In vitro post-antibiotic effect of three antifungal agent combinations on Candida albicans
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ABSTRACT

The occurrence of systemic fungal infections among immunocompromised patients has resulted in an increased number of studies involving antifungal agents. This study examines the in vitro post antibiotic effect (PAE) of three such antifungal agents on the opportunistic pathogen, Candida albicans. The MIC values for Amphotericin B, Fluconazole and 5-Fluocytosine on C.albicans were determined and each of the three drugs tested individually as well as in combinations for the induction of PAE. This was done by exposure of the yeasts in their logarithmic phase of growth to 5 x MIC concentrations of the antifungals for an hour and subsequent removal of the drugs by centrifugation and washing. The quantification of PAE was done using Miles and Misra technique of enumeration. All three antifungal agents individually induced a significant PAE. Amphotericin B and 5-flucysteine in combination induce a notable PAE while the amphotericin B - fluconazole combination presented a seemingly antagonistic effect, resulting in little or no suppression of growth. The in vitro study of drug-pathogen interactions such as PAE may have clinical implications with regard to the processes in vivo during the treatment of mycoses.

Introduction

Systemic fungal infections such are more frequent in immunocompromised patients. The antimicrobials administered in the treatment of such mycoses, depending upon the dosage given, can either the kill pathogen, and/or prevent further multiplication or reduce the number of organisms such that the manifestation of disease is prevented. The lowest concentration of an antimicrobial drug that prevents the growth of a particular strain of microorganism under a set of defined conditions in an agar or broth culture is referred to as the Minimum Inhibitory Concentration (MIC). The levels of antimicrobial in the serum falls below the MIC prior to the next dose, resulting in sub-inhibitory effects of the antimicrobial. This is discernible in either one or both of the following phenomena commonly described in bacteria and fungi:

(1) Sub-MIC effect. This is defined as the effect of subinhibitory concentrations of antimicrobials on microbes, resulting in a reduced rate of growth.

(2) The Post Antibiotic Effect (PAE) defined by McDonald, Craig and Kunin[i] (1977) as the suppression of bacterial growth that persists after short periods of exposure to antimicrobials.

Pathogenic yeasts can express various virulence factors ranging from mild to severe. The original aim of this project was to study the Sub-MIC effects of antifungal agents on cryptococcal virulence factors – namely the production of urease and a melanin-like pigment. A number of different broth systems and repeated attempts did not yield the production of any of virulence factors that could be detected. Hence we began work on a different project with the effect of PAE on Candida albicans as our area of focus. The post-antibiotic effect caused by the antifungals on the growth of C.albicans has been studied previously, with notable results seen with polyenes and DNA-analogs. We studied the post antibiotic effects of Amphotericin B, Fluconazole and 5-Flucytosine on one strain of Candida albicans in its logarithmic phase of growth. No studies have shown any sort of results involving a PAE effect using Flu as well as a

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mixture of AmB plus Flu. This effort, therefore examines the in vitro studies of the PAE effect and outcome induced by these three antifungals, both individually and in combinations.

**Materials and Methods:**

Stock solutions of AmB, Flu and 5FC were prepared. Amphotericin B and 5FC were supplied by Sigma. Fluconazole was supplied as Diflucan by Pfizer Inc. The MIC values of AmB, Flu and 5FC for *Candida albicans* and *Cryptococcus neoformans* var. neoformans in cation–adjusted Mueller Hinton Broth were determined. One strain of *Candida albicans* and one strain of *Cryptococcus neoformans* var. neoformans (ATCC 34871) were used in this project. The yeasts were grown in Mueller Hinton Broth (Becton Dickinson and Co., USA). Sabourauds Dextrose Broth (SAB) and Sabourauds Dextrose Agar (SDA) supplied by Oxoid, England, were used for MIC and colony counts respectively. The formation and presence of chlamydospores and germ tubes, characteristic of *C. albicans*, was observed under 100x and 400x magnification. The *Candida albicans* was inoculated into a carbohydrate assimilation kit, API 20C AUX (bioMérieux, USA) as per the manufacturer's instructions and the results are shown in figure 3.1. An overnight culture of *Candida albicans*, in 10 ml of MHB was incubated at 37°C. Two bottles containing 40 ml of fresh MHB broth each were labeled as Control (C) and Experiment (E) respectively, and 4 ml of the overnight culture was added into each of the two bottles. Both bottles were incubated for an hour at 37°C. The antifungal was added at a concentration of five times the MIC to the bottle labeled E, and both bottles were incubated for an hour at 37°C. The contents of C and E were poured into centrifuge tubes and spun at 3000 RPM for 10 mins. The supernatant was discarded and the pellet in each tube was resuspended in 40 ml of fresh Phosphate Buffered Saline (PBS, Oxoid). The contents were centrifuged again for 10 minutes at 3000 RPM and the PBS was decanted and the pellets were resuspended in 40ml of fresh MHB. Enumeration of colonies was done using Miles and Misra technique.

**Results**

![Figure 3.1 – Results of the API 20 C AUX on Candida albicans](image)

Table 3.1 – MIC values for (A) Cryptococcus neoformans var. neoformans and (B) Candida albicans in MHB

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>MIC ug/ml (A)</th>
<th>MIC ug/ml (B)</th>
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<tbody>
<tr>
<td>AmB</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>Flu</td>
<td>16</td>
<td>2.0</td>
</tr>
<tr>
<td>5FC</td>
<td>16</td>
<td>4.0</td>
</tr>
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Fig 3.2 PAE Fluconazole

Fig 3.3 PAE –Amphotericin B + 5FC

Fig 3.4 PAE –Amphotericin B + Flu
Discussion:

Yeasts from previous experiments had shown no change in growth kinetics subsequent to a brief exposure to azoles. A marked PAE with Fluconazole was noted (Figure 3.2). Although a number of previous experiments using other azoles gave mixed PAE results we feel that based on the strict protocol we followed, the patterns seen in Figures 3.9 and 3.10 seem to be more of an PAE effect of flucanazole on the growth of the Candida rather than the effect of Flu as a residual agent. Previous literature reviews also state that a positive PAE was almost always seen with 5FC and AmB and our results concur with the results obtained in previous experiments. Although a myriad of factors affect PAE, of which drug concentration and mechanism of action of action are of significant importance, the factors that govern the action of either one of the antifungals might not be necessarily the same. The use of a combination of the antifungals to study the PAE probably is of more importance than the study of the action of each individual drug in itself. Clinical prophylaxis or treatment may involve the administration of two antifungals either together or sequentially to suppress the manifestation of disease in numerous mycoses. The action of the two antifungals could be synergistic or antagonistic, with the implications for the patient being either positive or negative. AmB and 5FC (figure 3.3) produce a positive PAE, suggesting that they are synergistic. The outcome of the AmB+Flu combination (figure 3.4) is visibly different compared to the results obtained from either one of the drugs used. From the results obtained, though, administration of 5 x MIC of AmB and Flu results in the growth of Candida albicans, rather than suppression despite the presence of two antifungal agents. The experiments we carried out were with the main aim of observing a delineated post antibiotic effect by the action of AmB, Flu, 5FC and their combinations. It is imperative to repeat these experiments with other strains of C.albicans before inferring on the results obtained from these investigations, especially that of Fluconazole. Another crucial factor that would have been worth examining is the recovery period of Candida albicans in response to the treatment with the three antifungals and their combinations.

References: