Effect of Ansamitocins on Growth of Yeasts

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Ansamitocins in combination with amphotericin B produced synergistic inhibition on the growth of several yeasts in liquid cultures. Ansamitocin P-3 at 5 $\mu\text{g/ml}$ completely suppressed the growth of *Saccharomyces cerevisiae* whereas ansamitocin P-3 alone at 50 $\mu\text{g/ml}$ hardly affected growth. Ansamitocin P-4 and maytansine also showed synergistic activity with amphotericin B against *S. cerevisiae*. The synergism also occurred in cultures of *Candida albicans* and *Hansenula anomala*. Combinations of ansamitocin P-3 with various agents revealed that the synergism depended on the specific property of amphotericin B. Ansamitocins showed no interfering activity against regeneration of protoplasts of *S. cerevisiae*. These results suggest that the limited activity of ansamitocins against these yeasts is due to the membrane permeability barrier of these cells.

Ansamitocins, maytansinoid antitumor antibiotics purified from fermentation fluids of *Nocardia* sp. No. C-15003 (N-1),^{1,2)} have specific actions against microtubule systems in eukaryotic cells,^{3~5)} resulting in inhibition of growth of several fungi, protozoa and tumor cells, and suppression of the regeneration of cilia in deciliated *Tetrahymena* at low concentrations.³⁾ However, the antibiotics show slight activity against some fungi.²⁾ There are two possible reasons for the limited activity of ansamitocins against these fungi, i.e., the low affinity of the antibiotics to the cellular targets in these cells and obstruction by the permeability barrier of their cytoplasmic membrane.

In an attempt to clarify the action of ansamitocins on fungi, we studied the effect of the antibiotics in combination with a polyene antibiotic, amphotericin B, against several yeasts tolerant to ansamitocins, and further studies were made using protoplasts of *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Organisms. *Saccharomyces cerevisiae* IFO 0209, *Candida albicans* IFO 0583 and *Hansenula anomala* IFO 0118 were used throughout this work. These yeasts were maintained on agar slants of trypticase soy agar (BBL) containing 1% glucose.

Synergism studies. The susceptibility of each yeast to ansamitocin P-3, P-4 or maytansine combined with amphotericin B or other agents was determined by the broth dilution method with YMM medium (0.67% yeast-nitrogen base (Difco) and 2% glucose, pH 6.5). Each fungus was cultured for 20 hours at 28°C with shaking before inoculation. The culture was diluted 10-fold with the medium and a 50 μl portion was transferred to 5 ml of medium with each drug. The initial cell density in this medium was about $5 \times 10^4/\text{ml}$. After incubation for 20 hours at 28°C with shaking, growth was measured by the turbidity of the culture at 600 nm with a Hitachi spectrophotometer model 101.

Regeneration of protoplasts. Protoplasts of *S. cerevisiae* were prepared by the method of Yamamoto and Fukui.⁶⁾ The regeneration of protoplasts was carried out as follows: The protoplastized suspension was diluted with SET medium⁶⁾ (1.3 M sorbitol, 0.1 mM EDTA and 10 mM Tris-HCl, pH 7.4), or distilled water as the control, and the diluent was mixed with the regeneration medium kept at 50°C which contained 0.5% yeast extract, 0.5% peptone, 3% glucose, 2.5% agar, and 0.6 M KCl, pH 6.0. After addition of ansamitocin P-3, P-4 or maytansine, the mixture was poured into a plastic plate, and then the solidified plate was incubated for 66 hours at 28°C. The number of regenerated colonies was estimated by subtracting the number of colonies with dilution with distilled water from that with SET.

Chemicals. Ansamitocins P-3 and P-4 were purified from the culture fluid of *Nocardia* sp. No. C-15003 (N-1).^{1,2)} Maytansine used in this study was prepared in our research division. Amphotericin B, cyclo-

heximide, 5-fluorocytosine and deoxycholate were purchased from P-L Biochemicals Inc., USA, Sigma Chemicals Co., USA, Nippon Roche K.K., Japan, and Difco Co., USA, respectively. Antimycin was purified from the culture fluid of a streptomycete strain isolated in our laboratories.

