Post-antifungal effect of polyene, bistriazole and antimetabolite agents and their combinations against *Candida albicans*.

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**ABSTRACT**

Oropharyngeal Candidiasis (OPC) is an opportunistic oral infection caused by the yeast *Candida*. Many different therapeutic options for treatment of OPC have been tested in vivo and vitro procedures. In our study, we chose to assess the effectiveness of antifungal agents in vitro. For this, we evaluated the minimum inhibitory concentration (MIC) and the post-antifungal effect (PAFE) of 3 major families of drugs against a *Candida albicans* isolate. The yeast was exposed to 5xMIC of Amphotericin B, Fluconazole and 5-Fluorocytocine for a period of 1 h. Following subsequent removal of the drug, the growth rates were measured. For the combinations, two antifungals were added, each at concentrations of 5xMIC. Significant PAFEs were repeatedly observed for all 3 antifungal agents, though at different intensities. The combination between Amphotericin B and 5-fluorocytosine showed enhanced PAFE. Combination of Fluconazole and Amphotericin B did not induce a PAFE. These in vitro findings may have clinical relevance to the design of dosing regimens.

**INTRODUCTION**

*Cryptococcus neoformans* is a capsulated yeast, which causes infections in immunocompromised patients\(^1\). *Cryptococcus neoformans* has several defined virulence determinants including the capsule, urease and melanin production. The original aim of this project was to seek possible sub-MIC effects of antifungal agents on the expression of urease and melanin production. The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation under defined conditions in a broth or agar culture system. Sub-MIC effects occur when bacteria continue to grow when exposed to continuous fixed levels of an antimicrobial drug below the MIC.

Urease is a nickel metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbamate in the microorganism\(^2\). Presence of urease was determined through an ammonia assay. The principle of this method involved urea being hydrolyzed by urease.

After many carefully done assays, which gave erratic graphs, we discovered that the distilled water contained higher concentrations of ammonia ions compared to the extracted sample. *Cryptococcus neoformans* is relatively unique in its possession of an enzyme system that allows it to metabolize a variety of catechol precursors to melanin\(^2\). There was no known broth medium for the production of melanin by the microorganism. MHB at 37\(^\circ\)C gave the darkest colouration but later we found out that it was due to a chemical reaction between the broth medium and the caffeic acid. Due to the above difficulties in the protocols we had to abandon this experiment and move onto another related project.

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The new project was on the Post Antifungal Effects (PAFE) of an organism identified as Candida albicans. Post-antifungal effect (PAFE) is the suppression of growth that persists following limited exposure of fungi to antifungals and subsequent removal of the drug. Candida is of particular importance in patients who are already immuno-suppressed due to the presence of the HIV/AIDS virus. We evaluated in vitro the minimum inhibitory concentration (MIC) and the post-antifungal effect (PAFE) of Amphotericin B, 5-Fluorocytosine and Fluconazole against Candida albicans. As the frequency of fungal infections increases, there is a need for new combinations of antifungal agents and increased understanding of the pharmacodynamic properties of these combined agents.

The PAFE was determined as the difference between the growth of the drug free control and the drug exposed test cultures, following removal of the antifungal agent. Plots of averaged colony counts (log10 CFU per milliliter) versus time were constructed and compared against a growth control. The data we have elucidated reveals additional mechanisms by which antimycotics may operate in vitro to suppress candidal pathogenicity.

**METHODS AND MATERIALS**

Confirmation of Candida albicans strain was done by a biochemical test (API 20C AUX), Chlamydospore formation in Rice Tween and positive production of Germ tubes. MICs were determined for each isolate using the microdilution method. Inoculation of 1/3rd of a colony into 10ml test medium and incubating the tube at 37°C for 24h gave a culture in the logarithmic phase of growth. 4ml of the inoculum is then separated in to two centrifuge tubes containing 36ml of MHB each. The two tubes were then incubated for 1hr at 37°C. The 5xMIC antimicrobial concentration was added to one tube containing medium and the other is left as drug free control medium. The tubes are incubated at 37°C for the desired exposure period of 1h.

At the end of exposure period the antifungal is removed by washing method. Both cultures were centrifuged at 3000rpm for 10 minutes. A visible pellet was observed, supernatant was decanted and the pellet was resuspended into fresh drug free 40ml of PBS medium. This was centrifuged again at 3000rpm for 10 minutes. Supernatant removed and pellet resuspended in fresh MHB broth. After the drug removal procedure the tubes are maintained at 37°C. This is noted as zero time. Counts of CFU/mL were performed on all cultures at time zero, and every two hours there after. Cold sterile PBS is used for serial 10-fold dilutions (10⁻¹ to 10⁻⁶). Sabauroud dextrose agar plates were used for Miles and Misra method and read after 24hrs at 37°C.

**RESULTS AND DISCUSSION**

Only three graphs are shown as results due to space limit. There are 10 graphs in all. MIC results for Candida albicans and Cryptococcus neorformans

<table>
<thead>
<tr>
<th>Antifungal Agent</th>
<th>Candida albicans</th>
<th>Cryptococcus neorformans</th>
</tr>
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<tbody>
<tr>
<td>Amphotericin B</td>
<td>0.5µg/ml</td>
<td>0.125µg/ml</td>
</tr>
<tr>
<td>Flucytosine(5-FC)</td>
<td>4.0µg/ml</td>
<td>2.000µg/ml</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>2.0µg/ml</td>
<td>2.000µg/ml</td>
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</tbody>
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Fluconazole displays a measurable PAFE against *Candida albicans*. The azole inhibit cytochrome-P450 activity. This decreases conversion of 14-alpha-methylsterols to ergosterol, an important membrane component in fungi. Failure of ergosterol synthesis causes altered membrane permeability leading to loss of ability to maintain a normal intracellular environment.

5-FC produces a PAFE from 6hrs and peaks at 24h. 5-FC is believed to act by interfering with RNA, DNA, and protein synthesis. It is converted to 5-fluorouracil (5-FU) which inhibits thymidylate synthetase. Thymidine is required for DNA synthesis.

Amphotericin B gives a longer PAFE compared to the other two antifungal agents. The mode of action of amphotericin B, involves binding to ergosterol in the fungal cell membrane resulting in pores being produced causing cell leakage and ultimately cell death.

**Combinations of Antifungal agents**

**Amphotericin B and 5-Flourocytosine**

Combinations of 5-FC and Amphotericin B produced PAFEs ranging from zero to 24hrs. These PAFEs persisted longer than those achieved when each of the two agents was assayed separately. A synergistic PAFE was evident with combinations of the two drugs at 5 x MIC values.

**Amphotericin B+ Fluconazole**

These graphs show no apparent PAFE at any time. The membrane Ergosterol is the site of action for both these antifungals. Fluconazole inhibits Ergosterol synthesis thus target for Amphotericin B is lost.
REFERENCE

4. Lorian Victor Antibiotics in laboratory medicine Baltimore : Williams & Wilkins , c1991