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The Effect of Cilofungin (LY 121019) in Combination with Amphotericin B or Flucytosine Against *Candida* Species

Die Wirkung von Cilofungin (LY 121019) in Kombination mit Amphotericin B oder Flucytosin auf *Candida*-Arten

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Summary: Cilofungin was combined with amphotericin B or flucytosine to determine if synergistic inhibition or killing occurred against 50 strains of various *Candida* species. Synergistic inhibition of growth occurred only once with amphotericin B and cilofungin and only 2 times with flucytosine and cilofungin. Synergistic killing occurred in 5 strains with the amphotericin B-cilofungin combination and in 7 strains with the flucytosine-cilofungin combination. Antagonism occurred frequently with both the amphotericin B-cilofungin and the flucytosine-cilofungin combinations.

Zusammenfassung: Cilofungin wurde mit Amphotericin B oder Flucytosin kombiniert, um eine etwaige synergistische Wachstumshemmung oder Inaktivierung von 50 Stämmen unterschiedlicher *Candida*-Arten zu untersuchen. Eine synergistische Wachstumshemmung wurde nur einmal mit Amphotericin B und Cilofungin und zweimal mit Flucytosin und Cilofungin beobachtet. Eine synergistische Abtötung trat an 5 Stämmen mit der Amphotericin B-Cilofungin- und an 7 Stämmen mit der Flucytosin-Cilofungin-Kombination auf. Häufiger wurden bei beiden Kombinationen Antagonismen gefunden.

Introduction

LY 121019, now known as cilofungin, is a new antifungal agent which has been shown to be effective against several *Candida* species, especially *Candida albicans* and *C. tropicalis*, the major causes of disseminated candidosis (2–4, 7, 8). It has been suggested that the drug may be effective against *C. krusei* and *C. glabrata* although the minimum inhibitory concentration (MIC) is generally higher for these organisms (2, 7). Against

other *Candida* strains and other yeast-like fungi cilofungin does not appear to be as active (2).

Currently, the drug of choice for serious *Candida* infections is amphotericin B, despite its serious toxicity (6). In some instances, flucytosine can be added to synergistically treat *Candida* infections (5). Combination therapy with flucytosine and amphotericin B and Flucytosinehas the added advantage of allowing reduction of the daily amphotericin B and Flucytosinedose and thus decreasing the toxicity (1). We tested cilofungin with amphotericin B and Flucytosineand with flucytosine in vitro to determine if synergism might occur when this new anti-*Candida* drug was combined with the drugs used commonly in the treatment of candidosis.

Materials and Methods

Fungi

Fifty clinical isolates of *Candida* species maintained in our laboratory were examined. Organisms grown overnight on Sabouraud dextrose agar (Difco, Inc., Detroit, MI) were suspended in 0.9 % saline to a concentration of 2 x 10^7 CFU/ml, (0.55 optical density reading at 660 nm). The final inoculum was prepared in either Sabouraud dextrose broth (Difco, Inc.), or yeast nitrogen base broth (YNB) (Difco, Inc.), depending on which drug was to be studied.

Antifungal agents

Cilofungin (Lilly Research Laboratories, Indianapolis, IN) was solubilized in 50% ethanol to give a concentration of 1000 μg/ml. A further dilution was made in either Sabouraud dextrose broth or YNB to give a concentration of 80 μg/ml.

Amphotericin B and Flucytosine(Fungizone) (E.R. Squibb & Sons, Inc., Princeton, NJ) was solubilized in sterile distilled water to a concentration of 1000 μg/ml and further diluted in Sabouraud dextrose broth to a concentration of 20 μg/ml.

Flucytosine (Hoffman-LaRoche Inc., Nutley, NJ) was solubilized in sterile 0.9 % saline to give a concentration of 1000 μg/ml and further diluted in YNB broth to a concentration of 20 μg/ml.

