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Amplification of Vacuole-targeting Fungicidal Activity of Antibacterial Antibiotic Polymyxin B by Allicin, an Allyl Sulfur Compound from Garlic

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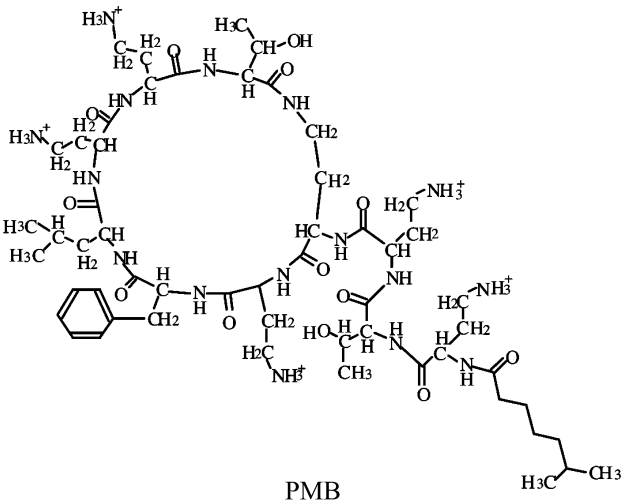
Abstract A cationic antibacterial peptide, polymyxin B (PMB), was evaluated as an antifungal antibiotic against various yeasts and filamentous fungi when used in combination with allicin, an allyl sulfur compound from garlic. Allicin was not lethal but could markedly amplify the fungicidal activity of PMB, which was weakly detected with the increase in the plasma membrane permeability in *Saccharomyces cerevisiae*. Their combined actions caused a dynamic structural damage to the yeast vacuole as judged by the disappearance of its swollen spherical architecture. The vacuole-targeting activity of PMB was similarly amplified in medium with *t*-butyl hydroperoxide as a substitute for the action of allicin. These findings suggest that the allicin-mediated lipoperoxide production in fungal plasma membrane is the cause of the enhancement in the cellular uptake of PMB as well as its action against the vacuole.

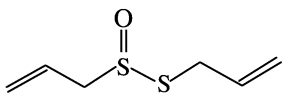
Keywords polymyxin B, allicin, *Saccharomyces cerevisiae*, vacuole, plasma membrane

Introduction

Polymyxin B (PMB, Fig. 1) is a decapeptide antibiotic characterized by a heptapeptide ring containing four 2,4-diaminobutyric acids. An additional peptide chain covalently bound to the cyclic peptide carries an aliphatic chain attached to the peptide through an amide bond. PMB is bactericidal to almost all Gram-negative bacteria at relatively low concentrations [1]. The antibiotic is known to disrupt a permeability barrier of the bacterial outer membrane by forming a complex with lipopolysaccharide [2, 3]. Therefore, PMB is expected to increase the



PMB



Allicin

Fig. 1 Structures of polymyxin B (PMB) and allicin.

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permeability of the outer membrane to a variety of molecules, including its own uptake [4]. PMB passing through the outer membrane interacts with the acidic phospholipids exposed on the plasma membrane, and thus, its lethal action is proposed to depend on the increase in the plasma membrane permeability to intracellular molecules [1, 5]. PMB can also interact with the plasma membranes of *Saccharomyces cerevisiae* and *Candida albicans*, as seen from its stimulatory effect on the membrane permeability to various antibiotics such as tetracycline, miconazole, and ketoconazole [6, 7]. However, it remains unknown why the fungicidal activity of PMB is much lower than its bactericidal activity [8, 9].

Allicin (diallyl thiosulfinate, Fig. 1) is the main biologically active component of freshly crushed garlic extract [10]. Allicin exerts various biological activities such as antimicrobial and anticancer activities in addition to the capacity to lower serum lipid levels, particularly cholesterol levels, and ocular pressure [11～15]. The antimicrobial activity of allicin is considered to depend on its inhibitory effects on certain thiol-containing enzymes *via* strong SH-modifying properties because of the production of *S*-allylmercaptocysteine from L-cysteine [12, 16]. L-Cysteine of the antioxidant glutathione may also be a target of allicin [16]. In the amoebic parasite *Entamoeba histolytica*, allicin was found to strongly inhibit various cysteine proteases, alcohol dehydrogenase and thioredoxin reductase [17]. In our previous study, allicin was found to amplify the fungicidal activity of the polyene macrolide antibiotic amphotericin B (AmB) and their combined actions depended on the inhibition of vacuole morphogenesis in *S. cerevisiae* [18]. Allicin may contribute to AmB-dependent therapy for fungal infectious diseases through proper administration.

