

Comparative studies on the postantifungal effect produced by the synergistic interaction of flucytosine and amphotericin B on *Candida albicans*

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Abstract

A turbidometric method was used to measure *Candida albicans* yeast cell growth and to quantitate the postantifungal effect (PAFE) after exposure to various concentrations of flucytosine and amphotericin B, alone and in combination, for 2 hr at 30 °C. The drug concentrations used in the PAFE assays were determined by initial MIC and FIC (fractional inhibitory concentration) evaluations. The PAFE was calculated by the difference in time (hr) required for growth of the control and test cultures to reach the 0.5 absorbance level following removal of the drug by dilution. A synergistic PAFE was evidenced with combinations of the two drugs at concentrations below their individual MICs. Combinations of flucytosine (0.012 to 0.049 µg ml⁻¹) and amphotericin B (0.195 to 0.39 µg ml⁻¹) produced PAFEs ranging from 6.3 to 21.8 hr. These PAFEs persisted from 0.3 to 14.7 hr longer than those achieved when each of the two agents was assayed separately.

Introduction

Recent in vitro susceptibility studies in our laboratory have indicated that a postantifungal effect (PAFE) can be induced by exposing *Candida albicans* yeast cells to certain antifungal agents for a short period of time [1, 2]. Numerous investigations on this phenomenon have been performed with bacteria (the postantibiotic effect; PAE) [3–6], but not with fungal organisms.

PAFE determinations provide susceptibility information to the clinician that most standard susceptibility tests do not provide. In the PAFE assay the drug-organism interaction is for a limited time period (0.5 to 2 hr) instead of continual exposure for 24 hr or more. Such assays may provide useful data with regard to treatment regimen decisions since the presence or absence of a PAFE provides a theoretical rationale for either less frequent or more frequent dosing. We have

used viable counts [1] and turbidometric methods [2] to measure and quantitate the growth of *C. albicans* yeast cells following exposure to flucytosine, amphotericin B and miconazole and subsequent removal of the drug by dilution. PAFEs were induced with the agents at concentrations lower than MICs or optimal peak serum levels.

We have recently initiated investigations to determine if combinations of certain antifungal agents, at concentrations considerably lower than their individual MICs, are able to induce PAFEs of longer duration than those achieved with either agent separately. There has been an increasing interest in utilizing drug combinations in attempts to broaden the antifungal spectrum and enhance the efficacy of the available agents. Many recent reports have been concerned with combination therapy and the potential beneficial effects derived from the synergistic interaction of certain drugs [7–13]. Many of the investigations have provided encouraging results and combination therapy may therefore allow for drugs to be used as concentrations which could be potentially more effective and less toxic than single-dose therapy. PAFE determinations, as above, may provide a rationale for an even greater reduction in the concentration of certain agents.

The aim of the present investigation was to determine if combinations of flucytosine and amphotericin B could interact synergistically and produce a PAFE. Flucytosine and amphotericin B have been widely used, alone and in combination, for the treatment of candidiasis and cryptococcosis, but treatment has been complicated with these agents due to severe renal, hematologic and gastrointestinal toxicity problems [10, 14, 15]. Thus it would certainly be desirable if these side effects could be minimized by less frequent administration of lower concentrations of each agent. Data from such assays may be useful for determining treatment regimens with the two drugs. In the present study a turbidometric method was used to evaluate and determine optimal PAFE parameters following exposure of *C. albicans* yeast cells to flucytosine and amphotericin B.