Susceptibility determinations

Initial studies were performed to determine the MIC each of the 50 isolates for cilofungin, amphotericin B, and flucytosine. Sabrouraud dextrose broth was used for amphotericin B and FlucytosineMICs; YNB was used for flucytosine MICs. MICs for cilofungin were determined for both media. Initially, 100 μl of the broth was added to each well except the first of a 96 well U bottom microtiter plate (Flow Laboratories, Inc., McLean, VA). For cilofungin, 100 μl of the 80 μg/ml solution was added to the first well of each row, and for amphotericin B and Flucytosineand flucytosine, 100 μl of the 20 μg/ml solution was added to the first well of each row. Serial twofold dilutions were made across the plate using a Costar octapette (Costar, Inc., Cambridge, MA). The final well received no drug, serving as a positive growth control. The concentration of cilofungin ranged from 0.04 μg/ml to 40 μg/ml, while that of amphotericin B and flucytosine ranged from 0.01 μg/ml to 10 μg/ml. Each different fungal isolate was added to the 12 wells in a given row so that the final inoculum was 1 x 10^3 CFU/well. Plates were incubated for 24 h at 30°C. The MIC was determined as the lowest concentration showing no visible turbidity using a microtiter plate reader.

After determining the MIC for each drug for each of the 50 organisms, synergy studies were performed in a similar manner by determining the MIC for each drug in the presence of a constant amount of the other drug added at a concentration four-fold less than the previously determined MIC. The MIC was read in the same manner as described above.

Table 1: Comparison of MIC of cilofungin (CF) alone or with amphotericin B (AmB) or flucytosine (5 FC) against 50 strains of *Candida*

Organism	Cumulative percentage of strains inhibited at indicated concentration (μg/ml)										
	.04	.08	.16	.31	.62	1.25	2.50	5	10	20	40
C. albicans (20)											
CF			25	55	100						
CF + AmB			20	95	100						
CF						10	95	100			
CF + 5 FC						35	100				
C. tropicalis (8)											
CF			25	37	62		75	87			100
CF + AmB			12	37	62			87			100
CF						25	62	75	87		100
CF + 5 FC					25	62		87		100	
C. glabrata (8)											
CF								50	100		
CF + AmB								87	100		
CF							12		100		
CF + 5 FC								25	100		
C. parapsilosis (8)											
CF			12	50					75	100	
CF + AmB				12	50					100	
CF					12		50			100	
CF + 5 FC							50			100	
Candida species (6)*											
CF					17		50	67		83	100
CF + AmB					17		33	50	67	83	100
CF							17	33	67	83	100
CF + 5 FC							33		50	83	100

* *C. krusei* (2), *C. pseudotropicalis* (2), *C. guilliermondii* (1), *C. lusitaniae* (1)

After reading the MIC for each isolate, 100 μl was taken from those wells which showed to turbidity and plated on Sabouraud dextrose agar for assays involving amphotericin B and Flucytosineand on YNB agar for those involving flucytosine. The plates were incubated at 30°C for 24 hrs and the minimum fungicidal concentration (MFC) was read as the lowest concentration of drug in which only one or no fungal colonies persisted.

Synergy was defined as a four-fold or greater reduction in the MIC or the MFC for the combination as compared with the value for each drug alone. Antagonism was defined as a four-fold or greater increase in the MIC or MFC for the combination as compared with the value for each drug alone.

Results

Table 1 shows MICs for cilofungin alone and when combined with either amphotericin B and Flucytosineor flucytosine. In no case was the addition of amphotericin B and Flucytosineto cilofungin synergistic; in only one isolate *(C. tropicalis)* did the addition of flucytosine to cilofungin produce synergistic inhibition of growth.

Antagonism was also uncommon, occurring only once when flucytosine was added to cilofungin.

