Mode of Antifungal Action of Benanomicin A in *Saccharomyces cerevisiae*

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The mechanism of fungitoxic action of an antifungal antibiotic benanomicin A was studied with intact cells and protoplasts of *Saccharomyces cerevisiae* as well as with its enzymic preparations. The results obtained are summarized as follows: (1) benanomicin A at relatively high concentrations (almost equal to MIC) was fungicidal and disrupted the cell permeability barrier, inducing leakage of intracellular K^+ and ATP in growing cells, while the antibiotic had none of these effects in non-growing cells; (2) no biosynthesis of any of several major cellular constituents in yeast cells was inhibited markedly or selectively enough to explain its fungitoxic activity; (3) whereas benanomicin A induced lysis of metabolically active yeast protoplasts incubated in the presence of glucose, inactive yeast protoplasts incubated without glucose were refractory to the lytic action of the antibiotic; (4) osmotically shocked yeast cells became feasible to the cidal action of benanomicin A; (5) benanomicin A substantially inhibited uptake of 6-deoxy-glucose by yeast cells; (6) liposomes composed of phospholipids and cholesterol were not susceptible to benanomicin A; and (7) benanomicin A inhibited *in vitro* activity of H^+-ATPase from yeast cell membranes to a greater extent than that for H^+-ATPase from yeast mitochondria or H^+-ATPase from yeast vacuolar membranes.

Based on these and our previous data that benanomicin A preferentially binds to mannan or mannoproteins constituting the cell wall and cell membrane of yeasts, such binding of the antibiotic is suggested to deteriorate the normal structure and function of those cell membranes of yeasts which are in a growing or metabolically active state, ultimately leading to cell death.

Treatment with a polyene amphotericin B and several azoles such as fluconazole is a mainstream of the chemotherapy for systemic fungal infections which has risen dramatically in recent years. However, these drugs have drawbacks associated with their safety in the former and their efficacy in the latter. Thus their clinical usefulness is considerably limited. To overcome this situation, the discovery and development of new classes of antifungal agents which show low toxicity and high efficacy is urgently required.

Benanomicin A produced by *Actinomadura sporax* is a new class of antifungal agent possessing a benzo-(α)-naphthacenequinone skeleton[1,2] and closely related with pradimicins in its structure and biological activity[3~7]. Our previous studies demonstrated that benanomicin A inhibited growth of a wide range of pathogenic fungi[8], and that either intravenous or subcutaneous doses of it were effective in protecting mice lethally infected with *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* from death, while the antibiotic was favorably tolerated by experimental animals[9]. It was also found that benanomicin A had a selective affinity to yeast and other fungal cells, particularly cell surface mannans but not to bacteria or mammalian cells[10]. This may, at least, partly explain the selective toxicity of this antibiotic.

In the present paper we describes the results of studies performed to determine the mechanism of action of benanomicin A by which it exerts a fungicidal action toward susceptible yeasts.

Materials and Methods

Compounds

Benanomicin A was prepared in the Pharmaceutical Research Center of Meiji Seika Kaisha, Ltd., and dissolved in distilled water for use. Amphotericin B (Sigma Chemical Co., St. Louis) was dissolved in dimethyl sulfoxide (DMSO) followed by dilution with distilled water.

Yeast Strain and Cultivation

Saccharomyces cerevisiae X2180-1A was used throughout the study. Cultures were grown at 27°C in yeast extract (1%)-peptone (1%)-glucose (1%) broth (YPG broth) with shaking overnight. Yeast cells were harvested by centrifugation and washed two or three times with distilled water.

Determination of MIC to Various Inoculum Sizes of Yeast

The MICs of benanomicin A were determined by the microdilution method using Yeast Nitrogen Base (Difco) (YNB broth) supplemented with 1% glucose (YNBG broth) on 96-well microplates. The range of final antibiotic concentration in the assay was from 0.2 to 200.0 μg/ml. Washed yeast cells were resuspended in fresh YNBG broth and adjusted to a concentration of 10^7 cells/ml with the aid of a Thoma's hemocytometer. This cell suspension was further diluted to varying concentrations from 10^7 to 10^2 cells/ml to prepare inocula. After inoculating the cell suspension into each well, all microplates were incubated at 27°C for 24 hours. The MIC was defined as the lowest concentration of antifungal showing no visible growth.

Counting of Viable Cells

Washed yeast cells were resuspended in YNB broth or YNBG broth at a cell concentration of 2×10^5 cells/ml. These cell suspensions were pre-incubated at 27°C for 2 hours with shaking, benanomicin A at a concentration of 0.2 μg/ml was then added, and incubation was continued. At intervals, samples were taken from each culture and inoculated on YPG agar plate to measure viable counts.