Materials and methods

Antifungal agents. Flucytosine (5-fluorocytosine) was generously provided by Nippon Roche, K.K. (Tokyo, Japan). An initial stock solution containing $1000\ \mu\text{g ml}^{-1}$ was prepared in saline and the solution sterilized by filtration ($0.22\ \mu\text{m}$ membrane filter; Nucleopore, Pleasanton, CA). Amphotericin B (AmpB; Squibb reference standard; obtained from Bristol-Myers Squibb Co. Ltd., Tokyo, Japan) was diluted in dimethylsulfoxide (less than 1% final concentration). Methanol (50%) was added to the first tube of the dilution series in order to sterilize the solution. Concentrations (two-fold serial dilutions) of flucytosine ranging from 6.25 to $0.006\ \mu\text{g ml}^{-1}$ and amphotericin B ranging from 3.125 to $0.049\ \mu\text{g ml}^{-1}$ were then prepared in sterile saline for use in the assays.

Organisms. Ten strains of *C. albicans* were used in these studies. Strain IFM 1001, which is used as a reference for susceptibility studies in our laboratory, and clinical isolates 86–23717, 11128–H99, 9319–H76, 9760–H85, 9490–H81, 2773–H26, 3532–K31, 11323–H101 and 1090–2–H17 were maintained on Sabouraud dextrose agar (Eiken Chemical Co. Ltd., Tokyo, Japan) slants. The yeast cells were transferred to yeast nitrogen base broth (Difco Laboratories, Detroit, MI) containing 1% glucose (YNB-G) and incubated for approximately 24 hr prior to use in the MIC and PAFE assays. Hemocytometer counts were made on the cell suspensions and the yeast cell number was adjusted to 1×10^5 cells ml^{-1} (MIC determinations) or 1×10^7 cells ml^{-1} (PAFE assays).

MIC and FIC determinations. Initially MIC determinations were made using flucytosine and amphotericin B, separately and in combination, to evaluate synergism between the two drugs. A previously described broth dilution method was used for the MIC assays with yeast cells from the 10 strains of *C. albicans* [16]. A checkerboard titration [17, 18] was performed in which two-fold

serial dilutions of flucytosine (6.25 to $0.006 \mu\text{g ml}^{-1}$) and amphotericin B (3.125 to $0.049 \mu\text{g ml}^{-1}$) were added to YNB-G broth in microdilution plates. Yeast cells (approximately 2.5×10^4 cells ml^{-1} final concentration) were added and the plates incubated for 48 hr at 30°C . This assay and the PAFE determinations were performed at 30°C since previous studies in our laboratory indicated that this temperature was optimal when using YNB-G and the above two drugs for susceptibility studies with *C. albicans*. Endpoints were determined both visually and by a spectrophotometric method (490 nm, Toyo ETY III plate analyzer, Tokyo, Japan). The MIC was defined as the lowest concentration of each drug which inhibited multiplication of the yeast cells, as indicated by the absence of turbidity. Fractional inhibitory concentrations (FIC) [17, 18] and FIC indices were calculated from the MIC values. The FIC is a measure of synergy or antagonism between the two drugs and is equal to the MIC of each drug alone divided into its concentration in a particular combination that inhibits growth. The index is equal to the sum of the two FICs. Sums of <1 , 1 and >1 indicate synergy, additivity and antagonism, respectively [17, 18]. These determinations provided us with data on approximate concentrations of each agent to use in the PAFE assays.

PAFE assay. Approximately 1×10^7 yeast cells ml^{-1} from strain IFM 1001 were added (0.2 ml) to tubes of YNB-G broth (1.6 ml) containing different concentrations (two-fold dilutions, 0.1 ml) of flucytosine (0.195 to $0.012 \mu\text{g ml}^{-1}$), amphotericin B (1.56 to $0.098 \mu\text{g ml}^{-1}$) or combinations of both drugs. A drug-free control tube containing 1.6 ml of YNB-G, 0.2 ml of yeast cells and 0.2 ml of saline was also used in the assay. The tubes were incubated at 30°C for 2 hr with shaking. Following the exposure period the drugs were removed by dilution (10^2 -fold) in sterile saline. Aliquots (0.1 ml) were added to duplicate wells of a microdilution plate containing 0.15 ml of YNB-G, placed in a Bioscreen C spectrophotometric instrument (Labsys-

tems, Tokyo, Japan) and incubated for 72 hr at 30°C [19]. Growth of the cells was monitored by measuring turbidity (absorbance at 600 nm) at 20 min intervals. The data was recorded by a computer interfaced with the analyzer and growth curves were generated from each cell suspension. Mean values were determined for each of the cultures from the duplicate growth curve data.