In this study, we examined the effect of allicin as an enhancer of the antifungal activity of a variety of agents, and found a predominant synergistic relationship only between this compound and a typical antibacterial antibiotic PMB. Allicin did not significantly influence PMB-induced plasma membrane disability but their combined actions caused a dynamic structural damage to the vacuoles of *S. cerevisiae*, which has been used as a model of eukaryotic microbial cell. We also found a difference in the mode of cooperation between allicin and each of PMB and AmB in the amplification of vacuole-targeting activity. Our finding may contribute to the improvement of antifungal therapy which has been achieved with a limited number of antibiotics.

Materials and Methods

Measurement of Cell Growth and Viability

MICs of PMB and allicin were determined against *Escherichia coli* IFO 3545, *Bacillus subtilis* IFO 3007, *S. cerevisiae* W303-1A, *C. albicans* IFO 1061, *Aspergillus fumigatus* IFO 5840, and *A. niger* ATCC 6275 by 2-fold broth dilution method applying the checkerboard technique [19]. Cells of bacterial strains were grown overnight in 3.0% nutrient broth (Nissui Co., Tokyo, Japan) with vigorous shaking at 30°C, and diluted with the same medium to 10^6 cells/ml. Overnight cultures of yeast strains in YPD medium (1.0% yeast extract, 2.0% peptone, 2.0% glucose) were diluted with the same medium to 10^6 cells/ml. Filamentous fungi were precultivated in 2.5% malt extract medium (Oriental Yeast Co., Tokyo, Japan) at 30°C for 24 hours and were diluted 100-fold with the same medium. Cells of each microbial strain were then incubated with PMB and allicin added at various concentrations at 30°C for 24 hours (bacteria) and 48 hours (yeasts and filamentous fungi) in a 96-well plate.

Unless otherwise stated, *S. cerevisiae* W303-1A was used in the following experiments, in which cells were grown overnight in YPD medium and were diluted into freshly prepared medium to 10^7 cells/ml. Cells were then incubated with vigorous shaking at 30°C in YPD medium in which PMB and allicin were added at various concentrations for the measurement of viable cell numbers as colony-forming units [20].

Assay of Plasma Membrane Permeability Change

The effects of PMB and allicin on plasma membrane permeability were examined by measuring the effluxes of both intracellular K^+ and UV-absorbing materials such as nucleotides as follows. Cells were harvested by centrifugation, washed with 50 mM Tris-HCl buffer (pH 7.4), and suspended in the buffer to obtain a density of 10^8 cells/ml. The cell suspensions were then shaken with PMB and allicin added at various concentrations at 30°C for 60 minutes. The supernatants obtained after cell removal by centrifugation were assayed for K^+ content using a K^+ assay kit (HATCH, Floriffoux, Belgium) based on the tetraphenylborate method [21]. The amount of UV-absorbing materials was measured as absorption at a wavelength of 260 nm.

Staining of Vacuole with FM 4-64

Vacuoles were visualized by staining with a fluorescent probe FM 4-64 according to the method of Vida and Emr [22]. Cells were inoculated in YPD medium containing

5.0 μM FM 4-64 at a density of 10^7 cells/ml. After incubation at 30°C for 30 minutes, cells were collected by centrifugation and washed various times with the medium to remove excess FM 4-64. Cells were then resuspended in YPD medium and further incubated with PMB and allicin added at various concentrations at 30°C for 60 minutes, and then observed under a phase-contrast and fluorescence microscope with excitation at 480 and emission at 530 nm.

HPLC Analysis of PMB

Cells were harvested by centrifugation, washed with 50 mM Tris-HCl buffer (pH 7.4), and suspended in the buffer to obtain a density of 10^8 cells/ml. The cell suspensions were then shaken with PMB and allicin added at various concentrations at 30°C for 120 minutes. The supernatants obtained after cell removal by centrifugation were assayed for PMB content by HPLC using a reverse-phase C_{18} column (4.7 by 250 mm; YMC-Pack ODS-AM, YMC Inc.). Elution was carried out by increasing the content of acetonitrile from 0 to 100% in a mobile phase of 0.15% trifluoroacetic acid in H_2O over 30 minutes at a flow rate of 1.0 ml/minute, and PMB was detected at an absorbance at 215 nm [23].

