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Classification of Polyene Antibiotics According to Their Synergistic Effect in Combination with Bleomycin A2 or Fusidic Acid

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Five polyene antibiotics were compared for their effects on colony formation of either Chinese hamster V79 or *Saccharomyces cerevisiae* cells. A 10 to 40 times higher concentration of amphotericin B (heptaene) or nystatin (degenerated heptaene) was necessary to inhibit colony formation of hamster cells than that needed to inhibit colony formation of yeast cells. In contrast, colony formation of both hamster and yeast cells was inhibited to the same extent by similar concentrations of filipin (pentaene), pentamycin (pentaene), or pimaricin (tetraene). The five polyene antibiotics were also compared for their effects on colony formation of either V79 or *S. cerevisiae* cells when combined with a nonpolyene antibiotic, fusidic acid or bleomycin A2. Amphotericin B or nystatin could augment the cytocidal effect of fusidic acid but not that of bleomycin A2, whereas pentamycin or pimaricin could augment the cytocidal effect of both fusidic acid and bleomycin A2 against hamster and yeast cells. Filipin was found to enhance the action of fusidic acid and bleomycin upon growth of mammalian cells, whereas the pentaene polyene significantly potentiated the action of fusidic acid, but not that of bleomycin A2, against *S. cerevisiae*. It was therefore suggested that these polyene antibiotics be classified into two groups: group 1 (pimaricin, pentamycin, and filipin) and group 2 (amphotericin B and nystatin).

Polyene antibiotics (8) have aroused interest in their pleiotropic effects on eucaryotic cells through interacting with cellular sterol molecules. The biological effects caused by the polyenes include enhanced membrane permeation of other combined agents (12), immunoadjuvant properties (17), modulation of macrophage tumoricidal capability (3), and enhanced production of interferon (2). Kotler-Brajtburg et al. (13) recently tried to correlate the chemical structures of polyenes and their biological properties, and they classified polyenes into two groups according to their ability to cause K^+ leakage and cell death.

We found previously that fusidic acid, an inhibitory antibiotic of protein synthesis (19), and bleomycin A2, an antitumor agent (20), were significantly potentiated by amphotericin B or other polyenes in cultured animal cells (11, 14, 15). Our recent study also indicated that bleomycin was potentiated by pimaricin or filipin, but not by amphotericin B (1). Amphotericin B, however, was shown to potentiate fusidic acid (10, 13). We therefore used bleomycin A2 and fusidic acid to determine whether polyene-induced synergism correlated with the chemical structure of polyene antibiotics. In this report, we examine the synergistic effect of various polyene antibiotics on animal and yeast cells when combined with fusidic acid or bleomycin A2 and suggest a possible classification of five polyenes into two groups in accordance with previous work by Kotler-Brajtburg et al. (13).

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MATERIALS AND METHODS

Cell culture of Chinese hamster V79 cells and yeast cells. Chinese hamster V79 cells were routinely cultured in monolayer in plastic plates in minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan) containing 0.1% peptone (Difco Laboratories, Detroit, Mich.), 10% fetal bovine serum (Microbiological Associates, Bethesda, Md.), kanamycin (100 μg/ml), and penicillin (200 U/ml). Colony formation was done under the conditions described previously (10; K. Hidaka, S. Akiyama, and M. Kuwano, Exp. Cell Res., in press). We used *Saccharomyces cerevisiae* 4450-1A (wild type), which was kindly donated by Pencho Venkov. *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 containing 1% yeast extract (Difco), 2% peptone (Difco), 2% dextrose, and 2% agar (Difco) (6).

Chemicals. Bleomycin A2 was obtained through Nippon Kayaku Co., Tokyo. Fusidic acid, amphotericin B, and nystatin were provided by Sankyo Co.,

Tokyo, and pentamycin was given by Nikken Chemical Co., Tokyo. Filipin was from The Upjohn Co., Kalamazoo, Mich., and pimaricin was from Torii Pharmaceutical Co., Tokyo.