The duration of the PAFE was calculated by a modification of a method used previously for PAE determinations with bacteria [3–6] and used in recent PAFE studies in our laboratory [1, 2]. The formula $\text{PAFE} = T - C$ was applied, where T was the time (hr) required for the turbidity of the drug-exposed cell suspension to reach the 0.5 absorbance level after removal of the drug by dilution, and C was the time required for the turbidity of the drug-free control culture to reach the same level. Previous studies indicated that approximately 1×10^7 viable cells ml^{-1} were present when the turbidity of the culture reached the 0.5 absorbance level. Thus $T - C$ denotes the time interval during which an antifungal agent exerts a PAFE on fungal growth.

Results

The initial susceptibility determinations indicated that combinations of flucytosine and amphotericin B inhibited growth of the 10 stains of *C. albicans* at concentrations considerably less than MICs of each agent separately (Table 1). Flucytosine MICs ranged from 0.098 to $6.25 \mu\text{g ml}^{-1}$ and amphotericin B MICs ranged from 0.78 to $1.56 \mu\text{g ml}^{-1}$ when assayed separately. In contrast, MICs as low as $0.012 \mu\text{g ml}^{-1}$ (flucytosine) and $0.39 \mu\text{g ml}^{-1}$ (amphotericin B) were obtained when the two drugs were used in combination. FIC index determinations, using the above MIC data, indicated that the two agents interacted synergistically (index values less than 1) with index values ranging from 0.26 to 0.75 with the 10 strains.

A comparative PAFE evaluation was then performed using concentrations of each agent as de-

Table 1. MICs and FIC indices of flucytosine (5-FC) and amphotericin B (AmpB) when assayed alone and in combination against 10 strains of *C. albicans*

Strain	MIC ($\mu\text{g ml}^{-1}$)				FIC index
	5-FC	AmpB	5-FC & AmpB		
IFM 1001	0.098	0.78	0.024	0.39	0.75
86-23717	0.195	0.78	0.024	0.39	0.62
11128-H99	0.195	0.78	0.098	0.195	0.75
9319-H76	0.098	0.78	0.049	0.195	0.75
9760-H85	0.098	0.78	0.012	0.39	0.62
9490-H81	0.098	1.56	0.049	0.195	0.63
2773-H26	0.098	1.56	0.049	0.39	0.75
3532-K31	6.25	1.56	0.049	0.39	0.26
11323-H101	0.098	0.78	0.024	0.39	0.74
1090-2-H17	6.25	0.78	0.024	0.39	0.50

MICs for the 5-FC: AmpB combination represent the concentrations of each agent which gave the lowest FIC index value.

terminated in the above MIC assays. *C. albicans* strain IFM 1001 was exposed for 2 hr at 30 °C to two-fold serial dilutions of flucytosine (0.195 to 0.012 $\mu\text{g ml}^{-1}$) and amphotericin B (1.56 to 0.098 $\mu\text{g ml}^{-1}$), both alone and in combination, at flucytosine:amphotericin B combination ratios of 1:64 to 1:0.5. The results, as shown in Table 2, indicated that flucytosine and amphotericin B tested alone produced PAFEs ranging from 3.8 to 13.5 hr and from 2.3 to 29.3 hr respectively. When the two drugs were combined synergism was noted with 13 combinations of concentrations with PAFEs ranging from 6.3 to 38.0 hr. One concentration of amphotericin B (1.56 $\mu\text{g ml}^{-1}$) greatly inhibited growth of the cells and the turbidity of the culture did not reach the 0.5 absorbance level during the evaluation period. It was therefore not possible to calculate PAFE values for this concentration. PAFEs ranging from 6.3 to 20.5 hr were evidenced with 7 combinations of the drugs (flucytosine:amphotericin B ratios from 1:2 to 1:32) when tested at concentrations below their individual MICs (Table 3). The PAFEs persisted from 0.3 to 14.7 hr longer than those induced by either agent alone. Typical growth curves of *C. albicans* strain IFM 1001 against flucytosine and amphotericin B or in combination of the both drugs documenting PAFEs, were shown in Fig. 1. PAFEs persisting for 14.7

