

## ORIGINAL ARTICLE

# Dependence of vacuole disruption and independence of potassium ion efflux in fungicidal activity induced by combination of amphotericin B and allicin against *Saccharomyces cerevisiae*

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**Allicin selectively enhances the fungicidal activity of amphotericin B (AmB). It also accelerates AmB-induced vacuole disruption but does not affect AmB-induced potassium ion efflux in *Saccharomyces cerevisiae* and *Candida albicans*. The fungicidal activity of AmB alone or combined with allicin was further evaluated based on the relationship among cell viability, vacuole disruption and potassium ion efflux in *S. cerevisiae*. Lethality and vacuole disruption caused by AmB alone were completely restricted when K<sup>+</sup> and Mg<sup>2+</sup> were added to the growth medium. On the other hand, in identical conditions, the combination of AmB and allicin induced both lethality and vacuole disruption. *S. cerevisiae*  $\Delta$ *erg6* cells, which lack ergosterol in plasma membrane, were mostly resistant to AmB as well as the combination of AmB and allicin against both lethality and vacuole disruption. The incorporation of AmB into the cytoplasm of  $\Delta$ *erg6* cells was significantly reduced in comparison with that in parent cells, regardless of the presence of allicin. Our results suggest that the fungicidal activity of AmB combined with allicin is involved in vacuole disruption but not in potassium ion efflux, and that the expression of allicin-mediated activity of AmB requires the presence of ergosterol in the plasma membrane.**

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## INTRODUCTION

Amphotericin B (AmB), a typical polyene macrolide antifungal antibiotic, is widely used for the treatment of many systemic mycoses. Its mechanism of action is generally accepted as follows. AmB binds to ergosterol in fungal plasma membranes, alters the permeability of plasma membrane (for example, by forming pores that leak potassium ions, resulting in loss of intracellular K<sup>+</sup>) and then causes the death of fungal cells.<sup>1,2</sup> Supply of K<sup>+</sup> and Mg<sup>2+</sup> from the external environment protects the AmB-treated cells from death,<sup>3</sup> indicating the direct involvement of intracellular K<sup>+</sup> in cell viability. On the other hand, AmB-induced loss of intracellular K<sup>+</sup> results in various secondary effects, including ATP depletion and subsequent inhibition of protein synthesis. Therefore, loss of intracellular K<sup>+</sup> might accelerate cell death.<sup>4</sup> In addition, AmB induced oxidative stress accompanying cell death in the pathogenic fungus *Candida albicans*.<sup>5</sup> Moreover, AmB was able to permeate through the plasma membrane and then to invade the fungal cytoplasm. Cytoplasmic AmB might fragment the vacuole membrane, thereby disrupting vacuoles.<sup>6</sup> Thus, the exact

fungicidal mechanism of AmB has not yet been fully understood; therefore AmB-induced lethality cannot be simply explained only by the disturbance in biophysical functions in the plasma membrane.<sup>7,8</sup>

Allicin, an allyl-sulfur compound from garlic, exerts various biological effects such as antimicrobial and anticancer activities.<sup>9,10</sup> We have recently reported that allicin selectively enhances the fungicidal activity of AmB. In addition, allicin accelerated AmB-induced vacuole disruption but did not affect AmB-induced leakage of intracellular K<sup>+</sup> in *Saccharomyces cerevisiae* and *C. albicans*.<sup>6,11</sup> Allicin alone did not induce the loss of intracellular K<sup>+</sup> nor the disruption of vacuole membrane.<sup>6,11</sup> Furthermore, we found that allicin enhanced AmB-induced vacuole disruption by inhibiting ergosterol trafficking from the plasma membrane to the vacuole membrane.<sup>12</sup> The constant ergosterol transport to the vacuole membrane may protect yeast cells from AmB-induced vacuole disruption.<sup>12</sup> The vacuole-targeting fungicidal activity, which depends on the structure of the macrocyclic ring, might elucidate at least in part the primary mechanism of the lethal action of AmB.<sup>13</sup> However, the transport mechanism

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of AmB into the cytoplasm is unclear, as well as the relationship between vacuole disruption and AmB-induced alteration of membrane permeability. In this study, we examined the relationship among cell viability, vacuole disruption and potassium ion efflux in cells treated with AmB in the presence or absence of allicin. Moreover, we discuss the significance of ergosterol content in plasma membrane on the vacuole-targeting activity of AmB to clarify the primary mechanism underlying AmB lethality.

