cell growth at 50 μM. However, their MICs only slightly decreased in the presence of AmB, reflecting the lack of effective synergy between the actions of alkylamine and this polyene macrolide antibiotic. MC8 showed no growth inhibitory activity with or without AmB. In contrast, the presence of AmB could significantly decrease the MICs of MC12 and MC16, which were commonly detected at 100 μM in the absence of 0.5 μM AmB. Our previous study suggests that the polyol lactone ring of NM functions as a carrier of the alkylguanidinium chain which enables the interaction of the chain with the corresponding molecular target [5]. This study rather supported the idea that the polyol lactone ring of NM can potentiate the fungicidal activity of the alkylguanidinium chain *via* the AmB-like effect even if the chain is not covalently bound to the ring. Such a potentiation of the antifungal activity of MC12 or MC16 could not be simply attributed to its enhanced incorporation into or across the plasma membrane due to AmB-induced plasma membrane permeability change, as seen from the failure of AmB in elevating the antifungal activity of alkylamine.

We next applied a checkerboard technique to evaluate whether MC12 and AmB can inhibit the growth of *S. cerevisiae* and other fungal strains in a synergistic manner. Typical isobolograms demonstrated the appearance of synergy between the actions of MC12 and AmB was greater against filamentous fungi such as *A. niger* and *R. oryzae* than against yeasts such as *S. cerevisiae* and *C. albicans*, as shown in Fig. 2. Although MC12 inhibits the growth of Gram-positive bacteria similarly to NM [3～5], its growth inhibitory activity against *B. subtilis* was scarcely enhanced in the presence of AmB (data not shown). These findings suggest that their combined actions fundamentally depend on the fungicidal activity of AmB, which has no molecular targets in bacterial cells.

In cultivation with shaking, MC12 decreased the growth rate of *S. cerevisiae* cells in a dose-dependent manner but caused no lethal damage even at the highest concentration (Fig. 3a) over the 4 hours incubation period. AmB was ineffective in inducing cell death at 1 μM, whereas this antibiotic could weakly decrease cell viability when its concentration was increased up to 2 μM (Fig. 3b) over the 4 hours period. However, their combined actions resulted in more serious cell death progression even when MC12 and AmB were added at the nonlethal concentrations of 50 μM and 0.5 μM, respectively. The lethal damage much more

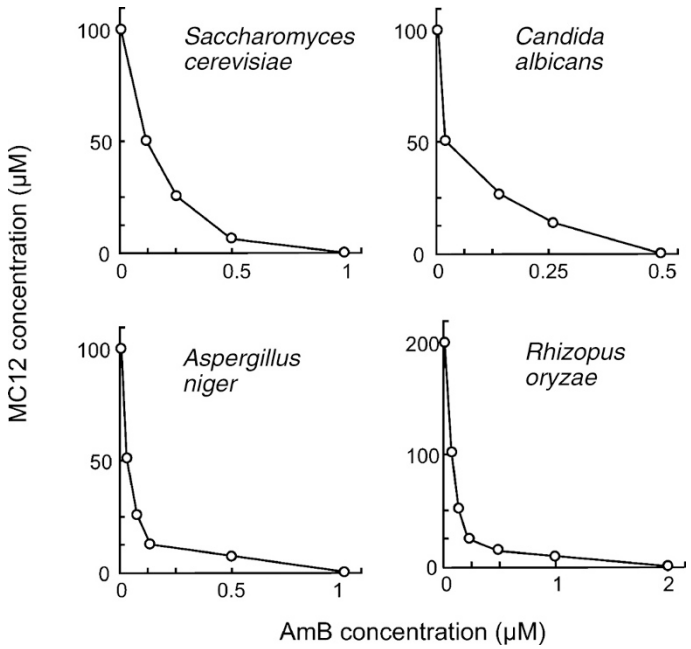

Fig. 2 Isobolograms demonstrating synergy of MC12 and AmB against *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger*, and *Rhizopus oryzae*.