Chemicals

PMB sulfate salt and AmB were purchased from Sigma Chemical Co. (St. Louis, MO, USA). FM 4-64 was a product of Molecular Probes (Eugene, OR, USA). The other chemicals used were of analytical reagent grade.

Results and Discussion

Synergy between PMB and Allicin

The checkerboard technique was used to examine the relationship between PMB and allicin in terms of their growth inhibitory activities against various bacteria, yeasts and filamentous fungi. As shown in Fig. 2, PMB inhibited the growth of *E. coli*, a Gram-negative bacterium, at the concentration of 3.0 μg/ml and the MIC remained unchanged with increasing concentration of allicin up to 250 μM. This means that allicin was not inhibitory to the bacterial growth even at this concentration. Unexpectedly, while *B. subtilis* was highly resistant to PMB, the bacterium was rendered relatively sensitive to the antibiotic as seen from the reduction of the original MIC (800 μg/ml) to 100 μg/ml in the presence of 125 μM allicin. Some *B. subtilis* strains are sensitive to PMB and are made more susceptible to the action of the antibiotic as a result of lysozyme treatment [24]. This may indicate the possibility that allicin can reduce the bacterial resistance to PMB by interacting with peptidoglycan thickly accumulated outside the plasma membrane of Gram-positive bacteria.

However, the synergy between PMB and allicin was more predominant in yeasts and filamentous fungi, in which cell walls are composed of various types of polysaccharides other than peptidoglycan. PMB could strongly inhibit the growth of *S. cerevisiae* with increasing concentration of allicin up to 125 μM so that the MIC was markedly reduced to 3.13 μg/ml. This antibiotic was mostly inactive against the pathogenic yeast and filamentous fungi,

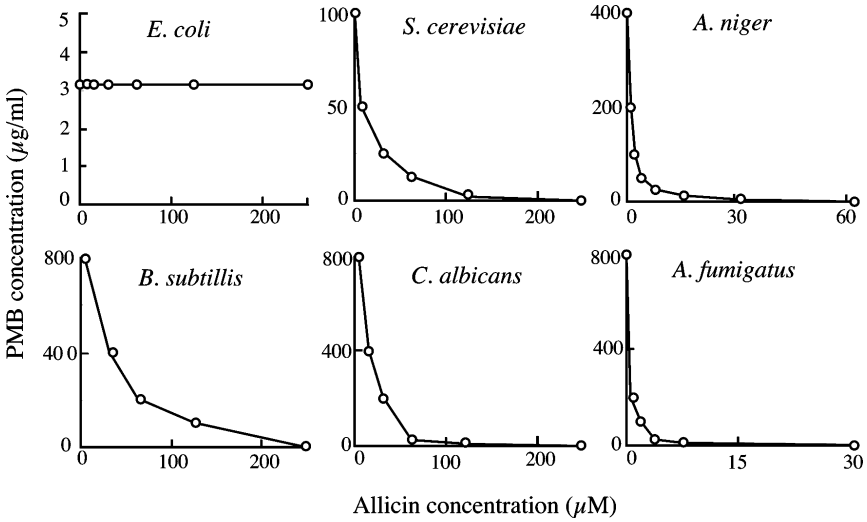

Fig. 2 Isobolograms demonstrating synergy of PMB and allicin.

Each point represents a combination of the MICs of PMB and allicin against *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger*, and *A. fumigatus*.