Measurements of cell survival of Chinese hamster V79 cells and synergistic study by colony formation. Cell survival of V79 cells was measured by colony formation. V79 cells were plated (150 to 200 each) in duplicate 60-mm dishes in the absence of any drug and incubated for 18 h. Then the cells were exposed to various doses of polyenes for measuring dose-response curves to polyenes alone. To measure the synergistic effect, the cells were exposed to bleomycin A2 or fusidic acid with or without polyenes. After incubation at 37°C for 8 days, colony numbers were counted when stained with Giemsa as described previously (1, 11). Fusidic acid and bleomycin A2 were freshly prepared by dissolving in sterile water. Polyene antibiotics used in this study were prepared by dissolving in dimethyl sulfoxide before each experiment, and all control experiments were done by adding the same amount of dimethyl sulfoxide alone.

Cell survival and synergistic study of yeast cells by colony formation. To test cellular dose response to polyenes, exponentially growing *S. cerevisiae* cells (5×10^6 to 12×10^6 cells per ml) in 1 ml of minimal medium were incubated with various doses of polyene antibiotics. To measure the synergistic effect, *S. cerevisiae* cells were exposed to bleomycin A2 or fusidic acid with or without polyene antibiotics. After incubation for 2 h with shaking at 33°C, cells were washed twice with saline by repeated centrifugation. Finally, the cell pellets were suspended and diluted in saline and plated on YPD agar (6). Numbers of visible colonies on the agar were counted after incubation for 2 days at 33°C.

RESULTS

Dose response of survival of hamster V79 and *S. cerevisiae* cells to polyene antibiotics. It was previously shown that growth of yeast cells was blocked by a much smaller amount of amphotericin B than was that of mammalian HeLa cells (16). To test whether other polyene antibiotics also inhibit cellular growth of yeast at lower concentrations than those required for cultured mammalian cells, we compared colony-forming ability of hamster and yeast cells in the presence of various doses of five polyene antibiotics, amphotericin B (heptaene), nystatin (degenerated heptaene), filipin (pentaene), pentamycin (pentaene), and pimaricin (tetraene) (8). Colony formation of *S. cerevisiae* was blocked by 50% of the initial value in the presence of 0.5 to 1.0 μg of amphotericin B or 1 to 1.5 μg of nystatin per ml (Fig. 1B), whereas colony formation of V79 cells was reduced to 50% of the initial value in the presence of 10 to 15 μg of amphotericin B or 70 to 80 μg of nystatin (Fig. 1A) per ml. In contrast, 1 to 2 μg of pentamycin, 3 to 4 μg of filipin, and 20 to 50 μg of pimaricin per ml blocked colony formation activity of both V79 and *S. cerevisiae* cells by 50% of the initial value (Fig. 1A and B). Dose-response curves of yeast to pentamycin, pimaricin, and filipin were found to be similar to those of mammalian cells (V79), but the extent of sensitivity to amphotericin B or nystatin differed by a factor of 10 to 40 between yeast and mammalian cells.

Synergistic effect of polyenes in combination with fusidic acid or bleomycin A2 on yeast and mammalian cells. To correlate the chemical structure of a polyene antibiotic with its synergistic effect, we examined whether the cytocidal effect of fusidic acid or bleomycin A2 was enhanced by each of five polyene antibiotics, amphotericin B, nystatin, pentamycin, filipin, and pimaricin. Within concentrations that cause 50 to 80% of cell killing of V79 cells (Fig. 1A), we tested the synergistic effect of a combination of two agents against Chinese hamster V79 cells. The cytocidal effect of fusidic acid was remarkably enhanced by all five polyene antibiotics tested, whereas each agent alone had little, if any, effect (Table 1). For example, the survival fraction was reduced to 10^{-2} of the initial value by 100 μg of fusidic acid per ml in combination with 6 μg of amphotericin B per ml, each agent alone at the test concentration did not affect the colony formation of V79 cells (Table 1). In contrast, Table 1 also indicated that bleomycin A2 was significantly potentiated by filipin, pentamycin, and pimaricin, but not by amphotericin B and nystatin.

