

Post-antifungal effect of polyene, azole and DNA-analogue agents against oral *Candida albicans* and *Candida tropicalis* isolates in HIV disease

S. Anil^{1,2}

A. N. B. Ellepola^{1,3}

L. P. Samaranayake¹

¹Oral Bio-Sciences, Faculty of Dentistry,
University of Hong Kong, Hong Kong

²Faculty of Dentistry, University of Kerala,
Trivandrum, Kerala, India

³Faculty of Dental Sciences, University of
Peradeniya, Sri Lanka

Abstract

Oropharyngeal candidiasis (OPC) is the most frequent AIDS-associated opportunistic infection, as up to 90% of HIV-infected individuals suffer at least one episode during the course of their disease. Various *in vivo* and *in vitro* procedures have been used to assess the effectiveness of antifungal agents used in HIV infection. In the present study, we evaluated *in vitro* the minimum inhibitory concentration (MIC) and the post-antifungal effect (PAFE) of two polyenes, two azoles and one DNA-analogue against 10 oral isolates of *Candida albicans* and 10 of *Candida tropicalis*, all from HIV-infected individuals, in order to obtain basic data on the pharmacodynamics of these drugs. One-hour exposure to twice the MIC of all the drugs, except fluconazole, elicited a consistently high PAFE in both *Candida* species. Furthermore, the PAFE elicited by the antifungals (except fluconazole) was significantly prolonged for *C. tropicalis* compared with *C. albicans*. This speedy recovery of *C. albicans* isolates exposed to transient low concentrations of antifungals appeared to reflect its virulence compared with lesser potent species, such as *C. tropicalis*. Taken together, the current data, while confirming the existence of PAFE in a non-*albicans* species of *Candida*, also provide further clues for the recalcitrance of *C. albicans* species in the face of antifungal therapy for oropharyngeal candidiasis.

Key words: antifungal agents; candidiasis; HIV infection; post-antifungal effect (PAFE)

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Correspondence to:

Lakshman P. Samaranayake
5/F, Oral Bio-Sciences, Faculty of Dentistry,
34 Hospital Road, Hong Kong
e-mail: lakshman@hkucc.hku.hk

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Oropharyngeal candidiasis continues to be the commonest opportunistic infection in patients with human immunodeficiency (HIV) disease and AIDS (1, 2). *Candida albicans* is the most common causative agent, although non-*albicans* species, such as *C. glabrata*, *C. tropicalis* and *C. krusei*, are becoming increasingly prevalent, possibly due to acquisition of drug resistance (3, 4). *Candida* species respond to a variety of topical and systemic antifungal agents (5). The topical agents are very popular in the management of oral

candidiasis and are available in a variety of forms, including oral rinses, creams, and slow releasing tablets (6).

The goal of treatment with antifungal agents is to maintain the drug concentration above the minimum inhibitory concentration (MIC) for almost the entire dosing period (7). Therefore, in treating candidal infections, it would be desirable if the clinicians could rely on *in vitro* susceptibility tests to obtain critical information about antifungal dosage regimens. But there is a lack of universally accepted, standardized procedures and criteria, which provide treatment guidelines that correlate well with the *in vivo* situation of the most effective dosage regimens (8). Furthermore, in determining the MIC, the fungi are continuously exposed to a constant level of the drug, whereas *in vivo* the organisms are exposed to fluctuating levels of the drug (9), which is further exacerbated in the oral cavity by the diluent effect of saliva and the cleansing effect of the oral musculature. Various *in vitro* procedures that closely simulate the *in vivo* environment have therefore been evaluated to assess the effectiveness of antifungal agents against particular organisms. One phenomenon that has been described recently in this context is the post-antifungal effect (PAFE), which refers to the suppression of fungal growth that persists following limited exposure of the organisms to antimicrobial agents (9). The clinical significance of PAFE is associated with the impact that it may have on the dosage regimen of a selected antimicrobial agent during clinical usage (10), since antimicrobials that induce long PAFE can be administered with longer dosing intervals than used previously against a particular organism without losing efficacy, and possibly with diminished adverse effects (11, 12).

