

## IN VITRO SUSCEPTIBILITY OF PARACOCIDIROIDES BRASILIENSIS YEAST FORM TO ANTIFUNGAL AGENTS

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### SUMMARY

A study was conducted to determine the susceptibility of *P. brasiliensis* yeast form to amphotericin B (A), ketoconazole (K), 5-fluorocytosine (5-FC) and rifampin (R). The three isolates tested produced minimal inhibitory concentrations (MICs) (mcg/ml) in the following range: A: 0.09-0.18; K: 0.001-0.007; 5-FC: 62.5-250 and R: 40-80. The minimal fungicidal concentrations (MFC) were several times higher than the corresponding MICs. Precise MFC for 5-FC were not obtained ( $\geq 500$  mcg/ml). Combination of K plus A proved synergic, with the fractional inhibitory concentration (FIC) indices revealing synergy when the drugs were combined at the 1 to 1 and 1 to 5 MIC ratios. R (40 mcg/ml) appeared to antagonize K. These results indicate promise for the combined use of K plus A as a therapeutical regimen.

### INTRODUCTION

The number of drugs that can be used for the treatment of paracoccidioidomycosis is quite limited; the list includes sulfonamides, alone or in combination with trimethoprim, amphotericin B and the imidazole derivatives, miconazole and ketoconazole<sup>3,14</sup>. This paucity of antifungals parallels the number of studies that have been conducted to determine the *in vitro* susceptibility of *Paracoccidioides brasiliensis* to antimycotics<sup>8,9,12,17</sup>. In part, this is due to the difficulties experienced when growing the fungus in a culture medium which would not, by itself, interfere with the drugs under study. Media appropriate for this type of testing have been utilized with *P. brasiliensis* only recently<sup>12,13</sup>.

Work with the etiologic agents of other systemic mycoses has revealed that some antifungal agents when combined, produce a synergistic response which may be reflected in a more effective therapy<sup>1,5,10,11</sup>. We present the results of studies aimed at determining the effects of various drugs, alone and in combi-

nation, on the yeast (tissue) form of *P. brasiliensis*.

### MATERIALS AND METHODS

**Organisms** — Three isolates of *P. brasiliensis* (WCH, LS, FG) were used; they differed on date (1979, 1980, 1981) and source of isolation (lymph node, skin lesions and sputum). For this study the isolates were maintained at 36°C in their yeast form by weekly transfers on agar slants of the chemically defined medium (DM) of DOUGHERTY et al.<sup>4</sup>. This medium has been formulated in order to avoid interference with antimycotic drugs<sup>4</sup>.

**Drugs** — Four drugs were used and supplied in the powdered form, as follows: amphotericin B (A) obtained from E.R. Squibb and Sons, Cali, Colombia; 5-fluorocytosine (5-FC) from Hoffman La Roche, Inc. Nutley, N. J.; ketoconazole (K), from R & D. Inc, New Brunswick, N.J. and rifampin (R) from Lepetit, S.A., São Paulo, Brazil.

**Susceptibility Testing** — Stock solutions of the drugs were prepared by dissolving the powders in the following fashion: A (36 mg/ml) in sterile dimethylsulfoxide; 5-FC (10.0 mg/ml) in distilled water; K (10 mg/ml) in HCL 0.2 N and then in water; R (10.0 mg/ml) was initially dissolved in ethanol and further in PBS, pH 7.2<sup>15</sup>. Susceptibility testing was performed in the same DM (4) used to grow the isolates, except for the agar. The medium was filter-sterilized and dispensed in 5.0 ml quantities into screw-capped tubed. Serial, working two-fold dilutions of the drugs were made in DM so as to have the following range in (mcg/ml): A from 0.90 to 0.009; K from 0.035 to 0.0001; 5-FC from 500 to 6.2 and R from 180 to 40<sup>15</sup>.

The inoculum consisted of the 3-day growth of the fungus in liquid DM, with cultures being incubated at 36°C in a rotary shaker (120 rpm). Turbidity was adjusted to 49% transmittance at 540 nm (Coleman Junior Spectrophotometer). Each tube with drug received 0.02 ml of this inoculum. The experiments were done in duplicate. Controls included uninoculated sterility control and drug — and solvent — free growth controls. Preliminary testing had demonstrated that at the concentrations attained by the solvents in the various drug dilutions, 0.2N HCL, DMSO and ethanol were not inhibitory.