Within the concentration of polyenes that cause cell killing of 50 to 80% of *S. cerevisiae* (Fig. 1B), we also tested the antifungal effect when bleomycin or fusidic acid was combined with each of the polyene antibiotics. Fusidic acid was potentiated remarkably when combined with the five polyene antibiotics (Table 2). In contrast, the antifungal effect of bleomycin was enhanced by pentamycin or pimaricin, but not by amphotericin B or nystatin (Table 2). It was also found that filipin had only a minimal (if any) synergistic effect against yeast when combined with bleomycin A2 (Table 2), but the same combination of filipin and bleomycin was found to be effective against animal cells (Table 1).

DISCUSSION

Our present study indicated a relatively higher sensitivity of *S. cerevisiae* to amphotericin B and nystatin than of mammalian V79 cells, whereas *S. cerevisiae* and V79 cells showed almost the same sensitivity to pentamycin, pimaricin, and filipin (Fig. 1). In addition, the heptaene antibiotics potentiated only fusidic acid, whereas the nonheptaene antibiotics potentiated both

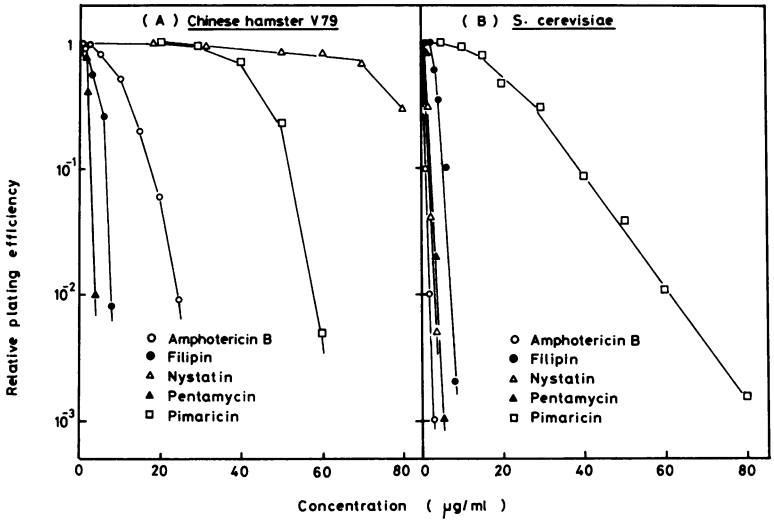

FIG. 1. *Dose response to polyene antibiotics of Chinese hamster V79 and S. cerevisiae cells. Both V79 and S. cerevisiae cells were exposed to various doses of amphotericin B, nystatin, filipin, pentamycin, and pimaricin. The initial count of V79 was 150 to 160 and that of yeast was* 8×10^6 *to* 12×10^6.

TABLE 1. *Comparison of synergistic effects of five polyene antibiotics in combination with fusidic acid or bleomycin A2 against colony formation of Chinese hamster V79 cells*

Polyene (μg/ml)	Fusidic acid (μg/ml)			Polyene (μg/ml)	Bleomycin A2 (μg/ml)		
	0	50	100		0	0.4	0.8
None	1.0[a]	0.9	0.8	None	1.0[a]	0.9	0.7
Amphotericin B				Amphotericin B			
6	1.0	0.6	1.3×10^{-2}	5	1.0	0.9	0.9
9	1.0	0.2	10^{-2}	10	1.0	0.8	0.8
Nystatin				Nystatin			
75	1.0	0.5	0.1	40	1.0	0.9	0.7
100	1.0	0.1	10^{-2}	80	1.0	0.9	0.6
Filipin				Filipin			
3	1.0	0.6	0.3	2	1.0	0.7	0.3
6	1.0	0.4	5×10^{-2}	4	1.0	10^{-2}	10^{-2}
Pentamycin				Pentamycin			
1	1.0	0.7	0.1	1	1.0	0.6	0.4
2	1.0	0.4	2×10^{-2}	2	1.0	10^{-2}	10^{-2}
Pimaricin				Pimaricin			
20	1.0	0.8	5×10^{-2}	20	1.0	0.6	0.4
40	1.0	0.1	10^{-2}	40	1.0	10^{-2}	10^{-2}

