Antifungal Drug Combinations for *Cryptococcus neoformans* and *Prototheca* spp.

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Abstract

Seventy-one isolates of *Cryptococcus neoformans* and 5 isolates of *Prototheca* spp. were tested for *in vitro* susceptibility against amphotericin B alone and against the combination of amphotericin B with each clinically relevant concentration of flucytosine (5-FC) and rifampin by broth dilution methods. The combinations of amphotericin B and rifampin produced greater effect on reduction of the minimal inhibition concentration (MIC) of amphotericin B than did either drug used individually. Flucytosine combined with amphotericin B produced little or no reduction of the MIC compared with amphotericin B alone.

Key word : *Cryptococcus neoformans, Prototheca* spp., Amphotericin B, Drug Combination

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Amphotericin B (Amp B) remains the drug of choice for treatment of most systemic fungal infections but its administration carries the risk of serious toxic reactions[1-3]. The use of amphotericin B in combination with other antifungal or antimicrobial agents may solve these problems by reducing toxicity through reduction of amphotericin B dosage. Previously, *in vitro* and *in vivo* studies [4-7] have indicated a possible role of combination drugs therapy between flucytosine (5-FC) or rifampin (RMP) with Amp B against *Aspergillus* spp., the fungus had prolonged survival in monotherapy with Amp B alone in a pulmonary aspergillosis patient and in a mouse model of disseminated *A. fumigatus* infection. Combination therapy with Amp B and 5-FC has long been recommended for the treatment of cryptococcal meningitis, but there is no evidence of *in vitro* combined drugs suscep-

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tibility performed with Thai isolates of *C. neoformans*. We, therefore, report here the *in vitro* activities of Amp B, 5-FC and RMP alone and their combinations against a pathogenic yeast, *Cryptococcus neoformans* and pathogenic achlorophyllous algae, *Prototheca* spp.

MATERIAL AND METHOD

Organisms : A total of 76 clinical isolates of pathogenic yeasts and achlorophyllous algae cultured from CSF, blood, urine, and tissue were tested. These included 71 isolates of *Cryptococcus neoformans* and 5 isolates of *Prototheca* spp. All were clinical isolates collected by several sources : Infectious Disease and Host Defence Unit, Research Center, Ramathibodi Hospital, Mycology Laboratory at Siriraj Hospital, Mahidol University; Faculty of Medical Technology, KhonKaen University and Faculty of Medicine, Prince of Songkhla University. All strains were maintained by subculture on Sabouraud dextrose agar.

Medium : Yeast Nitrogen Base 10 strength supplemented with 1 per cent glucose and 0.15 per cent asparagine was used as susceptibility test medium. The medium were sterilized by filtration through 0.2 μm pore size membrane filter (Gelman Sciences). This sterilized solution was stored at 4°C until used. Phosphate buffer was used as diluent in the test. The diluent, 0.01 M Phosphate buffer was prepared by mixing a solution of potassium hydrogen phosphate (0.01M K_2HPO_4) and potassium dihydrogen phosphate (0.01M KH_2PO_4) and adjusted to the pH 7.0, then sterilized by autoclaving and stored at room temperature until used. One volume of the 10X YNB medium and 9 volumes of the 0.01 M phosphate buffer were mixed together and used in each sensitivity test on the same day.

Drugs : Amphotericin B (Squibb, assay 891 μg/ml) was dissolved in dimethylsulfoxide (DMSO) and diluted with distilled water to obtain a two-fold dilution ranging from 32 μg/ml to 0.01 μg/ml. 5-FC (Hoffman-La Roche, Inc.) was dissolved in distilled water from 32 μg/ml to 0.05 μg/ml and rifampin (Le Petit) was dissolved in DMSO and diluted in distilled water from 1000 μg/ml to 10 μg/ml.

Each constant amount (per reaction tube) of 5-FC (1 and 5 μg/ml) and rifampin (10 and 25 μg/ml) was added in each dilution of amphotericin B as a combination reaction.

Susceptibility Test : Tests were performed with inocula prepared from 24-48 hours old *C. neoformans* or *Prototheca* spp. cultured on Sabouraud dextrose agar. A volume of 0.05 ml of the inoculum standardized to approximately $5x10^5$ cfu/ml were added to each tube. The tubes were incubated at 37°C for 24-48 hour or until growth in the control tubes was visible. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of drug in which no visible growth was observed. All tubes showing inhibition of growth were subcultured and the lowest concentration of drug with negative growth on subculture was determined as minimal fungicidal concentration (MFC).

RESULTS

The *in vitro* susceptibility of the 76 isolates to Amp B, 5-FC and RMP alone and their combinations are summarized in Tables 1 and 2. Seventy-one isolates of *C. neoformans* and 5 *Prototheca* spp. were susceptible to Amp B and most strains of *C. neoformans* were sensitive to 5-FC but all strains of *Prototheca* spp. were resistant to 5-FC. All strains of both *C. neoformans* and *Prototheca* spp. were highly resistant to RMP.

Evidence of synergy, as indicated by a fourfold or greater reduction of the MIC of Amp B in the presence of the fixed amount concentration of RMP, was seen in 60 of 71 isolates (84.5%) at the fungistatic level and 69 of 71 strains (97.2 %) at the fungicidal levels when added 25 μg/ml of RMP in the serial dilution of Amp B. When 10 μg/ml of RMP was added instead, synergy evidence was seen at the fungistatic and fungicidal levels in 56 of 71 isolates (78.8%) and 60 of 71 isolates (85.7%) respectively. The combination of Amp B and 5-FC did not show greater effect than did Amp B alone against both organisms but antagonistic evidence in some strains of the organisms was demonstrated.