## MATERIALS AND METHODS

### Measurement of yeast cell viability

*S. cerevisiae* W303-1A wild-type strain (provided by Dr T Nakamura, Osaka City University, Japan) and its *ERG6* gene deletion mutants (National BioResource Project, Japan) were used in the following experiments to examine the effects of AmB and allicin on the properties of cells, including viability. Cells were grown overnight in YPD medium containing 1% yeast extract (Difco Laboratories, Detroit, MI, USA), 2% Bacto-peptone (Difco Laboratories) and 2% glucose at 30 °C with vigorous shaking. After washing with distilled water, the cells were immediately diluted with distilled water to a density of  $1 \times 10^6$  cells per ml. The cells were then incubated in the presence or absence of each compound with vigorous shaking at 30 °C. Viable cell count was determined by counting the number of colony-forming units after 48 h of incubation at 30 °C in YPD medium containing 1.8% (w/v) agar.

### Leakage of potassium ions

Cells were harvested by centrifugation, washed with 50 mM Tris-HCl buffer (pH 7.4), and suspended in the same buffer to obtain a density of  $1 \times 10^8$  cells per ml. The cell suspensions were then shaken with 5  $\mu$ M AmB in the presence or absence of 120  $\mu$ M allicin at 30 °C for 60 min. The supernatants obtained after cell removal by centrifugation were assayed for K<sup>+</sup> content using a K<sup>+</sup> assay kit (HACH, Florifoux, Belgium) based on the tetraphenylborate method.<sup>14</sup>

### Vacuole staining

Vacuoles were visualized by staining with the fluorescent probe FM4-64 as follows.<sup>15</sup> Cells from the overnight culture in YPD medium were suspended in a freshly prepared medium to obtain a density of  $1 \times 10^7$  cells per ml. After incubation with 5  $\mu$ M FM4-64 at 30 °C for 30 min, the cells were collected by centrifugation, washed twice and then suspended in distilled water at a final density of  $1 \times 10^7$  cells per ml. The cells were incubated in the absence or presence of each compound with vigorous shaking at 30 °C for 60 min, and then observed under a phase-contrast microscope and a fluorescence microscope with excitation at 520–550 nm and emission at 580 nm.

### Measurement of AmB content by HPLC

Cells from the overnight culture in YPD medium were collected by centrifugation, washed twice with phosphate-buffered saline (PBS) and then suspended in PBS to obtain a final density of  $1 \times 10^8$  cells per ml. The cell suspension was incubated with 20  $\mu$ M AmB in the absence or presence of 120  $\mu$ M allicin at 30 °C for 60 min. The supernatant obtained after the removal of cells by centrifugation was used as the supernatant fraction. The cell pellets were washed twice with PBS and then suspended in PBS at a density of  $1 \times 10^8$  cells per ml. After the addition of yeast lytic enzyme at a final concentration of 6 mg ml<sup>-1</sup> and 0.5% 2-mercaptoethanol, the cells were incubated with gentle agitation at 30 °C for 60 min or longer to ensure complete cell lysis. The supernatant obtained after centrifugation (5000 g) of the cell lysate at 4 °C for 10 min was used as the cytoplasmic fraction. The pellets were washed twice with PBS. The final precipitate obtained was decomposed by repeated vortexing with a mixture of water/methanol/chloroform (30:20:50, v/v) at room temperature. The upper water-soluble layer was used as the plasma membrane fraction including phospholipids.<sup>6</sup>

The fractions obtained above were assayed for AmB content using HPLC with a reverse-phase column (4.6  $\times$  250 mm, COSMOSIL 5C<sub>18</sub>-MS-II, Nacalai Tesque, Kyoto, Japan). The chromatographic solvents were eluted at room temperature with a mobile phase consisting of a mixture of 0.1 M sodium acetate (pH 4.0)/acetonitrile (60:40, v/v) at a flow rate of 1.0 ml min<sup>-1</sup>. AmB was detected at 405 nm.<sup>16</sup>

