ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1975, p. 117-121

Vol. 8, No. 2
Printed in U.S.A.

In Vitro Studies with Combinations of 5-Fluorocytosine and Amphotericin B

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Received for publication 18 March 1975

Synergistic antifungal activity of 5-fluorocytosine (5-FC) and amphotericin B was studied using an abbreviated checkerboard titration scheme. 5-FC was titrated in twofold increments (100 to 0.05 μg/ml) in the absence and presence of graded increments of amphotericin B (1.0. 0.5, 0.1, 0.05, and 0.01 μg/ml) in buffered yeast nitrogen base. A limited number of experiments were performed using expanded dual titration checkerboard schemes and growth curve studies. Forty-eight isolates of yeastlike organisms were tested; two were inhibited by the buffer system. Evidence of synergy, as indicated by a fourfold or greater reduction of the minimal inhibitory concentration of 5-FC in the presence of subinhibitory concentrations of amphotericin B, was seen with 11 of 46 isolates, or 24%, at the fungistatic level and with three isolates, or 7% at the fungicidal level. Indifferent results were obtained for 44 and 74% of the isolates, respectively, at the fungistatic and fungicidal levels. Antagonism was observed with three isolates.

In 1971 Medoff et al. reported on the apparent synergistic antifungal activity of combinations of 5-fluorocytosine (5-FC) and amphotericin B against yeastlike organisms (5). Although the validity of these findings was questioned by some investigators, they nonetheless stimulated others to examine the possible therapeutic applications of this effect both in vivo in experimentally infected animals (2, 8, 10) and clinically in naturally acquired human cryptococcosis (I. L. Garriques, M. A. Sande, J. P. Utz, G. L. Mandell, J. F. Warner, R. F. McGehee, and S. Shadomy. Abstr. Prog. Intersci. Conf. Antimicrob. Agents Chemother., 13th, Washington, D.C., Abstr. 239, 1973).

Previously, we presented preliminary results from in vitro studies with combinations of 5-FC and amphotericin B which, to a degree, confirmed the original report of Medoff et al. (5) (S. Shadomy and B. A. Davis, Prog. Intersci. Conf. Antimicrob. Agents Chemother., 13th, Washington, D.C., Abstr. 238, 1973). Although we observed synergy in vitro with such combinations, we also noted that this phenomenon was restricted to a limited proportion of the organisms tested. The purpose of this communication is to elaborate on these earlier findings.

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MATERIALS AND METHODS

Cultures. A total of 48 clinical isolates of pathogenic and commensalistic yeastlike organisms were tested. These included 33 isolates of *Cryptococcus neoformans*, 8 of *Candida albicans*, and 7 of *Candida parapsilosis* and *Candida tropicalis*. All were identified on the basis of microscopic morphology, production of germ tubes, urease activity, growth at 37 C, carbohydrate fermentation and assimilation reactions, and nitrate assimilation reactions.

Susceptibility testing. A broth dilution susceptibility test procedure using yeast nitrogen base (Difco) supplemented with asparagine (1.5 g/liter) and dextrose (10.0 g/liter) was used. The medium was sterilized by filtration. To avoid inactivation of amphotericin B, a nonchelating buffer (P. D. Hoeprich, personal communication) was added (5 ml in 100 ml of yeast nitrogen base). The buffer consisted of 15.89 g of 2(*N*-morpholino) propane sulfonic acid and 10.08 g of 2-amino-2-(hydroxymethyl)-1,3-propane-diol combined in a final volume of 100 ml of distilled water, and was then sterilized by autoclaving.

Solutions of amphotericin B were prepared by dissolving standard material (E. R. Squibb and Sons, Inc., batch 91368-001, assay 875 μg/mg) in Me_2SO and then diluting the solution in sterile 5.0% Me_2SO in saline to give a solution containing 100 μg/ml. 5-FC (Hoffmann-La Roche, Inc., Nutley, N.J., lot PP-3) was dissolved in saline (10,000 μg/ml) and sterilized by filtration.

In most tests for synergy, 5-FC was titrated from 100 to 0.05 μg/ml in twofold increments in the absence and presence of graded amounts of amphotericin B.