Table 2: Comparison of MIC of amphotericin B (AmB) and flucytosine (5FC) alone or with cilofungin (CF) against 50 strains of *Candida*

Organism	Cumulative percentage of strains inhibited at indicated concentration (μg/ml)										
	.01	.02	.04	.08	.16	.31	.62	1.25	2.50	5	10
C. albicans (20)											
AmB					50	100					
AmB + CF				5	50	70	100				
5FC		5	10	30	60						100
5FC + CF			10	25	50	60					100
C. tropicalis (8)											
AmB						50	87	100			
AmB + CF						50	75	87	100		
5FC			12	62		75					100
5FC + CF		12		50	62	75					100
C. glabrata (8)											
AmB					25	87	100				
AmB + CF				25	37	50	100				
5FC		50	62	75							100
5FC + CF	12		37	62	75	100					
C. parapsilosis (8)											
AmB					37	75	100				
AmB + CF						50	100				
5FC		25	50	62	87						100
5FC + CF		12	25	50	75	87					100
Candida species (6)*											
AmB						17	83		100		
AmB + CF							33	100			
5FC		17	50	67		83		100			
5FC + CF				33	67		100				

* *C. krusei* (2), *C. pseudotropicalis* (2), *C. guilliermondii* (1), *C. lusitaniae* (1)

Table 2 shows MICs for amphotericin and flucytosine when used singly and when combined with cilofungin. Addition of cilofungin to amphotericin B and Flucytosinewas synergistic once *(C. glabrata)* and antagonistic in 5 strains (2 *C. tropicalis* isolates, and one each of *C. glabrata, C. albicans,* and *C. parapsilosis*). When cilofungin was added to flucytosine, synergism occurred only once *(C. albicans)* and antagonism occurred 8 times (3 *C. albicans* strains, 2 *C. glabrata,* one each of *C. tropicalis, C. guilliermondii,* and *C. lusitaniae*).

In every instance except one, antagonism and synergism were only four-fold higher or lower than the MIC for the single drug. In that one instance, a strain of *C. glabrata* had a MIC for the combination of flucytosine-cilofungin 8-fold greater than that for flucytosine alone.

Table 3 shows MFC's for cilofungin alone and when combined with either amphotericin B and Flucytosineor flucytosine. No synergism with cilofungin-amphotericin B and Flucytosinewas noted, and in only 3 instances *(C. tropicalis)* was synergism seen with the cilofungin-flucytosine combination. Antagonism, on the other hand, was seen more frequently.

The addition of amphotericin B and Flucytosineto cilofungin increased the MFC ≧ 4-fold in 2 *C. tropicalis* strains and 3 *C. albicans* strains. The addition of flucytosine to cilofungin was frequently antagonistic, as

Table 3: Comparison of MFC of cilofungin (CF) alone or with amphotericin B (AmB) or flucytosine (5 FC) against 50 strains of *Candida*

Organism	Cumulative percentage of strains at indicated concentration (μg/ml)										
	.04	.08	.16	.31	.62	1.25	2.50	5	10	20	40
C. albicans (20)											
CF				10	20	25					100
CF + AmB				5	10					15	100
CF							20	80	90	95	100
CF + 5 FC							25	50	60	70	100
C. tropicalis (8)											
CF				12	25	37			50	62	100
CF + AmB					12				25	50	100
CF							25	50	62		100
CF + 5 FC						25	50		75		100
C. glabrata (8)											
CF									62	100	
CF + AmB									50	100	
CF									50	62	100
CF + 5 FC									37	100	
C. parapsilosis (8)											
CF											100
CF + AmB											100
CF											100
CF + 5 FC							25	50			100
Candida species (6)*											
CF								17	50		100
CF + AmB								17		50	100
CF								17		33	100
CF + 5 FC								17	50		100

* *C. krusei* (2), *C. pseudotropicalis* (2), *C. guilliermondii* (1), *C. lusitaniae* (1)

noted in 7 *C. albicans*, 1 *C. tropicalis*, and 1 *C. parapsilosis* isolates.

