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HIV-1 ANTI-RETROVIRAL DRUG EFFECT ON THE *C. ALBICANS* HYPHAL GROWTH RATE BY A BIO-CELL TRACER SYSTEM

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ABSTRACT

Declining incidence of oropharyngeal candidosis and opportunistic infections over recent years can be attributed to the use of highly active anti-retroviral therapy (HAART). Infection with *C. albicans* generally involves adherence and colonization of superficial tissues. During this process, budding yeasts are able to transform to hyphae and penetrate into the deep tissue. Using the biocell tracer system, *C. albicans* hyphal growth was dynamically observed at the cellular level. Ritonavir was effective in the inhibition of hyphal growth with growth rate of 0.8 μm/min. This study showed the *in vitro* effect of HIV anti-retroviral drug on the growth rate of the *C. albicans* hyphae.

Key words: *Candida*, hyphal, protease inhibitor

INTRODUCTION

Oropharyngeal candidosis is a frequent opportunistic mycosis in immunocompromised patients. The main causative agent of this infection is *Candida albicans* (30). *C. albicans* is a dimorphic fungus with ability to transform between yeast and hyphal cells. Both forms are invariably present in lesions. Evidence suggests that the mycelial form is important in the pathogenesis of candidoses (30). Putative virulence factors of *C. albicans* include cell wall adhesion, phenotypic switching, hyphal formation, thigmotropism and secretion of proteases and others hydrolytic enzyme (36). Production of extracellular proteases in *Candida* was first reported by Staib, 1969 (22,33). *C. albicans* has the ability to secrete proteases facilitating the invasion of mucosal tissue (13). Declining incidence of oropharyngeal candidoses and opportunistic infections over recent years can be attributed to the use of highly active anti-retroviral therapy (HAART), including HIV protease inhibitor (PI), in the treatment of HIV-infected patient (8,19,26). This has been attributed to *Candida* proteases belonging the same protease class as HIV protease.

Recent studies suggest a correlation exist between high protease secretion and reduced susceptibility to some azoles by *C. albicans* isolates from HIV-infected patients before HAART (4,11,25,31,37). Kretschmar *et al.*, 1999 (22) demonstrated that both germ tubes and protease activity correlated with tissue damage in *C. albicans* infection. However little is known about the effect of HIV protease inhibitor on *Candida* hyphal growth. The main treatment of *Candida* infections has been based on azole and polyene therapy (20). Azoles have also been showed to interfere with respiration process, inhibition of the hyphal formation and activity of membrane-bound enzymes (7,23). This study investigated the effect of an HIV protease inhibitor on the growth rate of *Candida* single hyphal by a Bio-Cell Tracer system.

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MATERIALS AND METHODS

Yeasts: Candida albicans ATCC 90028 reference strain Material

Amphotericin B (AMB) (Bristol-Myers Squibb, UK), reagent grade was dissolved in dimethyl sulfoxide solvent (DMSO). Ritonavir (RT) (Abbott Co., USA) was dissolved in methanol. Other chemicals used included poly-L-lysine (Sigma Chemical Co., Ltda., St. Louis, Mo., USA), fetal calf serum 5% (GIBCO, Laboratories, USA), and RPMI-1640 medium (Nissui Pharmaceutical Co., Japan) which was buffered with morpholinepropanesulfonic acid (MOPS; Sigma Chemical Co., USA).

Antifungal susceptibility test

To determine the MIC of the strain, antifungal susceptibility tests were performed as previously described by the National Committee for Clinical Laboratory Standards (NCCLS, 1997) (28).