Although the PAFE values for *C. albicans* have been described previously by a few groups (13–15), there is scanty data on the PAFE of other pathogenic *Candida* species especially isolated from HIV-positive patients. Hence, in the present study, we evaluated the PAFE of five commonly used antifungal agents by using 10 oral isolates each of *C. albicans* and *C. tropicalis* from HIV-infected subjects. The antifungal agents tested were the polyenes (nystatin and amphoterecin-B), azoles (fluconazole and ketoconazole), and the DNA-analogue 5-fluorocytosine.

Material and methods

Isolates

Ten oral isolates of *C. albicans* and ten of *C. tropicalis*, all from HIV-infected subjects with oral candidiasis, were used in the study. All isolates were from patients attending the Queen Elisabeth Hos-

pital, Hong Kong, and were obtained by the oral rinse technique (16). The procedure involves the patient holding and rinsing 10 ml of sterile phosphate-buffered saline (0.01 M, pH 7.2) in the mouth for 60 s. The solution is then expectorated into a chilled container, immediately transported to the laboratory, and 50 µl of concentrate are inoculated onto Sabouraud's agar medium using a spiral plating system. After 24–48 h incubation at 37°C, the resultant growth is subcultured for purity and identified using the germ-tube test and the commercially available API-20C system (bioMérieux, Basingstoke, UK) (17). Stock cultures were maintained at –20°C.

After recovery these were maintained on Sabouraud's dextrose agar and stored at 4–6°C during the experimental period. *C. albicans* ATCC 90028 and *C. tropicalis* ATCC 13808 obtained from the American Type Culture Collection (Gaithersburg, MD, USA) were used as the reference strains for the determination of MIC.

Antifungal agents

Five antifungal agents obtained as reagent grade powders were tested: nystatin (Sigma, St. Louis, MO, USA) and amphotericin B (Sigma) were dissolved in dimethylsulphoxide (DMSO) and absolute ethanol (3:2 ratio), respectively. 5-fluorocytosine (Sigma) was dissolved in sterile distilled water. Ketoconazole (Janssen, Beerse, Belgium) was dissolved in dimethylsulphoxide, and fluconazole (Pfizer Inc., New York, NY, USA) was dissolved in absolute methanol. All agents were prepared initially as 10,000 µg/ml solutions and stored at –20°C until used.

Since the antifungal agents used were dissolved in DMSO, DMSO/absolute ethanol and absolute methanol, equivalent amounts of the latter chemicals were tested initially to ascertain whether they had an effect on the isolates tested. The minute volumes of the chemicals used did not have any effect on yeast growth when compared with the controls.

MIC determinations

Broth microdilution method

Antifungal susceptibility of all 20 isolates to nystatin was determined according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (18). The inoculum was prepared from 24-h cultures of species. Cell suspensions were prepared in RPMI-1640 medium and adjusted to give a final inoculum of 10⁵ CFU/ml. Testing was performed in 96-well round-bottomed microtitre plates. The plates were incubated at 37°C and read after 24 h. The MIC of nystatin was defined as the lowest concentration at which there was 100% inhibition of yeast growth.

E-test method

The MIC determinations of amphotericin-B, fluconazole, ketoconazole and 5-fluorocytosine were performed using the E-test. The E-test (AB BIODISK, Solna, Sweden) is a patented commercial method for the quantitative determination of MICs of antimicrobial drugs. Comparisons of the E-test method with the NCCLS broth dilution method have demonstrated high levels of agreement (19).

