

# Impact of Brief and Sequential Exposure to Nystatin on the Germ Tube Formation and Cell Surface Hydrophobicity of Oral *Candida Albicans* Isolates from Human Immunodeficiency Virus-Infected Patients

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## Key Words

*Candida albicans* · Cell surface hydrophobicity · Germ tubes · Nystatin

## Abstract

**Objective:** To evaluate the impact of brief and sequential exposure to nystatin on the germ tube formation and cell surface hydrophobicity of oral isolates of *Candida albicans* obtained from patients infected with human immunodeficiency virus (HIV). **Materials and Methods:** After determining the minimum inhibitory concentration of nystatin, 10 oral isolates of *C. albicans* from 10 different HIV-infected patients were briefly (1 h) and sequentially (10 days) exposed to subtherapeutic concentrations of nystatin. Following a subsequent drug removal, the germ tube formation and cell surface hydrophobicity of these isolates were determined via a germ tube induction assay and an aqueous hydrocarbon assay, respectively. The data obtained from these assays for the control (unexposed to nystatin) and nystatin-exposed isolates were analyzed using Student's t tests. **Results:** The mean percentage reduction in the germ tube formation and cell surface hydrophobicity of the nystatin-exposed isolates compared to the controls was  $30.12 \pm 1.99$  ( $p < 0.001$ ) and  $29.65 \pm 2.33$  ( $p < 0.001$ ), respectively. **Conclusion:** These data

elucidate the possible pharmacodynamic mechanisms by which nystatin might operate in vivo in the modulation of candidal virulence.

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## Introduction

More than 90% of human immunodeficiency virus (HIV)-infected individuals develop oral candidosis at some point during the disease, and it is by far the commonest oral manifestation in these patients. In HIV-infected patients, persistent debilitating pseudomembranous oral mucosal disease has been linked to oral *Candida* and has been postulated to be intimately associated with the level of host immunosuppression. Also, oral candidosis and the amount of *Candida* present have been postulated to be predictive of the HIV disease progression and viral load [1]. Among all *Candida* species, *Candida albicans* is considered the most virulent and pervasive fungal pathogen implicated in oral candidosis.

Multiple factors have been implicated in the potentiation of the *C. albicans* pathogenicity, and its adherence to host mucosal surfaces is a major determinant of successful microbial colonization and subsequent infection [2].

Adhesion enables the organisms to avoid dislodgement due to the cleansing action of mucosal secretions and facilitates infection. Germ tubes (GT), which mark the onset of hyphal growth, are a biological trait that has been implicated in the pathogenesis of oral candidosis, as these cylindrical extrusions are known to facilitate yeast adherence to epithelial cells and impart resistance to phagocytic killing [3, 4]. Furthermore, GT tend to promote the aggregation of yeast cells and bridging of adjacent hyphal elements, thereby bringing a large battery of organisms in intimate contact with the oral epithelium [5]. *Candida* hyphae have also been shown to penetrate dentinal tubules along the cracks of tooth surfaces, enabling the organisms to invade dental hard tissues [6]. In addition, microbial cell surface hydrophobicity (CSH), which contributes to hydrophobic interactions between cells and surfaces, is thought to be a nonbiological factor associated with the adherence of *Candida* to inert surfaces [7]. Studies have also shown that hydrophobic yeasts are more virulent than their hydrophilic counterparts [8, 9]. Significant correlations between CSH and candidal adhesion to buccal epithelial cells and denture acrylic surfaces have also been previously reported [10, 11].

Nystatin is a widely available antifungal agent for the topical treatment of oral candidosis. However, the diluting effect of saliva and the cleansing effect of the oral musculature in the oral cavity tend to reduce the availability of topically applied antimycotics below that of effective therapeutic concentrations, thereby compromising their therapeutic efficacy [12, 13]. Hence, the opportunistic yeasts may undergo brief and sequential exposure to topically applied antifungal drugs during therapy, a scenario which is all too common in the niches of the oral cavity [12, 13]. To our knowledge, there is no information on the impact of brief and sequential exposure to nystatin on the colonization traits of oral *C. albicans* isolates obtained from HIV-infected patients. Thus, the aim of this study was to determine the effect of such exposure to nystatin on the GT formation and CSH of oral isolates of *C. albicans* from HIV-infected patients.