Table 2. PAFE following exposure of *C. albicans* strain IFM 1001 to various concentrations of flucytosine (5-FC) and amphotericin B (AmpB)

Ratio 5-FC: AmpB	Conc. ($\mu\text{g ml}^{-1}$) 5-FC	AmpB	5-FC	PAFE (hr) AmpB	5-FC & AmpB
1:64	0.012	0.78	3.8	29.3	32.8
1:32	0.024	0.78	5.3	29.3	26.9
	0.012	0.39	3.8	7.1	7.4
1:16	0.049	0.78	7.5	29.3	33.6
	0.024	0.39	5.3	7.1	21.8
	0.012	0.195	3.8	2.6	6.5
1:8	0.098	0.78	10.9	29.3	17.9
	0.049	0.39	7.5	7.1	20.5
	0.024	0.195	5.3	2.6	6.3
	0.012	0.098	3.8	2.3	3.1
1:4	0.195	0.78	13.5	29.3	38.0
	0.098	0.39	10.9	7.1	10.6
	0.049	0.195	5.3	2.6	10.0
	0.024	0.098	5.3	2.3	4.5
1:2	0.195	0.39	13.5	7.1	17.8
	0.098	0.195	10.9	2.6	10.8
	0.049	0.098	7.5	2.3	8.4
1:1	0.195	0.195	13.5	2.6	15.3
	0.098	0.098	10.9	2.3	9.8
1:0.5	0.195	0.098	13.5	2.3	5.3

Table 3. PAFEs following exposure of *C. albicans* strain IFM 1001 to combinations of flucytosine (5-FC) and amphotericin B (AmpB) at concentrations below their individual MICs^a

Conc. ($\mu\text{g ml}^{-1}$) 5-FC	AmpB	Ratio 5-FC:AmpB	PAFE (hr) ^b 5-FC & AmpB
0.024	0.39	1:16	21.8 (14.7)
0.049	0.39	1:8	20.5 (13.0)
0.012	0.195	1:16	6.5 (2.7)
0.049	0.195	1:4	10.0 (2.5)
0.024	0.195	1:8	6.3 (1.0)
0.049	0.098	1:2	8.4 (0.9)
0.012	0.39	1:32	7.4 (0.3)

^a MICs of 5-FC and AmpB were 0.098 and 0.78 $\mu\text{g ml}^{-1}$ respectively when assayed against strain IFM 1001.

^b The figures in parentheses represent the increase in the duration of the PAFE (hr) produced by the synergistic interaction of 5-FC and AmpB in comparison to the maximum PAFE produced by either agent separately.

and 13.0 hr longer than those achieved with each agent separately were evidenced when 0.024 and 0.049 $\mu\text{g ml}^{-1}$ concentrations of flucytosine were combined with 0.39 $\mu\text{g ml}^{-1}$ of amphotericin B (Table 3). Therefore one might theoretically ex-

