Amphotericin B-Induced in vitro Postantifungal Effect on *Candida* Species of Oral Origin

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Key Words

Candida · Amphotericin B · Postantifungal effect

Abstract

Objective: The aim of this investigation was to measure the postantifungal effect (PAFE) of 6 different oral *Candida* species following exposure to amphotericin B. **Materials and Methods:** Five oral isolates each of *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, *Candida glabrata* and *Candida guilliermondii* (total of 30 isolates) were examined for the presence of PAFE after 1 h of exposure to the minimum inhibitory concentration of amphotericin B. The PAFE was determined as the difference in time (hours) required for the growth of the drug-free control and the drug-exposed test cultures to increase to 0.05 absorbance level following removal of amphotericin B. **Results:** The mean duration of amphotericin B-induced PAFE was lowest for *C. albicans* (5.91 ± 0.31 h) and greatest for *C. parapsilosis* (12.72 ± 0.11 h), while *C. guilliermondii* (8.32 ± 0.33 h), *C. glabrata* (8.43 ± 0.21 h), *C. krusei* (9.68 ± 0.23 h) and *C. tropicalis* (10.98 ± 0.18 h) elicited intermediate values. **Conclusion:** Even a limited exposure to sublethal concentrations of amphotericin B suppressed growth of *Candida* species of oral origin. The significant variation in amphotericin B-induced PAFE amongst different *Candida* species may have clinical implications in terms of amphotericin B regimens used in the management of oral candidiasis.

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Introduction

Opportunistic oral infections caused by *Candida albicans* and species of *Candida* other than *C. albicans* are particularly common in compromised patients either due to disease or usage of drugs such as broad-spectrum antibiotics, cytotoxic drugs and corticosteroids. Oral candidiasis is considered the commonest human fungal infection which manifests itself in a variety of clinical semblances. These include pseudomembranous and erythematous variants, *Candida*-induced denture stomatitis, median rhomboid glossitis and angular stomatitis. The vast majority of HIV-infected patients suffer from oral candidiasis during the course of the disease, which is the most common AIDS-associated oral infection. In fact, oral candidiasis and the amount of oral yeast present have been postulated to be closely associated with the level of host immunosuppression and to be predictive of HIV disease progression and viral load [1]. Moreover, in HIV-infected patients, the form of oral candidiasis seen is often resistant to treatment [1]. Although *C. albicans* is the most pervasive *Candida* pathogen, others such as *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, *Candida glabrata* and *Candida guilliermondii* are infrequently but consistently isolated from patients with either oral or systemic candidosis. For instance, *C. krusei* is an emerging pathogen often innately azole resistant [2], and *C. glabrata* has been reported to acquire resistance in vi-

tro and in vivo and is an important pathogen causing serious and persistent infection in the immunocompromised population [3]. Similarly, *C. parapsilosis* and *C. tropicalis* have been obtained from the oral cavity of AIDS patients [1, 4].

The postantifungal effect (PAFE) refers to suppression of fungal growth that persists following limited exposure of yeasts to antifungal agents and subsequent removal of the drug [5]. The clinical significance of in vitro PAFE is associated with the impact that it may have on the dosage regimen of a selected antimicrobial agent during clinical usage in vivo [5]. Antifungal agents inducing a longer PAFE can be administered at longer dosing intervals, whereas organisms exhibiting a shorter PAFE are more likely to be resilient to antifungal therapy, as they recover relatively more quickly than their counterparts which exhibit a longer PAFE [6].

Amphotericin B is a polyene antifungal agent that acts through an interaction with ergosterol, a fungal membrane sterol. This effect results in the loss of membrane-selective permeability and intracellular components, which in turn causes impairment of barrier functions, leakage of cellular components and cell death. Topical amphotericin B oral preparations (lozenges, mouthwashes, creams, ointments) are available for the treatment of oral candidosis. While topical therapy may be useful in its own right in primary oral candidosis, it could be used as an adjunct to parenteral therapy in secondary candidosis, which manifests both systemically as well as on mucosal surfaces [7]. However, intra-orally the diluent effect of saliva and the cleansing action of the oral musculature often tend to reduce the availability of such topical applications below the effective therapeutic concentration [8]. Hence it is likely that the organisms undergo only a limited exposure to the antifungal agent during such treatment. Thus, the drugs that are capable of inducing a longer PAFE and thereby perturbing the yeast growth would be of immense benefit in vivo in the management of oral candidiasis [6].

There are reports on amphotericin B-induced PAFE on *C. albicans*, *C. tropicalis* and *C. glabrata* species [9–11]. However, there are no reports on PAFE elicited by amphotericin B on other *Candida* species of oral origin. Also, there are no reports on a comparative study among oral *Candida* species on the amphotericin B-induced PAFE. Hence the main aim of the current investigation was to evaluate the PAFE of amphotericin B against oral isolates belonging to 6 different *Candida* species, comprising *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. glabrata* and *C. guilliermondii*.

Materials and Methods

Micro-Organisms

Five oral isolates of each of the species *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. glabrata* and *C. guilliermondii* were used in the study (total of 30 isolates). *C. albicans* ATCC 90028 and *C. tropicalis* ATCC 13803 were used as reference strains for determination of the minimum inhibitory concentration (MIC). All yeast isolates were tested for germ tube formation. Thereafter a presumptive identification of *Candida* isolates was performed on the basis of the characteristic colony colour on CHROMagar *Candida* medium (Becton Dickinson & Co., Sparks, USA). All the isolates were further identified based on their carbohydrate assimilation pattern by the API 20C AUX yeast identification system (Bio-Mérieux, France). Stock cultures were maintained at -20°C . After recovery these were maintained on Sabouraud's dextrose agar and stored at $4-6^{\circ}\text{C}$ during the experimental period.

Antifungal Agents and Media

As in previous PAFE studies [9–11], amphotericin B (Sigma, St. Louis, Mo., USA) was dissolved in dimethylsulphoxide and absolute ethanol (3:2 ratio), respectively, and prepared initially as 10,000- μg solutions and stored at -20°C before use. It was thereafter suspended in the following medium during the exposure period (1 h) of yeasts: RPMI 1640 medium, buffered with 0.165 M morpholinopropanesulphonic acid containing L-glutamine and lacking sodium bicarbonate (Sigma, USA), in 1 litre of sterile distilled water, adjusted to a pH of 7.2 and filter sterilized [9–11]. This liquid RPMI was stored at $2-8^{\circ}\text{C}$.

Since amphotericin B was dissolved in dimethylsulphoxide and absolute ethanol, equivalent amounts of the latter chemicals were tested initially as in previous studies using the same isolates to ascertain whether they had an effect on the isolates tested.

Determination of MIC

The MIC values of amphotericin B were determined by the broth dilution technique [6, 9] by performing twofold serial dilutions of the drug in microtitre plates using an inoculum of $1-5 \times 10^5$ colony-forming units/ml. The MIC was determined visually and spectrophotometrically at 595 nm following 24 h of incubation at 37°C . The MIC was defined as the lowest concentration of the drug that inhibited growth of yeast cells, as indicated by the absence of turbidity (optically clear). The MIC was read independently by two laboratory technicians. *C. albicans* ATCC 90028 and *C. tropicalis* ATCC 13803 were used as reference strains, as done in previous studies [6, 9–11]. All experiments were repeated on 2 separate occasions with duplicate determinations on each occasion.

Preparation of the Cell Suspension for the PAFE Assay

Yeast cells maintained on Sabouraud's dextrose agar were inoculated onto fresh plates and incubated overnight for 24 h prior to use. The organisms were harvested and a cell suspension was prepared in sterile PBS at 520 nm to an optical density of 1.5. From this cell suspension, 0.5 ml was added to tubes containing 2 ml of RPMI broth (control) and 2 ml of RPMI/amphotericin B solution (test). The drug concentration used was the MIC of amphotericin B as done previously with other polyene antifungal drugs [6]. This gave a cell suspension of 10^6-10^7 cells/ml in each assay tube.

The tubes were then incubated at 37°C for a period of 1 h in a rotary incubator. Following this limited exposure, the drugs were

removed by two cycles of dilution with sterile PBS and centrifugation for 10 min at 3,000 g. Afterwards the supernatant was completely decanted and the pellets were resuspended in 2.5 ml of sterile PBS as was done previously [6, 9, 11]. Viable counts of the control and the test were performed after drug removal. As the procedure of drug removal effectively eliminated any carry-over effect, there was virtually no difference on the viable counts of the control and the test specimens following exposure to already diluted subtherapeutic concentrations of the drug as observed in previous PAFE determination studies [6, 9–11].