RESULTS

Effect of ansamitocins on growth of yeasts in combination with amphotericin B

Ansamitocins showed no activity against several yeasts at concentrations up to 50 $\mu\text{g/ml}$. In an attempt to clarify whether their limited antifungal activity is due to the permeability barrier of the membrane of the tolerant cells, combination of ansamitocin P-3 with a polyene antibiotic, amphotericin B, was examined. As shown in Fig. 1, ansamitocin P-3 at 5 $\mu\text{g/ml}$ in combination with amphotericin B at 0.05 $\mu\text{g/ml}$ showed complete inhibition of growth of *S. cerevisiae*, whereas ansamitocin P-3 at 20 $\mu\text{g/ml}$ alone showed no inhibition. Combination of ansamitocin P-3 with amphotericin B at 0.02 $\mu\text{g/ml}$ or less exhibited no marked effect. When amphotericin B was added to the exponential culture in the presence of ansamitocin

FIG. 1. Inhibition of Growth of *S. cerevisiae* by Ansamitocin P-3 in Combination with Amphotericin B.

A 20 hour culture was diluted 10-fold with fresh medium and a 50 μl portion was transferred to 5 ml of medium with varying concentrations of ansamitocin P-3 and amphotericin B. After incubation for 20 hours at 28°C, the turbidity of the culture at 600 nm was measured. ○, without amphotericin B; ●, amphotericin B at 0.02 $\mu\text{g/ml}$; ▲, amphotericin B at 0.05 $\mu\text{g/ml}$.

FIG. 2. Inhibition of Exponential Growth of *S. cerevisiae* by Ansamitocin P-3 in Combination with Amphotericin B.

Ansamitocin P-3 at 10 $\mu\text{g/ml}$ was added at time zero. The arrow indicates the addition of amphotericin B at 0.05 $\mu\text{g/ml}$. ●, no drug; ▲, ansamitocin P-3 alone; ○, amphotericin B alone; △, ansamitocin P-3 plus amphotericin B.

P-3 at 10 $\mu\text{g/ml}$, growth was prevented after one hour of the subsequent division (Fig. 2.) Ansamitocin P-4 and maytansine, which has a structure⁷⁾ and function^{8~12)} closely related to ansamitocins, also showed synergistic inhibition with amphotericin B against *S. cerevisiae* (Fig. 3). The inhibitory activity of ansamitocin P-3 and maytansine combined with amphotericin B at 0.05 $\mu\text{g/ml}$ was weaker than that of ansamitocin P-4. Ansamitocins at 20 $\mu\text{g/ml}$ alone did not affect the growth of *Hansenula anomala* or *Candida albicans*. However, the growth of both organisms was completely suppressed by ansamitocin P-3 at 5 $\mu\text{g/ml}$ in

FIG. 3. Inhibition of Growth of *S. cerevisiae* by Ansamitocin P-4 and Maytansine in Combination with Amphotericin B.

Procedures and symbols are the same as described in Fig. 1.

FIG. 4. Inhibition of Growth of *C. albicans* and *H. anomala* by Ansamitocin P-3 in Combination with Amphotericin B.

Procedures are the same as described in Fig. 1. A, *C. albicans* IFO 0583; B, *H. anomala* IFO 0118. ○, without amphotericin B; ●, amphotericin B at 0.05 µg/ml.

combination with amphotericin B at 0.05 µg/ml (Fig. 4).

In order to clarify the specific property of amphotericin B, combinations of ansamitocin P-3 with other agents with different action mechanisms were examined. As shown in Fig. 5, cycloheximide, a specific inhibitor of protein synthesis in eukaryotic cells, and 5-fluorocytosine, an inhibitor of nucleic acid synthesis in fungal cells, showed no synergistic effect. Antimycin, an inhibitor of the respiratory mechanism, and deoxycholate, a surface activating agent, caused a synergistic effect

FIG. 5. Inhibition of Growth of *S. cerevisiae* by Ansamitocin P-3 in Combination with Various Agents.

