Morphological Alterations of *Saccharomyces cerevisiae* Induced by Benanomicin A, an Antifungal Antibiotic with Mannan Affinity

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**Abstract:** The effects of benanomicin A, a mannose-binding antifungal antibiotic, on yeast cells of *Saccharomyces cerevisiae* were studied by electron microscopy. Cytological studies using vital stain with methylene blue demonstrated that benanomicin A at 20 and 80 µg/ml killed buds in preference to parent cells. In confirmation, examination by TEM revealed that benanomicin A at 80 µg/ml damaged buds more severely than parent cells. The major effect on the ultrastructure was characterized by severe damage to the cell membrane. In addition, it caused expansion and vacuolation of the endoplasmic reticulum (ER), and partial fragmentation and disappearance of nuclear membranes. The membrane-disruptive activity of benanomicin A may be closely associated with its membrane affinity.

**Key words:** *Saccharomyces cerevisiae*, Benanomicin A, Antifungal agent, Electron microscopy

Benanomicin A is an antibiotic produced by *Actinomadura spadix* (6), which has a benzo[α]naphthacenequinone skeleton substituted for a disaccharide and D-alanine in its molecule (Fig. 1). It shows potent antifungal activity *in vitro* (9) and *in vivo* (4) against a wide range of pathogenic fungi except certain Zygomycetes. On the other hand, pharmacological and toxicological studies have demonstrated that benanomicin A is well tolerated with low toxicity in experimental animals (12). This highly selective toxicity of benanomicin A seems to be due to its specific affinity to mannan or related polysaccharides; that is, the antibiotic binds to fungal cells which possess mannoproteins on their surface, but not to those cells lacking in mannan, such as mammalian and most bacterial cells (8).

Further biochemical studies using *Saccharomyces cerevisiae* as a test organism revealed that benanomicin A was only active when the yeast cells are growing or in a metabolically active state. Moreover, the binding of benanomicin A to growing yeast cells caused the leakage of cellular materials and lysis of protoplasts, suggesting that all membrane is a target of benanomicin A (10).

This study was undertaken to confirm the membrane damage of growing yeast cells after binding with benanomicin A by morphological observation.

**Materials and Methods**

**Compounds.** Benanomicin A was prepared in the Pharmaceutical Research Center of Meiji Seika Kaisha, Ltd., Yokohama, Japan, and was dissolved in distilled water, filtered through a Millipore filter (0.45 µm) and stored at 4°C before use.

**Organism.** *Saccharomyces cerevisiae* X2180-1A was employed throughout this study.

**Preparation of synchronous cultures.** Synchronous cultures of the yeast were prepared according to previous literature (2) with slight modifications. Yeast cells were grown at 27°C overnight with shaking in YPG broth consisting of 1% yeast extract, 1% peptone and 2% glucose. For glucose starvation, the cultures were harvested and washed three times with sterilized distilled water, resuspended in 1/10 volume of yeast nitrogen base (YNB, Difco) without glucose, and incubated at 27°C for 18 hr. Yeast cells harvested from the stationary phase of growth were resuspended in normal YNB broth at a

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Abbreviations: ER, endoplasmic reticulum; TEM, transmission electron microscope; YNB, yeast nitrogen base; YPG, yeast peptone glucose.*
concentration of 10⁷ cells per milliliter.

Cytological measurement of fungicidal action of benanomicin A toward synchronized cultures. The viability of benanomicin A-treated or untreated cells was assessed microscopically using a vital stain with methylene blue. For experimental purposes, 10 ml of synchronized culture was transferred to L-tubes with 1% glucose and incubated at 27 C. After 0, 40 and 60 min of incubation, benanomicin A was added to a final concentration of 20 or 80 µg/ml in the synchronized cultures. After various incubation periods, a portion (100 µl) of the culture was removed and mixed with an equal volume of methylene blue solution consisting of 0.04% methylene blue (Wako) and 0.1 M phosphate buffer (pH 4.6). Yeast cells stained (non-viable) and not stained (viable) were differentially counted using a hemocytometer. Counting was conducted on ten samples, and averaged numbers were recorded.

Electron microscopy. A loopful of yeast cells from a 24 hr-slant culture was inoculated into an L-tube containing 10 ml of YPG broth and incubated at 27 C for 18 hr with shaking. Yeast cells were harvested by centrifugation, washed once with sterilized water, and suspended in a 20-fold volume of fresh YNB broth supplemented with 1% glucose and 0.1 mM calcium chloride. The incubation mixture (10⁷ cells/ml) was preincubated at 27 C for 1 hr, and then benanomicin A was added to final concentrations of 80 and 160 µg/ml. The incubation was continued, and samples were taken at 1, 2 and 3 hr. Yeast cells were collected by centrifugation, and prefixed at 4 C for 2 hr with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After washing with the same buffer, the cells were postfixed in 1.5% potassium permanganate at 4 C for 18 hr and washed several times with distilled water. Prestaining with 1% uranyl acetate followed, and the fixed specimens were dehydrated in an acetone series and embedded in Quetol 653. Ultrathin sections were prepared using Ultracut N (Reichert-Nissei), stained with uranyl acetate and lead nitrate, and examined with a Hitachi H-7000 electron microscope operated at 75 kV.

Results

Fungicidal Effect of Benanomicin A on Synchronized Cells

The percentage of non-viable cells, as determined by vital staining with methylene blue, in a culture growing synchronously is shown in Fig. 2.