The inoculum for the E-test was prepared from 24-h cultures of all *Candida* species tested. Cell suspensions were prepared in sterile distilled water and adjusted to a concentration corresponding to a 0.5 McFarland standard using a spectrophotometer set at 520 nm. The medium used was RPMI-1640 agar (1.5%) with 2% glucose buffered with MOPS (pH 7.0). The plates were inoculated by dipping a sterile swab into the appropriate cell suspension and streaking it across the entire surface of the agar in three directions; they were then dried at room temperature for 15 min before the E-test strips were applied. Afterwards, plates were incubated at 37°C and read after 24 h. The E-test MIC was read as the drug concentration at which the border of the elliptical inhibition zone intersected the scale on the antifungal test strip, according to the manufacturer's instructions.

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, 1 ml was added to tubes containing 4 ml of RPMI broth (control) and 4 ml of RPMI/drug solution (test). This gave a cell suspension of 10^6 – 10^7 cells/ml in each assay tube.

Twice the MIC of the drugs was used for all five antifungals investigated. 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 centrifugation for 10 min at 3000×g. Afterwards, the supernatant was completely decanted and the pel-

lets were resuspended in 2.5 ml of sterile PBS. Previous investigators have found that removal of 90% of the supernatant with two washings reduces antimicrobial concentration 100-fold, while complete decanting of the supernatant with two washings (as carried out in the current study) reduces the concentration 10,000-fold (20). Hence this method virtually eliminates any "carry-over effect" of the drug following its removal.

Viable counts of the control and the test were performed after drug removal, and control suspensions were reconstituted as needed to obtain a cell concentration comparable to the test, before the commencement of the PAFE assay.

The PAFE assay

The method used for determining the PAE of bacteria (21) was applied with minor modifications to evaluate the PAFE of yeasts (13). Aliquots of 100 µl from each cell suspension was added to a microtitre well containing 150 µl of RPMI broth. Then the microtitre plate was placed in a computerized spectrophotometric incubator (Spectramax 340: Tunable Microplate Reader, Molecular Devices Corp, Sunnyvale, CA, USA) and incubated at 37°C for 24 h. Growth of yeast cells was automatically monitored by the computerized instrument for the change in the turbidity (absorbance at 595 nm), at 30-min intervals, for a period of 18 h. The duration of PAFE was calculated by using the formula $PAFE = T - C$, where T is the time required for the relative optical density of the drug-exposed cell suspension to reach the 0.05 absorbance level after removal of the drug and C is the time required for the relative optical density of the drug-free control cell suspension to reach the same absorbance level (7, 22). Thus, $T - C$ expressed the period in which the antifungal agent was capable of causing growth suppression of the organism following limited exposure to the drug (i.e., post-antifungal effect). Each experiment with each isolate was performed in triplicate on three separate occasions.

The statistical analysis was done using the Tukey-Kramer multiple comparison test using the Instat® (GraphPad Software,

Table 1. The MIC (µg/ml) of five antifungal agents for *C. albicans* and *C. tropicalis* isolates studied

Isolate	Nystatin	Amphotericin-B	Ketoconazole	Fluconazole	5-Fluorocytosine
<i>Candida albicans</i>					
<i>C. albicans</i> (n=10)	0.78–1.56	0.19–0.38	0.012–0.016	0.125–0.38	0.094–0.125
<i>C. albicans</i> ATCC 90028	1.56	0.38	0.012	0.125	0.125
<i>Candida tropicalis</i>					
<i>C. tropicalis</i> (n=10)	0.78	0.25–0.38	0.064–0.125	0.25–0.50	0.094–0.125
<i>C. tropicalis</i> ATCC 13808	0.78	0.38	0.064	0.25	0.125

Table 2. The post-antifungal effect (in h \pm SEM) of five different antifungal agents on ten oral *Candida albicans* isolates following 1-h exposure to twice the MIC of the drugs