Each point represents a combination of both agents that is needed for minimum growth inhibitory activity. The concentrations of MC12 and AmB are expressed as the fraction of one MIC for each agent when used alone. An isobol that bows toward the coordinates indicates synergism, a straight line isobol indicates indifference, and an isobol that bows away from the coordinates indicates antagonism.

rapidly appeared in the medium in which the concentration of AmB was increased up to 1 μM (Fig. 3c).

Effects of MC12, AmB and Their Combination on Plasma Membrane Permeability

The fungicidal activity of AmB has been mainly elucidated on the basis of its promotive effect on the plasma membrane permeability change [11]. This antibiotic directly interacts with sterols constituting the plasma membrane of eukaryotic microbial cells and thus can cause a change in the permeability of fungal plasma membrane [12]. We therefore examined the mode of synergy between MC12 and AmB on the basis of their effects on plasma membrane permeability to K^+. As shown in Fig. 4, MC12 alone at 50 μM or even at 100 μM did not promote K^+ efflux from cells, as deduced from the loss of cell death induction (Fig. 3). AmB was effective in inducing the leakage of K^+ from cells even at 0.5 μM, and the rate of AmB-induced K^+ efflux was slightly higher in the medium containing this antibiotic at 1 μM. However, cells characteristically showed an extremely high rate of K^+ efflux at the initial step of cell death induced by the

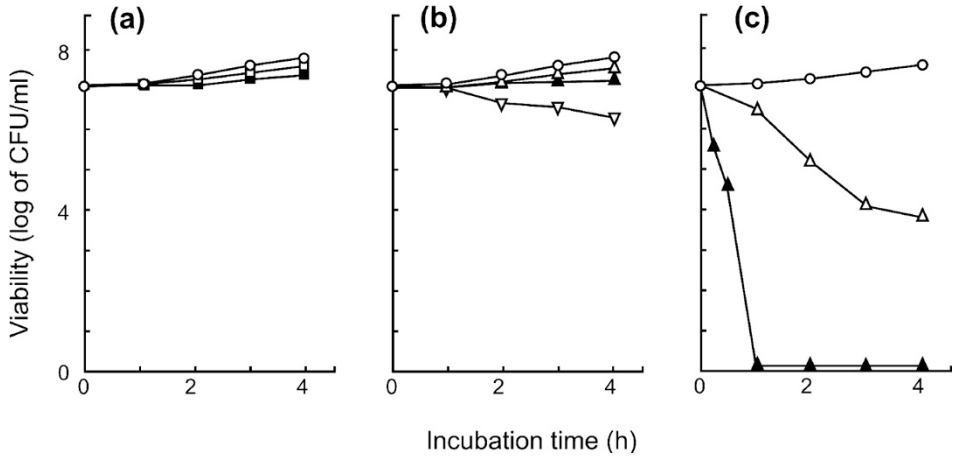

Fig. 3 Effects of MC12, AmB and combination of MC12 and AmB on growth of *Saccharomyces cerevisiae* cells.

For (a), cells (10^7/ml) were incubated in YPD medium containing MC12 at 0 (○), 50 (□), and 100 μM (■). For (b), cells were incubated in YPD medium containing AmB at 0 (○), 0.5 (△), 1 (▲), and 2 μM (▽). For (c), cells were incubated in YPD medium containing 50 μM MC12 and AmB at 0 (○), 0.5 (△) and 1 μM (▲).

