Generation of Novel Fungicidal Activity by the Combined Actions of Hygromycin B and Polymyxin B

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***Abstract*– The aminoglycoside antibiotic hygromycin B (HgB) has been widely used in veterinary medicine and in cell culture selection. HgB kills bacteria, fungi, and higher eukaryotic cells by inhibiting protein synthesis. A marked synergistic relationship was observed between HgB and the bactericidal antibiotic polymyxin B (PMB) in their ability to cause cell death of *Saccharomyces cerevisiae*, but this was not observed when *S. cerevisiae* cells were treated with PMB and a different aminoglycoside selective for prokaryotic organisms. However, the combined lethal actions of HgB and PMB did not depend on the inhibition of 80S ribosomal protein synthesis, even if the ribosome-binding OH of HgB is similarly involved in its PMB-dependent fungicidal activity. Our findings could have implications for improving the use of HgB in veterinary medicine.**

Keywords- Saccharomyces cerevisiae; Hygromycin B; Polymyxin B; Fungicidal Activity

I. INTRODUCTION

Hygromycin B (HgB, Fig 1A) is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* that is characterized by a unique molecular structure consisting of a dual ester linkage between 2 of its 3 sugar moieties [1, 2], and it has functional properties related to protein biosynthesis inhibition [3]. This antibiotic was developed in the early 1950s for use in veterinary medicine, and it is still used as a feed additive to control roundworms, nodular worms, and whip worms in swine and large round worms, cecal worms, and capillary worms in poultry [1]. The mechanism of HgB-mediated protein synthesis inhibition has been extensively studied in *Escherichia coli*. These studies have shown that HgB interferes with ribosomal subunit formation, as well as the translocation of mRNA and tRNA [4, 5]. The unique molecular structure of HgB may also contribute to its inhibitory effect on the activity of ribosomal ATPase [6]. Additionally, HgB resistance is used as the most common dominant selectable marker in the genetic manipulation of bacteria, fungi, plants, insects, and mammalian cells, in which the *E. coli* HgB-phosphotransferase confers the cellular resistance to the antibiotic [7–10]. HgB can indeed inhibit the growth of *Saccharomyces cerevisiae*, whereas its Δ*vps* mutants, which lack a vacuole protein-sorting function, are hypersensitive to the antibiotic, supporting a linkage between HgB sensitivity and vacuolar function defects [11–13].

Polymyxin B (PMB, Fig. 1B) is a cyclic bactericidal antibiotic that can selectively inhibit the growth of gram-negative bacteria and can weakly inhibit the growth of fungal cells such as *S. cerevisiae* [11, 14–16]. This antibiotic has re-emerged in clinical practice owing to the dry antibiotic development pipeline and the increasing prevalence worldwide of nosocomial infections caused by multidrug-resistant gram-negative bacteria [16]. PMB passing through the bacterial outer membrane interacts with acidic phospholipids exposed on the plasma membrane, and thus, its bactericidal action likely depends on an increase in the permeability of the plasma membrane to intracellular molecules [16, 17]. PMB can also interact with the plasma membrane of *S. cerevisiae* and other fungal cells to a less extent; therefore, PMB-induced plasma membrane damage might be a primary cause of its fungicidal action [18–21]. We recently found vacuole membrane damage in PMB-treated cells from *S. cerevisiae* and some filamentous fungi, suggesting this intracellular event to be a more direct cause of its fungicidal activity [22]. The vacuole-targeting fungicidal activity of PMB can be markedly enhanced in the presence of allicin, an allyl-sulfur compound from garlic, as well as zwiebelane A, an organosulfur compound isolated from onion [22–24]. Ionophores such as salinomycin and monensin are also effective enhancers of the vacuole-disruptive action of PMB [25].

It is highly probable that *S. cerevisiae* cells are rendered hypersensitive to the action of HgB in PMB-supplemented medium, based on the assumption that PMB can interfere with a certain vacuole-related function even at a non-lethal concentration, as is the case with the HgB hypersensitivity of various Δ*vps* mutants [12, 13]. In accordance with this postulation, a typical synergistic relationship was observed between HgB and PMB in their growth-inhibitory activities against *S. cerevisiae*. In this study, we characterized the newly found lethal action on the basis of a relationship between the activity of protein synthesis inhibition and the molecular structure essential for ribosome binding. Our findings could have implications for improving the use of aminoglycoside antibiotic in veterinary medicine.