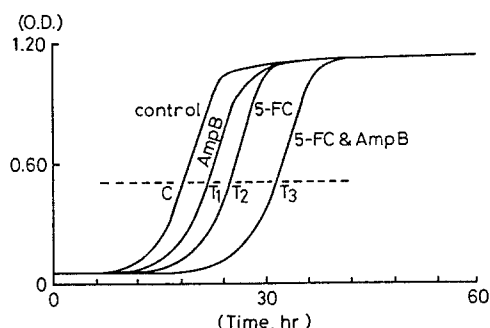


Fig 1. Typical growth curves of *Candida albicans* strain IFM 1001 exposed for 2 hr at 30°C to flucytosine (5-FC, 0.049 $\mu\text{g ml}^{-1}$) and amphotericin B (AmpB, 0.195 $\mu\text{g ml}^{-1}$) both alone and in combination. Postantifungal effect (PAFE) was determined by following formula: $\text{PAFE} = T_n(\text{hr}) - C(\text{hr})$, $n = 1, 2$ or 3.

pect that a PAFE of approximately 21 hr duration, lasting approximately 14 hr longer than one produced by either agent alone, could be produced by the synergistic interaction of flucytosine and amphotericin B at concentrations of approximately 0.04 and 0.4 $\mu\text{g ml}^{-1}$ respectively.

Discussion

Several new antifungal drugs have been developed in recent years, but clinicians are still plagued with efficacy and toxicity problems associated with the currently available agents. With this in mind, investigations concerned with combination therapy have increased considerably recently in hope of reducing some of the detrimental aspects related to antifungal drugs [7–13]. Flucytosine and amphotericin B have been used both alone and in synergistic combinations for the treatment of candidiasis and other infections, but clinicians have learned to expect adverse reactions with these agents in many patients [7, 10, 15]. Therefore attempts are being made to broaden the spectrum, improve the efficacy and minimize the side effects by using combinations of two or more drugs at reduced concentrations.

In conjunction with these in vivo approaches, many in vitro determinations have been aimed

at developing improved laboratory methods for determining susceptibility profiles of fungal organisms that will aid clinicians in decisions on optimal dosing regimens [20]. In vitro MIC assays are subject to many test variables including media composition, incubation temperature, exposure time, etc. [16, 20] and such procedures have suffered from a lack of a universally accepted standardized testing methodology. In most standard in vitro susceptibility assays measurements are made after continual exposure of the organism to the drug for a period of 24 hr or longer, but in the in vivo situation organisms are not exposed to constant levels of the drug. Thus it seems reasonable to assume that results from an in vitro assay in which the drug is not in constant contact with the organism might better approximate the in vivo response than results from the standard susceptibility assays. In addition studies are required in order to determine whether the drug level needs to be continually maintained above the MIC for optimal dosing efficacy.

We have performed several studies recently in which we have been able to demonstrate in vitro suppression of *C. albicans* growth following only limited exposure of the organism to certain antifungal agents. With this assay the organism is exposed to the drug for only a short period of time and then the suppressive effect is quantitated. PAFEs were produced by flucytosine and amphotericin B separately [2] and by combinations of flucytosine and fluconazole [unpublished observations].

In this present study we have demonstrated PAFE production by certain combinations of flucytosine and amphotericin B. It was shown that the synergistic interaction of the two drugs, at concentrations less than their individual MICs, produced PAFEs of longer duration than those produced by either agent alone. An optimal PAFE was produced when 0.024 $\mu\text{g ml}^{-1}$ of flucytosine was combined with 0.39 $\mu\text{g ml}^{-1}$ of amphotericin B. These concentrations, which produced a PAFE of 21.8 hr, were less than MIC values (0.098 $\mu\text{g ml}^{-1}$, flucytosine; 0.78 $\mu\text{g ml}^{-1}$, amphotericin B) and optimal peak serum levels

(35–100 $\mu\text{g ml}^{-1}$, flucytosine; 0.5–3.5 $\mu\text{g ml}^{-1}$, amphotericin B) [14, 15] of these two agents.

The results from this PAFE investigation are encouraging and this type of assay may be useful as an adjunct to other in vitro susceptibility assays. Further in vitro and in vivo studies will be required, however, in order to assess the utility of this procedure with regard to providing clinicians with susceptibility profile information necessary to formulate optimal dosing regimens.

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