Isolate	Nystatin	Amphotericin B	Ketoconazole	Fluconazole	5-Fluorocytosine
HK1KD	12.87 \pm 0.40	6.44 \pm 0.26	0.51 \pm 0.11	-0.37 \pm 0.06	2.18 \pm 0.13
HK2OB	13.33 \pm 1.20	9.40 \pm 0.82	0.51 \pm 0.17	-0.70 \pm 0.16	2.50 \pm 0.26
HK4RB	11.95 \pm 0.32	11.11 \pm 0.54	1.22 \pm 0.23	-0.23 \pm 0.11	2.45 \pm 0.35
HK5SD	10.07 \pm 0.71	9.03 \pm 0.29	1.85 \pm 0.55	0.05 \pm 0.18	3.80 \pm 0.31
HK6SC	12.78 \pm 0.14	10.46 \pm 0.23	1.02 \pm 0.26	-0.37 \pm 0.06	2.13 \pm 0.22
HK8CA	12.73 \pm 0.18	10.51 \pm 0.28	1.39 \pm 0.29	-0.37 \pm 0.06	2.08 \pm 0.14
HK9TB	12.27 \pm 0.39	10.92 \pm 0.36	1.25 \pm 0.24	-0.03 \pm 0.11	2.32 \pm 0.20
HK10OD	12.36 \pm 0.28	10.60 \pm 0.33	2.09 \pm 0.56	-0.36 \pm 0.06	3.15 \pm 0.35
HK36SC	11.81 \pm 0.45	11.02 \pm 0.28	0.42 \pm 0.36	-0.19 \pm 0.21	1.48 \pm 0.43
HK39RE	12.96 \pm 0.56	9.77 \pm 0.22	1.25 \pm 0.19	-0.09 \pm 0.14	1.57 \pm 0.18
Mean	12.31 \pm 0.2	9.93 \pm 0.21	1.14 \pm 0.12	-0.27 \pm 0.05	2.37 \pm 0.12

Table 3. The post-antifungal effect (in h \pm SEM) of five different antifungal agents on ten oral *Candida tropicalis* isolates following 1-h exposure to twice the MIC of the drugs

Isolate	Nystatin	Amphotericin B	Ketoconazole	Fluconazole	5-Fluorocytosine
HK1KA	14.63 \pm 0.23	11.62 \pm 0.59	1.76 \pm 0.21	-0.14 \pm 0.14	4.17 \pm 0.24
HK1KE	12.55 \pm 0.89	10.05 \pm 0.44	1.44 \pm 0.30	0.09 \pm 0.14	2.92 \pm 0.43
HK4LA	15.14 \pm 0.59	12.64 \pm 0.47	2.27 \pm 0.39	0.05 \pm 0.11	6.44 \pm 0.93
HK5LG	14.54 \pm 0.36	12.08 \pm 0.27	1.95 \pm 0.37	-0.47 \pm 0.06	4.54 \pm 0.24
HK5LF	14.17 \pm 0.57	12.32 \pm 0.51	2.13 \pm 0.23	-0.05 \pm 0.15	4.72 \pm 0.39
HK9LF	15.19 \pm 0.33	13.24 \pm 0.28	1.80 \pm 0.21	-0.09 \pm 0.06	3.01 \pm 0.29
HK9LG	15.33 \pm 0.47	12.73 \pm 0.29	2.59 \pm 0.26	-0.42 \pm 0.06	5.51 \pm 0.42
HK36LA	15.23 \pm 0.50	13.33 \pm 0.57	2.59 \pm 0.28	-0.05 \pm 0.09	4.40 \pm 0.15
HK44TD	17.50 \pm 0.46	14.81 \pm 0.67	1.94 \pm 0.41	0.05 \pm 0.11	4.44 \pm 0.14
HK44LF	14.07 \pm 0.46	11.34 \pm 0.53	1.80 \pm 0.25	-0.14 \pm 0.10	3.93 \pm 0.23
Mean	14.83 \pm 0.22	12.42 \pm 0.21	2.03 \pm 0.10	-0.12 \pm 0.04	4.41 \pm 0.18

Inc., San Diego, CA, USA) programme. A *P* value less than 0.05 was deemed significant.