For synergy studies, serial two-fold dilutions of one of the drugs (main drug) were made in DM broth as previously described; 3 of such dilution series were prepared with one receiving the MIC of the second drug, (combining drug), the second 1/10 and the third, 5 times that MIC. The fungal inoculum, at the concentration described above was then added.

In all experiments, the inoculated tubes were incubated at 36°C with continuous agitation (120 rpm) for a period of 5 days.

The minimal inhibitory concentration (MIC) was determined by the spectrophotometric method of Galgiani and Stevens and expressed as the 50% inhibitory concentration (IC/50)<sup>7</sup>. The minimal fungicidal concentration (MFC) was obtained after determining the MIC, by separately transferring to duplicate plates with DM agar, 0.05 ml quantities of the drug free control, the MIC tubes, and the next two tubes above such MIC. These plates were incubated at 22-25°C for 3 weeks. The plate with the minimal amount of drug which showed less than 3 colonies was accepted as the MFC<sup>16</sup>.

The effect of the combination of A plus K on growth of *P. brasiliensis* was also calculated, using the fractional inhibitory concentration (FIC) index, according to Elion, SINGER & HITCHINGS<sup>6</sup>. By this formulation, an index of 1.0 represents an additive effect,  $\leq 2.0$  shows antagonism while an index  $\geq 1.0$  equals synergy.

## RESULTS

*In vitro* testing of *P. brasiliensis* indicated a comparable pattern of susceptibility for the 3 isolates (Table I). In mcg/ml, the MICs ranged from 0.09 to 0.18 for A; 0.001 to 0.007 for K; 62.5 to 250.0 for 5-FC and 40 to 80 for R. The MFC's were higher than the corresponding MIC's; 5-FC required large concentrations (over 500 mcg/ml) to kill the fungus; consequently this drug was not tested further. Determination of MFCs for R were not attempted.

T A B L E I  
*In vitro* susceptibility of *P. brasiliensis* yeast form to various antimycotic drugs

Isolate	Susceptibility (*) in mcg/ml to							
	Amphotericin B		Ketoconazole		5-Fluorocytosine		Rifampicin	
	MIC (**)	MFC (***)	MIC	MFC	MIC	MFC	MIC	MFC
WCH	0.09	0.36	0.007	0.01	62.5	> 500	40.0	ND
LS	0.18	0.36	0.001	0.07	250.0	> 500	60.0	ND
FG	0.09	0.36	0.003	0.01	125.0	> 500	80.0	ND

(\*) Expressed as IC/50

(\*\*) Minimal inhibitory concentration

(\*\*\*) Minimal fungicidal concentration

ND Not determined

As shown in Fig. 1 the combination of A plus K produced decreased A MIC values when the combining drug (K) was employed in concentrations equivalent to 1 (0.001 — 0.007 mcg/

ml) and 5 (0.005 — 0.035 mcg/ml) times the K MIC of the respective isolate. However, this was not the case when only 1/10 (0.0001 — 0.0007 mcg/ml) of the K MIC was used.