Procedures are the same as described in Fig. 1. A: ●, without cycloheximide (CYH); ○, CYH at 0.02 µg/ml; ▲, CYH at 1.0 µg/ml. B: ●, without 5-fluorocytosine (5FC); ○, 5FC at 0.005 µg/ml; ▲, 5FC at 0.02 µg/ml. C: ●, without antimycin (ANM); ○, ANM at 0.5 µg/ml; ▲, ANM at 1.0 µg/ml. D: ●, without deoxycholate (DOC); ○, DOC at 20 µg/ml; ▲, DOC at 40 µg/ml.

with ansamitocin P-3, but to a much lesser extent than amphotericin B. These results suggest that the synergism depends on the specific property of amphotericin B.

Effect of ansamitocins on the regeneration of protoplasts of *S. cerevisiae*

If ansamitocins cannot penetrate the membrane of tolerant fungi, the regeneration of protoplasts into vegetative cells in these organisms might not be affected. In fact, the regeneration of protoplasts of *S. cerevisiae* to form colonies was hardly affected by ansamitocins (Table I). Although the growth was completely suppressed by 5 µg/ml of ansamitocin P-3 combined with 0.05 µg/ml of amphotericin B (Fig. 1), a high regeneration value was obtained even at 100 µg/ml of ansamitocin P-3 alone. Ansamitocin P-4 and maytansine also showed only slight activity against the regeneration of protoplasts at a

TABLE I. EFFECT OF ANSAMITOCINS ON THE REGENERATION OF YEAST PROTOPLASTS

Protoplasts were prepared from *S. cerevisiae* IFO 0209 by the method of Yamato and Fukui.⁶⁾ The regeneration of protoplasts was carried out by the method described in MATERIALS AND METHODS. The number of regenerated colonies was estimated by subtracting the number of colonies with dilution with distilled water from that with SET medium. The value of relative regeneration was estimated relative to the number of regenerated colonies without ansamitocins, which was taken as 1.0.

Compound	Concn.	Total colonies	Regenerated colonies	Relative regeneration
Ansamitocin P-3	0 μ g/ml	1.18×10^8 /ml	9.19×10^7 /ml	1.00
	1	1.13	8.72	0.95
	10	1.06	8.36	0.91
	100	0.72	4.66	0.51
Ansamitocin P-4	10	1.12	8.12	0.88
Maytansine	10	1.09	8.29	0.90

concentration of 10 μ g/ml, the same as ansamitocin P-3. It is therefore clear that ansamitocins and maytansine can hardly penetrate the membrane of *S. cerevisiae* and have no interfering activity against the formation of the cell wall in this organism.

DISCUSSION

Ansamitocins are specific inhibitors for microtubule systems in eukaryotic cells. Although some eukaryotic microorganisms show tolerance to ansamitocins,²⁾ their mitotic process might also be susceptible to the antibiotics because of the closely related properties of tubulin molecules among higher and lower eukaryotic organisms.¹³⁾ As shown in this communication, ansamitocins show a synergistic effect with amphotericin B against some yeasts that are relatively resistant to ansamitocins when used alone. The polyene antibiotics, such as amphotericin B, are well-known to interact with sterol-containing membranes of eukaryotic cells¹⁴⁾ resulting in leakage of cellular constituents and permeability changes.^{15~18)} Therefore, the basis of this synergism is most likely amphotericin B-induced penetration of ansamitocins into these tolerant yeasts, resulting in the inhibition of the functions of their microtubule systems. In the same way the antifungal property of maytansine may be enhanced by amphotericin B. This presumption is supported by the results which show that ansamitocins and maytansine did not suppress the regeneration of protoplasts of

S. cerevisiae into vegetative cells. This observation indicates that these compounds can neither penetrate the membrane of the yeast nor interfere with the formation process of its cell wall.