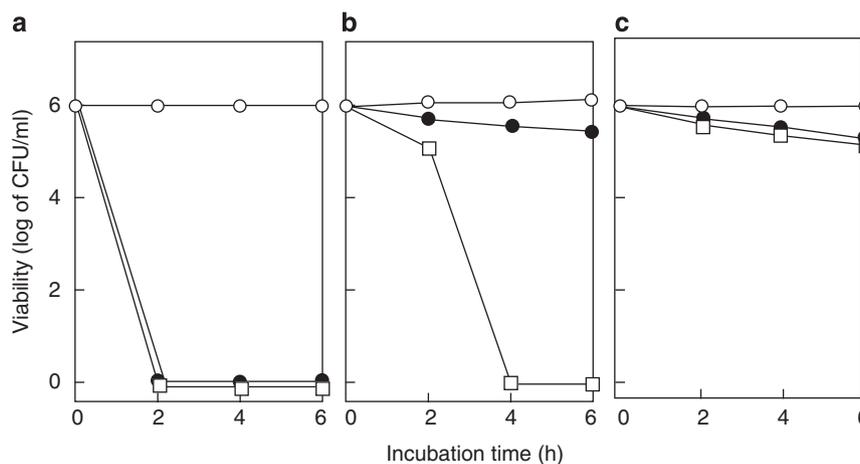
### Chemicals

AmB was purchased from Sigma Aldrich (St Louis, MO, USA). Allicin was obtained from LKT Laboratories (St Paul, MN, USA). Yeast lytic enzyme and FM4-64 were from ICN Biomedicals (Aurora, OH, USA) and Molecular Probes (Eugene, OR, USA), respectively. The other chemicals used were of analytical reagent grade and purity.

## RESULTS AND DISCUSSION

### Fungicidal activity of AmB and allicin

The addition of K<sup>+</sup> into the medium preserves intracellular K<sup>+</sup> content in AmB-treated cells of *S. cerevisiae*,<sup>3</sup> whereas the addition of Mg<sup>2+</sup> maintains membrane integrity and cell metabolism.<sup>17,18</sup> Therefore, co-addition of both K<sup>+</sup> and Mg<sup>2+</sup> markedly restricts both the growth-inhibitory and lethal effects of AmB.<sup>3</sup> On the other hand,  $\Delta$ *erg6* cells cannot convert zymosterol to fecosterol in the ergosterol biosynthetic pathway, leading to a reduction of ergosterol content in the plasma membrane and thereby increasing the resistance to polyene antibiotics such as AmB.<sup>19</sup> We first examined whether allicin could enhance the



**Figure 1** Fungicidal activity of amphotericin B (AmB) in the absence or presence of allicin against parent (a, b) and  $\Delta$ *erg6* (c) cells of *S. cerevisiae*. Cells ( $1 \times 10^6$  cells per ml) were incubated with none (○), 5  $\mu$ M AmB (●) and a combination of 5  $\mu$ M AmB and 120  $\mu$ M allicin (□) in distilled water, in the absence (a, c) or presence (b) of 85 mM KCl and 45 mM MgCl<sub>2</sub> at 30 °C.

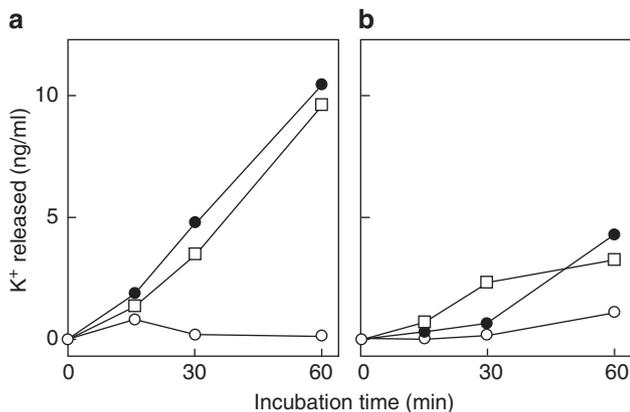
fungicidal activity of AmB in the presence or absence of  $K^+$  and  $Mg^{2+}$  against parent and  $\Delta erg6$  strains of *S. cerevisiae* W303-1A. Five micromolars of AmB, as well as the combination of  $5 \mu M$  AmB and  $120 \mu M$  allicin, showed fungicidal activity against the parent cells in the absence of  $K^+$  and  $Mg^{2+}$  (Figure 1a). The parent cells were resistant to  $5 \mu M$  AmB in distilled water supplemented with  $K^+$  and  $Mg^{2+}$  (Figure 1b). This supported the previous report<sup>17</sup> that the addition of both  $K^+$  and  $Mg^{2+}$  could decrease the lethal effect of AmB. In contrast, the combination of  $5 \mu M$  AmB and  $120 \mu M$  allicin showed fungicidal activity in the presence of  $K^+$  and  $Mg^{2+}$  (Figure 1b). Namely, AmB expressed fungicidal activity in combination with allicin even when the intracellular concentration of  $K^+$  was maintained at normal levels in  $K^+$ -enriched medium. On the other hand,  $\Delta erg6$  cells were resistant to AmB combined with allicin in addition to AmB alone (Figure 1c). These results suggest that the allicin-mediated fungicidal activity of AmB probably depends on the ergosterol content in plasma membrane, as well as the case of the activity of AmB alone, but not on the loss of intracellular  $K^+$  due to dysfunction of the plasma membrane.

### Potassium ion efflux induced by AmB and allicin

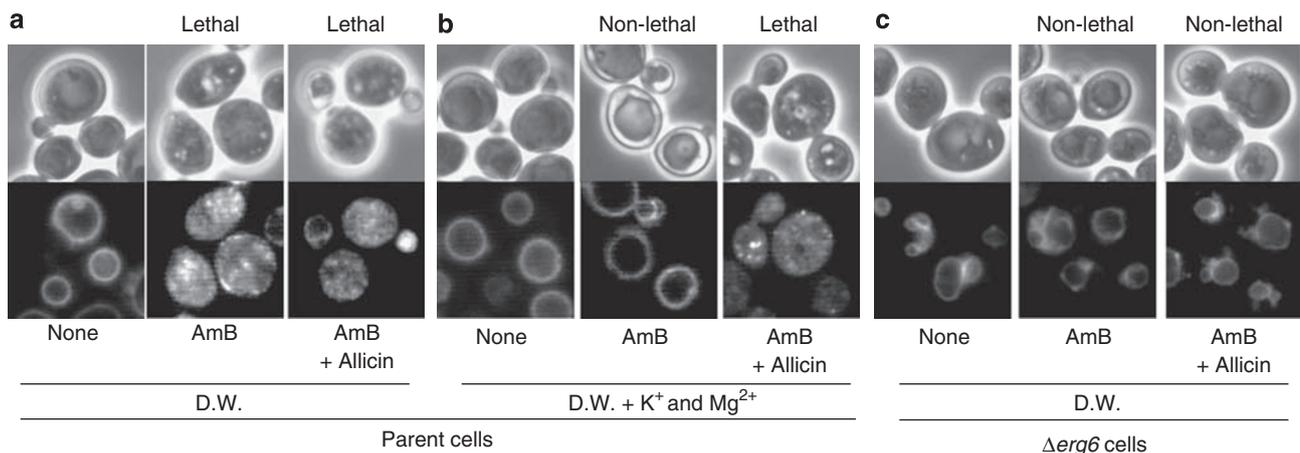
Ion-permeability change by AmB in fungal cells occurs because of the pores formed by ergosterol directly bound to AmB in the plasma membrane.<sup>20</sup> In  $\Delta erg6$  cells, the reduced content of ergosterol decreases the number of target molecules for AmB.<sup>19</sup> As shown in Figure 2a, the rate of  $K^+$  release caused by AmB in parent cells was similar in conditions with or without allicin, indicating the ion-permeability change induced by AmB. Allicin does not seem to have a role in this  $K^+$  efflux. Contrary to the results obtained with parent cells,  $\Delta erg6$  cells did not exhibit such a damage as the ion-permeability change caused by AmB, even if allicin was added simultaneously with AmB (Figure 2b). The fungicidal activity of AmB combined with allicin was expressed under conditions with or without  $K^+$  on parent cells (see Figure 1b). These results indicate that cell death induced by the combination of AmB and allicin was unrelated to the ion-permeability change caused by AmB.