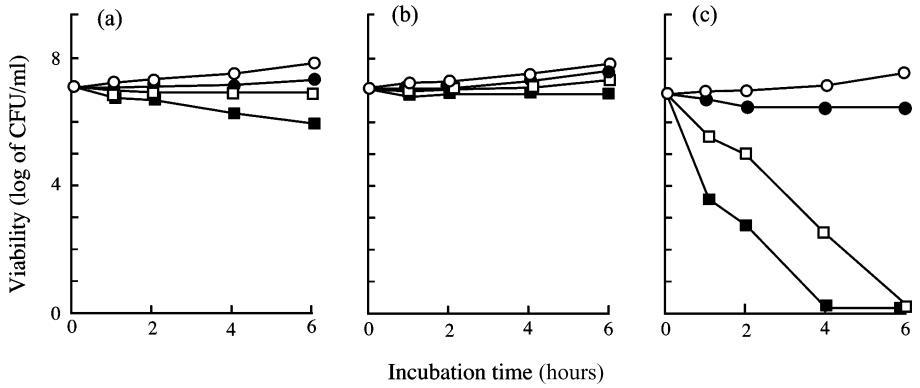

Fig. 3 Effects of PMB, allicin and combination of PMB and allicin on the growth of *S. cerevisiae* cells.

For (a), cells (10^7/ml) were incubated in YPD medium containing PMB at 0 (○), 30 (●), 60 (□), and 120 μg/ml (■). For (b), cells were incubated in YPD medium containing allicin at 0 (○), 60 (●), 120 (□), and 240 μM (■). For (c), cells were incubated in YPD medium containing 60 μM allicin and PMB at 0 (○), 30 (●), 60 (□), and 120 μg/ml (■).

C. albicans, *A. niger* and *A. fumigatus*, but the presence of allicin could render each of these fungal strains extremely sensitive to the antibiotic. The MIC of PMB against *A. fumigatus* (800 μg/ml) could be most effectively reduced to 6.25 μg/ml with the aid of the lowest concentration of allicin (7.5 μM). These findings suggested that PMB has a selective molecular target in these fungal cells, which is generally protected against direct action of the antibiotic in the absence of allicin.

Allicin-dependent Fungicidal Activity of PMB against *S. cerevisiae*

We then examined the modes of actions of PMB, allicin, and their combination on *S. cerevisiae* as a model of eukaryotic microbial cells. As shown in Fig. 3a, PMB inhibited the growth of *S. cerevisiae* cells in a dose-dependent fashion and weakly reduced the viable cell number at 120 μg/ml. Allicin was also weakly inhibitory to the growth of the yeast cells at 60 μM and the maximum growth inhibition was achieved at 240 μM. The viable cell number remained unchanged throughout the incubation in medium with 240 μM allicin, representing the static growth inhibition pattern of this compound (Fig. 3b). Although PMB and allicin were not evaluated as potent antifungal agents when individually administrated, the presence of allicin rendered the yeast cells susceptible to the lethal action of PMB even at 60 μg/ml (Fig. 3c). In this experiment, PMB and allicin were required at the higher concentrations than expected from their MIC values (Fig. 2), since the cell density was adjusted to the higher levels such as 10^7/ml and even 10^8/ml essential for the following physiological experiments. We also measured MICs of PMB and allicin using the cell suspension of 10^7/ml with the aid of a checkerboard-type synergy test and found

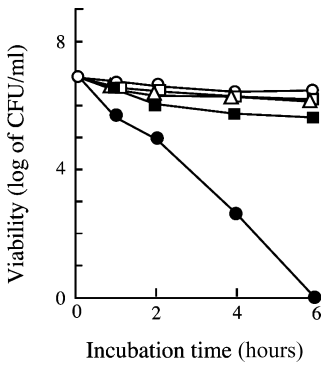

Fig. 4 Effects of various sulfur-containing compounds on the fungicidal activity of PMB against *S. cerevisiae* cells.

Cells (10^7/ml) were incubated in YPD medium containing 60 μg/ml PMB alone (○), and the medium containing 60 μg/ml PMB and 60 μM each of allicin (●), diallyl disulfide (□), diallyl trisulfide (■), and *N*-acetyl cysteine (△).

that 60 μM allicin was required for absolutely inhibiting the cell growth by PMB at 60 μg/ml. Among various related compounds, diallyl trisulfide was slightly effective in enhancing the fungicidal activity of PMB, whereas diallyl disulfide or *N*-acetyl cysteine showed no stimulatory effects even at the concentration of 60 μM (Fig. 4). Therefore, allicin could be evaluated as an active principle of enhancing the PMB-induced lethal event despite that diallyl disulfide and diallyl trisulfide may be produced as its degradation products [25].

Effects of PMB and Allicin on Plasma Membrane Permeability

PMB dissipates the K^+ gradient of the bacterial plasma membrane [26]. We therefore examined the effects of PMB,

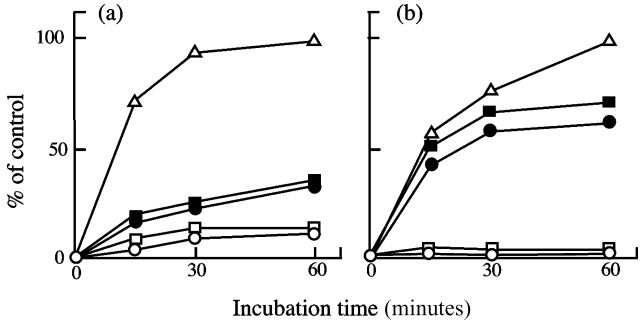

Fig. 5 Promotive effects of PMB, allicin, and combination of PMB and allicin on the leakage of UV-absorbing materials (a) and K^+ (b) from *S. cerevisiae* cells.

Cells (10^8/ml) were incubated in 50 mM Tris-HCl buffer (pH 7.4) alone (○), the buffer containing 60 μg/ml PMB (●), the buffer containing 60 μM allicin (□), and the buffer containing both PMB and allicin at these concentrations (■). The extents of UV-absorbing materials and K^+ leakages are expressed as the percentages of the value obtained after a 60 minutes incubation with 0.2% Triton X-100 (a) or 10 μM AmB (b), respectively (△).

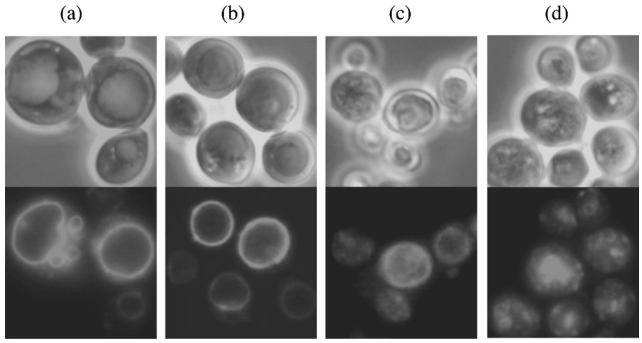

Fig. 6 Effects of PMB, allicin, and combination of PMB and allicin on vacuole morphology of *S. cerevisiae* cells.

After FM 4-64 fluorescent dye treatment, cells (10^7/ml) were incubated in YPD medium alone (a), the medium containing 120 μM allicin (b), the medium containing 120 μg/ml PMB (c), and the medium containing 60 μg/ml PMB and 60 μM allicin (d). Cells were observed under a phase-contrast (top) and fluorescence microscope (bottom).

allicin, and their combination on the leakages of UV-absorbing materials and K^+, which are commonly used as indicators of plasma membrane permeabilization [27, 28]. As shown in Fig. 5, allicin did not induce a plasma membrane permeability change in yeast cells, as judged from the extents of their leakages at the control levels. In agreement with the previous reports [6, 7], PMB enhanced the release of UV-absorbing materials to a considerable extent, but its release was kept at lower level than cells treated with Triton X-100 (Fig. 5a). PMB could more markedly enhance the leakage of K^+ although the antibiotic was apparently less effective in enhancing the ion efflux than AmB, which is believed to be the most effective agent for causing the plasma membrane permeability to the ion (Fig. 5b). The presence of allicin was mostly ineffective or only weakly effective in further accelerating the releases of these components despite its stimulatory effect on PMB-induced cell death (see Fig. 3c). This suggested the involvement of a lethal event other than the plasma membrane permeability change in the combined fungicidal actions of PMB and allicin.

Effects of PMB and Allicin on Vacuole Morphology

We recently found a stimulatory effect of allicin on the fungicidal activity of the polyene macrolide antibiotic AmB, in which the vacuole was evaluated as the target of their combined actions [18]. Vacuolar membranes were clearly fragmented into small discrete patches when *S. cerevisiae* cells were subjected to a lethal damage caused by AmB at a nonlethal concentration in combination with allicin. Various hydrolytic enzymes including proteases and nucleases are thought to accumulate in the vacuole so that the collapse of the organelle is considered to be a critical step for cell death induction [29]. It was therefore determined whether such vacuole-targeting activity is involved in the combined fungicidal actions of PMB and allicin. Vacuoles were visualized as swollen spherical architectures in both untreated and allicin-treated cells with the aid of a fluorescent dye, which is selectively incorporated into the vacuolar membrane (Fig. 6a, b). Unexpectedly, the dye was detected throughout the organelle in most cells (*ca.* 62%), reflecting the vacuolar membrane permeability change, when cells were treated with PMB alone at a lethal concentration (Fig. 6c). In more than 90% of cells, vacuoles absolutely lost membrane-enclosed architectures so that the dye was detected in various sizes of smaller compartments with weakly stained background when cells were treated with PMB at a nonlethal concentration in combination with allicin (Fig. 6d). These findings revealed that such a structural damage of the vacuole is closely related to the amplification of the fungicidal activity of PMB.

Dependence of the Vacuole-targeting Fungicidal Activity of PMB on Phospholipid Peroxidation

Our previous study demonstrated the inhibitory effect of allicin on the cell surface localization of alkyl hydroperoxide reductase 1, which functions as a defense against phospholipid peroxidation, in *S. cerevisiae* [30]. This was strongly supported by the direct detection of lipoperoxide in the plasma membrane of allicin-treated

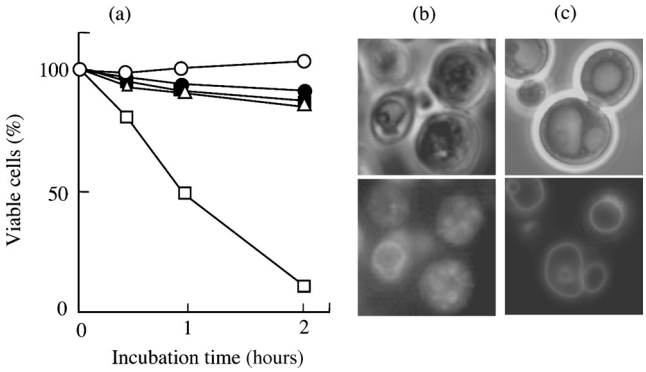

Fig. 7 Effects of *t*-butyl hydroperoxide (*t*-BOOH) on the vacuole-targeting fungicidal activities of PMB and AmB.

For (a), cells (10^7/ml) were incubated in YPD medium alone (○), the medium containing 60 μg/ml PMB (●), the medium containing 60 μg/ml PMB and 300 μM *t*-BOOH (□), the medium containing 2.0 μM AmB (■), and the medium containing 2.0 μM AmB and 300 μM *t*-BOOH (△). After FM 4-64 fluorescent dye treatment, cells (10^7/ml) were incubated in YPD medium containing 60 μg/ml PMB and 300 μM *t*-BOOH (b) and the medium containing 2.0 μM AmB and 300 μM *t*-BOOH (c). Cells were observed under a phase-contrast (top) and fluorescence microscope (bottom).

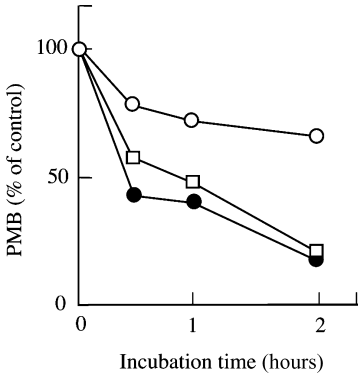

Fig. 8 Effects of allicin and *t*-BOOH on the cellular uptake of PMB.

Cells (10^8/ml) were incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 60 μg/ml PMB (○), the buffer containing 60 μg/ml PMB and 60 μM allicin (●), and the buffer containing 60 μg/ml PMB and 300 μM *t*-BOOH (□).