Measurement of Radioactivity Incorporated into Major Cellular Constituents

Washed yeast cells were resuspended in YNBG broth at a concentration of 1×10^7 cells/ml. An aliquot of the cell suspension thus prepared was dispensed into tubes which received the indicated concentration of benanomicin A, along with [^{14}C]adenine, [^{14}C]uracil, [^{14}C]-leucine or [^{14}C]acetate, to study the effect of the antibiotic on biosynthesis of DNA, RNA, protein and lipids, respectively. The incorporation of [^{14}C]glucose into glucan, mannan and chitin in yeast cells was also performed under the same experimental conditions, but with the use of YNB broth in place of YNBG broth to study the effect of benanomicin A on biosynthesis of these cell wall polysaccharides. All tubes were incubated at 27°C and after 30, 60 and 90 minutes samples were taken from the incubation mixture for fractionation and subsequent radioactive assay according to the methods reported[11~13].

Measurement of Potassium Leakage

Washed yeast cells were resuspended at a concentration of 10^7 cells/ml in MM medium (0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.01% NaCl) supplemented with 20 mM HEPES buffer (pH 6.4), 0.1 mM $CaCl_2$ and 2% glucose. The cell suspension was dispensed into 15 ml-disposable tubes which received the indicated concentrations of benanomicin A. All tubes were incubated at 27°C under mild shaking. One-ml of sample was taken from each tube at 0, 10, 30 and 60 minutes of incubation and filtered through a Millipore filter. Potassium content in the filtrate was measured with an atomic absorption spectrophotometer (Hitachi Z-6100). The total cellular potassium content was determined using samples obtained after boiling the untreated control cell suspension at 95°C for 10 minutes with subsequent filtration. Percent of potassium leakage was calculated on the basis of the total cellular potassium content.

Measurement of ATP Leakage by HPLC

Washed yeast cells were resuspended in YNBG broth at a concentration of 1×10^8 cells/ml. The cell suspension in a volume of 50 ml was dispensed into 200 ml-flasks which received the indicated concentration of benanomicin A or amphotericin B. Each flask was incubated at 27°C with mild shaking, from which 25-ml samples were taken at 0, 30 and 60 minutes and filtered through a Millipore filter. To the filtrate was added trichloroacetic acid to a final concentration of 5% for the extraction of nucleotides and other acid-soluble cellular constituents. The supernatant was then treated with an equal volume of Alamin-freon (1 : 1) solution to remove trichloroacetic acid, concentrated by freeze-drying, and then analyzed with Hitachi HPLC system (Type 638-50) according to the method of SHIBATA *et al.*[14].

Determination of Uptake of [^{14}C]Deoxy-glucose in Yeast Cells

Washed yeast cells were resuspended with YNB broth supplemented with 1 mM glucose to give a cell suspension at a concentration of 1×10^8 cells/ml. Five-ml of the cell suspension was dispensed into 15-ml test tubes which received the indicated concentration of benanomicin A, along with 1 mM [^{14}C]deoxy-D-glucose (0.1 μCi/ml), and

incubated at 27°C on a shaker. Samples of 1 ml were taken every 10 minutes, and mixed with 0.5 ml of 3 mM uranyl nitrate (pH 4.0) in an ice-cooled bath to terminate the reaction. The mixture was poured onto a glass fiber paper (Whatman GF/A) to collect the cells, which were then washed once with 3 mM uranyl nitrate and twice with distilled water. The glass fiber papers were dried and measured for radioactivity in a scintillation counter.

Determination of the Effect of Osmotic Shock on Susceptibility to Benanomicin A

Washed yeast cells suspended in YNB broth supplemented with 1.2 M sorbitol and DULBECCO's phosphate buffered saline (pH 7.5; Sigma) were incubated statically at room temperature for 1 hour with or without 100 μg/ml of benanomicin A. Then the suspension was rapidly diluted with 10-fold volume of distilled water (for osmotic shock) or 1.2 M sorbitol (for non-osmotic shock) at a cell concentration of 4×10^7 cells/ml. Both experimental cell suspensions were dispensed into test tubes with or without the indicated concentrations of benanomicin A and incubated at room temperature for 30 minutes. Viable counts were measured as described above.