Concentrations of the latter were 1.0, 0.5, 0.1, 0.05, and 0.01 μg/ml. In a limited series of experiments using a classical checkerboard titration scheme, both 5-FC and amphotericin B were serially diluted in twofold increments and all concentrations were combined. Concentrations ranged from 100 to 0.05 μg/ml for 5-FC and from 5.0 to 0.005 μg/ml for amphotericin B.

Tests were performed in duplicate with inocula prepared from 48- to 72-h-old cultures grown on Sabouraud agar slants. Inoculum size was adjusted to approximately 10^6 cells/ml. Incubation was at 30 C for 48 h. After incubation, the tubes were examined visually for turbidity and minimal inhibitory concentrations (MIC) were recorded. All tubes showing inhibition of growth were then subcultured to drug-free Sabouraud agar plates which were incubated at 30 C for 48 h before examining for minimal fungicidal concentrations (MFC). Growth curve studies were performed with two isolates of *C. neoformans*.

Interpretation of results. In the absence of a commonly agreed upon definition for antimicrobial synergy, the following criteria were used in evaluation of results. A reduction of the MIC for 5-FC by a fourfold factor or greater in the presence of an otherwise subinhibitory concentration of amphotericin B was regarded as synergistic potentiation of the former drug by the latter. A twofold reduction of the 5-FC MIC was regarded as possible potentiation. A twofold or greater increase in the MIC of 5-FC was regarded as antagonism. Similar criteria were used in evaluation of changes in MFC end points.

RESULTS

C. neoformans. One isolate of *C. neoformans* was inhibited by the buffer system and, as a result, eliminated from the study. Three isolates were resistant to 100 μg of 5-FC per ml and remained resistant to 5-FC in subinhibitory concentrations of amphotericin B (Table 1). Synergy was seen with six isolates. Less than fourfold decreases were seen with two isolates. Possible antagonism was seen with two isolates. No evidence of either synergy or antagonism was seen in the remaining 19 isolates.

In most instances, MFC end points of 5-FC were not altered by amphotericin B. However, three isolates gave evidence of a synergistic killing effect; two of these were not killed by 5-FC in the absence of amphotericin B.

It is impractical to present all the data from the studies with *C. neoformans*. However, some of the more unique examples warrant presentation. One isolate for which synergy was detected was reexamined using the checkerboard scheme. This isolate, *C. neoformans* 9.282, was inhibited separately by 25 μg of 5-FC per ml and by 0.16 μg of amphotericin B per ml; in the presence of 0.01, 0.02, 0.04, and 0.08 μg of amphotericin B per ml, respectively, the MIC values for 5-FC were 12.5, 6.25, 1.56, and 0.78 μg/ml. An isobologram of these results is presented in Fig. 1. Another example of the synergistic action of the two drugs against *C. neoformans* is shown in Fig. 2. Synergism was frequently observed with isolates of *C. neoformans* partially resistant to 5-FC. With one clinical isolate, the MIC values for amphotericin B and 5-FC alone were 1.0 and 100 μg/ml, respectively. In the presence of 0.5 μg of amphotericin B per ml, this isolate was inhibited by 12.5 μg of 5-FC per ml.

In contrast to the above, a recent clinical isolate of *C. neoformans*, 9.298, was inhibited by 6.25 μg of 5-FC per ml alone; in the presence of 0.5 μg of amphotericin B per ml, the MIC for 5-FC was 50 μg/ml. Similar antagonism observed with a second isolate, 9.143, is depicted in Fig. 3. In the growth curve experiments, yeasts were incubated for several days in the presence or absence of 50 μg of 5-FC per ml and 0.5 μg of amphotericin B per ml separately and in combination, and quantitative dilution plate

TABLE 1. *Results of combined titrations of 5-FC and amphotericin B[a] on inhibitory and fungicidal concentrations of 5-FC*

Organisms (No. tested)	Synergistic potentiation[b]		Possible potentiation		Indifference		Antagonism		Totally resistant
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	
Cryptococcus neoformans (32)	6	3	2		19	25	2		3
Candida albicans (7)	2		1		1	3			3
Candida tropicalis and *C. parapsilosis* (7)	3		2			4	1		1

[a] 5-Fluorocytosine titrated in twofold increments (100 to 0.05 μg/ml) in buffered yeast nitrogen base in presence of fixed amounts of amphotericin B (1.0, 0.5, 0.1, 0.05, 0.01 μg/ml). Incubation was at 30 C for 48 h.