[a] Relative plating efficiencies of Chinese hamster V79 cells were obtained by plating the cells and normalizing the ratio of colonies that appeared under synergistic conditions to those in the absence of any drug or in the presence of each polyene alone.

fusidic acid and bleomycin (Tables 1 and 2). Although the synergistic effect of filipin and bleomycin was minimal against yeast cells (Table 2), these two independent assays tempted us to classify the five polyene antibiotics into two groups: a heptaene group (amphotericin B and nystatin) and a nonheptaene group (filipin, pentamycin, and pimaricin). Our data so far consistently support an idea suggested by Kotler-Brajtburg et al. (13) that heptaene antibiotics and other smaller polyene antibiotics such as triene, tetraene, pentaene, and hexaene can be classified into two functionally different groups. According to the classification, group 1 (nonheptaene group) caused K^+ leakage and cell death at the same doses of added polyenes, and group 2 (heptaene group) caused K^+ leakage at low doses and cell death at high doses (13). On the other hand, macrophage tumoricidal activity was stimulated in the presence of amphotericin

TABLE 2. *Comparison of synergistic effects of five polyene antibiotics in combination with fusidic acid or bleomycin A2 against colony formation of S. cerevisiae*

Polyene (μg/ml)	Fusidic acid (μg/ml)			Polyene (μg/ml)	Bleomycin A2 (μg/ml)		
	0	50	100		0	25	40
None	1.0[a]	0.9	0.8	None	1.0[a]	0.5	0.3
Amphotericin B				Amphotericin B			
0.2	1.0	0.2	0.1	0.5	1.0	0.3	0.3
0.5	1.0	10^{-2}	10^{-2}	1.0	1.0	0.3	0.3
Nystatin				Nystatin			
0.6	1.0	0.1	9×10^{-2}	0.6	1.0	0.3	0.2
1.0	1.0	10^{-2}	10^{-2}	1.2	1.0	0.2	0.2
Filipin				Filipin			
3	1.0	0.7	0.4	3	1.0	0.4	0.2
6	1.0	0.2	10^{-2}	6	1.0	0.2	0.1
Pentamycin				Pentamycin			
2	1.0	10^{-2}	10^{-2}	3	1.0	10^{-2}	10^{-2}
3	1.0	10^{-2}	10^{-2}				
Pimaricin				Pimaricin			
20	1.0	0.7	0.4	10	1.0	6×10^{-2}	10^{-2}
30	1.0	8×10^{-2}	2×10^{-2}	40	1.0	10^{-2}	10^{-2}

[a] Relative plating efficiencies of *S. cerevisiae* were obtained by plating the cells and normalizing the ratio of colonies that appeared under synergistic conditions to those in the absence of any drug or in the presence of each polyene alone.

B, but not in the presence of pimaricin or filipin (3). Relevant work also indicated immunoadjuvant activities of amphotericin B, but not of filipin or etruscomycin (tetraene) (9, 17). These immunological studies also suggest differential biological functions of heptaene and nonheptaene polyenes. According to a model proposed by De Kruijff and colleagues (4, 5), amphotericin B and nystatin make 0.8-nm aqueous pores in the membrane and filipin produces disruptions larger in area (20 to 25 nm). One might argue that the extent of the membrane distortion by polyene antibiotics is somehow correlated with their functional differences.

Filipin is known to alter the cellular permeability of mammalian cells (1, 7), but it failed to potentiate bleomycin A2 on yeast cells (Table 2). Damage of yeast membranes caused by filipin might be partly cured, and therefore bleomycin, but not fusidic acid, is not supposed to permeate the cells treated with the pentaene. If all of the polyenes interact with sterols (4, 8) and the cellular content of sterol per phospholipid is important in determining the cellular sensitivity to the polyenes (10, 11, 18), the different sensitivities of yeast and animal cells might be due to disparity in sterol molecules between yeast (ergosterol) and hamster (cholesterol) cells. Alternatively, use of another component(s) besides sterols might be considered for determining the cellular sensitivities to these polyenes.

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