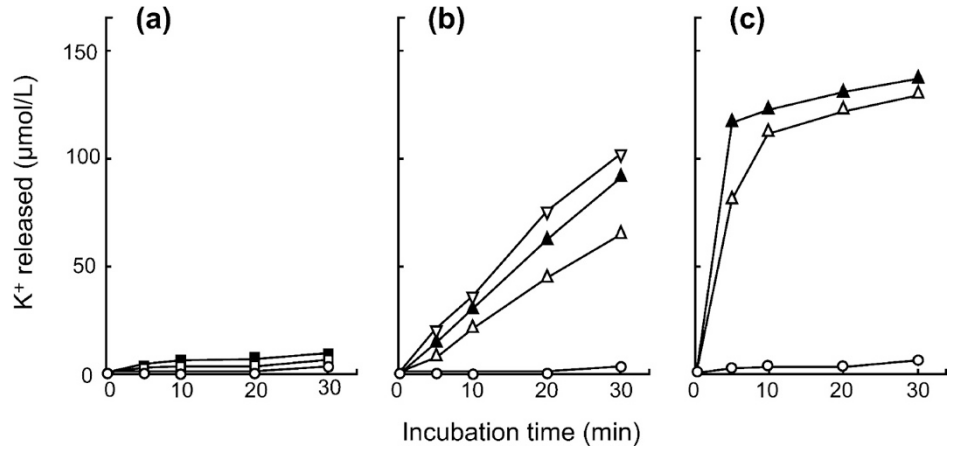

Fig. 4 Promotive effects of MC12, AmB, and combination of MC12 and AmB on leakage of K^+ from *Saccharomyces cerevisiae* cells.

For (a), cells (10^8/ml) were incubated in 50 mM succinate buffer (pH 6.0) containing MC12 at 0 (○), 50 (□), and 100 μM (■). For (b), cells (10^8/ml) were incubated in 50 mM succinate buffer (pH 6.0) containing AmB at 0 (○), 0.5 (△), 1 (▲), and 2 μM (▽). For (c), cells (10^8/ml) were incubated in 50 mM succinate buffer (pH 6.0) containing 50 μM MC12 and AmB at 0 (○), 0.5 (△), and 1 μM (▲).

combined actions of MC12 and AmB each at the nonlethal concentration. Such a marked change in plasma membrane permeability suggests that MC12 enhances the formation of a complex between AmB and ergosterol for K^+ channel formation across the plasma membrane.

Effects of MC12, AmB and Their Combination on Cellular ROS Production

The involvement of oxidative damage has been suggested for the fungicidal activity of AmB against *C. albicans* and its lytic activity on mammalian cells [13～15]. On the contrary, the antibiotic itself is evaluated as an antioxidant, and furthermore the antibiotic is able to exhibit a synergistic fungicidal activity against *C. albicans* when added with an antioxidant such as gallate [16～18]. We first applied a selective fluorescent probe for detection of ROS in *S. cerevisiae* cells in order to characterize the difference in the mode of fungicidal activity between AmB and NM, and found ROS predominant production only in NM-treated cells but not in AmB treated cells [4]. The alkylguanidinium chain was thus considered to be a constituent responsible for cellular ROS production and related plasma membrane disruptive damage. We likewise examined whether MC12 accelerated this cytotoxic event by its own action or in combination with AmB. As shown in Fig. 5, ROS production was promoted in the medium with MC12 to a significant extent and was markedly accelerated in cells treated simultaneous with MC12 and AmB each at the nonlethal concentration. This agrees with the role of AmB as an enhancer of MC12-dependent fungicidal activity.

Effects of MC12, AmB and Their Combination on Vacuole Morphology and Function

The vacuole is an organelle essential for plant and fungal cell function including osmotic adaptation [19]. *nss1* mutants of *S. cerevisiae*, which are characterized by severe vacuolar protein sorting and vacuolar morphological defects, are highly sensitive to polyene macrolide antibiotics [20]. On the basis of this finding, we have recently determined the yeast vacuole as a target of the fungicidal activity of AmB when cells are incubated under hypoosmotic

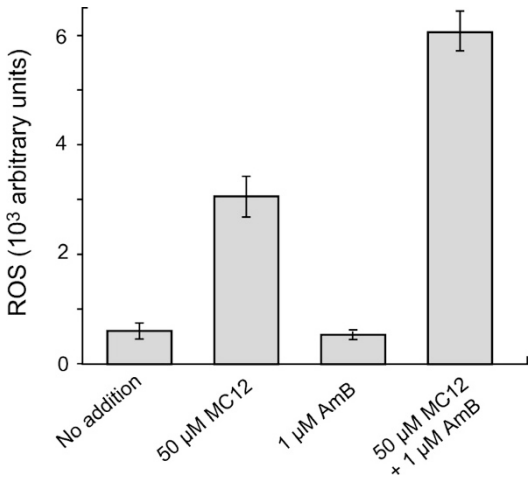

Fig. 5 Promotive effects of MC12, AmB, and combination of MC12 and AmB on ROS production of *Saccharomyces cerevisiae* cells.