When benanomicin A (20 and 80 µg/ml) was added to synchronized cultures of S. cerevisiae cells at various times during incubation (0, 40 or 60 min), the percentage of non-viable cells rose quickly during 60 to 100 min of incubation, irrespective of the time the antibiotic was added. In these cultures, 60 and 90% of the cells became non-viable after 100 min and 150 min, respectively. After 3 hr of incubation, cell death was 100% (Fig. 2).

Light microscopic examination of these cultures revealed that yeast cells were not stained with methylene blue at 0 time (Fig. 3a), but the buds were stained preferentially at 20 min of incubation when treated with 80 µg/ml of benanomicin A (Fig. 3b). After 1 hr of incubation, buds and some of the parent cells were stained (Fig. 3c), and 2 hr later, all of the buds and parent cells were stained indicating that fungicidal action had occurred (Fig. 3d).
Observation of the Thin-Sectioned Cells

The ultrastructural appearance of untreated control cells grown for 3 hr is shown in Figs. 4 and 5. They were ellipsoidal in shape and the average diameter was 3 to 5 μm. Yeast cells were surrounded by a cell wall of about 100 nm in thickness and their cell membrane was closely attached to this wall. A nucleus, mitochondria, endoplasmic reticulum (ER) and vacuoles were visible in the cytoplasm (Fig. 4). Bud scars and buds which contained mitochondria and ER were frequently observed (Fig. 5).

Ultrastructures of the cells treated with 80 μg/ml of benanomicin A for 3 hr are shown in Figs. 6 and 7. The cell membrane of the bud was severely disrupted and partially dissolved (Fig. 6). Small vesicles originating from the cell membrane were located in the space between the cell wall and membrane (Fig. 6). In the parent cells, the cell membrane which was affected to a lesser extent had become wavy, partly detached from the cell wall and formed a pit or trench-like invagination (Fig. 6, arrows).

One of the most notable alterations observed in benanomicin A-treated cells was the vacuolar enlargement of ER, which was seen in the cytoplasm of both bud and parent cells (Figs. 6 and 7). Slight expansion of the membranes was seen in the ER located in the central region of the cytoplasm, and this was more marked in the ER connected to the nuclear membrane, leading to vacuolization (Fig. 7, arrow). Furthermore, a part of the nuclear membrane was either fragmented or had disappeared (Figs. 6 and 7). Morphological changes caused by 160 μg/ml benanomicin A were essentially the same as those by 80 μg/ml, although the cellular damage was more marked (data not shown).

Discussion

It was shown in this study that the killing action of benanomicin A was highest in bud cells in synchronized cultures. The preferential death of the rapidly growing
Fig. 4. TEM image of untreated *S. cerevisiae* X2180-1A cell grown for 3 hr. N, nucleus; M, mitochondria; ER, endoplasmic reticulum; V, vacuole; CM, cell membrane; CW, cell wall. Bar: 1 µm.

Fig. 5. TEM image of an untreated *S. cerevisiae* X2180-1A cell grown for 3 hr. Bud and bud scar (BS) were observed in the cell. Bar: 1 µm.
Fig. 6. TEM image of *S. cerevisiae* X2180-IA cell treated with 80 µg/ml of benanomicin A for 3 hr. Note the invagination of the cell membrane of the mother cell (arrows) and destruction of that of the young bud (arrowhead). Bar: 1 µm.

Fig. 7. TEM image of a *S. cerevisiae* X2180-IA cell treated with 80 µg/ml of benanomicin A for 3 hr showing expansion of the ER (arrow). Fragmentation of the nuclear membrane is also seen. Bar: 1 µm.
buds, shown light microscopically by staining with methylene blue, and the severe damage such as waving and disruption of the cell membrane, shown electron microscopically, are in accordance with previous findings that this antibiotic exhibited fungicidal action only when cells were growing (10). The high sensitivity of buds to the action of benanomicin A may be due to the fragile structure of rapidly growing cells, in which a delicate balance of synthesis and hydrolysis of the cells is easily disturbed by binding with the antibiotic. It is also possible that the expanding nascent cell wall might be more sensitive to the effect of benanomicin A binding than the mature wall, because the former has a structure and mechanical properties different from the mature wall (11).

One of the most notable findings in this study was the ultrastructural change of ER; the narrow cisternae of which were expanded to form vacuoles by the action of benanomicin A. This was an unexpected finding, and such a drastic change in the morphology of ER of fungal cells in response to any known antifungal agent has not been reported before. Similar membrane damage to that caused by benanomicin A was observed in Candida albicans cells treated with BMY 28864, which has a structure not unlike that of benanomicin A (3), but no mention was made of the alteration of ER. The synthesis of mannoproteins in Saccharomyces cerevisiae is known to be initiated in the rough ER, followed by processing and transglycosylation in the smooth ER and a whole array of different types of small vesicles carrying wall components (5). It was also reported that, in S. cerevisiae cells treated with concanavalin A-ferritin, both outer and inner surfaces of ER were stained (7) and that concanavalin A bound to the membrane of vacuoles that was derived from ER as well as to yeast cell membrane (1). This suggested that benanomicin A is capable of binding not only with cell membrane but also with ER. However, it is unclear at this stage how the alteration of ER contributes to the fungicidal action of benanomicin A. Likewise, the cytological effect of benanomicin A on nuclear membrane, causing its fragmentation and disappearance, remains to be clarified in regard to the action of the antibiotic.

In summary, benanomicin A exerts a fungicidal action on Saccharomyces cells, especially young growing cells, primarily by damaging the membranes of the cell, ER and nucleus. This damage might be induced by the binding of benanomicin A to the mannan moiety of these membranes.

References