Results

The minimum inhibitory concentration of 10 isolates of *C. albicans* and 10 isolates of *C. tropicalis* to nystatin, amphoterecin-B, ketoconazole, fluconazole and 5-fluorocytosine are given in Table 1. All the strains tested were susceptible to the antifungal agents tested according to the NCCLS criteria. The two-fold MIC concentrations chosen for the current study were based on these results.

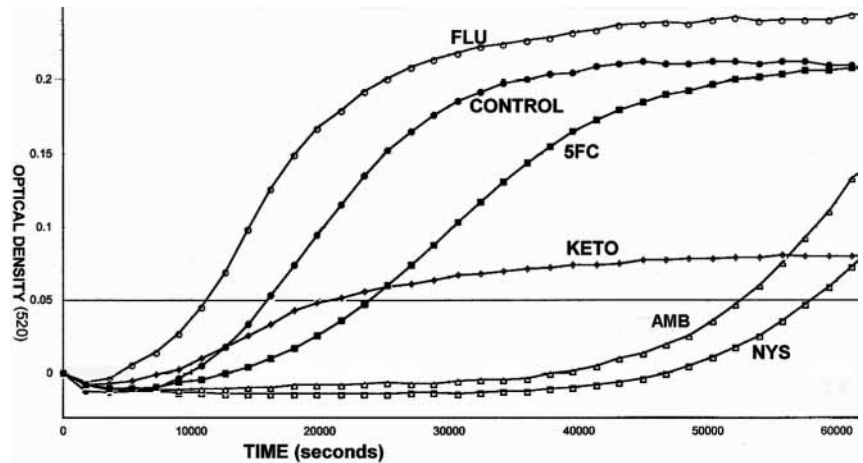
The *in vitro* PAFE exerted by the five antifungal agents on *C. albicans* and *C. tropicalis* isolates after 1-h exposure to twice MIC values is shown in Tables 2 & 3. The kinetic pattern of yeast growth induced consequent to the brief antifungal exposure and subsequent removal of the drug is depicted in Fig. 1. With all the control organisms tested, the lag phase of growth was approximately 1 h, after which the exponential phase of growth was seen for up to 3 h, and it took up to 15 h to reach the stationary phase (Fig.

1). On the other hand, nystatin and amphotericin B had the greatest impact on yeast growth in general and elicited the highest PAFE compared with the other drugs. In the case of fluconazole, normal growth resumed immediately after drug removal with all the isolates. Ketoconazole and flurocytosine elicited growth inhibition, and hence a PAFE intermediate between the polyenes and the fluconazole (Fig. 1).

Significant PAFE were induced in all 10 oral *C. albicans* and all 10 oral *C. tropicalis* isolates by nystatin, amphotericin-B, ketoconazole and 5-fluorocytosine. In contrast, the fluconazole elicited a negative PAFE in both *C. albicans* and *C. tropicalis*. The mean PAFE of nystatin for *C. albicans* and *C. tropicalis* were 12.31 h and 14.83 h, respectively. While exposure to amphotericin B elicited a PAFE of 9.93 h and 12.42 h for *C. albicans* and *C. tropicalis*, respectively. On the other hand the mean PAFEs elicited by ketoconazole and 5-fluorocytosine were relatively shorter and lasted for 1.14–2.03 h, and 2.37–4.4 h for both *Candida* species, respectively. Taken together consistent and prolonged growth suppression was seen with the polyenes for all the isolates tested.

Interestingly, significant inter-species variations in PAFE were noted with all drugs, except fluconazole (Table 4). Ketoconazole and

Fig. 1. Growth curve demonstrating the PAFE elicited by five different antifungals ($2\times$ MIC) against *Candida*. Note the prolonged PAFE elicited by polyenes, and the negative PAFE of fluconazole. (FLU=fluconazole, 5FC=5-fluorocytosine, KETO=ketoconazole, AMB=amphotericin B, NYS=nystatin).