After DCFH-DA treatment, cells (10^7/ml) were incubated in YPD medium alone, the medium containing 50 μM MC12, the medium containing 1 μM AmB, and the medium containing both 50 μM MC12 and 1 μM AmB at 30°C for 60 minutes.

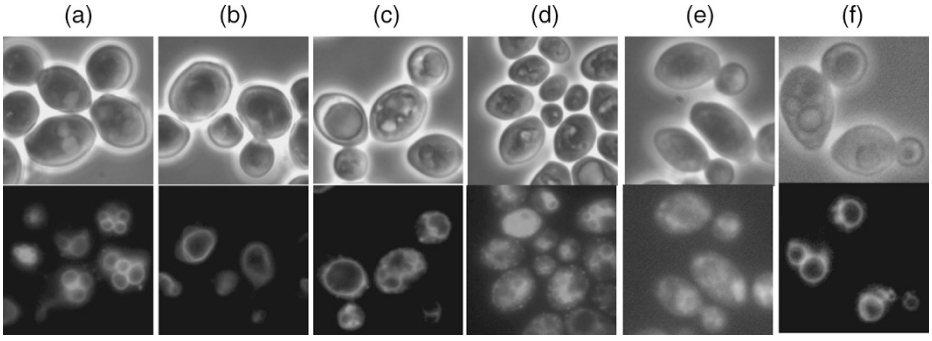

Fig. 6 Effects of MC12, AmB, NM, and combination of MC12 and AmB on vacuole morphology of *Saccharomyces cerevisiae* cells.

After FM4-64 fluorescent dye treatment, cells (10^7/ml) were incubated in YPD medium alone (a), the medium containing 50 μM MC12 (b), the medium containing 1 μM AmB (c), the medium containing both 50 μM MC12 and 1 μM AmB (d), the medium containing 4 μM NM (e), and the medium containing 10 μM AmB (f) at 30°C for 30 minutes. Cells were observed under a phase-contrast microscope (top) and a fluorescence microscope (bottom).

condition [21]. We thus examined the relationship between the fungicidal activity of NM and that arising from the combination of MC12 and AmB in terms of their effects on vacuole function and morphology. The yeast vacuoles were visible as a set of spherical membrane-enclosed compartments in untreated cells (Fig. 6a). They were observed as enlarged prominent architectures in cells treated with either MC12 or AmB alone at the nonlethal concentration (Fig. 6b, c). The AmB-induced morphological change of vacuoles seemed to reflect their fusion or expansion to a larger spherical structure, which is possibly effective for maintaining the cytoplasmic osmolarity balance. MC12 should promote vacuolar membrane fusion or expansion *via* a mechanism other than cellular osmoregulation, because MC12 did not cause plasma membrane permeability change at any of the concentrations tested. Vacuoles were not clearly visible in cells treated simultaneously with MC12 and AmB each at the nonlethal concentration, in which a fluorescence from FM4-64 was observed throughout the cytoplasm (Fig. 6d). NM similarly caused vacuolar membrane disability at its lethal concentration (Fig. 6e) as cytoplasmic distribution of the dye suggested leakage from the vacuolar membrane. In contrast, AmB-treated cells were characterized by intact vacuoles without the leakage of FM4-64 in medium even when the concentration of the antibiotic was increased up to 10 μM (Fig. 6f). These findings support the idea that the combined actions of MC12 and AmB resulted in the generation of NM-like inhibitory activity on the machinery involved in vacuole morphogenesis or the maintenance of vacuolar membrane integrity.