Agents for the specific disruption of the mitotic apparatus are available for studying the relationship between growth and nuclear division in fungi. For instance, colcemid was used for investigating the growth of yeasts.^{19~21)} Since ansamitocins have specific characters that inhibit mitosis resulting in metaphase arrest of susceptible cells,^{1,4)} combinations of ansamitocins with amphotericin B may also be useful for studying nuclear division in fungi.

REFERENCES

- 1) E. Higashide, M. Asai, K. Ootsu, S. Tanida, Y. Kozai, T. Hasegawa, T. Kishi, Y. Sugino and M. Yoneda, *Nature*, **270**, 721 (1977).
- 2) S. Tanida, T. Hasegawa, K. Hatano, E. Higashide and M. Yoneda, *J. Antibiotics*, **33**, 192 (1980).
- 3) S. Tanida, E. Higashide and M. Yoneda, *Antimicrobial Agents and Chemother.*, **16**, 101 (1979).
- 4) K. Ootsu, Y. Kozai, M. Takeuchi, S. Ikeyama, K. Igarashi, K. Tsukamoto, Y. Sugino, T. Tashiro, S. Tsukagoshi and Y. Sakurai, *Cancer Res.*, **40**, 1707 (1980).
- 5) S. Tanida, E. Higashide and M. Yoneda, *J. Gen. Microbiol.*, **118**, 411 (1980).
- 6) M. Yamamoto and S. Fukui, *Agric. Biol. Chem.*, **41**, 1829 (1977).
- 7) S. M. Kupchan, Y. Komoda, W. A. Court, G. J. Thomas, R. M. Smith, A. Karim, C. J. Gilmore, R. C. Haltiwanger and R. F. Bryan, *J. Am. Chem. Soc.*, **94**, 1354 (1972).

- 8) S. Remillard, L. I. Rebhun, G. A. Howie and S. M. Kupchan, *Science*, **189**, 1002 (1975).
 - 9) M. K. Wolpert-DeFilippes, V. H. Bono, Jr., R. L. Dion and D. G. Johns, *Biochem. Pharmacol.*, **24**, 1735 (1975).
 - 10) F. Mandelbaum-Shavit, M. K. Wolpert-DeFilippes and D. G. Johns, *Biochem. Biophys. Res. Commun.*, **72**, 47 (1976).
 - 11) B. Bhattacharyya and J. Wolf, *FEBS Lett.*, **75**, 159 (1977).
 - 12) R. K. Johnson, T. Inouye and M. K. Wolpert-DeFilippes, *Biochem. Pharmacol.*, **27**, 1973 (1978).
 - 13) R. E. Stephens and K. T. Edds, *Physiological Reviews*, **56**, 709 (1976).
 - 14) J. M. T. Hamilton-Miller, *Adv. Appl. Microbiol.*, **17**, 109 (1974).
 - 15) G. Medoff, G. S. Kobayashi, C. N. Kwan, D. Schlessinger and P. Venkov, *Proc. Natl. Acad. Sci. U. S.*, **69**, 196 (1972).
 - 16) G. S. Kobayashi, S. C. Cheung, D. Schlessinger and G. Medoff, *Antimicrobial Agents and Chemother.*, **5**, 16 (1974).
 - 17) W. C. Chen, D. Chou and D. S. Feingold, *Antimicrobial Agents and Chemother.*, **13**, 914 (1978).
 - 18) J. Palacios and R. Serrano, *FEBS Lett.*, **91**, 198 (1978).
 - 19) S. Lederberg and G. Stetten, *Science*, **168**, 485 (1970).
 - 20) J. E. Haber, J. G. Peloquin, H. O. Halvorson and G. G. Borisy, *J. Cell Biol.*, **55**, 355 (1972).
 - 21) S. Lederberg, R. L. Gourse and D. L. Sackett, *J. Bacteriol.*, **129**, 198 (1977).
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