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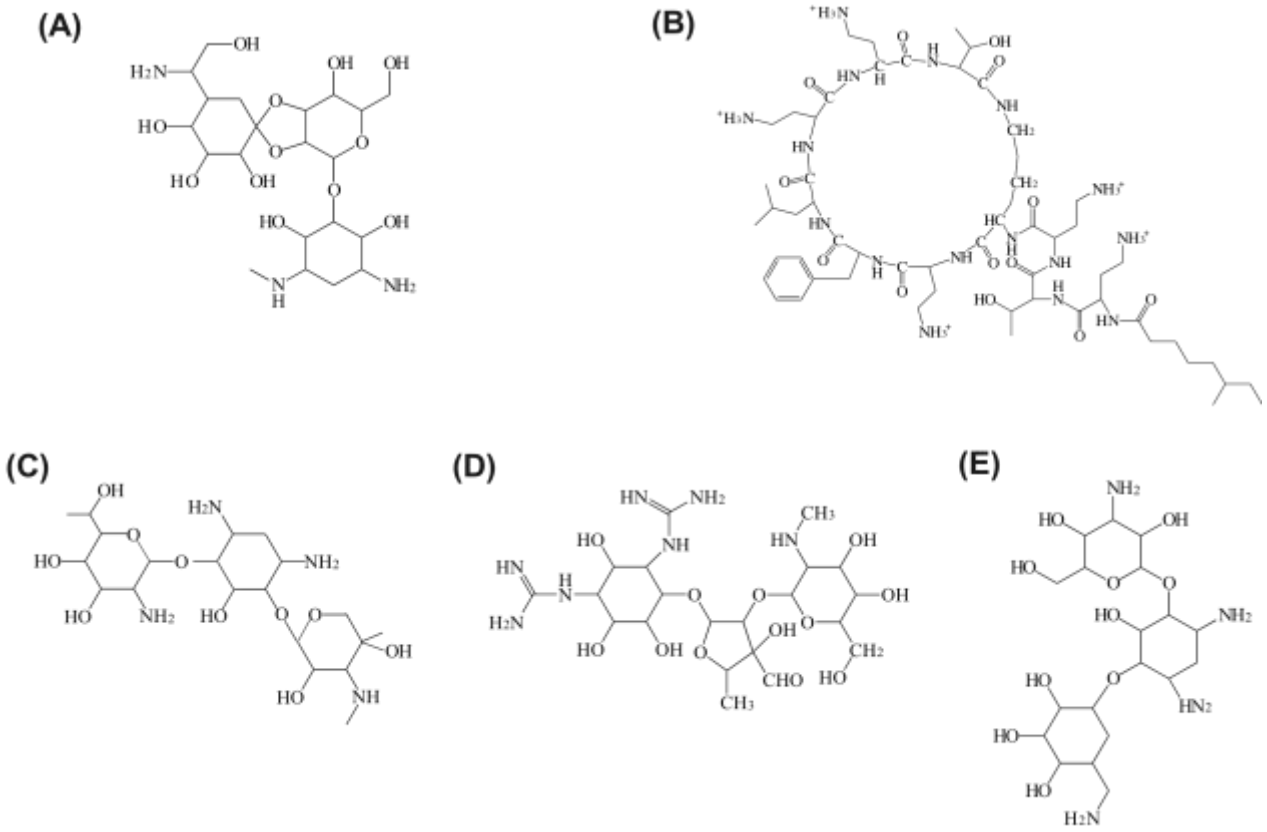

Fig. 1 Structures of hygromycin B (A), polymyxin B (B), geneticin (C), kanamycin (D), and streptomycin (E)

II. MATERIALS AND METHODS

A. Measurement of Yeast Cell Growth and Viability

S. cerevisiae BY4741 and its isogenic strain transformed with a plasmid carrying the HgB-phosphotransferase gene were used [26]. Unless otherwise stated, the yeast cells were grown overnight in YPD medium, which contained (per liter) 10 g of yeast extract, 20 g of peptone, and 20 g of D-glucose, with vigorous shaking at 30 ℃. Cells were then inoculated into freshly prepared YPD medium at a density of 10^6 cells/ml or 10^7 cells/ml and incubated with or without each of the various aminoglycosides and PMB at 30 ℃. The minimum growth inhibitory concentrations (MICs) were determined by the serial broth dilution method, as described in our previous study [22]. Viable cell number was measured by counting colony-forming units after 48-h incubation at 30 ℃ in YPD medium containing 1.8% (w/v) agar [22].

