Synergistic Action of Amphotericin B and 5-Fluorocytosine Against Yeast-Like Organisms[1] (35943)

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(Introduced by C. G. Harford)

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In spite of the discovery of several potent antifungal agents (1), amphotericin B still remains the single most effective antibiotic for the treatment of systemic mycotic diseases. Adequate therapy of most serious fungal infections requires high doses of this drug given over a long period of time (2). Treatment regimens are empirically derived, carry with them a significant incidence of toxicity, and in many cases the infection recurs once therapy is discontinued (2). In view of the seriousness of disseminated mycotic diseases and the toxic potential of amphotericin B, several workers have investigated different approaches to amphotericin B therapy (3–5). Unfortunately, none of these therapeutic regimens increases the effectiveness of treatment and in some the toxicity of amphotericin B remains an important problem (6). Clearly, it is important to develop more effective antifungal treatment regimens.

One method by which antibacterial therapy has been improved is through the use of two antibiotics which together act synergistically against a particular organism. This has been accomplished most dramatically in the treatment of enterococcal infections with the combination of penicillin and streptomycin (7). The action of these two drugs against the enterococcus is based on disruption of the cell walls of actively dividing organisms by penicillin which then enables a greater concentration of streptomycin to enter the cell and bind to the ribosomes (8). We have been investigating the *in vitro* action of antifungal agents used in combination against fungi, with the intention of increasing the effectiveness of therapy and reducing or eliminating any toxic side effects of the drugs under study. The present report describes our observations of antibiotic synergy of amphotericin B and 5-fluorocytosine against several yeasts. For the purpose of this study the term synergy is defined as a decrease, after a prescribed period of observation, of 10-fold or more colony forming units caused by the combination compared to the drugs used alone.

Methods. Saccharomyces cerevisiae (Hoffman-La Roche, Nutley, NJ) and clinical isolates of *Cryptococcus neoformans, Candida albicans,* and *Candida tropicalis* were employed in this study. All cultures were maintained on Sabouraud's dextrose agar (4% glucose, 1% peptone, 2% agar) and transferred at weekly intervals to fresh culture medium. Initially studies were performed in parallel with yeast morphology agar (Difco) and Sabouraud's dextrose broth to ensure the adequacy of Sabouraud's dextrose broth as supporting medium for these studies. No differences in total viability were detected. Tube dilution sensitivities of each fungus to amphotericin B and 5-fluorocytosine were performed. Approximately 10^5 organisms, as determined by hemocytometer counts and confirmed by colony counts, from a 24 hr culture grown in Sabouraud's dextrose broth were dispensed in 8 ml portions to sterile screw capped tubes. Various concentrations of the test antibiotics were added to each tube in 2 ml amounts. Turbidometric studies (530 nm, Bausch and Lomb Spectronic 20) were performed to follow the relative increases in population of the yeast. Viable particle counts were determined at 72 hr for *S. cerevisiae* and *C. albicans* and at 7 days

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for *C. neoformans*, and *C. tropicalis* by plating a sample of each test dilution. All viability studies were performed in triplicate on Sabouraud's dextrose agar.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of each drug that completely inhibited multiplication of the fungus as measured turbidometrically and by colony counts at the time period indicated for each organism. The clinical isolates used in this study were selected primarily for their relative resistance to amphotericin B and 5-fluorocytosine. Concentration of amphotericin B and 5-fluorocytosine, less than half the MIC, and shown to be relatively ineffective against the yeast when tested alone, were chosen for the synergism studies. The yeasts were incubated in these concentrations of the drugs used singly and in combination and plated as described above. All studies were carried out at 24–26°.

Results. 5-Fluorocytosine at the concentrations tested (0.25 to 200 μg/ml) had no killing effect on the fungi and only partially inhibited their growth. The minimum inhibitory concentration of 5-fluorocytosine against the sensitive organism *S. cerevisiae* was 6.25 μg/ml; the concentration of 2.5 μg/ml used against this yeast, however, allowed growth of the organism when used alone (Table I). The minimum inhibitory concentrations of 5-fluorocytosine against the other yeasts were in excess of 200 μg/ml.

The concentration of amphotericin B employed in each of the synergy studies were determined by titration and were less than half of the minimum inhibitory concentrations for each organism. At these concentrations, amphotericin B only partially inhibited growth of the yeasts and never evidenced a killing effect. Doubling the concentrations of amphotericin B and 5-fluorocytosine only slightly increased the growth inhibitory effects.

The combined use of amphotericin B and 5-fluorocytosine was synergistic at several different concentrations where no killing effect could be observed when the agents were used alone. In all cases the concentrations of drugs in the combinations were less than one half the MIC of the drugs used alone. The concentrations of the combined drugs which demonstrated the most marked synergistic effect are shown in Table I. Amphotericin B and 5-fluorocytosine, when used in combination at these concentrations, produced a marked killing effect. In all cases the differences between the drugs used alone and in combination were greater than tenfold, and, in one case, the combination was fungicidal.