### Enhancement effects of allicin on vacuole disruption

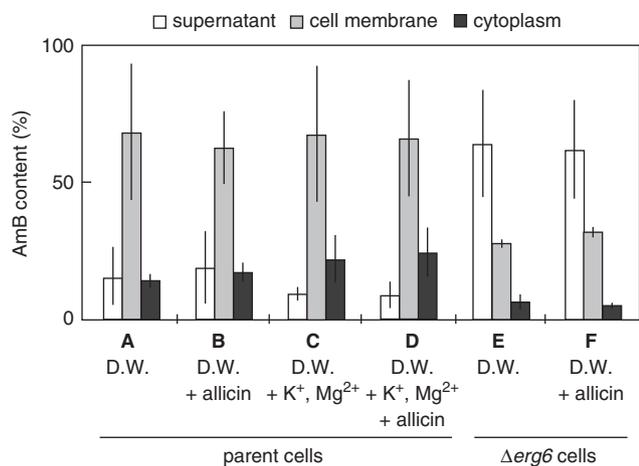
We further examined whether allicin could amplify the vacuole-disruptive activity of AmB when the intracellular  $K^+$  concentration was maintained by external addition of  $K^+$  and  $Mg^{2+}$  in parent cells and  $\Delta erg6$  cells. In these experiments, conditions with  $K^+$  and  $Mg^{2+}$  were applied only to parent cells because  $K^+$  did not leak in  $\Delta erg6$  cells treated with AmB (see Figure 2b). AmB disrupted vacuoles in the absence of  $K^+$  and  $Mg^{2+}$  (Figure 3a), and eventually caused cell death. The coexistence of  $K^+$  and  $Mg^{2+}$  maintained the normal morphology of vacuoles in parent cells treated with AmB alone (Figure 3b). Cells remained viable in this treatment (see Figure 1b). These suggested that the maintenance of intracellular  $K^+$  concentrations might restrict the cell death, which was induced by both the plasma membrane permeability change and the vacuole disruption caused by AmB alone. On the other hand, the combination of AmB and allicin disrupted vacuoles as well as the case of AmB alone in the absence of  $K^+$  and  $Mg^{2+}$ , followed by cell death, under conditions with or without  $K^+$  and  $Mg^{2+}$  (Figures 3a and b). These results indicated that the inhibition of ergosterol trafficking from plasma membrane to vacuoles caused by allicin was able to overcome the vacuole stabilization provided by adding  $K^+$  and  $Mg^{2+}$ . On the other hand, vacuoles of  $\Delta erg6$  cells were not disrupted by both AmB alone and the combination of AmB and allicin (Figure 3c). Although lethality induced by



**Figure 2** Promotive effects of amphotericin B (AmB) and the combination of AmB and allicin on leakage of  $K^+$  from parent (a) or  $\Delta erg6$  (b) cells of *S. cerevisiae*. Cells ( $1 \times 10^8$  cells per ml) were incubated in 50 mM Tris-HCl buffer (pH 7.4) containing none (○), 5  $\mu M$  AmB (●), and a combination of 5  $\mu M$  AmB and 120  $\mu M$  allicin (□) at 30 °C.



**Figure 3** The effects of amphotericin B (AmB) and the combination of AmB and allicin on vacuole morphology in parent (a, b) and  $\Delta erg6$  (c) cells of *S. cerevisiae*. After treatment with the fluorescent dye FM4-64, cells ( $1 \times 10^7$  cells per ml) were incubated in distilled water (a, c), or in 85 mM KCl and 45 mM  $MgCl_2$  added to distilled water (b), each containing none, 5  $\mu M$  AmB or a combination of 5  $\mu M$  AmB and 120  $\mu M$  allicin at 30 °C for 60 min. Cells were observed under a bright-field microscope (top) and a fluorescence microscope (bottom).



