POTENTIATION OF ANTIFUNGAL EFFECT OF AMPHOTERICIN B BY SQUALENE, AN INTERMEDIATE FOR STEROL BIOSYNTHESIS

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The antifungal effect of a polynye antibiotic amphotericin B (AMB) was almost completely abrogated by exogenous addition of 0.1 μg/ml ergosterol in the medium. The cytocidal effect of AMB on Saccharomyces cerevisiae was synergistically enhanced when cultured for more than 4 hours with squalene, an obligatory intermediate molecule for sterol formation. However, we could not find significant increase in cellular level of ergosterol content in the yeast cells fed with squalene.

Polyene antibiotic, through interacting with membranous sterols, has shown to alter membrane permeability.1-2) Antifungal effects of various drugs such as 5-fluorocytosine, rifampicin and fusidic acid were potentiated when combined with polynye antibiotics.3-6) Larger polynye antibiotics such as amphotericin B (AMB) or nystatin showed much higher affinity for fungi than for mammalian cells.3-6) Synergism induced by the polynye antibiotics has been evaluated as an invaluable antifungal therapy.7) Ergosterol8) or fatty acid9) is supposed to involve susceptibility of fungi polynye antibiotics, we examined whether the antifungal effect of AMB is stimulated when combined with squalene, an obligatory intermediate for sterol formation.10) In this report, we describe a new synergism of a polynye antibiotic AMB and squalene.

Materials and Methods

Chemicals  
As AMB, we used Fungizone (E. R. Squibb and Sons, Inc., Princeton, N.J.). As squalene, Same-milon (Nissel-Marine Inc., Tokyo) whose purity is more than 99% was used through this study. Ergosterol (Ishizu Chemical Co., Osaka) whose purity is more than 99% was used.

Yeast and Cell Culture  
Saccharomyces cerevisiae 4450-1A (wild type) was employed in this study. S. cerevisiae was cultured in synthetic minimal medium containing 0.67% yeast nitrogen base (Difco) without amino acids and 2% dextrose, and colonies were made on YPD agar as described previously.3)

Synergistic Study by Colony Formation  
Exponentially growing yeast cells (5×10⁶~1×10⁷ cells/ml) were incubated in synthetic medium without or with various dose of squalene and AMB for 4 hours. After the treatment, the cells were washed by centrifugation and diluted samples were plated on YPD agar. Colonies on the agar were scored after incubation at 37°C for 2 days as described previously.3)

Total Lipid Extraction  
Yeast cells were cultured in 500 ml of synthetic medium with or without 50 μl/ml squalene for

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4 hours. Some $5 \sim 6 \times 10^8$ cells were harvested and washed three times with 0.15 m KCl. The cells were suspended in chloroform - methanol (2:1, v/v) containing 50 µg lanosterol acetate (as standard marker) and stirred overnight at room temperature. The chloroform - methanol fraction was evaporated at 40°C without foaming as described previously.\textsuperscript{11,12}

Gas Chromatographic Analysis

The lipid extract was dissolved in trimethylsilylimidazole (100 µl) and the mixture was heated at 60°C for 10 minutes. The solution (0.5 µl) was injected to gas chromatograph. A Shimadzu GC-7A was used in this study. The column, 100 cm x 3 mm i. d., was packed with 1.5% OV-17 on Shimalite W (80~100 mesh). The column temperature was programmed from 250°C to 290°C in a rate of 4°C/minute. The retention times were 1.9 minutes for squalene, 4.4 minutes for ergosterol-TMSi, and 11.3 minutes for lanosterol-acetate (internal standard).

Results

In eucaryotic cell system, various biological effects of polyene are exerted through the cellular level of sterol contents,\textsuperscript{11~14} and ergosterol is a major sterol molecule in yeast cells. As shown in Fig. 1, various dose of AMB was combined with sterol to test the effect upon colony formation of \textit{S. cerevisiae} (4450-1A, wild type) cells. The cytocidal effect of AMB was found to be abrogated almost completely by 0.1 µg/ml to 1.0 µg/ml of ergosterol (Fig. 1).