cells as well as allicin-like effect of *t*-butyl hydroperoxide (*t*-BOOH), a kind of organic hydroperoxide [31]. We next determined whether the addition of *t*-BOOH can similarly amplify the vacuole-targeting fungicidal activity of PMB in order to clarify the physiological significance of allicin. As shown in Fig. 7a, the presence of *t*-BOOH was not inhibitory to the growth of the yeast cells at 300 μM but could render the cells susceptible to the fungicidal action of PMB. In association with the amplification of fungicidal activity, their combined actions resulted in exactly the same type of damage to the vacuole morphogenesis (Fig. 7b). In contrast, the simultaneous addition of α-tocopherol, a typical antioxidant protective against the plasma membrane phospholipid peroxidation, at 120 μM was slightly competitive with allicin, thereby increasing its minimum concentration (60 μM) essential for PMB-induced (60 μM) cell death up to 120 μM. Unlike the case with PMB, however, the fungicidal activity of AmB was scarcely amplified by the presence of *t*-BOOH, and indeed, their combined actions did not accompany any morphological change in the yeast vacuoles (Fig. 7a, c). These findings supported the relationship of allicin-mediated phospholipid peroxidation to its stimulatory effect on the vacuole-targeting fungicidal activity of PMB, but not to the corresponding stimulatory effect of allicin on the vacuole-targeting fungicidal activity of AmB.

As expected from such a difference between the actions of allicin and *t*-BOOH, allicin was found to enhance the vacuole-targeting fungicidal activity of AmB *via* the event other than phospholipid peroxidation, such as the inhibition of ergosterol trafficking from the plasma membrane to the vacuolar membrane (unpublished results). This ergosterol-related event was not provoked upon *t*-BOOH treatment, being distinguished from plasma membrane phospholipid peroxidation, which is commonly observed with allicin- and *t*-BOOH-treated cells. We also found an aberrant vacuole morphogenesis in *S. cerevisiae* when cells were treated with AmB in the presence of *N*-methyl-*N*″-dodecylguanidine, a constituent of the polyol macrolide antibiotic niphimycin [32]. In their combined actions, AmB could serve as an enhancer of the vacuole-targeting activity, which primarily depends on *N*-methyl-*N*″-dodecylguanidine, rather than an active principle of directly interacting with the organelle.

Effects of Phospholipid Peroxidation on Cellular Uptake of PMB

It was finally determined whether the plasma membrane of allicin-treated cells is more permeable to PMB than that of untreated cells so that vacuoles are more susceptible to the action of the antibiotic. PMB was indeed lost from the supernatant when cells were incubated with the antibiotic alone, but the rate of its disappearance was reduced in a time-dependent fashion, reflecting its binding with a cell surface component, possibly acidic phospholipid, as shown in Fig. 8. The presence of allicin more significantly accelerated the disappearance of PMB from the supernatant although the agent did not cause plasma membrane permeability change (see Fig. 5a, b). This supports the idea that allicin accelerated the cellular uptake of PMB into the

cytoplasm by increasing the acidic lipoperoxide content of phospholipids [31]. The addition of *t*-BOOH similarly increased the rate of cellular uptake of PMB, in agreement with the above idea on the relationship between allicin-mediated phospholipid peroxidation and an increase in the rate of cellular uptake of this antibiotic.

Although early clinical reports suggested a high rate of toxicity associated with PMB, recent studies have suggested that the toxicities associated with the antibiotic may be less severe and less frequent than already reported [33]. Various clinical trials have evaluated PMB to be a drug effective for the treatment of multidrug-resistant Gram-negative organisms. On the other hand, different kinds of mycoses have become an important public health problem as their incidence has increased considerably in the last decades. Current therapy for invasive mycoses uses a relatively reduced number of antifungal drugs, such as AmB and fluconazole [34]. Echinocandin, an inhibitor of (1,3)-β-D-glucan synthesis, has been used as a new class of antifungal drugs. Some mutants of *S. cerevisiae* are viable in spite of a genetic defect in the vacuole morphogenesis, suggesting suppression of the impaired vacuolar function by another physiological event [35]. In PMB-treated cells, however, the sudden fragmentation of vacuolar membrane should be a cause of excretion of proteases, nucleases, and other hydrolytic enzymes, which are generally entrapped in the organelle, into the cytoplasm as well as their unregulated actions on the corresponding substrates essential for normal cellular functions. The sudden disappearance of membrane-enclosed architecture of vacuole should also result in the failure in protection against the cytoplasmic osmotic imbalance due to the leakage of intracellular K^+ by the plasma membrane-targeted action of PMB [29]. Clinical applications of PMB and allicin may be valuable in antibacterial therapy that additionally requires protection against the emergence of opportunistic fungal infection.

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