[b] Interpretive criteria: synergistic potentiation, fourfold or greater reduction of 5-FC MIC in presence of subinhibitory concentrations of amphotericin B; possible potentiation, a twofold or less relation of the 5-FC MIC; indifference, no change in the 5-FC MIC; antagonism, a twofold or greater increase in the 5-FC MIC; totally resistant, MIC of 5-FC > 100 μg/ml under all test conditions.

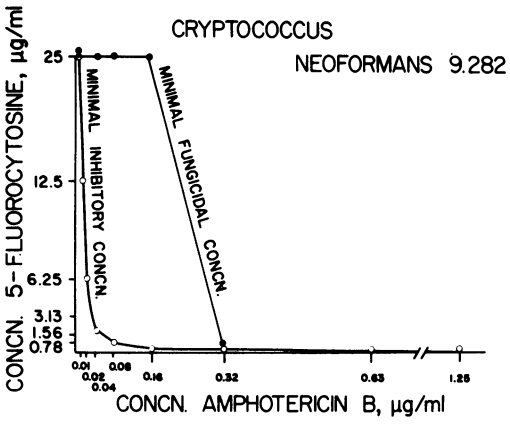

FIG. 1. *Isobologram depicting synergistic results of combined effects of 5-FC and amphotericin B against C. neoformans isolate 9.282. Levels of activity for each drug as measured separately: 5-FC, MIC, and MFC = 25 µg/ml; amphotericin B, MIC = 0.16 µg/ml, MFC = 0.32 µg/ml.*

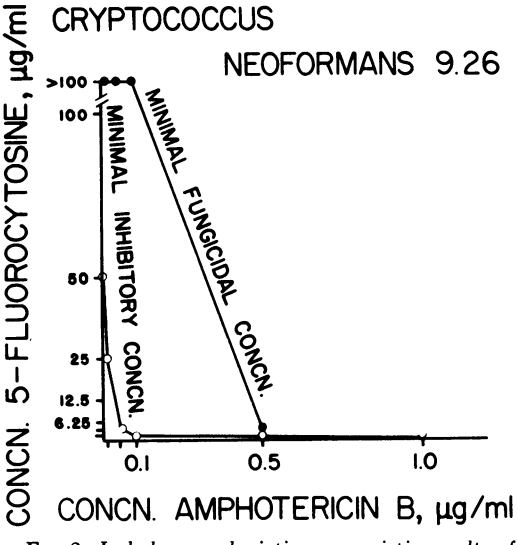

FIG. 2. *Isobologram depicting synergistic results of combined effects of 5-FC and amphotericin B against C. neoformans isolate 9.26. Levels of activity for each drug as measured separately: 5-FC, MIC = 50 µg/ml, MFC > 100 µg/ml; amphotericin B, MIC and MFC = 0.5 µg/ml.*

counts were made periodically. Results with one isolate (Fig. 4) showed that while neither drug alone was totally inhibitory, the combination was partially so.

Candida sp. One isolate of *C. albicans* was inhibited by the buffer system. Three isolates of *C. albicans* and one of *C. parapsilosis* were totally resistant to 5-FC under all test conditions (Table 1). Two isolates of *C. albicans* and

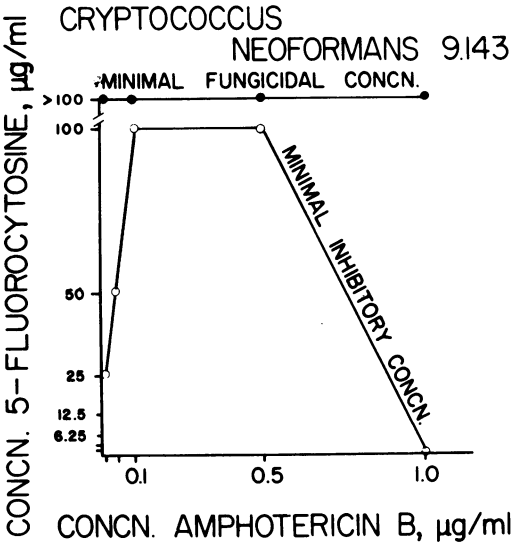

FIG. 3. *Isobologram depicting antagonistic results of combined effects of 5-FC and amphotericin B against C. neoformans isolate 9.143. Levels of activity for each drug as measured separately: 5-FC, MIC 25 µg/ml, MFC > 100 µg/ml; amphotericin B, MIC and MFC = 1.0 µg/ml.*