B. Assay of Protein Synthesis

S. cerevisiae cells were grown overnight in YPD medium and were then washed with SD medium, which contained (per liter) 6.7 g of yeast nitrogen base without amino acids, 5 g of casamino acid, 20 g of D-glucose, 2 mg of L-histidine, 2 mg of L-leucine, and 2 mg of uracil, and suspended in the same medium at a density of 10^7 cells/ml. The cell suspension was further supplemented with L-[^{35}S] methionine at a final concentration of 14.8 kBq/ml, and incubated in the absence or presence of each compound with vigorous shaking at 30 ℃. After 20 min, an aliquot of each cell suspension (100 μl) was withdrawn and mixed with an equal volume of 10% trichloroacetic acid (TCA). TCA-insoluble precipitates were collected on cellulose acetate filters, and then, the filters were washed 3 times with 5% TCA. Radioactivity on the filter was measured with the addition of 5 ml of a toluene-based cocktail using a liquid scintillation counter LS6500 (Beckman Coulter, Inc., Brea, CA).

C. Vacuole Staining

Vacuoles were visualized by staining with the fluorescent probe FM4-64 according to our previously described method [22]. Briefly, cells from the overnight culture in YPD medium were suspended in freshly prepared YPD medium to obtain a cell density of 1×10^7 cells/ml. After incubation with 5 μM FM4-64 at 30 ℃ for 30 min, the cells were collected by centrifugation, washed twice with YPD medium, and suspended in the same medium at a cell density of 1×10^7 cells/ml. The cells were then incubated in the absence or presence of each compound with vigorous shaking at 30 ℃ for 60 min and were observed under phase-contrast microscopy and fluorescence microscopy with excitation at 520–550 nm and emission at 580 nm.

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D. Assay of Vacuolar pH

Vacuolar pH was measured using the fluorescent dye 2′-7′-bis-(2-caboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethylester (BCECF-AM) [27] according to the method of Ziegelbauer *et al*. [28] with the following minor modifications. Cells from overnight culture were washed and resuspended in freshly prepared YPD medium at a cell density of 1×10^7 cells/ml, and the cell suspension was then incubated in the absence or presence of each compound with vigorous shaking at 30 ℃ for 60 min. After several washes with freshly prepared YPD medium, a portion (200 μl) of each suspension was mixed with BCECF-AM at a final concentration of 50 μM in 96-well microtiter plate, and the fluorescence was detected using a Tecan GENios Fluorescence Detector (MTX Lab Systems, Inc., Vienna, VA) at the excitation wavelength of 480 nm and the emission wavelength of 595 nm.

E. HgB Phosphotransferase Gene Manipulation

pAG32, which contains a bacterial gene conferring resistance to HgB (*hph*, encoding HgB phosphotransferase) was obtained from EUROSCARF (Frankfurt, Germany). The 1.7-kb *Sac*I–*Sal*I *hph* fragment was excised from pAG32 and ligated into the same site of pAUR112 (Takara Bio, Shiga, Japan) including *URA3* to yield a recombinant plasmid pAUR112-*hph*. After transformation with pAUR112-*hph*, *S. cerevisiae* BY4741 cells were spread on a uracil-free SD agar plate and incubated at 30 ℃ for 48 h. Several colonies on the SD agar plate were then replica-plated onto a YPD agar plate containing 800 μM HgB in order to examine their HgB-resistant phenotype at 30 ℃ for 24 h.

F. Chemicals

HgB, PMB, geneticin, kanamycin, streptomycin, and cycloheximide were purchased from Sigma (St. Louis, MO). FM4-64 was a product of Molecular Probes (Eugene, OR). L-[^{35}S]-methionine (>37 TBq/mmol) was purchased from Muromachi Yakuhin Kaisha Ltd. (Tokyo, Japan). BCECF-AM is a product of Life Technologies (Grand Island, NY). Other chemicals were of analytical reagent grade.

G. Statistic Methods

The statistical evaluation was analyzed by using Student's *t*-test, in which $P < 0.05$ was considered statistically significant.