Measurement of Lytic Activity against Yeast Protoplasts and Erythrocytes

Saccharomyces protoplasts were prepared by the method reported[10]. Protoplasts were suspended in 1/15 M sodium phosphate buffer (pH 7.0) supplemented with 1.2 M sorbitol and with or without 1% glucose at a concentration of 1×10^6 cells/ml. The protoplast suspension was dispensed into tubes which received the indicated concentration of benanomicin A and was incubated at 27°C. After 15, 30 and 60 minutes, a sample was taken from each tube and centrifuged at 2000 rpm for 5 minutes to remove the supernatant. The amount of protein in the supernatant was determined using protein assay kit (Bio-Rad Laboratories). The total amount of soluble proteins in the protoplast was determined using the supernatant following bursting of the untreated control protoplasts by the addition of distilled water. Percent of lysis was calculated on the basis of the total cellular protein content.

The experimental procedure to determine erythrocyte hemolysis was that reported[10].

Determination of Glucose Release from Liposomes Encapsulating Glucose

Presome PPG-I (phosphatidylcholine : cholesterol : phosphatidyl-glycerol = 1 : 1 : 0.2; Nihon Seika Co.) was used to prepare liposomes by the method of KINSKY *et al.*[15] and NAKAJIMA *et al.*[16]. Briefly the liposomes and 0.3 M glucose were mixed and homogenized vigorously at 20,000 ~ 25,000 rpm for 6 minutes at 45°C to obtain multilamellar liposomes containing glucose. The glucose-trapped liposomes thus prepared were passed through a column of Sephadex G-50 (80 ml), and developed with 0.15 M NaCl solution to remove untrapped glucose. The liposomes were suspended in saline and incubated with the indicated concentration of benanomicin A, tioconazole or amphotericin B at 25°C for 1 hour and then the supernatant was collected by centrifugation.

The amount of glucose of the supernatant was assayed essentially as described by KINSKY *et al.*[15], by measuring the reduction of $NADP^+$ in the presence of hexokinase and glucose-6-phosphate dehydrogenase. Shortly, to the supernatant of the reaction mixture was added 5 mM Veronal buffer (pH 7.5) containing 0.15 M NaCl, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$ to increase the volume, and then 250 μl of assay reagent consisting of 100 mM Tris buffer (pH 7.5), 64 mM NaCl, 3.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 2 mM ATP, 1 mM $NADP^+$, 2.5 units of glucose-6-phosphate dehydrogenase and 5 units of hexokinase. After incubation for 5 minutes at 22°C, the absorbance at 340 nm due to the NADPH produced by consumption of glucose was measured. The total amount of glucose trapped in the liposomes was measured after disrupting them with dimethyl ether. Values were corrected for a control value assayed with reagent containing no ATP, $NADP^+$, or enzymes. The percentage release of glucose was calculated from the following equation: percentage glucose release $= (a-b)/(c-b) \times 100$, where a, b, and c are absorbances of reaction mixtures containing drug-treated liposomes, intact liposomes and dimethyl ether-treated liposomes (total glucose trapped), respectively.

Assays of H^+-ATPases

Cell membrane H^+-ATPase, mitochondrial F_1-ATPase and vacuolar membrane H^+-ATPase prepared from yeast cells by the method of UCHIDA *et al.*[17] were obtained through the courtesy of Dr. Y. OHSUMI, University of Tokyo. Assay of the enzymatic activities was also done according to the method of his group of investigators[17]. Cell membrane H^+-ATPase was mixed with 25 mM MES/Tris buffer (pH 6.0), 5 mM ATP, 5 mM $MgCl_2$ and 5 mM sodium azide, to which was then added the indicated concentrations of benanomicin A. The reaction was started by addition of Mg^{2+}-ATP and, after 2 to 20 minutes of incubation at 30°C, was stopped by adding 5% (w/v) SDS. Inorganic phosphate liberated by

this enzyme was measured by the method of OHNISHI *et al.*[18]. Assay conditions of mitochondrial F_1-ATPase and vacuolar membrane H^+-ATPase were the same as those for assay of cell membrane H^+-ATPase, except that pH of the reaction mixture was 8.8 (for mitochondrial F_1-ATPase) or 6.0 (for vacuolar membrane H^+-ATPase).

Results

Effect of Inoculum Size and Phase of Growth on Anti-*Saccharomyces* Activity of Benanomicin A