DISCUSSION

Antifungal agents have inherited potentially lethal side effects, especially in patients who already have serious underlying diseases. Antifungal drug combinations might prove to be therapeutically useful by reducing the dosage of antifungal agents. Hugues, et al[5] previously demonstrated a

Table 1. Concentrations (μg/ml) of amphotericin B (Amp B), and Flucytosine alone and in combinations against *Cryptococcus neoformans* and *Prototheca* spp.

Drugs (μg/ml)	range	MIC_{50}	MIC_{90}	MFC_{90} (range)
C. neoformans (71 strains)				
Amp B	0.06 - 1	0.25	0.5	4 (0.5 - 4)
5-FC	2 - 16	4	8	32 (2 – 32)
Amp B + 5 μg/ml 5-FC	0.25 - 4	4	4	4 (1 -> 4)
Amp B + 1 μg/ml 5-FC	0.25 - 4	4	4	4 (2 - > 4)
Prototheca spp. (5 strains)				
Amp B	0.125 - 0.25	0.25	0.25	2 (1-2)
5-FC	-	-	32	-
Amp B + 5 μg/ml 5-FC	0.125 - 0.25	0.125	0.25	4
Amp B + 1 μg/ml 5-FC	0.25 - 0.5	0.5	0.5	4

Table 2. Concentrations (μg/ml) of amphotericin B (Amp B), and rifampin alone and in combinations against *Cryptococcus neoformans* and *Prototheca* spp.

Drugs (μg/ml)	range	MIC_{50}	MIC_{90}	MFC_{90} (range)
C. neoformans (71 strains)				
Amp B	0.06 - 1	0.25	0.5	4 (0.5 - 4)
Rifampin (RMP)	-	-	>1,000	-
Amp B + 25 μg/ml RMP	0.01 - 0.25	0.06	0.125	0.5 (0.06 - 2)
Amp B + 10 μg/ml RMP	0.03 - 0.5	0.06	0.125	1 (0.125 - 2)
Prototheca spp. (5 strains)				
Amp B	0.125 - 0.25	0.25	0.25	2 (1-2)
Rifampin (RMP)	500 - 1,000	1,000	1,000	1,000
Amp B + 25 μg/ml RMP	0.01 - 0.06	0.06	0.06	0.5 (0.125-1)
Amp B + 10 μg/ml RMP	0.03 - 0.125	0.06	0.125	1 (0.06-1)

reduction of MIC of Amp B against the genus *Aspergillus* with the addition of RMP. A recent study by Tuker, et al[8] showed synergy between itraconazole (Itr) and RMP *in vitro* against *C. neoformans* isolates from 5 patients receiving this combination. Coker et al[9] reported a relapse in 3 patients with cryptococcus meningitis treated by fluconazole (Flu) with RMP but all patients were successfully treated with Amp B. From our study in yeast and algae, we found that the combination of Amp B and 5-FC gave no greater effect than did Amp B alone, whereas, the combination of Amp B and RMP at the relevant clinical dosage produced greater effect on reduction of the MIC of Amp B than did either drug used individually. Medoff and Beggs[10,11] previously reported *in vitro* synergy between Amp B and RMP against several pathogenic fungi and they hypothesized that Amp B altered the permeability barrier of the cytoplasmic membrane of the organisms to allow increased penetration of RMP, which inhibits the fungal RNA polymerase. Therefore, the therapeutic amount of Amp B can be reduced its toxicity. From the results of this study, this pair of drugs have been shown to be a potential chemotherapeutic alternative for the treatment of systemic mycoses.

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การศึกษาฤทธิ์ของยาแอมโฟเทอริซิน บี ร่วมกับฟลูไซโตซีน หรือร่วมกับไรแฟมปิน ต่อยีสต์และสาหร่าย ในหลอดทดลอง

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การศึกษาในหลอดทดลองของการทดสอบความไวของเชื้อ *C. neoformans* 71 สายพันธุ์ และเชื้อ *Prototheca* spp. 5 สายพันธุ์ ต่อยา Amphotericin B (Amp B) ร่วมกับยา flucytosine (5-FC) หรือร่วมกับยา rifampin (RMP) โดยวิธี Broth dilution ผลปรากฏว่า เมื่อใช้ยา Amp B ร่วมกับ RMP จะทำให้ความเข้มข้นต่ำสุดของ Amp B ที่ใช้ในการยับยั้งเชื้อ (MIC) หรือฆ่าเชื้อ (MBC) ต่ำกว่าที่จะใช้ยา Amp B เพียงอย่างเดียว แต่เมื่อใช้ 5-FC ร่วมกับ Amp B พบว่าความเข้มข้นต่ำสุดของ Amp B ที่ใช้ในการยับยั้งเชื้อและฆ่าเชื้อลดลงเพียงเล็กน้อยหรือไม่ลดลงเลยเมื่อเทียบกับการใช้ Amp B เพียงอย่างเดียว

คำสำคัญ : *คริพโตคอคคัส นีโอฟอร์แมนส์, โปรโตทีกา* สปีชี, แอมโฟเทอริซิน บี, การใช้ยาหลายขนานร่วมกัน

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