5-fluorocytosine were significantly more potent and elicited 78–86% growth suppression of *C. tropicalis* isolates, as compared with *C. albicans*. This inter-species difference in PAFE, though significant, was much less (17–20%) with the polyene drug.

Discussion

Oropharyngeal candidiasis is a notable cause of morbidity among HIV-infected and AIDS patients (23). While *C. albicans* is generally considered to be the most pathogenic of the species, a number of other species of this genus, notably *C. krusei*, *C. glabrata*, *C. tropicalis* and *C. dubliniensis*, have been cited as the causative agents of an increasing number of infections (24, 25).

As the frequency of fungal infections increases, there is a concomitant need for increased understanding of the pharmacodynamic properties of the antifungals available and those recently introduced. A number of effective antifungal agents are currently used for the treatment of oropharyngeal candidiasis. However, their efficacy and failure is dependent upon many factors. In the mouth, the diluent effect of saliva and the cleansing action of the oral musculature often tend to reduce the availability of the drug below that of the effective therapeutic concentration. Thus the organisms un-

dergo only a limited exposure to the antifungal agents during treatment, and the concentration of the drug tends to vary in different niches of the oral cavity. The local environmental factors are likely to have an impact on the therapeutic efficacy, but are given little regard in drug delivery regimens.

Nystatin, amphotericin B, 5-fluorocytosine, ketoconazole and fluconazole have all been used for treatment of *C. albicans* infections. The action of these agents has been described as either fungicidal or fungistatic (26). Amphotericin B, is described as having a concentration dependent fungicidal activity compared to azoles, which have a concentration independent fungistatic activity (27). Although the goal of treatment with antifungal agents is to maintain the drug concentration above the MIC for almost the entire dosing period (7), whether this is achieved intra-orally is questionable due to the aforementioned factors. Therefore, in treating candidal infections, it would be desirable if the clinicians could rely on *in vitro* susceptibility tests to obtain critical information about antifungal dosage regimens (28).

The post-antifungal effect (PAFE) is the suppression of fungal growth that persists after limited exposure to an antifungal agent. The PAFE may therefore have clinical relevance in the design of dosing regimens of the antifungal agents. Antimicrobials with a prolonged PAFE may be given less frequently than those with a short PAFE, as the latter may require more frequent administration.

Table 4. The relative PAFE (in h \pm SEM) of five different antifungal agents against ten oral isolates of *C. albicans* and 10 of *C. tropicalis*

	Nystatin	Amphotericin B	Ketoconazole	Fluconazole	5-Fluorocytosine
<i>C. albicans</i> (n=10)	12.31 \pm 0.2	9.93 \pm 0.21	1.14 \pm 0.12	-0.27 \pm 0.05	2.37 \pm 0.12
<i>C. tropicalis</i> (n=10)	14.83 \pm 0.22	12.42 \pm 0.21	2.03 \pm 0.10	-0.12 \pm 0.04	4.41 \pm 0.18
Percentage difference in PAFE values	17	20	78	-	86
P value	<0.001	<0.001	<0.001	NS	<0.001

NS=not significant.

Even though PAFE can be used as a useful indicator for this purpose, limited literature is available and a standard protocol is yet to be devised. Most of the previous studies highlighted the effect of PAFE and the concentrations of the drugs on a limited number of isolates. Hence, in the present study, we investigated the PAFE of 10 isolates of *C. albicans* and 10 of *C. tropicalis* obtained from HIV-infected subjects. To our knowledge PAFE of *C. tropicalis* isolates has not been described previously.