## Subjects and Methods

### Organisms

The following oral *C. albicans* isolates were studied: HK1 Kd (N1), HK3 Ob (N2), HK4 Rb (N3), HK5 Sd (N4), HK6 Sc (N5), HK8 Ca (N6), HK9 Tb (N7), HK10 Od (N8), HK36 Sc (N9), and HK39 Re (N10). These isolates were derived from 10 different HIV-infected patients attending the AIDS Unit of the Department of Health (Hong Kong, SAR, China). *C. albicans* ATCC 90028 was

used as the reference strain. Initially, all of the yeast isolates were tested for GT formation and thereafter they were identified by their carbohydrate assimilation profiles obtained using API 20C Aux yeast identification kits (API System, Vercieu, France) at the Prince Philip Dental Hospital (Hong Kong, SAR, China). Stock cultures were maintained at  $-20^{\circ}\text{C}$ . After recovery, these were maintained on Sabouraud's dextrose agar (SDA), stored at  $4-6^{\circ}\text{C}$ , during the experimental period.

### Antifungal Agents and Media

As described previously [12–15], nystatin (Sigma, St. Louis, Mo., USA) was dissolved in dimethyl sulfoxide (DMSO) and absolute ethanol (3:2 ratio), respectively, and was initially prepared as a 10,000- $\mu\text{g}$  solution and stored at  $-20^{\circ}\text{C}$  before use. It was thereafter suspended in the following medium during the period of exposure to yeasts (1 h): Rosewell Park Memorial Institute (RPMI) 1640 medium buffered with 0.165 M morpholinopropane-sulfonic acid (MOPS) containing L-glutamine and lacking sodium bicarbonate (Sigma) in 1 liter of sterile distilled water adjusted to a pH of 7.2 and filter sterilized [12–15]. This liquid (RPMI) was stored at  $2-8^{\circ}\text{C}$ . The stock solution was used to obtain the drug concentrations [i.e.  $2\times$  the minimum inhibitory concentration (MIC)] used in the experiments.

Since nystatin was dissolved in DMSO and absolute ethanol, equivalent amounts of the latter chemicals were tested initially as was done in previous studies using the same isolates to ascertain whether they had an effect on the isolates tested. Minute amounts of the chemicals used in this study did not have any effect on yeast survival/growth when compared to the controls.

### Determination of the MIC

Prior to the brief and sequential exposure to nystatin, the MIC values of nystatin were determined via the broth dilution technique as described previously [12–15] by performing 2-fold serial dilutions of the drug in microtiter plates using an inoculum of  $1-5 \times 10^5$  CFU/ml. The MIC was determined visually and spectrophotometrically at 595 nm following 24 h of incubation at  $37^{\circ}\text{C}$ . The MIC was defined as the lowest concentration of the drug that inhibited the growth of yeast cells, as indicated by the absence of turbidity (optically clear). The MIC was read independently by two independent observers. *C. albicans* ATCC 90028 was used as a reference strain. All experiments were repeated on two separate occasions with duplicate determinations on each occasion.

### Preparation of the Cell Suspension for the GT Assay and the Relative CSH Assay

A previously described method was used for this purpose [12–15]. Briefly, yeast cells maintained on SDA were inoculated onto fresh plates and incubated overnight at  $37^{\circ}\text{C}$  for 24 h prior to use. The organisms were harvested and a cell suspension was prepared in sterile PBS with a pH of 7.4 to an optical density (OD) of 1.5 at 520 nm. One milliliter of this cell suspension was added to tubes containing 4 ml of RPMI broth (control) and 4 ml of RPMI/drug solution (test), in which the drug concentrations were twice the MIC values. This yielded a cell suspension of  $10^6$  cells/ml in each assay tube. The tubes were then incubated at  $37^{\circ}\text{C}$  for a period of 1 h in a rotary incubator. Following this limited exposure, the drugs were removed by 2 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 5 ml

**Table 1.** Impact of brief (1 h) and sequential (10 days) exposure to nystatin (2× the initial MIC) on the susceptibility of oral *C. albicans* isolates to this drug