III. RESULTS

A. Synergy Between HgB and PMB

As expected from the hypersensitivity of various *S. cerevisiae* Δ*vps* mutants to HgB [10], the isobologram represented a typical synergistic relationship between HgB and PMB in their growth-inhibitory activities against cells of the parent strain (Fig. 2A). Geneticin (Fig. 1C) could also work in synergy with PMB (Fig. 2B). However, the isobologram represented a typical indifference pattern for the relationship between PMB and either kanamycin (Fig. 1D) or streptomycin (Fig. 1E), which inhibit protein synthesis only in prokaryotic organisms (Fig. 2C, D).

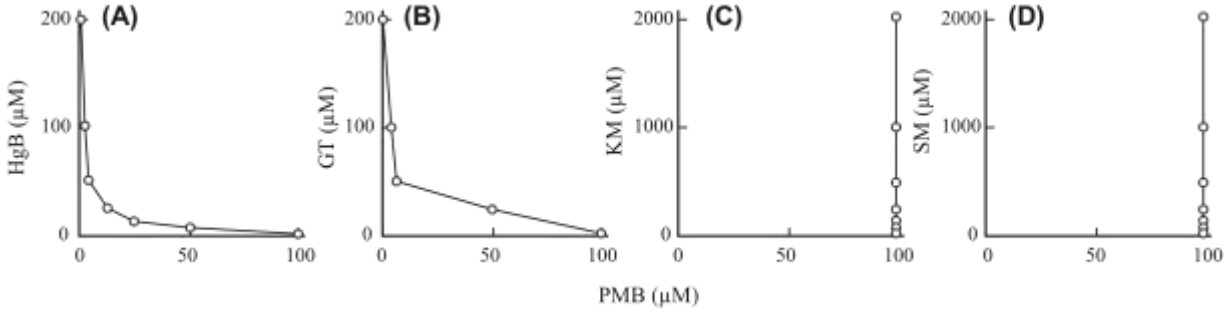

Fig. 2 Isobologram demonstrating synergistic effects of polymyxin B (PMB) and each of aminoglycosides on the growth of *S. cerevisiae*. Cells were incubated in a medium containing PMB and hygromycin B (HgB) (A), geneticin (GT) (B), kanamycin (KM) (C), or streptomycin (SM) (D) at 30 ℃ for 24 h. Each data point is expressed as the mean of triplicate assays.

B. Mode of Combined Actions of HgB and PMB

We then examined how HgB and PMB affect the growth of *S. cerevisiae* cells individually or in combination, by counting the number of viable cells. As shown in Fig. 3A, HgB inhibited yeast cell growth in a dose-dependent fashion, thereby slightly reduced the viable cell number when added at the MIC value of 200 μM. PMB was also weakly lethal to the yeast cells when added at the MIC value of 100 μM (Fig. 3B). Unlike the cells treated with either HgB or PMB alone, a significant increase in the rate of cell death progression was observed when cells were incubated with both HgB and PMB, even at non-lethal concentrations (Fig. 3C). These results indicate that PMB enhances the cellular incorporation of HgB into the cytoplasm, enabling this aminoglycoside antibiotic to interfere with the ribosomal protein synthesis reaction at a lower concentration than that expected from its own MIC value.

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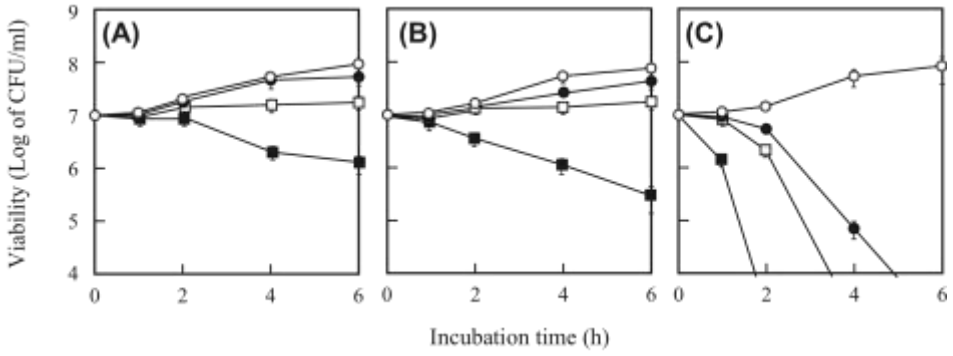

Fig. 3 Effects of hygromycin B (HgB), polymyxin B (PMB), and a combination of HgB and PMB on the growth of *S. cerevisiae*. In (A), cells were incubated in a medium containing HgB at 0 (○), 50 (●), 100 (□), or 200 μM (■) at 30°C. In (B), cells were incubated in medium containing PMB at 0 (○), 25 (●), 50 (□), or 100 μM (■) at 30°C. In (C), cells were incubated in medium alone (○) or in medium containing 25 μM PMB and HgB at 25 (●), 50 (□), or 100 μM (■) at 30 ℃. Data are expressed as the mean ± standard deviation in triplicate assays.