It is critical to determine the MIC of a drug prior to evaluating the PAFE, in order to ascertain the exposure concentration of the drug for the latter assay. The MIC of nystatin was determined using the broth microdilution method, according to National Committee for Clinical Laboratory Standards guidelines (M27-A) (18). The MIC of amphotericin B, 5-fluorocytosine, ketoconazole and fluconazole were estimated using the E-test. The E-test is a reliable, quality controlled commercial method for the quantitative determination of antimicrobial MICs. Multicentre studies have shown a high level of agreement between the NCCLS broth microdilution reference method and the E-test (19). The MIC values of five antifungal agents used against all 20 isolates were within the range of NCCL standards (18), and none of the organisms was therefore deemed to be resistant isolates.

The PAFE of antifungal agents is evaluated either by using viable counts (15) or turbidometric measurement of fungal growth (7). We used the turbidometric assay method for this purpose. The Spectramax machine (Spectramax 340: Tunable Microplate Reader) employed enabled easy, automatic and reliable quantification of yeast growth in terms of increased turbidity, as reported for bacteria and fungi in previous studies (13, 14). The sensitivity of the machine was good, as replicate experiments yielded only minimal growth differentials in the tested organisms and did not yield high standard errors of means usually witnessed in conventional techniques that rely on surface growth and colony-forming unit (CFU) determinations (29).

In contrast to the previous PAFE studies where the drug concentration varied from 2 to 8 times the MIC, we used a standard drug concentration, i.e., twice the MIC and the exposure time was limited to 1 h. The polyenes, nystatin and amphotericin B, elicited a prolonged PAFE with all the isolates belonging to two different species tested. This observation is in agreement with the previous reports of Ellepola & Samaranayake (14) where a number of *C. albicans* isolates exhibited this property. The polyene alters the permeability of the cytoplasmic membrane by binding sterols, principally ergosterol and fungisterol (30).

Perhaps the most important observation in the current study is the consistency and significantly high PAFE ($P < 0.001$) of *C. tropicalis* with four of five drugs tested, as compared with *C. albicans*.

This could be regarded as another indicator of the higher virulence potential of *C. albicans* compared with *C. tropicalis*, as the former recovers in a relatively short period after an antifungal insult. This result was obtained in spite of the absolute concentration of the antifungals used for pre-exposure of the organisms (i.e., $2 \times \text{MIC}$) being significantly less than that for *C. tropicalis* compared with *C. albicans*. In an earlier study, using nystatin alone, we reported similar observations with non-*albicans* strains (14). Whether this is the case with other non-*albicans* *Candida* species is not known and hence further studies are necessary to substantiate this observation.

Fluconazole did not produce a measurable PAFE against *C. albicans* or *C. tropicalis*. This observation is consistent with previously published reports (15, 22, 31). The azoles cause alterations in fungal cell membranes by blocking the 14 α -demethylation step in the biosynthesis of ergosterol. The consequent depletion of ergosterol and accumulation of 14 α -methyl-sterols leads to alterations in a number of membrane-associated functions (32). The lack of a PAFE when exposed to these agents may be due to the fact that the above effect is readily reversible or that the limited exposure period of 2 h is inadequate for the drugs to produce a lasting effect.