Isolate	Initial MIC, µg/ml	MIC following exposure to nystatin, µg/ml
N1	0.78	1.56
N2	0.78	1.56
N3	1.56	1.56
N4	1.56	1.56
N5	1.56	0.78
N6	1.56	0.78
N7	1.56	1.56
N8	0.78	1.56
N9	1.56	0.78
N10	1.56	0.78

of sterile PBS. One hundred microliters of this cell suspension were then inoculated onto SDA plates and incubated at 37°C for 24 h. Thereafter, the organisms were harvested and once again exposed to the drug as mentioned previously. This procedure was performed for 10 consecutive days, after which the cultures of the drug-treated *Candida* (test) and the *Candida* unexposed to nystatin (control) were inoculated onto SDA plates and incubated at 37°C for 24 h. Thereafter, these cultures were maintained on SDA and stored at 4–6°C prior to being used for the GT and CSH assays. Hence, this procedure allowed the *Candida* exposed to the drug to recover and grow after the final treatment with nystatin.

The aforementioned procedure for drug removal has been shown to reduce the concentration of the drug by as much as 10,000-fold, thereby minimizing any carryover effect of the drug following its removal [12–18]. Viable counts of the control and the test were performed after each procedure of drug removal. As the procedure of drug removal effectively eliminated any carryover effect, there was virtually no difference between the viable counts of the control and tests following exposure to the already diluted sub-therapeutic concentrations of the drug as observed in previous studies [12–18].

#### Microscopic Quantification of GT Forming Cells (GT Assay)

A previously used method for GT induction was employed [15, 16]. RPMI 1640 medium with L-glutamine (Sigma) was chosen for the assay because it effectively induces GT formation. Yeast suspensions of the control and test (following brief and sequential exposure) were prepared in sterile PBS at 520 nm to an OD of 1.5. For GT induction, 250 µl of the yeast suspensions of the control and test (following brief and sequential exposure) were added to 1 ml of RPMI 1640 medium with L-glutamine and incubated at 37°C for 90 min.

Then, the tube was vortex mixed for 10 s and a drop of each cell suspension was placed on a Neubauer hemocytometer chamber and covered with a coverslip for GT quantification. Thereafter, 300 yeast cells in contiguous fields were counted (under a ×40 magnification) and the percentage of GT-forming cells was calculated. The following previously used criteria were employed for counting [15, 16]: (a) only yeast cells with a GT, without constriction at the

junction between the cell and the elongation, were counted; (b) clumped cells with GT were excluded, and (c) pseudohypha-forming yeast cells were excluded.

#### Relative CSH Assay

A biphasic aqueous hydrocarbon assay previously used for the assessment CSH in oral *Candida* species was used in the current study [14, 17, 18]. In brief, 5 ml of the control and test (following brief and sequential exposure) yeast suspensions were adjusted to an OD of 0.442–0.448 at 520 nm to obtain a cell concentration of McFarland standard 7.0 (Beckman DU 530 spectrophotometer; Life Science, USA). For each organism tested (with and without exposure to nystatin), 2.5-ml volumes of suspension were added to 2 sterile glass test tubes (16 × 150 mm; 20 ml), representing 1 test and 1 control. Xylene (0.5 ml) was added to each test suspension. The test and controls were placed in a water bath at 37°C for 10 min to equilibrate; they were then taken and vortex mixed for 30 s and returned to the water bath for a further 30 min to allow the immiscible xylene and aqueous phases to separate. The lower, aqueous phase of the sample was carefully removed using a pipette and transferred to a clean test tube. Any traces of contaminating xylene that may have been carried over in the pipette or bound to the yeast were removed by bubbling air through the suspension at a rate of 180 ml/min for 2 min. The absorbance was measured as before at 520 nm (Beckman DU 530 spectrophotometer; Life Science) after vortex mixing for 5 s. Hydrophobicity was expressed as the percentage reduction in OD of the test suspension compared to the control [14, 17, 18]. Thus, the greater the change in absorbance was, the greater the shift in yeasts from the bulk medium to the interface was (i.e. the more hydrophobic the yeast strain). Suspensions without xylene were used as the negative controls.