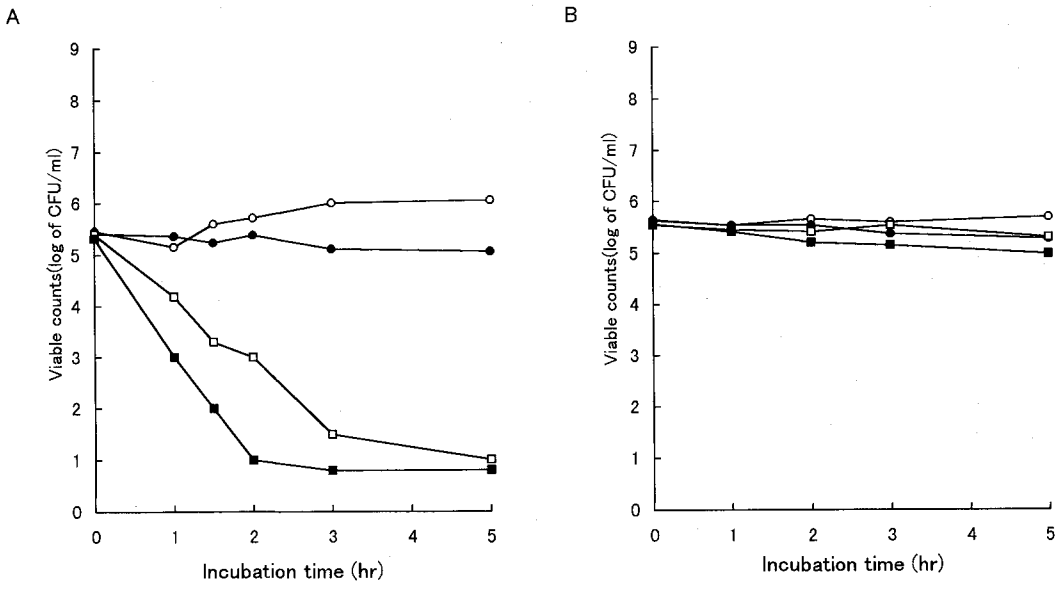
In seeking to explain fungistasis or cell death in terms of the loss of a particular cellular function, it is important to compare the effect of antifungals used at the same cell concentration on various cellular functions. This holds true especially for those antifungal agents which are taken up by fungal cells in large quantity, as has been observed for benanomicin A. However, because we had to use cell suspensions with different cell concentration in some experiments to optimize assay conditions, we first studied how the sensitivity to benanomicin A of the testing organism was influenced by the inoculum size. As shown in the Table 1, the MIC of benanomicin A against yeast cultures grown on YNBG broth increased with increasing inoculum sizes, the value being 1.6, 6.4 and 80 μg/ml with inocula of $10^2 \sim 10^4$, 10^6 and 10^7 cells/ml, respectively. This indicated that benanomicin A-sensitivity of cultures decreased with increase in inoculum size or depended on the ratio of a number of target cells to drug molarity ratio, especially in the case where the inoculum size exceeded 10^6 cells/ml. Thus, this marked effect of initial cell concentration of cultures on the antifungal activity of benanomicin A was taken into consideration when the experiments were conducted using a cell suspension at a concentration of 10^7 cells/ml or higher.

Next, we compared the effect of benanomicin A on the viability of growing cultures with that of non-growing cultures to examine whether or not the benanomicin A sensitivity depends on the phase of growth of a culture. As shown in Fig. 1, when cells were incubated in a growth-supporting glucose-containing medium (YNBG broth), viable numbers were lowered by benanomicin A in a dose-dependent manner at concentrations above 5 μg/ml. By contrast, in glucose-depleted medium (YNB broth), benanomicin A had no effect on viable cell counts at drug concentrations up to 20 μg/ml. These data suggest that benanomicin A exerts a fungicidal action toward

Table 1. Effect of the inoculum size on the anti-*S. cerevisiae* activity of benanomicin A in YNBG broth.

Inoculum size (cells/ml)	MIC (μg/ml)
10^2	1.6
10^3	1.6
10^4	1.6
10^5	3.2
10^6	6.4
10^7	80

Fig. 1. Effect of benanomicin A on growing cultures (A) and non-growing cultures (B) of *S. cerevisiae* in YNBG broth.



Benanomicin A was added at concentrations of 0 (○), 2.5 (●), 5 (□) and 20 μg/ml (■) at zero time of incubation.

growing or metabolically active cells, but is without such an effect on non-growing or metabolically inactive cells.

Effect of Benanomicin A on Biosynthesis of Major Cellular Constituents in Yeast Cells

The above result that benanomicin A appears to be active exclusively against growing or metabolically active yeast cell, led us to the possibility that the antibiotic acts preferentially on some anabolic metabolism of cellular constituents of vital importance, such as the biosynthesis of protein, RNA, DNA, lipid or cell wall polysaccharides (glucan, chitin and mannan) in this yeast. Table 2 shows the results of experiments in which the effect of the antibiotic on the incorporation of several radioactive precursors into their respective cellular fractions was examined. With 80 μg/ml of benanomicin A (approx. 1 MIC equivalent), the extent of inhibition of incorporation of all species of precursors employed was maximally 55%, suggesting that none of the anabolic metabolisms of major cellular constituents is preferably sensitive to benanomicin A.