Evaluation of PAFE

The method previously used for determining the PAFE [6, 9–11] was applied in the current study. In brief, aliquots of 100 µl from each cell suspension were added to microtitre wells containing 250 µl of RPMI broth. Then the microtitre plate was placed in a computerized spectrophotometric incubator (Spectramax 340: Tunable Microplate Reader, Molecular Device Corp., Sunnyvale, Calif., USA) and incubated at 37°C for 24 h. Growth of yeast cells was automatically monitored by the instrument in terms of change in turbidity (absorbance at 595 nm), at 30-min intervals. The duration of the PAFE was calculated by using the formula, $PAFE = T - C$ as used previously where T was the time required for the relative optical density of the drug-exposed cell suspension to reach the 0.05 absorbance level after drug removal and C was the time required for the relative optical density of the drug-free control cell suspension to reach the same absorbance level. Thus $T - C$ expressed the time in which the antifungal agent was capable of causing growth suppression of the organism following limited exposure to the drug (i.e. PAFE). All experiments were repeated on 3 separate occasions with duplicate determinations on each occasion.

Statistical Analysis

The variation of PAFE between the 6 different *Candida* species was analyzed with all the raw data by ANOVA using Tukey-Kramer multiple comparison (post-hoc) tests [6]. Regression analysis was used to determine the relationship between MIC and the PAFE of the *Candida* species. A p value of <0.05 was considered statistically significant.

Results

The MIC (µg/ml) values of amphotericin B in RPMI broth were: for *C. albicans*, 0.06–0.5; *C. tropicalis*, 0.06–0.5; *C. krusei*, 0.5–1; *C. parapsilosis*, 0.06–0.5; *C. glabrata*, 0.25–1; *C. guilliermondii*, 0.06 (table 1).

The in vitro PAFE of amphotericin B on oral *Candida* isolates is shown in table 1. A substantial PAFE was induced on all 6 different oral *Candida* species: *C. albicans*, 5.91 ± 0.31 h; *C. tropicalis*, 10.98 ± 0.18 h; *C. krusei*, 9.68 ± 0.23 h; *C. parapsilosis*, 12.72 ± 0.11 h; *C. glabrata*, 8.43 ± 0.21 h; *C. guilliermondii*, 8.32 ± 0.33 h. The analysis of the relationship between the PAFE of 6 different *Candida* species showed a statistically significant difference of amphotericin B-induced PAFE among all *Candida*

Table 1. The in vitro PAFE (in h; SEM in parentheses) of amphotericin B on *Candida* species of oral origin following limited exposure to MIC and subsequent removal of the drug

Isolate	MIC	PAFE	Mean PAFE
<i>C. albicans</i>			
1	0.06	5.87 (0.34)	5.91 (0.31)
2	0.06	6.68 (0.24)	
3	0.12	6.54 (0.32)	
4	0.5	5.12 (0.06)	
5	0.5	5.35 (0.16)	
<i>C. tropicalis</i>			
1	0.25	11.23 (0.14)	10.98 (0.18)
2	0.25	10.45 (0.21)	
3	0.5	11.08 (0.06)	
4	0.25	11.45 (0.12)	
5	0.06	10.67 (0.07)	
<i>C. krusei</i>			
1	0.5	9.87 (0.56)	9.68 (0.23)
2	0.5	9.58 (0.14)	
3	1	9.23 (0.45)	
4	1	10.46 (0.09)	
5	0.5	9.24 (0.47)	
<i>C. parapsilosis</i>			
1	0.06	12.78 (0.17)	12.72 (0.11)
2	0.06	12.95 (0.62)	
3	0.12	12.45 (0.08)	
4	0.5	12.48 (0.68)	
5	0.06	12.92 (0.55)	
<i>C. glabrata</i>			
1	0.5	8.08 (0.37)	8.43 (0.21)
2	1	9.23 (0.16)	
3	0.5	8.46 (0.22)	
4	0.5	8.22 (0.18)	
5	0.25	8.18 (0.06)	
<i>C. guilliermondii</i>			
1	0.06	7.94 (0.03)	8.32 (0.33)
2	0.06	8.56 (0.03)	
3	0.06	8.92 (0.45)	
4	0.06	8.97 (0.26)	
5	0.06	7.21 (0.74)	

species except for the difference between *C. glabrata* and *C. guilliermondii*. Analysis of the relationship between the MIC and PAFE of *Candida* species did not reveal any correlation between these two parameters ($r = -0.019$, $p = 0.919$).