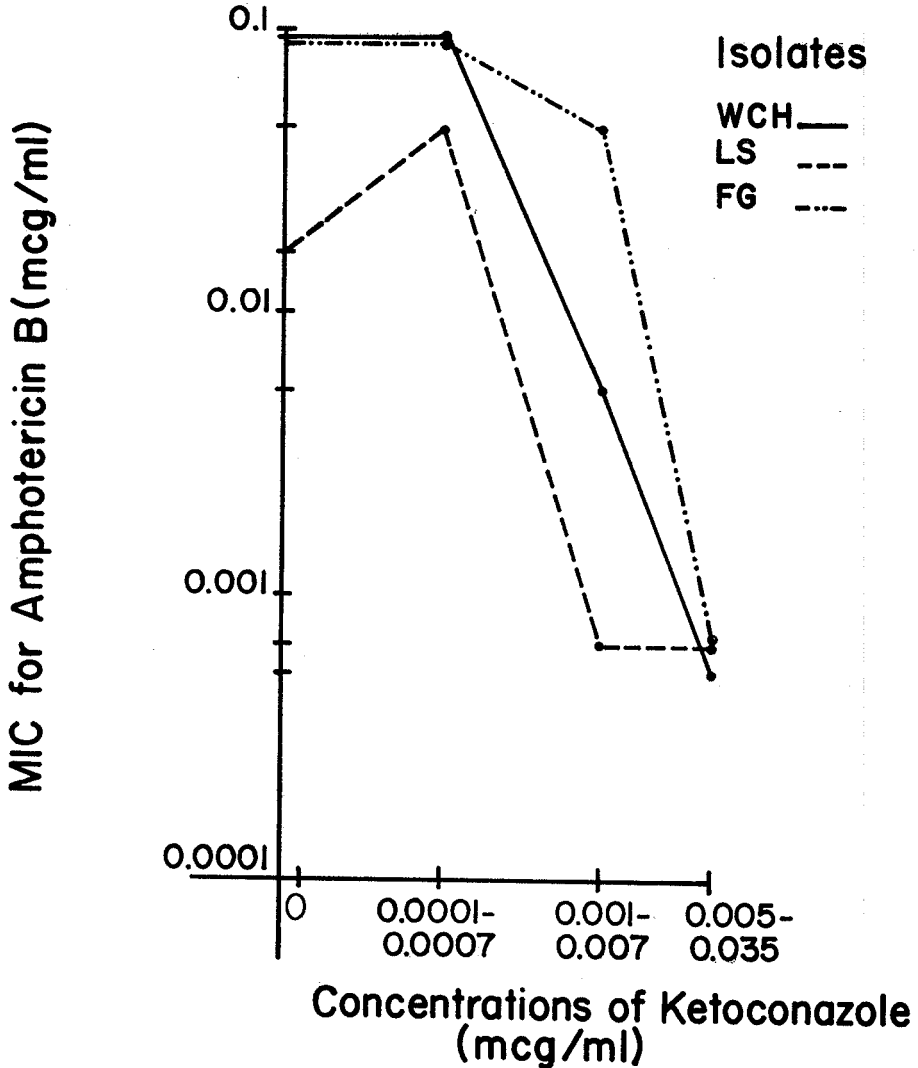


Fig. 1 — Effect of Ketoconazole on Amphotericin B MICs of 3 isolates of *P. brasiliensis*

The effect of A on the K MIC's revealed a somewhat different picture (Fig. 2). A employed in concentrations equivalent to 1/10 the MIC (0.009-0.018 mcg/ml) proved either indifferent (isolates WCH and LS) or resulted in an increased K MIC (isolate FG). When the MIC of A (0.09-0.18 mcg/ml) was used, the respective K MIC's were either reduced (isolates FG and WCH) or indifferent (isolate LS). Higher A concentrations, equivalent to five times the A

MIC's (0.45 — 0.9 mcg/ml) reduced the original K MIC's for the three isolates.

The effects of R on A and K MIC's are shown in Table II. Two isolates (WCH, LS) exhibited reduced A MIC's when in the presence of 40 and 20 mcg/ml of R, while the remaining isolate showed higher values with 40 but stationary with 20 mcg/ml R. Five mcg/ml of the latter drug produced either increased or station-