C. Effects of HgB and PMB on Protein Synthesis

HgB can inhibit the growth of *S. cerevisiae* at a concentration higher than the concentration that can inhibit protein synthesis catalyzed by the isolated polysome fraction [27] (Fig. 3). Such a difference in HgB sensitivities between *in vivo* and *in vitro* assays should depend on the concentration of HgB required for its incorporation into the cytoplasm. We therefore compared the concentration of HgB that inhibits protein synthesis in intact *S. cerevisiae* cells with the concentration that inhibits yeast cell growth. Cycloheximide is a commonly used protein synthesis inhibitor in eukaryotic cells, and thus, it could completely inhibit protein synthesis in *S. cerevisiae* cells in parallel with the inhibition of cell growth (Fig. 4B). The rate of protein synthesis was kept at a level comparable to that in untreated cells when cells were incubated in a medium with either 200 μM HgB or 100 μM PMB, in which the cell viability was only slightly lost after 1-h incubation (Fig. 4C, D; Fig. 3). Protein synthesis was detectable at a slightly increased rate, as measured by the rate of L-[^{35}S] methionine incorporation during the initial 20-min incubation, in which cells were kept mostly viable even in a medium containing both 100 μM HgB and 25 μM PMB (Fig. 4E). These findings suggest that in the presence of PMB, HgB can interact with an unidentified molecular target at a lower concentration than required for the usual mode of protein synthesis inhibition.

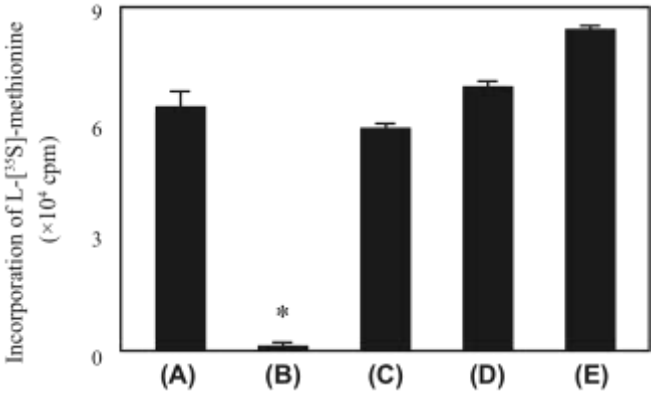

Fig. 4 Effects of hygromycin B (HgB), polymyxin B (PMB), and a combination of HgB and PMB on the protein synthesis of *S. cerevisiae*. Cells were incubated in a medium containing no additive (A), 50 μM cycloheximide (B), 200 μM HgB (C), 100 μM PMB (D), and both 100 μM HgB and 25 μM PMB (E) at 30 ℃ for 20 min. Data are expressed as the mean ± standard deviation in triplicate assays and were statistically analyzed by Student's *t*-test, in which $P < 0.05$ was considered statistically significant (*).

D. Effects of HgB and PMB on Vacuole Morphology and Function

As deduced from the enhancement effects of various compounds on the fungicidal activity of PMB, HgB is likely to enhance the vacuole-disruptive activity of PMB [21, 23, 24]. We therefore examined the vacuole morphology in cells treated with either HgB or PMB alone at the lethal concentration, as well as in cells treated with both compounds together. As shown in Fig. 5, the yeast vacuoles were visible as fragmented particles in cells treated with the lethal concentration of PMB alone, whereas the organelles appeared to have normal rounded architecture in cells treated with the lethal concentration of HgB alone. The organelles were also observed with normal rounded architecture even if cells were subjected to highly lethal damage in a medium containing both HgB and PMB.

Vacuole pH is normally maintained at a mildly acidic value of approximately 6.2 [26], and it increased to 6.7 when cells were incubated in a medium containing both 100 μM HgB and 25 μM PMB. However, such an increase in the vacuole pH cannot be considered a primary cause of the combined lethal actions of these compounds, because vacuole pH similarly increased when cells were treated with 25 μM PMB alone. Therefore, the newly generated lethality could not be attributed to

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an enhancement effect of HgB on the vacuole-disruptive action of PMB or even on its effect of the pH-regulatory function of the organelle.