Discussion

All 30 isolates in the current study elicited a high amphotericin B-induced PAFE, irrespective of the variation seen among the different *Candida* species on this attri-

bute. *C. albicans* produced the least PAFE, whereas *C. parapsilosis* elicited the largest PAFE. Other investigated species produced intermediate values. Similar observations have been reported with another polyene antifungal drug, nystatin, against the same isolates used in this study [6]. Similarly, *C. albicans* also produced the lowest nystatin-induced PAFE, *C. parapsilosis* elicited the highest PAFE and other species produced intermediate values. The existence of a significant variation in amphotericin B-induced PAFE among different *Candida* species (as well as in isolates of the same species) seen in the current study could have been due to subtle changes in the cell wall composition produced by the pharmacodynamics of amphotericin B on different *Candida* species (as well on isolates within the same species) resulting in the difference in interspecies and intraspecies genomic profiles. However, taken together, the findings of the current study on amphotericin B-induced PAFE and the previous study on nystatin-induced PAFE on *Candida* species indicate that both polyene antifungal agents elicited a high PAFE on all *Candida* species but with the least effect on *C. albicans* isolates. Hence, it seems that the latter is collectively the most resilient, having the ability to recover after drug exposure much sooner than the other species. This finding adds further credence to the fact that *C. albicans* is the most virulent and pervasive of all *Candida* species.

The high PAFE elicited by amphotericin B in the current study, as well as by nystatin, another polyene antimycotic agent, in a previous study [6], may be related to the mechanism of action of polyene antifungals on the yeast cell membrane. Polyenes prevent the biosynthesis of ergosterol in the fungal cell membrane, which contributes to a variety of cellular functions. Ergosterol is important for the fluidity and integrity of the membrane and for the many membrane-bound enzymes, including chitin synthetase, important for proper cell growth and division [12]. Hence, it is not surprising that polyene-mediated changes in the cell wall structure would affect active budding and multiplication, thus suppressing growth and eliciting a long-lasting PAFE.

In the current study, the MIC values of amphotericin B on oral *Candida* species were within the range of the reference strains, which implied that the isolates tested were susceptible to the drug. Furthermore, these values were within the MIC range obtained in previous investigations for susceptible *Candida* isolates [10, 11]. However, the analysis of the correlation between MIC and PAFE of amphotericin B did not show any correlation between them. The probable explanation is that MIC denotes a concentration of a drug that inhibits growth in the pres-

ence of a drug, whereas PAFE is a phenomenon which denotes inhibition of growth following removal of the drug (i.e. in the absence of the drug). Hence, though both these parameters are determinants of effectiveness of a drug on the organisms concerned, the MIC is a measurement in the presence while PAFE is a measurement in the absence of the drug. Hence, these findings highlight the possible need for determining not only the MIC, but also the PAFE in the design and usage of antimycotics in the future.

Amphotericin B has proved to be effective in modulating many pathogenic attributes of *Candida* both in vivo and in vitro. For instance, it has been shown that amphotericin B is capable of inhibiting candidal adhesion to buccal epithelial cells and to denture acrylic surfaces, as well as suppressing the ability of *C. albicans* isolates to form germ tubes [12–14]. Furthermore, amphotericin B has also been shown to suppress the cell surface hydrophobicity of *Candida*, which is considered a non-biological force attributed to its adhesion [14, 15]. In addition, the current study indicates that the antifungal activity of amphotericin B may probably be realized even when administered at extended intervals due to the PAFE elicited by this drug. However, since the PAFE depends upon the organism in question, the concentration, exposure time and mode of action of the tested drug and other environmental factors [11, 16–18], further studies with a larger battery of organisms are needed to determine the relationship between the PAFE of amphotericin B and its dosage regimen applicable in vivo.

Conclusion

Even a limited exposure to sublethal concentrations of amphotericin B suppressed the growth of *Candida* species of oral origin. The significant variation in amphotericin B-induced PAFE amongst different *Candida* species may have clinical implications, in terms of amphotericin B regimens used in the management of oral candidiasis.

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