Table 4 shows MFCs for amphotericin B and flucytosine when used alone and when cilofungin was added. Addition of cilofungin to amphotericin B and Flucytosinewas synergistic for 5 strains — 2 *C. tropicalis* and 3 *C. glabrata*. This same combination was antagonistic for 7 strains — 3 *C. parapsilosis*, 2 *C. tropicalis*, and one each of *C. albicans* and *C. glabrata*. When cilofungin was added to flucytosine, synergism occurred 4 times, in one strain each of *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. krusei*. This combination was antagonistic in 16 of the 50 strains (32 %) — 6 *C. parapsilosis*, 4 *C. glabrata*, and one each of *C. albicans*, *C. tropicalis*, *C. krusei*, *C. guilliermondii*, *C. pseudotropicalis*, and *C. lusitaniae*.

In the 12 instances of synergism noted when MFCs were compared for single vs. two drugs, 7 were only 4-fold decreases in the MFC. Antagonism, which occurred in 37 of the 200 combinations tested, was at the 4-fold level in 18 of the 37 instances.

Discussion

Cilofungin is a new antifungal agent that has a mechanism of action different from other antifungal agents in that it inhibits synthesis of beta-1,3-glucan leading to cell wall damage (3). It is possible that ci-

Table 4: Comparison of MFC amphotericin B (AmB) and flucytosine (5FC) alone or with cilofungin (CF) against 50 strains of *Candida*

Organism	Cumulative percentage of strains at indicated concentration (μg/ml)										
	.01	.02	.04	.08	.16	.31	.62	1.25	2.50	5	10
C. albicans (20)											
AmB							75	100			
AmB + CF						5	60	95		100	
5FC				5	10	30	50			55	100
5FC + CF				10	20	30	40	45			100
C. tropicalis (8)											
AmB							12	50	62	87	100
AmB + CF								50	62	75	100
5FC						12	25	37			100
5FC + CF				12	25	37					100
C. glabrata (8)											
AmB							25	50	87	100	
AmB + CF					37				87		100
5FC			25	50	62	75					100
5FC + CF			12		37	50	62	75			100
C. parapsilosis (8)											
AmB							12	37	75	100	
AmB + CF							12		25	50	100
5FC			12	37	50	87					100
5FC + CF					12	37	50	75	87		100
Candida species (6)*											
AmB							17	67		83	100
AmB + CF								33	67		100
5FC			17	33		50			83		100
5FC + CF						17	50	67	83		100

* *C. krusei* (2), *C. pseudotropicalis* (2), *C. guilliermondii* (1), *C. lusitaniae* (1)

lofungin might act synergistically with other antifungal agents, especially flucytosine, which acts, not on the cell wall, but at the level of DNA replication (5). Although it is possible to get synergistic action with two agents active at the level of the cell wall and cell membrane, such as cilofungin and amphotericin B, it seems less likely that this combination will result in synergism.

We found that, indeed, cilofungin added to amphotericin B and Flucytosineshowed only minimal synergistic inhibition or killing of any of the *Candida* species tested. When the corresponding studies were performed, which looked at the effect of subinhibitory concentrations of amphotericin B added to cilofungin, synergy did not occur in any strain tested.

We also found that synergistic inhibition or killing of *Candida* strains was rarely noted with the combination of cilofungin and flucytosine. In fact, antagonism was quite common with this combination. In no instance did a resistant strain become susceptible to cilofungin or flucytosine when the second drug was added.

The usefulness of cilofungin in *Candida* infections is not known at this time. It would appear to be active against *C. albicans* and *C. tropicalis* by in vitro tests (2, 3, 7). We could not verify by our in vitro assays a potential role for combination therapy with amphotericin B-cilofungin or flu-

cytosine-cilofungin. However, studies comparing cilofungin alone with combination therapy in experimental *Candida* infections could possibly show an in vivo synergistic effect not detected by in vitro assays.

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