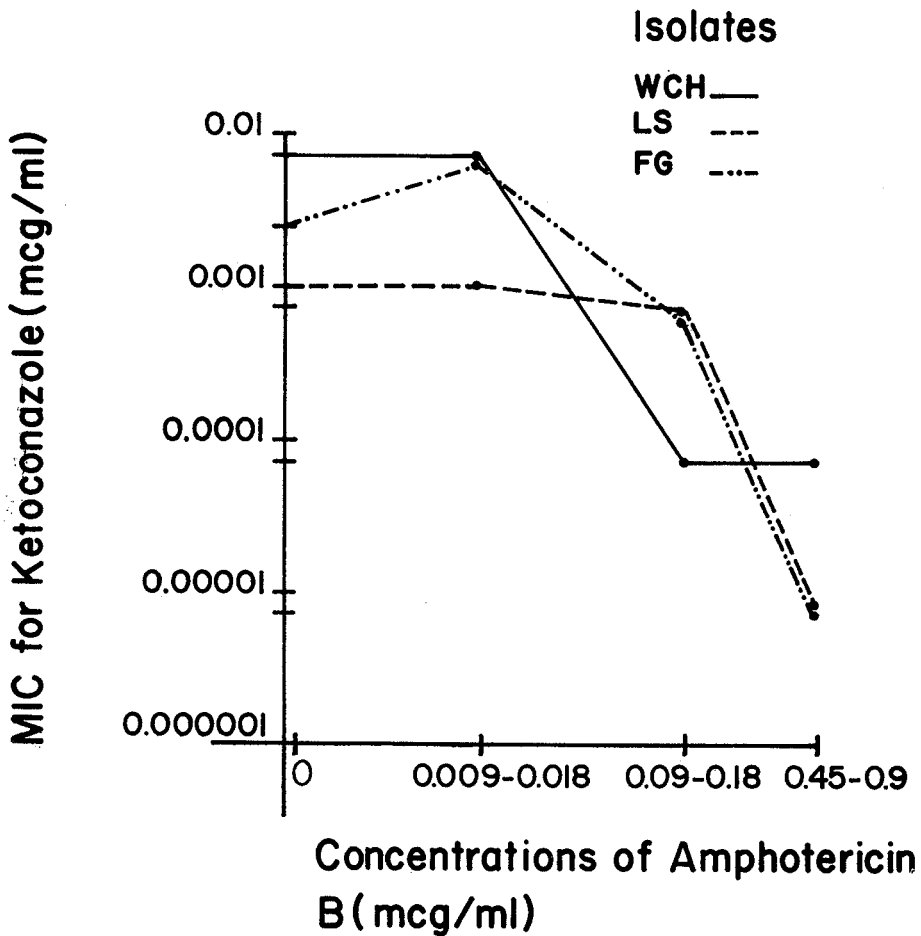


Fig. 2 — Effect of Amphotericin B on Ketoconazole MICs of 3 isolates of *P. brasiliensis*

T A B L E II  
Effect of rifampin on the amphotericin B and ketoconazole MIC's of three isolates of *P. brasiliensis*

Isolate	MIC cg/ml							
	Amphotericin B			Ketoconazole				
	Alone	Plus Rifampin (mcg/ml)			Alone	Plus Rifampin (mcg/ml)		
		40	20	5		40	20	5
WCH	0.09	0.045	0.045	0.09	0.007	0.07	0.01	0.007
LS	0.18	0.045	0.045	0.36	0.001	0.01	0.001	0.001
FG	0.09	0.18	0.09	0.36	0.003	0.07	0.03	0.003

nary A MIC's. The combination K plus R produced increased K values with the highest R concentration (40 mcg/ml) in all three isolates. Twenty mcg/ml R had a similar antagonistic effect on 2 of the 3 isolates. Lower R concentrations did not modify the original MIC's.

The interaction of K on the A MFC and vice versa, were also investigated (Table III). Concentrations equivalent to 5 times the MIC of K reduced the A-MFCs of all 3 isolates; similar results were obtained with the inverse combination. On the other hand, no changes occurred in the individuals MFCs of either K or

T A B L E III

Minimal fungicidal concentrations of amphotericin B and ketoconazole in combination for 3 isolates of *P. brasiliensis* yeast form

Isolate	MFC (mcg/ml)							
	Amphotericin B				Ketoconazole			
	Alone	Plus Ketoconazole			Alone	Plus Amphotericin B		
		0.005-0.035(*)	0.001-0.007(**)	0.0001-0.0007(***)		0.45-0.90(*)	0.09-0.180(**)	0.009-0.018(***)
WCH	0.36	0.18	0.36	0.36	0.01	0.001	0.01	0.01
LS	0.36	0.045	0.36	0.36	0.07	0.001	0.07	0.07
FG	0.36	0.09	0.36	0.36	0.01	0.001	0.01	0.01

(\*) Range of concentrations encompassing 5 times the MIC of each isolate.

(\*\*) Range of concentrations encompassing the MIC of each isolate.

(\*\*\*) Range of concentrations encompassing 1/10 the MIC of each isolate.

T A B L E IV

Fractional inhibitory concentration (FIC) indices for ketoconazole (K) and Amphotericin B (A) for three isolates of *P. brasiliensis* yeast form

Isolate	FIC index at given MIC combinations K : A		
	1:5	1:1	1:1/10
WCH	0.06	0.06	2.0
LS	0.09	0.72	1.2
FG	0.28	0.73	3.3

A when the combining drug was used at concentrations equivalent to 1 or 1/10 its MIC.

In order to better determine the effects of the K-A interaction, the FIC indices for these drugs were calculated (Table IV). When concentrations representing one K-MIC were combined with the respective A-MIC (1:1 ratio), the 3 isolates showed synergic effects (values of  $\cong 1.0$ ). Synergy was also obtained when one F-MIC was combined with 5 times the A-MIC. On the other hand, 1/10 of the A-MIC proved antagonic (values over 12).