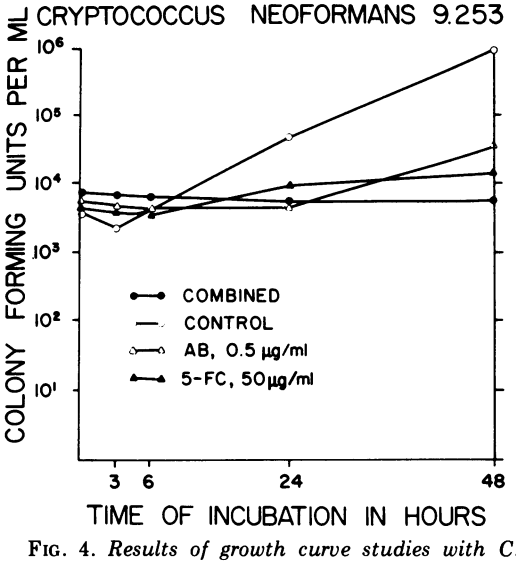

FIG. 4. *Results of growth curve studies with C. neoformans isolate 9.253 incubated in the absence of any drug and in the presence of 50 µg of 5-FC per ml and 0.5 µg of amphotericin B (AB) per ml separately and in combination.*

three of *C. parapsilosis* showed evidence of synergy. For example, *C. albicans* 7.65 was inhibited separately by 12.5 µg of 5-FC per ml and 1.0 µg of amphotericin B per ml; in the presence of 0.1 µg of amphotericin B per ml the

MIC for 5-FC was 3.13 μg/ml. An isobologram for another of these isolates is depicted in Fig. 5. As with *C. neoformans*, synergy often was seen in partially resistant isolates of *Candida* species. The most profound example of this occurred with an isolate of *C. parapsilosis* resistant to less than 100 μg of 5-FC per ml. In the presence of a subinhibitory concentration of amphotericin B, the MIC for 5-FC was reduced to 0.78 μg/ml. Less than fourfold decreases were observed with one isolate each of *C. albicans*, *C. tropicalis*, and *C. parapsilosis*. No evidence of drug interaction was seen with one isolate of *C. albicans*; antagonism was seen with one isolate of *C. tropicalis*. No evidence of synergy was seen with any of the *Candida* isolates at the fungicidal level.

DISCUSSION

Antimicrobial synergy is difficult to define. Barry and Sabath (1) state that a combination of antibiotics is synergistic when "... the effect observed with the combination is greater than the sum of the effects observed with the two drugs independently." Jawetz appears to be more conservative in his view that synergy is mostly concerned with the enhanced bactericidal activity of combined antimicrobial agents (4). Our criterion, the reduction of inhibitory end points of one drug when titrated in the presence of subinhibitory concentrations of a second drug, strikes toward the middle ground between the two above opinions.

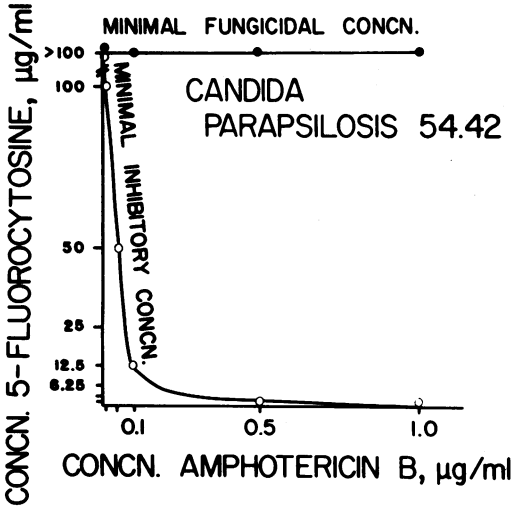

FIG. 5. *Isobologram depicting synergistic results of combined effects of 5-FC and amphotericin B against C. parapsilosis isolate 54.42. Levels of activity for each drug as measured separately: 5-FC MIC = 100 μg/ml, MFC > 100 μg/ml; amphotericin B MIC = 1.0 μg/ml, MFC > 1.0 μg/ml.*