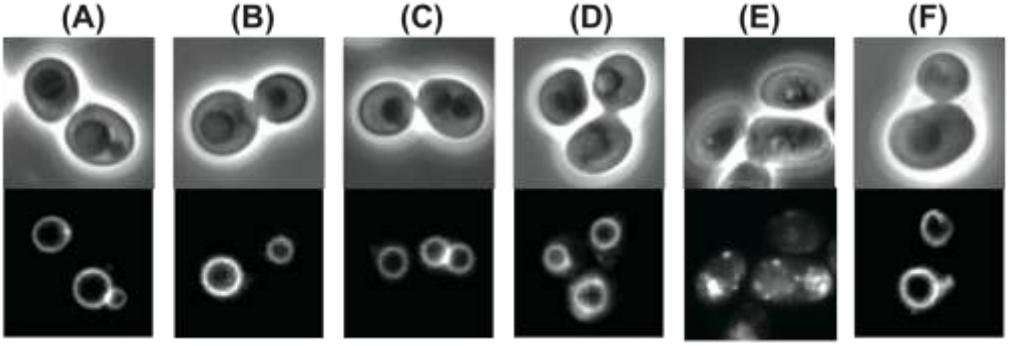

Fig. 5 Effects of hygromycin B (HgB), polymyxin B (PMB), and a combination of HgB and PMB on the vacuole morphology of *S. cerevisiae*. Cells were incubated in a medium containing no additive (A), 50 μM HgB (B), 200 μM HgB (C), 25 μM PMB (D), 100 μM PMB (E), and both 100 μM HgB and 25 μM PMB (F) at 30 ℃ for 60 min. Cells were then observed by phase-contrast microscopy (top) and fluorescence microscopy (bottom).

E. Effect of HgB Phosphotransferase Gene Expression on the Combined Lethal Actions of HgB and PMB

HgB is completely inactivated by ATP-dependent phosphorylation of the 4-OH on its hyosamine moiety by aminoglycoside phosphotransferase-(4)-Ia [28, 29]. This phosphotransferase gene from *E. coli* can be effectively expressed in eukaryotic cells, including the yeast *S. cerevisiae* [30]. We therefore examined whether the corresponding phosphate is essential for generating the combined lethal action of HgB and PMB. HgB showed growth inhibition or a slightly lethal action at a concentration of approximately 200 μM against the HgB-sensitive parent strain (see Fig. 2 and 3), whereas cells of the HgB-resistant strain grew even in a medium containing 8 mM HgB (Fig. 6A). This result suggests that these cells not only produce HgB phosphotransferase but also successfully phosphorylate HgB. Cells of the HgB-resistant strain were still viable in a medium containing both 8 mM HgB and 25 μM PMB, even though cell growth was fully repressed by treatment with PMB alone (Fig. 6B and 6C). These findings indicate that the enzymatic phosphorylation of HgB results in the absolute loss of its original lethality, depending on the protein synthesis inhibition, as well as the lethality generated in combination with PMB.

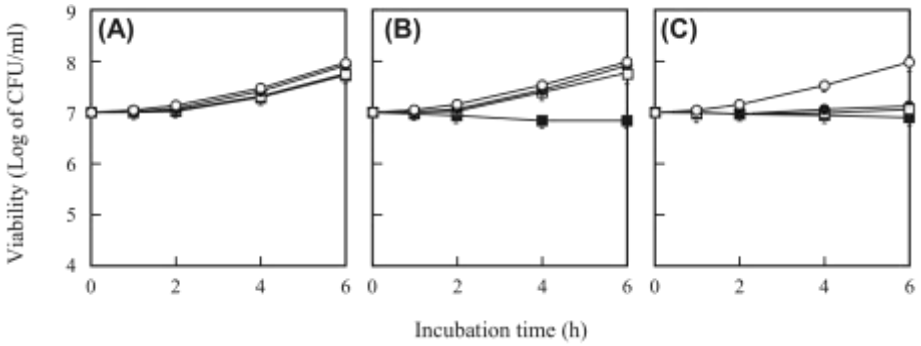

Fig. 6 Effects of hygromycin B (HgB), polymyxin B (PMB), and a combination of HgB and PMB on the growth of HgB-resistant *S. cerevisiae* cells. In (A), cells were incubated in a medium containing HgB at 0 (○), 2 (●), 4 (□), or 8 mM (■) at 30°C. In (B), cells were incubated in a medium containing PMB at 0 (○), 25 (●), 50 (□), or 100 μM (■) at 30°C. In (C), cells were incubated in a medium alone (○) or in a medium containing both 25 μM PMB and HgB at 2 (●), 4 (□), or 8 mM (■) at 30°C. Data are expressed as the mean ±standard deviation in triplicate assays.