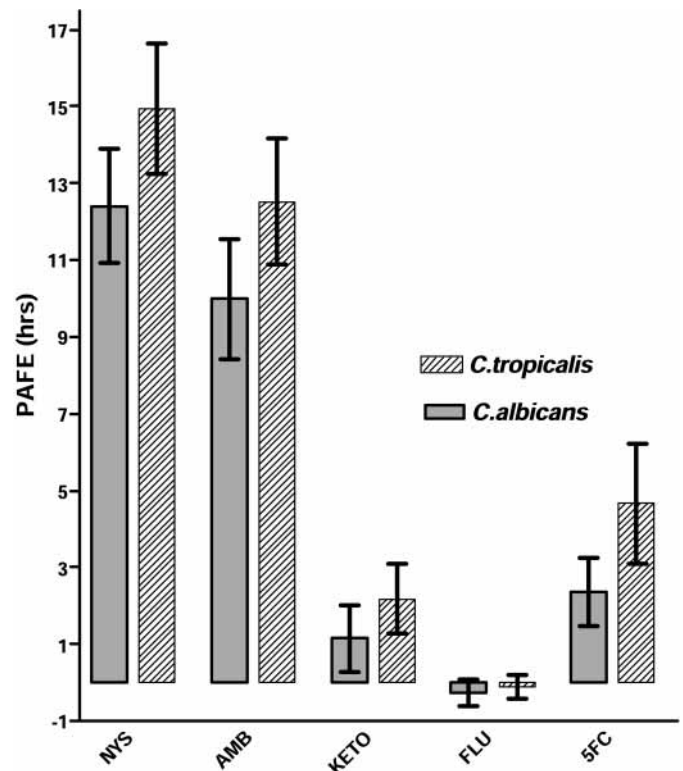


Fig. 2. The mean PAFE (\pm SD) of 10 isolates of *C. albicans* and 10 of *C. tropicalis* exposed to twice the MIC concentrations of five different antifungal agents. (FLU=fluconazole, 5FC=5-fluorocytosine, KETO=ketoconazole, AMB=amphotericin B, NYS=nystatin).

Ketoconazole, as compared with fluconazole, did exert a PAFE, but to much lesser degree than the polyenes (Fig. 2). Although all the azoles act on cell membranes of *Candida*, the pharmacokinetics of ketoconazole, which is an imidazole, differs from the recently introduced fluconazole, which are classified as bis-triazoles (33, 34).

The PAFE of 5-fluorocytosine was found to be significantly higher than that of ketoconazole, and was also significantly prolonged for *C. tropicalis* compared with *C. albicans*, resembling the effect observed with the polyenes. The agent is a DNA analogue and once transported into the fungal cell it is converted into 5-fluorouracil, which is incorporated into RNA in place of uracil, with resultant abnormalities of protein synthesis (32). It also inhibits DNA synthesis by binding to thymidylate synthetase (35). The long PAFE observed with 5-fluorocytosine (although shorter than cell wall acting polyene agents) may represent the period in which DNA synthesis is suppressed before cell division commences (15). Though not used in the management of oral candidiasis, 5-fluorocytosine is widely used in systemic *Candida* infections, particularly in the immunocompromised.

When the intra-species differences were compared, the two polyenes, 5-fluorocytosine and ketoconazole elicited significant intra-species variations in PAFE. This finding adds credence to the fact that the properties of each isolate tested could determine the PAFE, irrespective of the other environmental conditions and the drug being tested. Hence results from a single isolate should not be extrapolated to define a PAFE, for an individual species of *Candida*, and a large number of isolates should be evaluated as in the case of MIC determinations. As we used 10 isolates of *C. albicans* and 10 of *C. tropicalis* for the PAFE assessment, the current data could be regarded as baseline information for future workers in this area.

In clinical terms, the current findings imply that the optimal antifungal activity of polyenes may be realized even if they are administered at extended intervals, while this would not be the case for azoles. However, how this translates into clinical practice needs to be further evaluated in view of the *in vivo* and *in vitro* paradoxical MIC results that have been described for fluconazole in particular (36). Further studies with varying concentration of the drugs may perhaps help extrapolate the dose dependence of PAFE. Interestingly, in a recent study, Ernst et al. (31) failed to display any measurable PAFE after exposing *C. albicans* to varying concentrations of fluconazole, which agrees with the current data. They also found prolonged PAFE with varying concentrations of the novel echinocandin antifungal MK-0991, *in vitro*.

To conclude, the forgoing is the first comprehensive report on PAFE for two different *Candida* species, with five different antifungal agents. Further studies with other non-*albicans* species are required to broaden the PAFE database of these common opportunist

yeasts that cause recalcitrant oral infections in compromised populations.

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