Benanomicin A-induced Leakage of Potassium Ion and ATP from Yeast Cells

As shown Fig. 2 A, when growing yeast cells were exposed to 30 to 100 μg/ml of benanomicin A (approx. 1/3 to 1 MIC equivalent), there occurred a dose- and time-dependent leakage of cellular K^+ outside of the cells; in the presence of 100 μg/ml of the antibiotic, approx. 30 and 50% of intracellular content was released after 30 and 60 minutes, respectively. By contrast, as shown Fig. 2 B, when resting cells were treated with the same concentrations of benanomicin A, a much lower extent of leakage was observed.

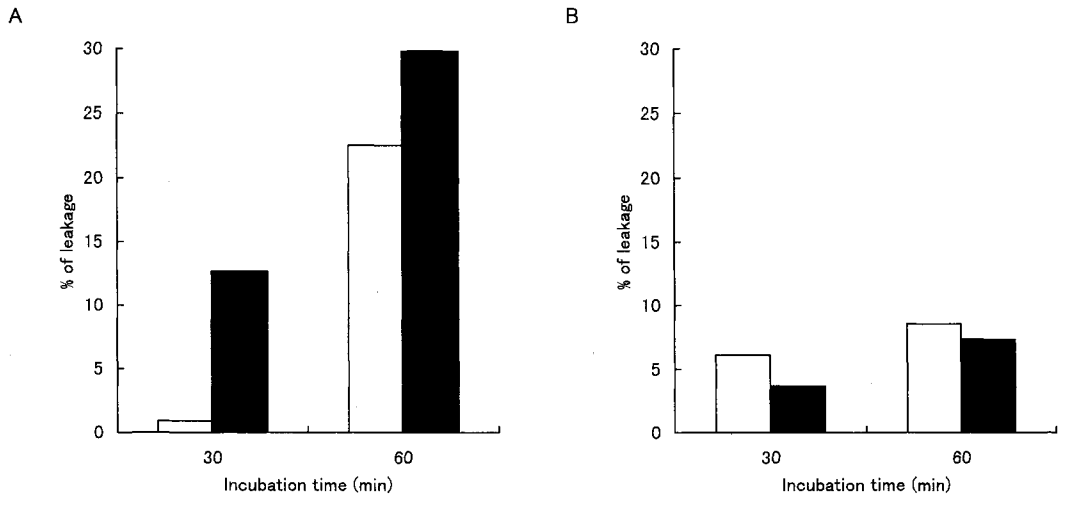
Similarly, benanomicin A at a concentration equivalent to MIC induced a marked leakage of cellular ATP from growing yeast cells: the amount released outside of the cells reached approx. 60% of its intracellular content after 30 minutes of treatment, while ATP was not or was only slightly released from non-growing cells exposed to the same concentration of the antibiotic (data not shown).

Table 2. Effect of benanomicin A on incorporation of radioactive precursors into major cellular constituents in *S. cerevisiae*.

Cellular constituent	Percent inhibition of uptake of radioactivity by benanomicin A at a concentration of 80 μg/ml (or 1 MIC equivalent)
Protein	48
RNA	31
DNA	0
Total lipid	55
Mannan	12
Alkali-insoluble glucan +chitin	34

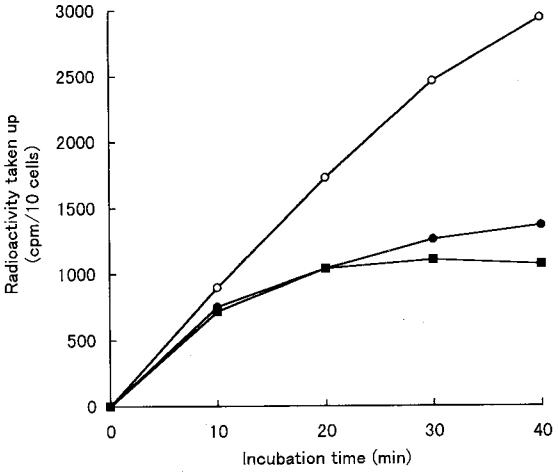
Yeast cells suspended in YNBG broth (approx. 1×10^7 cells/ml) were incubated with the radioactive precursor at 27°C for 30 minutes in the absence (for control) or presence of 80 μg/ml of benanomicin A.

Fig. 2. Effect of benanomicin A on leakage of potassium ion from growing cells (A) and non-growing cells (B) of *S. cerevisiae*.