**Figure 4** Subcellular localization of amphotericin B (AmB) in parent (A-D) and  $\Delta erg6$  (E and F) cells of *S. cerevisiae*. Cells ( $1 \times 10^8$  cells per ml) were incubated in distilled water (A, B, E and F) or in distilled water added with 85 mM KCl and 45 mM  $MgCl_2$  (C and D), each containing  $20 \mu M$  AmB in the absence (A, C and E) or presence (B, D and F) of  $120 \mu M$  allicin at  $30^\circ C$  for 60 min. The AmB content in the supernatant (open bar), plasma membrane (gray bar) and cytoplasmic fractions (black bar) was determined by HPLC analysis. Data are expressed as the mean (s.d.) of the AmB content (% of the total amount added to the cells) measured in triplicate assays. The AmB content of each fraction was statistically analyzed by Student's *t*-test, in which  $P < 0.05$  was considered statistically significant. Significant differences of AmB content were identified on each fraction in parent and  $\Delta erg6$  cells.

AmB is known to depend on the leakage of intracellular  $K^+$ , lethality in yeast cells seems to be related with vacuole disruption. Moreover, the existence of ergosterol in the plasma membrane is needed for vacuole disruption induced by AmB with or without allicin.

#### Cellular uptake of AmB

In our recent study using cells of *C. albicans*, we added AmB to the cell suspension to determine cellular uptake of AmB. AmB was mostly absent in the supernatant of the cell suspension after incubation for 60 min. However, in the plasma membrane fractions, AmB was detected at a ratio of more than 80% of the initial quantity at 0 min.<sup>6</sup> Further, the remaining AmB was quantitatively found in the cytoplasmic fraction. These results suggest that AmB permeates through the plasma membrane and invade the cytoplasm. In this study, we determined the subcellular uptake of AmB in parent and  $\Delta erg6$  cells in the presence or absence of  $K^+$  and  $Mg^{2+}$ . In this experiment, the concentrations of AmB were increased up to  $20 \mu M$  with increasing cell density up to  $1 \times 10^8$  cells per ml, owing to the sensitivity of HPLC analysis, which required the volume of AmB above a certain level. In agreement with our previous results,<sup>6</sup> the major proportion of AmB was detected in the plasma membrane fraction of parent cells treated with AmB, regardless of  $K^+$  and  $Mg^{2+}$  supplementation (Figure 4, A and C). The addition of allicin did not affect the subcellular uptake of AmB in parent cells (Figure 4, B and D). On the other hand, in  $\Delta erg6$  cells, the major proportion of AmB markedly remained in the supernatant; that is, the contents of AmB in the fractions of both plasma membrane and cytoplasm in  $\Delta erg6$  cells were significantly reduced in comparison with those in parent cells ( $P < 0.05$ ), regardless of the presence of allicin (Figure 4, E and F). AmB could directly disrupt the isolated vacuoles of *S. cerevisiae* and *C. albicans*.<sup>6,12,13</sup> Thus, taken together, these results suggest that the incorporation of AmB into the cytoplasm is at least needed for direct

vacuole disruption and is restricted by the reduction of ergosterol in the plasma membrane; however, it is not affected by the presence of allicin or the intracellular levels of  $K^+$ .

Our findings suggest that vacuole disruption correlates with cell death but not with changes in intracellular concentrations of  $K^+$ . Vacuole disruption induced by the combination of AmB and allicin at least depended on the presence of ergosterol in the plasma membrane for the initial interaction with AmB. Our previous reports showed that allicin might enhance the fungicidal activity of AmB by inhibiting ergosterol trafficking from plasma membrane to vacuoles.<sup>12</sup> Vacuole disruption induced by the combination of AmB and allicin may require both the reduction of ergosterol in the vacuole membrane and the presence of ergosterol in the plasma membrane. Moreover, these results might indicate that AmB, as a vacuole-targeting anti-biotic, cannot directly penetrate the plasma membrane but can be transported into the cytoplasm via interaction with ergosterol in the plasma membrane.