Transmission Electron Microscopy of Cells Treated with MC12, AmB, and Their Combination

We finally observed cells under a transmission electron microscope to precisely analyze the effects of MC12, AmB and their combination on the morphologies of vacuoles and other subcellular architectures. Vacuoles were visible as various membrane-enclosed compartments together with a nucleus in untreated cells (Fig. 7a), whereas MC12-treated cells were characterized by the appearance of an enlarged prominent vacuole (Fig. 7b). The vacuole was visible as highly expanded spherical architecture in AmB-treated cells (Fig. 7c). These transmission electron microscopy observations agreed with the fluorescence microscopy observations (Fig. 6). The most surprising feature of the combined actions of MC12 and AmB was the appearance of many vesicle-like compartments in addition to small vacuoles throughout the cytoplasm (Fig. 7d). The failure in clearly visualizing vacuoles with FM4-64 may be due to such an aberrant vacuole morphogenesis. Vacuole protein

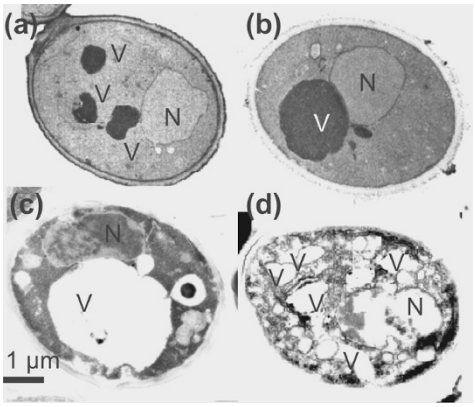

Fig. 7 Transmission electron microscopy of *Saccharomyces cerevisiae* cells incubated in YPD medium alone (a), medium containing 50 μM MC12 (b), medium containing 1 μM AmB (c), and medium containing both 50 μM MC12 and 1 μM AmB (d) for 60 minutes at 30°C.

N and V indicate nucleus-like and vacuole or vacuole-like architectures, respectively.

sorting (*vps*) mutants of *S. cerevisiae* exhibit defects in the sorting and processing of multiple vacuolar hydrolases, and are assigned to five distinct classes depending on vacuolar morphology and dynamics [10, 22]. Class C mutants lack any organelles resembling normal vacuoles, but accumulate vesicle-like aberrant architectures and are also characterized by the failure in their osmotic adaptation. The vesicle-like organelles seemed to be formed in cells treated with MC12 and AmB may result due to an impairment of the mechanism regulating vesicle assembly into vacuoles, as in the case of these mutants with the corresponding genetic defects, or in the corresponding cellular functions [23～25]. Unlike the case with these mutants, however, the membranous architectures of vacuoles and vesicle-like organelles were disrupted a least in part as judged from the image representing the leakage of FM4-64 (Fig. 6d). Nucleus-like organelles were also visible with abnormal morphology together with a disruptive damage in cells treated with MC12 and AmB. These findings indicated the occurrence of severe fatal damage widely over the plasma membrane and intracellular membranous architectures as a result of the combined actions of MC12 and AmB.

This study reveals that the synergistic fungicidal activities of MC12 and AmB depend on the combination of various lethal events corresponding to those induced by NM including plasma membrane permeability change or plasma membrane disruption, ROS production, and aberrant vacuole morphogenesis. These cytotoxic events are not actually involved in the fungicidal activity of AmB when fungal cells are grown or incubated in nutrient

medium under normal osmotic condition. Therefore, it is highly likely that the alkyl guanidinium chain of NM plays a major role in the fungicidal activity of NM in cooperation with the polyol lactone ring as its enhancer. Apoptosis-like events are induced in *S. cerevisiae* cells by the depletion of glutathione or by a low external dose of H_2O_2 [26]. Although ROS production is not necessarily a cause of cell death induction in *S. cerevisiae*, the fungicidal activity of NM could be attributed at least in part to its promotive effect on cellular ROS production *via* glutathione depletion [4, 8]. Vacuoles contain several hydrolytic enzymes so that their leakage into the cytoplasm can be a cause of cell death progression [27]. The combined actions of MC12 and AmB on vacuoles may be a primary cause of inducing various downstream lethal events including ROS production. On the basis of the results of this study, we also propose that AmB can be a probe for developing vacuole-targeting antifungal agents.

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