S. cerevisiae were grown in MM medium buffered with 20 mM HEPES (pH 6.4) plus 0.1 mM $CaCl_2$, with and without glucose (2%), respectively. Benanomicin A was added to the cultures at concentrations of 30 (□) and 100 (■) μg/ml at zero time of incubation.

Fig. 3. Effect of benanomicin A on uptake of [^{14}C]6-deoxy-glucose in growing *S. cerevisiae* cells.



Yeast cells suspended in YNB broth supplemented with 1 mM glucose (approx. 1×10^7 cells/ml) were incubated at 27°C for 30 minutes, and then dispensed into tubes which received 0 (○), 20 (●) or 80 (■) μg/ml of benanomicin A, along with 1 mM [^{14}C]6-deoxy-glucose (0.1 μCi/ml) and incubated at 27°C. At intervals, samples were removed to assay the radioactivity taken up by cells as described in Materials and Methods.

Effect of Benanomicin A on Influx of [^{14}C]Deoxy-glucose in Yeast Cells

To learn whether benanomicin A affects permeability of some essential substrate such as glucose in yeasts, we studied the effect of the antibiotic on influx of 6-deoxy-glucose because it is an unmetabolizable analog of glucose. Yeast cells suspended in YNBG broth containing 1 mM of glucose and 1 mM [^{14}C]6-deoxy-glucose were incubated with the indicated concentration of benanomicin A. As shown in Fig. 3, the radioactive substrate was taken up in the untreated control cells at a steady rate for 40 minutes of the incubation period, whereas in the presence of benanomicin A at concentrations of 20 and 80 μg/ml (1/4 and 1 MIC equivalent, respectively), the level of 6-deoxy-glucose uptake was 50% lower 20 minutes after the onset of incubation, at which time the uptake was almost completely leveled off.

Effect of Benanomicin A on Resistance to Osmotic Shock of Yeast Cells

Since we found earlier that benanomicin A has a selective affinity for mannan[10], we postulated that through this interaction the antibiotic alters the physicochemical properties of cell walls and/or cell membranes. Thus experiments were conducted to see whether or not benanomicin A has an effect on yeast cell resistance to osmotic shock. The yeast cells suspended in a hypertonic YNB broth supplemented with 1.2 M sorbitol were treated or untreated with 100 μg/ml of benanomicin A (approx. 1 MIC equivalent) and then the incubation mixture was abruptly diluted with 10 times as much distilled water or 1.2 M sorbitol solution as an osmotic stabilizer to render the cells osmotically shocked or not. As shown in Table 3, the viability of yeast cells was scarcely or only slightly affected when they were subjected to either osmotic shock or benanomicin A-treatment, but when benanomicin A-treated yeast cells were subjected to osmotic shock, viability was markedly reduced.

Table 3. Effect of benanomicin A on resistance to osmotic shock of *S.cerevisiae* cells

Treatment with benanomicin A (100 μg/ml)	Subjected to osmotic shock	Viable counts per ml
No	No	3.5×10^7
No	Yes	3.3×10^7
Yes	No	1.6×10^7
Yes	Yes	3.4×10^6

One volume of yeast cell suspension (3.5×10^7 cells/ml) in a hypertonic solution (1.2 M sorbitol) in the presence or absence of 100 μg/ml of benanomicin A (approx. 1 MIC equivalent) was incubated at room temperature for 30 minutes and then rapidly diluted 10-fold with distilled water (for osmotic shock) or 1.2 M sorbitol (for unshocked control) to determine viable counts.

Lytic Effect of Benanomicin A on Yeast Protoplasts

The membrane-active property of benanomicin A was more directly examined by measuring the lytic effect of the antibiotic on yeast protoplasts suspended in phosphate buffered 0.8 M-sorbitol as an osmotic stabilizer. As shown in Fig. 4, in the presence of glucose, yeast protoplasts suspended in glucose-supplemented medium were susceptible to benanomicin A; when the yeast protoplasts were exposed to 20 and 80 μg/ml of benanomicin A (1/4 and 1 MIC equivalent, respectively), approx. 20 and 60% lysis, respectively, was observed in 30 to 60 minutes. The yeast protoplasts suspended in glucose-depleted medium, however, were far less susceptible to benanomicin A despite there being no significant difference in the amount of the antibiotic bound to the protoplasts between these two suspensions. Sheep erythrocytes lacking in affinity for benanomicin A were highly resistant to its lytic action irrespective of the presence or absence of glucose (data not shown).

Fig. 4. Lytic effect of benanomicin A (BNM) on yeast protoplasts in 1/15 M phosphate buffer (pH 7.0) and 0.8 M sorbitol with (■) or without (□) 1% glucose and incubated for 30 minutes (A) and 60 minutes (B).