All experiments were repeated on 3 separate occasions, with duplicate determinations on each occasion.

#### Statistical Analysis

The data obtained from the GT and CSH assays performed thrice in duplicate were analyzed using Student's t tests (SPSS version 19.0), which treat one group as a control (unexposed to nystatin) and compare the other group (exposed to nystatin) against it.  $p < 0.05$  was considered statistically significant.

## Results

The MIC (µg/ml) values of the 10 isolates of *C. albicans* to nystatin prior to exposure to nystatin and following brief and sequential exposure to this drug are given in table 1. In both instances, the MIC values ranged from 0.78 to 1.56. Hence, all isolates were susceptible to nystatin, with no apparent change in MIC values. These values remained within the susceptible range for this drug.

The mean percentage of GT-positive cells in the 10 *C. albicans* isolates unexposed to nystatin and following brief and sequential exposure to this antifungal, drug removal, and the subsequent observation of GT formation

**Table 2.** GT-positive cells and mean reduction in the GT formation of oral *C. albicans* isolates following brief (1 h) and sequential (10 days) exposure to nystatin (2× the initial MIC)

Isolate	GT-positive cells in unexposed controls <sup>a</sup> , %	GT-positive cells following exposure to nystatin <sup>a</sup> , %	Reduction in GT formation <sup>b</sup> , %
N1	26.42±1.24	18.34±0.71	30.58
N2	29.54±1.00	24.96±0.67	15.50
N3	29.78±0.60	21.72±0.94	27.07
N4	32.16±1.13	20.25±0.62	37.03
N5	28.32±0.66	18.76±0.64	33.76
N6	24.58±0.64	16.14±0.66	34.34
N7	31.23±1.01	20.76±0.68	33.53
N8	21.59±0.96	14.75±1.17	31.68
N9	22.36±0.86	14.93±1.07	33.23
N10	30.78±0.54	23.26±0.42	24.43
Mean	27.68	19.38	30.12
SEM	1.19	1.09	1.99
p			<0.001

<sup>a</sup> Mean data ± SEM of 3 experiments done in duplicate.

<sup>b</sup> Obtained by comparing the mean values of drug-exposed isolates to those of unexposed isolates.

**Table 3.** Relative CSH and mean percentage reduction in the CSH of oral *C. albicans* isolates following brief (1 h) and sequential (10 days) exposure to nystatin (2× the initial MIC)

Isolate	CSH of unexposed controls <sup>a</sup>	CSH following exposure to nystatin <sup>a</sup>	Reduction in CSH <sup>b</sup> , %
N1	19.67±0.52	11.78±0.88	40.11
N2	14.04±1.32	8.56±0.36	39.03
N3	19.58±0.40	14.58±1.27	25.53
N4	16.26±0.59	10.57±0.62	34.99
N5	16.78±0.76	13.52±0.71	19.42
N6	12.36±0.96	10.02±0.58	18.93
N7	15.78±0.69	10.68±0.63	32.32
N8	20.56±0.91	14.26±1.37	30.64
N9	21.32±1.10	14.93±1.36	29.97
N10	12.52±1.03	9.32±0.51	25.56
Mean	16.89	11.82	29.65
SEM	1.04	0.74	2.33
p			<0.001

<sup>a</sup> Mean data ± SEM of 3 experiments performed in duplicate.

<sup>b</sup> Obtained by comparing the mean values of drug-exposed isolates to those of unexposed isolates.

was 27.68 ± 1.19 (range 21.59–32.16) and 19.38 ± 1.09 (range 14.75–24.96), respectively, and the mean percentage reduction was 30.12 ± 1.99% (range 15.5–37.03; p < 0.001; table 2).

The mean CSH of the 10 *C. albicans* isolates unexposed to nystatin and following brief and sequential exposure to nystatin, drug removal, and the subsequent determination of CSH via a biphasic aqueous hydrocarbon assay was 16.89 ± 1.04 (range 12.36–21.32) and 11.82 ± 0.74 (range 8.56–14.93), respectively, and the mean percentage reduction was 29.65 ± 2.33% (range 18.93–40.11; p < 0.001; table 3).