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- Ghannoum, M. A. & Rice, L. B. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* **17**, 501–517 (1999).
- Carrillo-Muñoz, A. J., Giusiano, G., Ezkurra, P. A. & Quindós, G. Antifungal agents: mode of action in yeast cells. *Rev. Esp. Quimioter.* **19**, 130–139 (2006).
- Brajtburg, J., Medoff, G., Kobayashi, G. S. & Elberg, S. Influence of extracellular  $K^+$  or  $Mg^{2+}$  on the stages of the antifungal effects of amphotericin B and filipin. *Antimicrob. Agents Chemother.* **18**, 593–597 (1980).
- Alonso, M. A., Vázquez, D. & Carrasco, L. Compounds affecting membranes that inhibit protein synthesis in yeast. *Antimicrob. Agents Chemother.* **16**, 750–756 (1979).
- Okamoto, Y., Aoki, S. & Mataga, I. Enhancement of amphotericin B activity against *Candida albicans* by superoxide radical. *Mycopathologia* **158**, 9–15 (2004).
- Borjihan, H., Ogita, A., Fujita, K., Hirasawa, E. & Tanaka, T. The vacuole-targeting fungicidal activity of amphotericin B against the pathogenic fungus *Candida albicans* and its enhancement by allicin. *J. Antibiot.* **62**, 691–697 (2009).
- Baginski, M., Czub, J. & Sternal, K. Interaction of amphotericin B and its selected derivatives with membranes: molecular modeling studies. *Chem. Rec.* **6**, 320–332 (2006).
- Liu, T. T. *et al.* Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. *Antimicrob. Agents Chemother.* **49**, 2226–2236 (2005).
- Ankri, S. & Mirelman, D. Antimicrobial properties of allicin from garlic. *Microbes Infect.* **1**, 125–129 (1999).
- Oommen, S., Anto, J. R., Srinivas, G. & Karunakaran, D. Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells. *Eur. J. Pharmacol.* **485**, 97–103 (2004).
- Ogita, A., Fujita, K., Taniguchi, M. & Tanaka, T. Enhancement of the fungicidal activity of amphotericin B by allicin, an allyl-sulfur compound from garlic, against the yeast *Saccharomyces cerevisiae* as a model system. *Planta Med.* **72**, 1247–1250 (2006).
- Ogita, A., Fujita, K. & Tanaka, T. Enhancement of the fungicidal activity of amphotericin B by allicin: effects on intracellular ergosterol trafficking. *Planta Med.* **75**, 222–226 (2009).
- Ogita, A., Fujita, K., Usuki, Y. & Tanaka, T. Targeted yeast vacuole disruption by polyene antibiotics with a macrocyclic lactone ring. *Int. J. Antimicrob. Agents.* **35**, 89–92 (2010).
- Ramotowski, S. & Szczesniak, M. Determination of potassium salt content in pharmaceutical preparations by means of sodium tetraphenylborate. *Acta. Pol. Pharm.* **24**, 605–613 (1967).
- Ogita, A. *et al.* Synergistic fungicidal activities of amphotericin B and *N*-methyl-*N'*-dodecylguanidine: a constituent of polyol macrolide antibiotic niphimycin. *J. Antibiot.* **60**, 27–35 (2007).
- Esposito, E., Bortolotti, F., Menegatti, E. & Cortesi, R. Amphiphilic association systems for amphotericin B delivery. *Int. J. Pharm.* **260**, 249–260 (2003).
- Romero, P. J. The role of membrane-bound magnesium in the permeability of ghosts to  $K^+$ . *Biochim. Biophys. Acta.* **339**, 116–125 (1974).
- Suomalainen, H. & Oura, E. in *The Yeasts*. Yeast nutrition and solute uptake, physiology and biochemistry of yeasts (eds Rose, A.H. & Harrison J.S.) 3–74 (Academic Press Inc., New York, 1971).
- Young, L. Y., Hull, C. M. & Heitman, J. Disruption of ergosterol biosynthesis confers resistance to amphotericin B in *Candida lusitanae*. *Antimicrob. Agents Chemother.* **47**, 2717–2724 (2003).
- Baginski, M., Sternal, K., Czub, J. & Borowski, E. Molecular modelling of membrane activity of amphotericin B, a polyene macrolide antifungal antibiotic. *Acta. Biochimica. Polonica.* **52**, 655–658 (2005).