Cellular yeast growth

Ritonavir and amphotericin B were tested at concentration ranging from 0.125 to 64 μg/mL. Single colonies of *C. albicans* ATCC 90028 strain was inoculated into 10-mL aliquots of YNB (yeast nitrogen broth, Difco, USA) medium containing 2% glucose (Difco, USA). These were incubated at 30ºC for 24 h with shaking at 250 rpm. Cells were harvested by centrifugation at 3500 rpm for 5 min, at 4ºC, washed twice with YNB medium and resuspend in 10 ML of YNB medium. Cells densities were adjusted spectrophotometrically to an optical density (OD_{600}) with value of 0.42 at 600 nm and then diluted to a final concentration of 2×10^3 cells/mL in YNB medium containing 2% glucose. Preparation of antifungal drugs and dilution schemes were performed in accordance with the National Committee for Clinical Laboratory Standards (NCCLS, 1997). Specific growth rates ($cells.h^{-1}$) of the strain were determined in aerobic batch cultures at 37ºC, 48 h using a Bioscreen C Analyser (Oy Growth curves AB Ltda., Helsinki, Finland) (16).

Monitoring of single hyphal growth by the Bio-Cell Tracer system

Cells were pre-cultured in RPMI-1640 medium at 37ºC with shaking at 150 rpm for 24h. Cells were washed 3 times with saline solution by centrifugation at 2000 rpm and cell count adjusted to approximately 1×10^6 cells/mL. Plastic tissue culture dishes (35 x 10 mm, Nunc, Denmark) were used as culture vessels. The inner surface of this vessel was covered with 0.01% poly-L-lysine. Cells suspension (1 mL) was inoculated onto the culture vessel and kept for 1 hour at room temperature. Using this procedure, cells not adhered to the poly-L-lysine on culture dishes were removed and 1 mL RPMI 1640 supplemented with 5% fetal calf serum was added. The culture vessel was set on the microscope chamber stage at 35ºC to get up to 90% hyphal growth. Fifteen to twenty hyphal tips were selected and monitored by the Bio-Cell Tracer system (BCT, Hidan Co., Ltd, Chiba Japan). This automatic system consists of a microscope (Olympus; IMT-2) and a digital image analyser (Flovel, Hidan Co., Ltd, Chiba Japan) using a computer program that traces individual hyphal tips. The analytical precision was 0.01 $\mu m.min^{-1}$. The apparatus can trace growing hyphal tips at speeds in the range of 0.5 to 20 $\mu m.min^{-1}$. Growth rates of hyphal tips were measured for 10 min intervals. After stable growth, approximately 1 hour, the medium from the culture vessel was removed and fresh RPMI medium containing the drug to be tested was added or control no drug added. The drugs were tested in separate sets in which AMB was used at concentration of ¼ MIC, 0.0125 μg/mL, and Ritonavir at concentration of 58 μg/mL. The growth rate was monitored for 2-4 h.

RESULTS AND DISCUSSION

Protease inhibitors (PI) caused a revolution in treating HIV infection when they were introduced in 1996. The introduction of highly active antiretroviral therapy (HAART) including PI has been accompanied by a reduction in the frequency of many of the secondary infections caused by HIV infection, including oral lesions (2,8-10,12,19). Infection by *C. albicans* generally involves adherence and colonization of superficial tissues (13,22,24). During this process, budding yeast cells are able to transform to hyphae and penetrate into the deep tissue (29).

In the present study the antifungal susceptibility tests for the ATCC 90028 strain gave a MIC to AMB of 1 μg/mL. The effect of the drugs on the yeast form growth rate ($cells.h^{-1}$) of the ATCC 90028 strain was determined in aerobic batch cultures using a Bioscreen C Analyser. Ritonavir and amphotericin B were tested at concentration ranging from 0.125 to 64 μg/mL. AMB inhibited 80% of growth at a concentration of 1 μg/mL and was fungicidal at a concentration >1 μg/mL. In contrast Ritonavir showed a progressive inhibitory effect on the yeast growth rate at higher concentrations, inhibiting 85% of the cell growth at concentrations of 0.25 μg/mL. However, at concentrations of 64 μg/mL, Ritonavir was not fungicide.