Discussion. Amphotericin B and other polyene antibiotics induce permeability alterations in fungi by binding with sterols which are present in the cell membrane (9). The available information suggests that 5-fluorocytosine, an antifungal agent effective against a variety of yeasts, inhibits nucleic acid synthesis in these organisms through its effect on the pyrimidine biosynthetic pathway (10). It is becoming increasingly clear that resistance to 5-fluorocytosine is common among many yeasts and particularly those cultured from patients treated with this fluoropyrimidine (11). There is experimental evidence that this resistance is based on a failure of the pyrimidine analog to penetrate the resistant yeast (10). We have shown that the combined use of amphotericin B and 5-fluorocytosine against a variety of yeasts (Table I) demonstrates a synergistic activity. This effect is dose related in that it is less striking at lower concentrations of amphotericin B. At every concentration tested, however, it is more than could be attributed to an additive effect of the two drugs. When amphotericin B was used at greater than the minimum inhibitory concentration for each organism, synergism was difficult to evaluate because of the killing effect of amphotericin B alone. Whether the mechanism of the synergy is similar to that of penicillin and streptomycin against the enterococcus, that is whether the cell membrane effect of amphotericin B increases the uptake of 5-fluorocytosine in the resistant cells, is presently unknown. The number of clinical isolates that is responsive to this synergistic combination is being determined.

The striking effect of amphotericin B and 5-fluorocytosine in our *in vitro* studies indicates that the combination offers great prom-

TABLE I. Synergism of Amphotericin B and 5-Fluorocytosine Against a Variety of Yeasts.

Organism	Minimum inhibitory conc for amphotericin B (μg/ml)	Treatment	Viable counts/ml		
			0 time	72 hr	7 days
S. cerevisiae	1.5	None	2.2×10^5	2.2×10^7	
		Amphotericin B, 0.5 μg/ml		6.0×10^6	
		Amphotericin B, 1.0 μg/ml		2.5×10^6	
		5-Fluorocytosine, 2.5 μg/ml		4.2×10^6	
		5-Fluorocytosine, 5.0 μg/ml		2.1×10^6	
		Combination (0.5 μg + 2.5 μg)		1.0×10^4	
C. albicans (374)	0.6	None	1.4×10^5	5.0×10^6	
		Amphotericin B, 0.2 μg/ml		1.1×10^6	
		Amphotericin B, 0.4 μg/ml		8.0×10^5	
		5-Fluorocytosine, 100 μg/ml		3.0×10^6	
		5-Fluorocytosine, 200 μg/ml		2.1×10^6	
		Combination (0.2 μg + 100 μg)		6.4×10^3	
C. neoformans (Handy)	1.5	None	1.4×10^5		5.0×10^6
		Amphotericin B, 0.5 μg/ml			1.4×10^6
		Amphotericin B, 1.0 μg/ml			9.0×10^5
		5-Fluorocytosine, 100 μg/ml			1.3×10^6
		5-Fluorocytosine, 200 μg/ml			1.0×10^6
		Combination (0.5 μg + 100 μg)			0
C. neoformans (Johnson)	3.0	None	1.2×10^5		1.0×10^7
		Amphotericin B, 0.78 μg/ml			1.0×10^7
		Amphotericin B, 1.56 μg/ml			5.0×10^6
		5-Fluorocytosine, 100 μg/ml			5.5×10^6
		5-Fluorocytosine, 200 μg/ml			4.2×10^6
		Combination (0.78 μg + 100 μg)			1.8×10^2
C. neoformans (Wilson)	2.5	None	1.0×10^5		1.2×10^7
		Amphotericin B, 0.78 μg/ml			3.2×10^6
		Amphotericin B, 1.56 μg/ml			1.0×10^6
		5-Fluorocytosine, 100 μg/ml			3.5×10^6
		5-Fluorocytosine, 200 μg/ml			2.0×10^6
		Combination (0.78 μg + 100 μg)			3.0×10^5
C. tropicalis (Overton)	4.0	None	1.0×10^5		1.9×10^7
		Amphotericin B, 0.78 μg/ml			9.0×10^6
		Amphotericin B, 1.56 μg/ml			6.0×10^6
		5-Fluorocytosine, 100 μg/ml			4.0×10^6
		5-Fluorocytosine, 200 μg/ml			3.0×10^6
		Combination (0.78 μg + 100 μg)			2.0×10^3

ise for the therapy of infections caused by *C. neoformans* and yeast-like organisms of the genus *Candida*. Documentation of its effectiveness and also of its toxicity will have to await *in vivo* studies.

Summary. The efficacy of amphotericin B and 5-fluorocytosine used in combination against several different isolates of *Cryptococcus neoformans, Candida albicans, Candida tropicalis,* and *Saccharomyces cerevisiae* has been investigated *in vitro*. In every case, the difference in killing between the drugs in combination and used alone was greater than tenfold. Against one of the six isolates the combination of drugs was fungicidal. The clinical implications of these findings regarding the synergistic activity of amphotericin B and 5-fluorocytosine are being evalu-

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