

Amphotericin B-metronidazole combination against *Candida* spp

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Summary

Interaction between amphotericin B and metronidazole was studied against *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, and *Candida lusitanae* strains. Minimum inhibitory concentrations (MICs) of the drugs alone and in combination were determined by means of the checkerboard method on YNB supplemented agar. Minimum fungicidal concentrations (MFCs) were determined on Sabouraud dextrose agar. Based on the MIC and MFC values, fractionary indices were determined respectively for inhibitory and lethal activities of the amphotericin B-metronidazole combination. These indices showed occurrence of additive and synergistic interactions between the drugs, but the synergism was predominant against the studied strains.

Key words

Amphotericin B, Metronidazole, Drug synergism, *Candida* spp, Combined therapy

Combinación de anfotericina B y metronidazol contra *Candida* spp

Resumen

Fueron estudiadas las interacciones resultantes de la combinación entre anfotericina B y metronidazol contra cepas de *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, and *Candida lusitanae*. Determinaciones de las concentraciones inhibitoras mínimas de las drogas aisladas y asociadas fueron realizadas por medio de la titulación bidimensional en agar YNB enriquecido y las concentraciones fungicidas mínimas lo fueron en agar Sabouraud dextrosa. Con base en los resultados obtenidos, fueron calculados índices fraccionarios para la actividad inhibitora y letal de la asociación anfotericina B-metronidazol, cuyos valores mostraron la ocurrencia de interacciones sinérgicas y aditivas, con predominio de las primeras frente a las cepas en estudio.

Anfotericina B, Metronidazol, Sinergismo, *Candida* spp, Terapia combinada

Amphotericin B has been the drug of choice in the treatment of systemic fungal infections for nearly 40 years [1,2]. However, due to its high toxicity, new formulations [2-4] and drug combinations [5-10] have been investigated. An important aspect of combined therapy, as determined by *in vitro* studies, is the use of lower concentrations of the drugs employed in the association, yielding nonetheless similar antifungal effect and lower toxicity, as compared with the drugs alone [11,12].

From previous research [13] synergistic or additive interactions between amphotericin B (AmB) and metronidazole (Me) in combination have been verified against 17 strains of *Candida albicans*. Confirmation of these results with a higher number of *C. albicans* strains and the study of the combined drugs against others *Candida* species were the aim of the present work.

MATERIAL AND METHODS

Microorganisms. *Candida* spp., including 27 strains of *C. albicans*, five of *Candida tropicalis*, five of *Candida parapsilosis*, two of *Candida krusei* and one of *Candida lusitanae* were studied. The strains were isolated from patients, without previous treatment with the studied drugs of Escola Paulista de Medicina, Hospital das Clínicas da UNICAMP and Hospital Universitário de Mato Grosso de Sul. All isolates were maintained on Sabouraud dextrose agar [15] at 25°C. Successive subcultures were performed each 15 days. For susceptibility tests, subcultures were performed each 24 h and incubated at 30°C [13,15].

Drugs and dilutions. Commercially available amphotericin B (Fungizone®, Bristol-Myers Squibb, São Paulo-SP, Brasil) and metronidazole (Flagyl®, Rhodia Farma, São Paulo-SP, Brasil) in injectable form were employed. From drug solution, serial two fold dilutions [15] were made in yeast nitrogen base (Difco Laboratories, Detroit, Michigan, USA), prepared in phosphate buffer 0.01 M and supplemented with 0.15% L-asparagine and 1% glucose, pH 7.0 [13,15]. The final concentrations of AmB and Me ranged respectively from

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4.0 mg/ml to 0.03 mg/ml and from 16 mg/ml to 0.03 mg/ml on minimum inhibitory concentration testing.

Antifungal activity of combined and isolated drugs

Growth media. Yeast nitrogen base agar (1%) prepared in phosphate buffer and supplemented [13,15] (YNBP agar) was employed in the minimum inhibitory concentration (MIC) testing. Sabouraud dextrose agar (SDA) was used for the minimum fungicidal concentration (MFC) testing.

Inoculum. From the 24 h growth on SDA, suspension of each strain was prepared in phosphate buffer with 0.05% Tween 80. The turbidity of these suspensions was adjusted to contain about 10⁶ cels/ml [3].

Minimum inhibitory concentration testing. Antifungal susceptibility determination was done by classical checkerboard titration [15,16]. This testing was performed four times in duplicate.

In order to study the activity of the drug by itself, 1ml of each dilution was transferred to a tube containing 19ml of YNBP agar kept at 45°C. The study of combined drugs was performed by mixing 1ml of each dilution of one drug with 1ml of each one dilution of the other drug in 18ml of YNBP agar at 45°C. Positive and negative growth controls for each test were performed by adding 1ml of YNBP broth and 1ml of 0,5% formaldehyde respectively to 19ml of the YNBP agar. Each medium was plated and allowed to gel. Yeast suspensions were inoculated with a Steer's replicator and the plates were kept at 30°C for 24 h or until visible growth of the positive control. MIC was considered as the lowest drug concentration that prevented visible yeast growth [17].

Minimum fungicide concentration testing. Inocula which did not yield growth in MIC testing as well as their respective positive control were subcultured on SDA and incubated at 30°C for 48 h. MFC was considered the lowest drug concentration that prevented yeast growth in subculture [15].

Interpretation of results. MICs and MFCs of each drug both alone and in combination were determined through the geometrical mean of the values found in 8 repeated tests. From such results, fractionary inhibitory concentration (FIC) and fractionary fungicidal concentration (FFC) of each associated drug were determined. The FIC of one drug is calculated by dividing its MIC value when combined by the MIC value when tested alone. The sum of the FICs of the combined drugs provides the FIC index.