IV. DISCUSSION

In addition to protein synthesis in bacteria, HgB can inhibit the ribosomal protein synthesis reaction, including the translocation of mRNA and tRNAs, in fungi and higher eukaryotic organisms. HgB was needed at a relatively high concentration (380 μM) to completely inhibit the growth of *S. cerevisiae* cells in a nutrient medium, and this is mostly in agreement with the finding that cellular protein synthesis could be fully inhibited when the yeast spheroplasts were treated with HgB at 450 μM [29]. Interestingly, a much lower concentration of HgB (2 μM) was effective in inhibiting the peptidyl-tRNA translocation together with peptide elongation when these reactions were assayed using the isolated polysome fraction *in vitro* [30]. Such a difference in the effective concentrations of HgB agrees with its energy-dependent permeability across the plasma membrane of the yeast cells.

Both prokaryotic 70S and eukaryotic 80S ribosomes are inhibited by HgB [5]. Because PMB can increase plasma membrane permeability, treatment with PMB may enhance cellular uptake of HgB by *S. cerevisiae* cells, resulting in the inhibition of 80S ribosomal protein synthesis at a concentration lower than the MIC of HgB itself. However, 80S ribosomal protein synthesis reaction cannot be evaluated as a target of the combined lethal actions of HgB and PMB, because the rate of L-[^{35}S]-methionine incorporation into the TCA-insoluble fraction was slightly increased during the initial incubation step (see

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Fig. 4). This may be also supported by the observation that cells of various Δ*vps* mutants are hypersensitive to HgB, because the corresponding gene deletion is not likely to result in an increase in plasma membrane permeability to HgB [12].

The ribosome-binding ability of HgB is completely lost because of enzymatic phosphorylation at the 4-OH of its hyosamine moiety [30, 31]. Furthermore, a synergistic relationship was observed not only between PMB and HgB but also between PMB and geneticin, which can similarly inhibit the protein synthetic reaction in eukaryotic organisms. These findings still support the idea that the combined lethal actions of HgB and PMB depend on an inhibition of the highly coordinated process of ribosomal protein synthesis reaction. However, it is unknown whether the ribosomal peptide elongation proceeds normally to yield mature and functional proteins in cells treated with both HgB and PMB even if L-[^{35}S]-methionine incorporation into the TCA-insoluble fraction occurs at a slightly increased rate (see Fig. 4). Davis [32] proposed a unified theory for the bactericidal action of aminoglycosides, suggesting that the binding of the aminoglycoside to the ribosome results in misreading of the mRNA and the formation of misfolded, non-functional proteins. This assumption may be applied to the combined lethal actions of HgB and PMB.

In mammalian cells, aminoglycosides have pleiotropic effects: K^+ efflux, transient stimulation of RNA synthesis, putrescine excretion, mistranslation of RNA to yield aberrant proteins, depletion of polyribosomes, loss of cell membrane integrity, and blockade of the initiation of DNA replication [8, 33]. The influence of aminoglycosides on cyclic AMP and guanosine pentaphosphate levels might explain some of these pleiotropic effects. One of these HgB-induced events may be alternatively enhanced when cells are treated with PMB at a non-lethal concentration, as in the case with a genetic lack of Vps34p, Vps45p, Vps52p, or Vps54p, resulting in a defect in the vacuole function [12].

HgB has been in use for a long time in veterinary medicine and was approved by the Food and Drug Administration (FDA). Aminoglycoside antibiotics are indispensable for treatment of serious bacterial infections, and despite careful attention to dosage regimens, nephrotoxicity and ototoxicity still cause concern [34]. In this study, we demonstrated that PMB exerts a potent enhancing effect on the fungicidal activity of HgB against *S. cerevisiae* cells. This enhancing effect by PMB can reduce the consumption of HgB. Although the mechanism of the fungicidal action remains unclear, our findings may have significant implications regarding the use of aminoglycoside antibiotics in veterinary medicine.

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