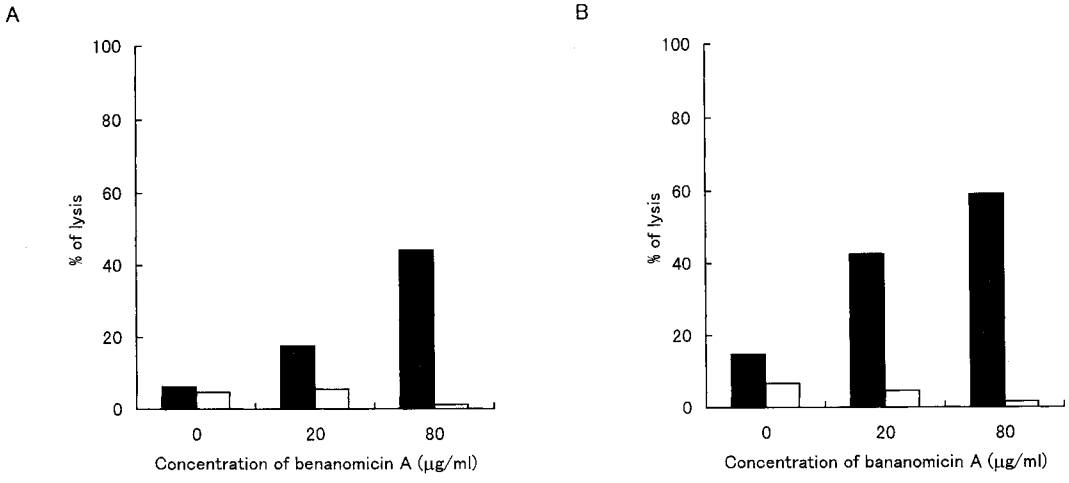


Table 4. Effect of benanomicin A, tioconazole and amphotericin B on glucose entrapped liposomes.

Treatment with (μg/ml)		Glucose release (%)
Benanomicin A	80	7.4
	320	9.0
Tioconazole	40	77.3
	80	100
Amphotericin B	10	13.2
	40	60

Liposomes prepared from phosphatidylcholine : cholesterol : phosphatidylglycerol = 1 : 1 : 0.2 with encapsulating glucose were incubated at 25°C for 60 minutes with the indicated drugs.

Table 5. Effect of benanomicin A (20 μg/ml) on the *in vitro* activity of three different types of H^+-ATPases from *S. cerevisiae* cells.

Incubation time (minutes)	% of inhibition of activity of:		
	Cell membrane H^+-ATPase	Vacuolar membrane H^+-ATPase	Mitochondrial ATPase
2	58.5	0	7.6
5	50.0	0	10.6
10	41.4	0	5.9

See Materials and Methods for assay conditions.

Effect of Benanomicin A on Liposomes

To learn whether the membrane-active property of benanomicin A is due to its direct interaction with some lipid components of the yeast cell membrane resulting in membrane damage or disruption, the effect of the antibiotic on glucose-trapped liposomes prepared from phospholipids and cholesterol was examined. As shown in Table 4, while tioconazole (80 μg/ml) or amphotericin B (40 μg/ml) which are known to have selective affinity for phospholipids and sterols, respectively[24,25], induced release of large amounts of trapped glucose from liposomes, benanomicin A up to a concentration of 320 μg/ml did not induce substantial glucose release, indicating that the liposomes were basically insusceptible to the antibiotic.

Effect of Benanomicin A on the Activities of ATPase from Yeast Cells

In addition to the above-mentioned data, the selective affinity of benanomicin A for mannan suggests to us that some mannoproteins localized on the cell membrane may be the target of benanomicin A action. To test this possibility, yeast cell membrane H^+-ATPase (P-ATPase) was chosen as one such mannoprotein and the benanomicin A susceptibility of this enzyme was compared with that of vacuolar membrane H^+-ATPase (V-ATPase) and mitochondrial H^+-ATPase (F_1-ATPase) from yeast, both of which are known to be mannan-free enzymes. As shown in Table 5, 20 μg/ml of benanomicin A inhibited P-ATPase activity by approx. 50%, whereas only slightly or not at all inhibited V-ATPase activity or F_1-ATPase activity.

Discussion

Benanomicin A exerted a fungicidal action toward growing yeast cells but had no such effect on resting yeast cells, suggesting that only metabolically active cells are susceptible to the antibiotic action. Thus there is a possibility that benanomicin A may affect some anabolic function, in particular, biosynthesis of a major cellular component (or components). However, the results of experiments with radioactive precursors demonstrate that biosynthesis of none of several major cellular constituents, such as protein, DNA, RNA, lipids or cell wall polysaccharides, in growing cultures of this yeast was inhibited to an extent high enough to explain the antifungal activity of benanomicin A.