The FIC index value is related to the kind of interaction between the associated drugs. Thus, for a FIC index < 1, the interaction is considered synergistic; = 1, additive; and >1, antagonis if either associated drug has FIC >1, otherwise, it is indifferent [10,12,16]. Similar calculations can be performed from FIC values to obtain the FFC index.

For calculation purposes, MICs and FICs of metronidazole > 16 mg/ml, were established as 32mg/ml; values of AmB > 4 mg/ml and 0,03 mg/ml, were fixed as 8 mg/ml and 0,03 mg/ml, respectively.

Statistical Analysis

Student's t-test was employed to determine the significance of the differences between the geometrical means of MICs and MFCs values for drugs alone and in combination against those *Candida* species whose number of strains were statistically sufficient to be analyzed.

RESULTS

Metronidazole did not show antifungal activity in concentrations up to 16 mg/ml. Combined with AmB, this azole showed fungistatic and fungicidal actions in low concentrations. The potentiation of AmB activity was a phenomenon that varied with the strain studied. Only synergistic and additive interactions were observed, but the synergism occurred against most of the yeasts (Table 1). The statistical study showed that the MICs of associated AmB against the strains of *C. albicans* (p<0.05) and *C. parapsilosis* (p<0.05) were significantly lower than those of AmB alone. The mean value of the MFCs of the polyene against *C. albicans* strains was also lower for combination (p<0.05) than for the drug alone. Nevertheless, no significant differences between MICs and MFCs of AmB alone and in combination (p>0,05) were observed against *C. tropicalis* strains.

As statistical comparison of Me in combination against *C. albicans* and *C. tropicalis* strains, showed that its MFC values were higher than the MIC values. Nevertheless, for *C. parapsilosis* strains, MFCs were lower than MICs (Figure 1). For AmB in combination, the MFCs were comparatively higher than those of MICs against the same three *Candida* species.

Table 1. Interactions between amphotericin B and metronidazole against *Candida* spp.

Yeast	N° of isolates for which the interaction was:			
	Synergic to:		Additive to:	
	inhibitory action	lethal action	inhibitory action	lethal action
<i>C. albicans</i>	17/27	19/27	10/27	8/27
<i>C. tropicalis</i>	1/5	1/5	4/5	4/5
<i>C. parapsilosis</i>	5/5	1/5	-	4/5
<i>C. krusei</i>	2/2	2/2	-	-
<i>C. lusitaniae</i>	1/1	1/1	-	-

Inhibitory action was based on FIC values and lethal action was based on FFC values.

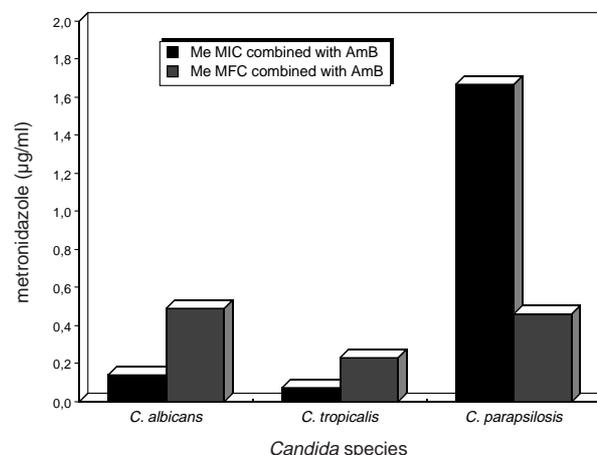


Figure 1. Mean values of minimum inhibition and minimum fungicidal concentrations of metronidazole (Me) combined with amphotericin B (AmB) against *Candida* spp.

DISCUSSION

Combined therapy has been a primary concern of physicians in attempting to decrease the incidence of emergent resistant strains, improve dosage regimens and reduce drug toxicity. Although definitions of interactions

resulting from drug combinations have been offered consistently in literature, the limits established by different authors can vary. The reasons for these discrepancies, especially in studies employing double dilution, are related to the fact that a single experimental mistake in one of the dilutions, can decrease the MIC or MFC drug value to half or increase to double [18]. Under these conditions, it is important that multiple repetitions of the *in vitro* experiment are performed before final conclusions about the kind of the combined drugs can be made.

In agreement with our previous study [13], we observed additive or synergistic interactions between AmB and Me against *C. albicans* strains. Moreover, it was verified that the AmB-Me combination can possess a wider spectrum of action, since it shows the same favorable interactions against the other species of *Candida* studied. Such findings can be related to the mechanisms of action of the drugs; thus, the alteration by AmB of the fungal cellular membrane [5] may facilitate the penetration and hence the activity of Me against specific molecules. Mainly for Me, it is possible that other events or more complex mechanisms may be involved in the observed

interactions. Reports about the mechanism of action of this azole have only included studies with bacteria and protozoa. According to some authors, Me enters the bacterial cell more rapidly under anaerobic conditions. After reduction by more polar metabolites, probably carrier proteins of low redox potential, such as ferredoxin, it binds to and degrades cellular DNA [19]. As the reduction of this azole is essential for its activity [20], it is possible that similar enzymes present in *Candida* species [6], are able to promote such reaction. This could be better clarified through more comprehensive studies that can be supported on the basis of the interactions already observed for AmB-Me combination, which have also positively pointed to the performance of future experiments employing animal models.

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