In our earlier studies with 5-FC and *C. neoformans*, we noted that this drug was nearly totally inactive in vitro in the presence of such common ingredients of nonsynthetic culture media as peptones, yeast extracts, and Casamino Acids (9). This was attributed to competitive inhibition by cytosine. This observation was confirmed in the elucidation of the mode of action of 5-FC in susceptible yeasts. In brief, 5-FC enters yeast cells via a permease enzyme, is deaminated for 5-fluorouracil intracellularly and then enters the pathway for synthesis of ribonucleic acid (8). This pattern can be antagonized by the natural pyrimidine analogue as well as by other purines, pyrimidines, and nucleosides at both the uptake and deamination steps. Thus, it was most surprising when Medoff et al. first reported that subinhibitory concentrations of amphotericin B and 5-FC combined in a nonsynthetic medium containing antagonists to the latter drug were synergistic in inhibiting growth of *C. albicans* and *C. neoformans* (5).

The original findings of Medoff and co-workers were disputed. Using a synthetic chemically defined medium Hoeprich and Finn (Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, Mm 42, p. 135) tested combinations of amphotericin B and 5-FC against 21 isolates of *C. neoformans* and *C. albicans* and reported that such combinations were antagonistic. Hamilton, using growth rates and oxygen consumption in vitro and survival rates in experimentally infected mice in vivo as parameters found in vitro but not in vivo evidence of only an additive effect with a 5-FC susceptible isolate of *C. neoformans* and both in vitro and in vivo evidence of antagonism with a 5-FC-resistant isolate (3).

It appears difficult to explain these contradictions regarding synergy between 5-FC and amphotericin B. The report of Hoeprich and Finn (Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, Mm 42, p. 135) presented only statistically summarized data; thus, comparison with our results or with those of Medoff and co-workers is impossible. The in vitro studies reported by Hamilton used maximum concentrations of only 10 μg of 5-FC per ml and 0.1 μg of amphotericin B per ml in combinations; thus, he may have been working with inadequate amounts of drug. In our study, potentiation of 5-FC by amphotericin B was seen most frequently in the presence of higher concentrations of the two drugs although with one isolate of *C. neoformans* the combination of 0.05 μg of am-

photericin B per ml and 3.13 μg of 5-FC per ml was synergistic.

Several factors emerge from the study reported here which may explain these contradictory findings. First, whereas such synergy is a valid phenomenon as recently confirmed by Medoff et al. (6) and more recently by Polak (7), it is restricted in that it is not demonstrable with all isolates of yeastlike organisms. This is borne out by the fact that in our study evidence of synergy at the fungistatic level was obtained with only 24% of 46 isolates and at the fungicidal level with only 7%. In contrast, indifference was seen with 44 and 74% of the isolates, respectively, at the fungistatic and fungicidal levels. Second, we observed synergy most frequently in isolates characterized by a moderate degree of resistance to 5-FC alone, i.e., with MIC values of 25 to 50 μg/ml. This finding is similar to that of Polak who reported synergy between the two drugs in "partially 5-FC resistant" isolates of *Candida* (7). The most plausible explanation for synergistic potentiation of 5-FC by amphotericin B in these organisms, as suggested by Medoff et al. (6), would be the increased penetration of the first drug as a result of changes in membrane permeability induced by the second. In such isolates, one can postulate the presence of either a modified permease system or one which does not recognize 5-FC together with functional deaminase and kinase systems. Further, in those isolates which remain totally resistant to the combined drugs, one can postulate either the absence of a functional deaminase or the failure of 5-fluorouracil to enter the pathway for ribonucleic acid synthesis via uridine kinase. Antagonism is more difficult to explain but it is reasonable to believe that this may also be a result of modification in membrane function induced by amphotericin B.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI 07791 from the National Institute of Allergy and Infectious Diseases and by a Brown-Hazen Grant from Research Corp.

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