Another possible mechanism of benanomicin A action is that, for some unknown reason, the antibiotic preferentially impairs the structure and/or function of the cell membrane of yeast cells in a growing phase. This postulation was supported by the finding that benanomicin A induced a rapid and marked release of cellular K^+ and ATP in a dose-dependent manner from growing cells, without such effects on resting cells. There are a number of papers reporting that a polyene antifungal amphotericin B[19,20] and imidazole antifungals such as tioconazole and miconazole[21~23] are active in inducing leakages of various cellular constituents including K^+ and ATP from yeast cells. Both sterols and phospholipids are major cell membrane lipid components, which exist not only in yeast and other fungal cells but also all eukaryotic cells including mammalian cells. It is also known well that polyenes and imidazoles have a specific affinity for sterols[24] and phospholipids[25], respectively, and that through direct drug-lipid interaction, these two classes of antifungals disrupt the permeability function of the cell membrane of yeasts, irrespective of their phase of growth. The lipid-affinity of polyenes and imidazoles could also explain their lytic action towards mammalian, erythrocytes[26~29] and liposomes[24,25] as was also demonstrated in this paper. Contrary to these lipophilic antifungals, benanomicin A was virtually without such effect. More characteristically, benanomicin A-induced leakage of cellular materials occurring in growing yeast cells was not observed in resting yeast cells. A selective and high benanomicin A-susceptibility of the cell membrane of growing yeast cells was confirmed by the finding that yeast protoplasts incubated in the glucose-supplemented medium were much more highly susceptible to the lytic action of benanomicin A than those incubated in the glucose-depleted medium. This also supports the postulation that the cell membrane of growing yeast cells or metabolically active yeast protoplasts is different in physico-chemical properties from that of resting yeast cells or metabolically inactive yeast protoplasts, and that only the former is susceptible to the detrimental action of benanomicin A.

Although such a unique and selective membrane action of benanomicin A appears to be primarily associated with its selective affinity for mannan or related polysaccharides of the yeast cell membrane, it remains to be answered why the cell membrane becomes susceptible to benanomicin A only when the yeast cells are in the growing phase or metabolically active state.

It is known that in a growing phase, yeast cells become swollen and the tension of the cell membrane increases. Thus there is a possibility that the binding of benanomicin A to the cell membrane lowers its resistance to an increased intracellular pressure. This hypothesis is supported by the result of experiments that osmotically shocked swollen yeast cells were more highly susceptible to the antibiotic than unshocked normal yeast cells. Presumably, benanomicin A- bound cell membranes would lose their flexibility and, as a result, become more fragile so that they could not resist the increasing internal pressure.

In addition to such physico-chemical and mechanical changes, changes of some functions of the yeast cell membrane, such as transport or internalization of essential substrates and activity of membrane associated enzymes also appear to be induced by benanomicin A. In the present study it was demonstrated that benanomicin A substantially inhibits transport of 6-deoxyglucose in yeast cells. Among mannoproteins associated with the cell membrane of yeasts is cell membrane ATPase (phosphorylated ATPase; P-ATPase) which belongs to a class of H^+-ATPase. Yeast cells have two other members of H^+-ATPase, namely mitochondrial ATPase (F_1-ATPase) and vacuolar-ATPase (V-ATPase) located on the mitochondrial inner membrane and vacuolar membrane, respectively[30]. Different from P-ATPase, both F_1-ATPase and V-ATPase are known to lack mannan moiety in their molecule. The present study, comparing the effect of benanomicin A on these three types of H^+-ATPase from yeasts, showed that P-ATPase was preferentially susceptible to the antibiotic. However, because the P-ATPase inhibitory activity of benanomicin A appears to be much lower that of omeprazole, which is known to be a potent fungicidal and to selectively inhibit the P-ATPase activity[31], the antifungal activity of the antibiotic likely is not solely

based on its inhibition of P-ATPase activity. All of the results obtained in the present study suggest to us that detrimental changes in the structure and/or function of cell membrane induced by benanomicin A, probably resulting from its interaction with not only "sparking" mannoproteins such as P-ATPase but also "bulk" mannoproteins of the cell membrane, would be primarily involved in the antifungal action of the antibiotic. The mechanism of such membrane action of benanomicin A, although not clarified on the molecular level, seems to be unique and distinct from that of any existing antifungal agents so far known. Considering this and other biological properties of benanomicin A including its favorable antifungal, pharmacokinetic and toxicological profiles, the antibiotic could be a promising candidate for clinical development as a useful antifungal drug.

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