## Discussion

In this study, the MIC values of nystatin before and after brief and sequential exposure to this drug were within the range of the reference strain (0.78–1.56 µg/ml), which implied that the isolates tested were susceptible to the drug despite multiple episodes of drug exposure. Furthermore, these values were within the MIC range obtained in previous investigations of *C. albicans* isolates [12, 14, 15]. Therefore, it could be speculated that such brief (1 h) and sequential (10 days) exposure to subthera-

peutic concentrations of nystatin does not have a significant impact on the susceptibility of the isolates and is unlikely to induce the development of drug resistance despite such exposure.

The reduction in GT formation of 30.12% following limited and sequential exposure to nystatin seen in this study may be related to the mechanism of action of this drug on the *Candida* cell wall. Polyenes bind avidly to the sterol components in the cell wall of *Candida*, thereby making it more permeable and leading to the impairment of barrier function, the leakage of cellular components, and metabolic disruption [12, 13]. Also, it has been suggested that the formation of sterols or their precursors may be inhibited by polyenes [12, 13]. Other investigations [15, 19] have shown internally collapsed cells with an intact cell wall that leave behind ‘ghosts-like’ cells and deflated *Candida* cells following exposure to subtherapeutic concentrations of nystatin [15]. Studies have also demonstrated dynamic changes in the ultrastructural features of the cell wall during morphogenic transformation to GT and have shown that the cell wall of GT possesses a stratification comparable to that of the blastospore wall [19]. Therefore, it is not surprising that nystatin-induced changes in the cell wall structure of *C. albicans* isolates affect active budding and multiplication, thus suppress-

ing the GT formation following brief and sequential exposure to nystatin.

Similar to that in GT formation, a reduction in CSH of 29.65% following a limited and sequential exposure to nystatin was also seen in this study. The microbial structures that contribute to CSH include outer membrane proteins, lipoproteins, phospholipids, lipopolysaccharides, and fimbriae [20]. Thus, drugs that modify these structural features have been shown to reduce the CSH of microbes [21]. In the case of *C. albicans*, it has been shown that CSH correlates with the concentration of fibrils in the exterior layer of the cell wall. Hence, the observed reduction in CSH elicited by nystatin in *C. albicans* may be related to the aforementioned pharmacodynamics of nystatin on the cell wall of *C. albicans*. Therefore, it is reasonable to speculate that by affecting the cell wall structure nystatin may be capable of suppressing the CSH of this *Candida* species.

In previous studies, we demonstrated that a single brief exposure to nystatin induced a 97.68% reduction in GT formation and a 27.14% reduction in CSH in oral isolates of *C. albicans* [14, 15]. This almost complete abrogation of GT formation (97.68% reduction) may have been due to the fact that these studies were conducted soon after drug removal, when the isolates were in a state of nystatin-induced metabolic shock, whereas the current study was conducted after a recovery period of these isolates, resulting in less of an impact on GT formation (30.12%). In contrast, there was virtually no difference in the impact of nystatin on CSH between these previous studies (27.14% reduction) and the current study (29.65% reduction), which may be due to the lower sensitivity of nystatin on nonbiological traits such as CSH compared to biological attributes such as GT formation. In fact, a re-

cent study on the effect of nystatin on the adhesion attributes of *C. dubliniensis* isolates failed to show a relationship between these two adhesion characteristics following exposure to nystatin [22]. Irrespectively of these differences, it is reasonable to venture that by affecting the cell wall structure nystatin may be capable of subduing both biological (GT formation) and nonbiological (CSH) adhesion traits of *Candida* species.

The resistance of *C. albicans* to azoles and echinocandin antifungal agents has been documented [23], which may have important implications for antifungal therapy in the management of oral candidosis in HIV infection and indicates the need for alternative therapies. In this context, the current findings enhance the usage of nystatin as a mainstay topical agent in the management of oral candidosis.

## Conclusion

The current results show that brief and sequential exposure to nystatin is capable of modulating *C. albicans* adhesion by reducing the GT formation as well as its CSH and thereby adds further credence to the effectiveness of nystatin against these colonization attributes of *C. albicans*.

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