We examined whether the cellular sensitivity to AMB was enhanced in the cells cultured with squalene, an intermediate molecule for sterol formation. Time course for the effect of squalene and AMB was tested against colony forming ability of \textit{S. cerevisiae} cells (Fig. 2 a). AMB (0.75 µg/ml) significantly affected the cell survival when incubated for 2 or 4 hours with 50 µl/ml squalene; 0.25 µg/ml AMB in combination with 50 µl/ml squalene had only slight effect when incubated for 4 hours (Fig. 2 a). Fig. 2b shows dose-response of AMB in the absence or presence of 50 µl/ml squalene when incubated for 4 hours. Within the dose of 1 µg/ml of AMB, the polyene alone had only slight effect, if any, on the cell survival, while the survival fraction was significantly reduced when both AMB and squalene were present (Fig. 2 b). Dose-response curves to squalene with 0, 0.25 and 0.75 µg/ml AMB are shown in Fig. 2 c where the cells were incubated for 4 hours. The antifungal effect of AMB was enhanced as a function of squalene concentration (Fig. 2 c). Squalene, below 100 µl/ml, \textit{per se} had no effect on the cell survival. However, the antifungal synergism was apparently observed when 0.25 or 0.75 µg/ml AMB was combined with higher dose of squalene (50 to 100 µl/ml) (Fig. 2 c). Almost the similar data as seen in Fig. 2 were observed when pure AMB was employed instead of Fungizone (data not shown).
Fig. 2. (a) Time course for exposure of *S. cerevisiae* cells to squalene. *S. cerevisiae* cells were incubated initially without or with AMB (0.25 μg/ml or 0.75 μg/ml) for 4 hours. During the total incubation time of 4 hours, 50 μl/ml of squalene was added at various time as indicated in figure after the addition of AMB (time 0). After incubation for 4 hours, samples were immediately diluted and plated on YPD-agar to score colony formation.

(b) Dose-response to AMB of *S. cerevisiae* cells in the absence or presence of squalene. The cells were exposed to various doses of AMB without or with 50 μl/ml squalene for 4 hours.

(c) Dose-response to squalene of *S. cerevisiae* cells in the absence or presence of AMB. The cells were exposed to various dose of squalene without or with AMB (0.25 or 0.75 μg/ml) for 4 hours.

![Graphs showing colony number vs. time for AMB and squalene exposure](image)

Gas-liquid chromatographic analysis was carried out to compare sterol molecules in lipid fraction between untreated cells and squalene-treated cells (Fig. 3). Peak indicates lanosterol acetate as a standardized marker which was added just before lipid extraction. Fig. 3 a shows a typical chromatogram of our lipid extracts derived from untreated yeast cells. With the aid of reference compounds, squalene (peak A) and ergosterol (peak B) were identified. As shown in Fig. 3 b, a peak corresponding to squalene remarkably increased in squalene-treated cells. However, in comparison with chromatogram of control sample, no significant increase of ergosterol peak was observed in the cells fed with squalene (Fig. 3 b).

**Discussion**

We observed a new antifungal synergism of AMB and squalene. This synergism might be an useful therapeutic method for mycotic infection because the amounts of AMB can be lessened and uptake of high dose of squalene (10 ml/day for a few months) also appears not to show significant side effect in human (T. Kihara, personal communication).

In fungi, ergosterol is one of the main target molecules for polyene antibiotics⁹) (see Fig. 1). In mammalian cells, AMB sensitivity of mouse cells was also enhanced by liposome containing ergosterol.¹⁵)
One might therefore expect that the enhanced sensitivity of yeast cells treated with squalene to the polyene antibiotic AMB is due to increased cellular level of ergosterol. Gas-chromatographic analysis, however, indicates that this is not the case; there is no increase in sterols or their precursor function other than squalene in squalene-treated cells. Our preliminary study suggests that squalene per se affects membrane permeability (unpublished data). Further study is necessary to elucidate underlying mechanism of the synergistic effects.

References