Ritonavir shows mean maximum concentrations in serum (C_{max}s) of 0.058 mg/mL after oral administration doses of 100 mg/day. Using the BCT system, cell culture after 1h showed up to 90% hyphal growth then the hyphal tips were exposured to 58 μg/mL of ritonavir (Fig.1). Figs. 2 and 3 show the time measurement in minutes and the growth rate ($\mu m.min^{-1}$) of single hyphae. In the post-exposure period the hyphal growth rate in the presence of Ritonavir was 0.8 ± 0.33 μm/min. In contrast AMB at a sub inhibitory concentration (0.125 μg/mL) caused only a slight reduction in hyphal growth (Fig. 3) with a growth rate of 2.8 ± 0.6 μm/min. The mean growth rate of the untreated hyphae was constant at approximately 2.5 μm/min at 37ºC.

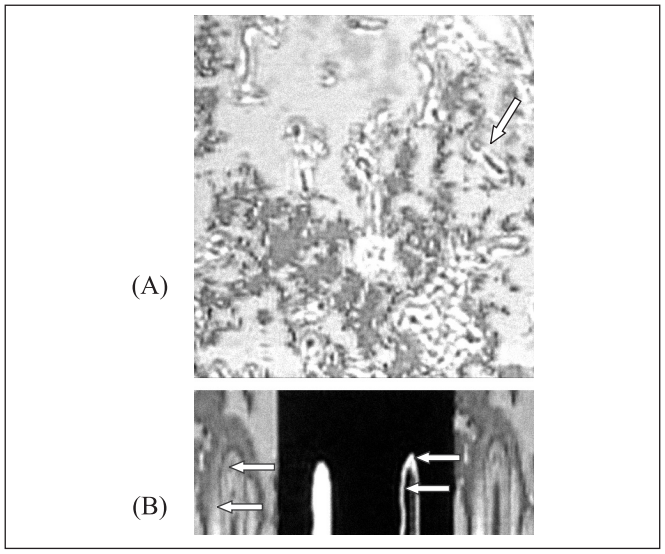

Figure 1. Photomicrographs of dynamic hyphal tip growth, (a) main screen showing full germination and (b) the tracing process of hyphal tip growth.

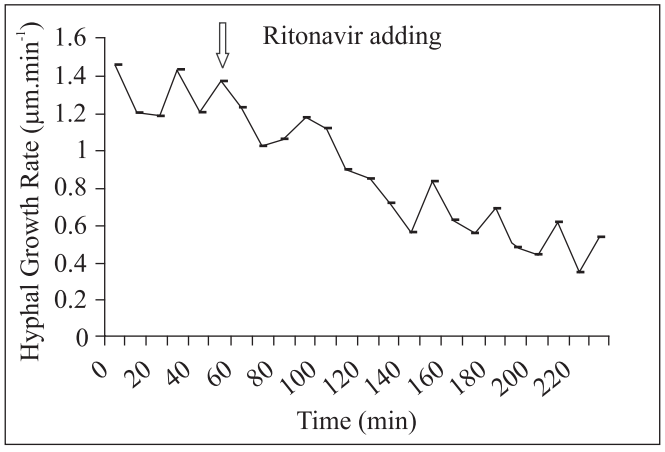

Figure 2. Time course of the growth rate of individual hyphae of *C. albicans.* Ritonavir at concentration of 58 µg/mL was added after 60 min of stable growth rate of hyphal tip.