## DISCUSSION

Therapy of paracoccidioidomycosis with either amphotericin B or ketoconazole has proven effective<sup>3,14</sup>. Our *in vitro* data corroborate the *in vivo* findings as *P. brasiliensis*, the etiologic agent of this disorder, is quite susceptible to both drugs. The fungus shares with the other dimorphic pathogens a similar range of susceptibility to amphotericin B (0.05-0.18-mcg/ml)<sup>16</sup>. A previous report on the susceptibility of *P. brasiliensis* to the latter drug indicated that larger concentrations (0.243 mcg/ml) were needed for inhibition<sup>8</sup>. However, the Authors used

complex media (Sabouraud dextrose agar) and this fact may explain the differences with the present results. Susceptibility of *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Coccidioides immitis* to ketoconazole varies from 0.1 — 0.8 mcg/ml; on the other hand, *P. brasiliensis* requires lower concentrations (0.001 to 0.007 mcg/ml), as previously reported<sup>2</sup>.

*P. brasiliensis* minimal fungicidal concentrations for amphotericin and ketoconazole, were 0.36 and 0.01 — 0.07 mcg/ml, respectively; they are below the range of achievable serum levels for both drugs (1.0 — 4.0 mcg/ml)<sup>16</sup> and as such they could, theoretically, exert an *in vivo* fungicidal effect. However, due to the high protein binding affinity of both drugs, a direct correlation between *in vivo* and *in vitro* findings cannot be attempted<sup>16</sup>.

The results of our studies indicate that the combination of ketoconazole and amphotericin B may result in a more effective therapeutic regimen, one that should be explored by appropriate experimental animal studies. Concentrations within the range of the amphotericin B-MICs (0.09-0.18 mcg/ml) resulted in reduced ketoconazole-MICs, to the extent of synergism (FIC indices of  $\cong 1.0$ ). The contrary was also true but it would appear easier on the patient to maintain the oral drug at the usual dose and decrease amphotericin concentration to reduce toxicity, such as done for cryptococcosis<sup>1</sup>. DROUHET and DUPONT have also demonstrated synergism for the K-A combination in certain mycoses<sup>5</sup>.

The combination of rifampin (40 mcg/ml) and ketoconazole resulted in increased K MIC's.

Lower R concentrations were either indifferent or antagonistic, depending on the isolate. This observation is of practical importance as the association of paracoccidioidomycosis with tuberculosis is relatively frequent (approximately 13%) in the mycosis endemic areas<sup>3</sup>. Consequently, care should be taken when the corresponding medications are to be prescribed simultaneously. With other fungi (*Candida spp.* and *Torulopsis glabrata*) ketoconazole and rifampin have also been shown antagonistic<sup>10</sup>.

The combination of amphotericin B plus rifampin produced lower, higher or stationary A MICs, depending on the isolate and the concentration of the latter drug. This suggests that the patient with paracoccidioidomycosis may or may not benefit from a combined therapy. However, as stated by ODDS<sup>11</sup>, drug interactions both *in vivo* and *in vitro* are complex events. Even an additive interaction *in vitro* may be clinically significant if it entails lowering the dosage to reduce toxicity. Consequently, in problem patients the corresponding fungal isolate should be tested to determine the effect of the above combination of drugs.

We were interested in determining the effect 5-fluorocytosine on *P. brasiliensis*, as previous observations have indicated that 250 mcg/ml of this drug when added to culture media markedly inhibited growth of the fungus<sup>15</sup>. Although the present results show that 5-fluorocytosine produces MICs between 62.5 and 250 mcg/ml, these concentrations are either too close or above the maximum permissible levels of the drug<sup>16</sup>. Consequently, its potentials for therapeutic usage in paracoccidioidomycosis are minimal.

## RESUMEN

### Susceptibilidad *in vitro* de la forma levaduriforme de *Paracoccidioides brasiliensis* a los agentes antifúngicos

Se realizó un estudio con el objeto de determinar la susceptibilidad de la fase levaduriforme del *P. brasiliensis* a la Anfotericina B (A), el Ketoconazol (K), la 5-fluorocitosina (5-FC) y la rifampicina. Las 3 cepas estudiadas tuvieron las siguientes concentraciones inhibitorias mínimas (MIC) (mcg/ml) A: 0.09-0.18; K: 0.001-0.007; 5-FC: 62.5-250 y R: 40-80. Las concentraciones fungicidas mínimas (MFC) resultaron

más altas que las MICs correspondientes. En el caso de la 5-FC no se obtuvo una cifra MFC precisa ( $\geq 500$  mcg/ml). La combinación de K más A mostró ser sinérgica al combinarse las drogas en relación 1:1 y 1:5 de los MICs respectivos. R (40 mcg/ml) se mostró antagonista del K. Los resultados indican que la combinación A + K pudiera constituir un adecuado régimen terapéutico en ciertos pacientes.

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