Therefore the hyphal growth was progressively reduced after the Ritonavir had been added, indicating hyphal sensitivity to Ritonavir. Several antifungal susceptibility tests such as microdilution (NCCLS), agar diffusion (17) and flow cytometry are designed to work primarily with yeasts and yeast-like fungi. However, for filamentous fungi or hyphal invasion, these standard antifungal susceptibility tests do not accurately determine the effectiveness of a drug as an antifungal agent. The main treatment of *Candida* infections has been based on

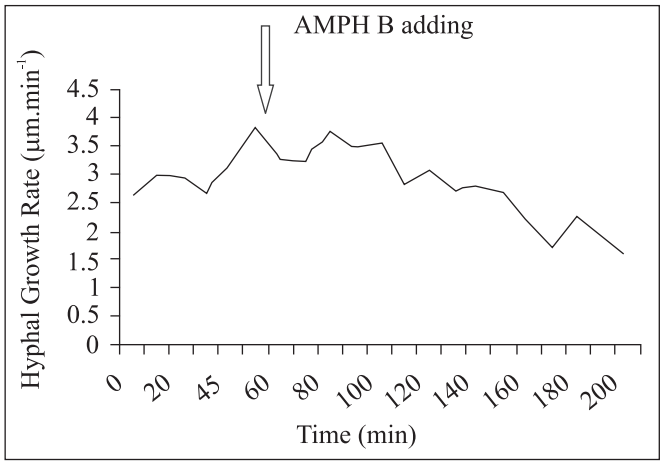

Figure 3. Time course of the growth rate of individual hyphae of *C. albicans.* AMB at concentration of 0.125 µg/mL was added as indicated by the arrow.

azole and polyene therapy (20). Although amphotericin B shows high toxicity, it is still the drug of choice for systemic mycosis (14). Amphotericin B act at the level of ergosterol by binding to this molecule. Azoles such as fluconazole, itraconazole or voriconazole inhibit the cytochrome P450 responsible for the 14α demethylation of lanosterol (CYP51) and thus block ersgosterol biosynthesis (21). Inhibition of ergosterol biosynthesis in *C. albicans* causes a variety of functional alterations in the cell membrane such as permeability changes, leakiness and disruptive interactions with non-sterol and lipid components. Ergosterol biosynthesis is more sensitive to azoles in mycelial cultures than yeast cultures, and this observation has been used to justify the efficacy of azoles *in vivo* (13,18,35).

Recent studies *in vitro* suggest that HIV- protease inhibitors cause inhibition of growth with *Pneumocystis carinii* (2), *Candida albicans* (8), and *Toxoplasma gondii* (12). Indinavir caused an insignificant inhibitory effect in line with that of AZT and Saquinavir was only lethal to *Toxoplasma* at concentrations cytotoxic to the human host cells. Nelfinavir and Ritonavir, however, blocked parasite growth at concentrations that were sub-lethal to human host cells. The main mechanism of pathogenicity in *Candida* infection is by hyphal growth (15,18). The major treatment of *Candida* infections has been the use of azole and polyene drugs (20) which inhibit hyphal growth and therefore prevent candidosis development. The effect of HIV protease inhibitors on *Candida* hyphal growth is unclear.

In studies using scanning and transmission electron microscopy, some antifungal drugs caused inhibition of growth and morphological changes in *Candida albicans* and *Aspergillus fumigatus* (1,3,34). These structural alterations were attributed to depletion of ergosterol (32). Hyphal-deficient

mutants are known to be avirulent in infections (13,24). *C. albicans* extracellular proteolytic activity due to secreted aspartic proteases has been purposed as putative virulence factor during the tissue invasion process by hyphal cells. Felk *et al.*, 2002 (4) showed that strains that produced hyphal cells but lacked hyphal-associated proteases were less invasive. Thus the hyphal morphology *per si* seems not make the fungus invasive (13). Several studies (5,6,8,27) showed inhibitory effects of Indinavir and Ritonavir on the yeast growth of *Candida albicans*. They established that a particularly virulent form of *C. albicans* associated with HIV infection produces a secretory aspartyl protease. This protease is inhibited by the HIV protease inhibitors. Using an experimental mouse model of vaginal candidosis, De Bernardis *et al.*, 1999 (8) demonstrated that the PIs had a therapeutic efficacy comparable to that of fluconazole.

The present study was succeeded in showing the inhibitory effect of ritonavir on a single hyphae tip growth of *C. albicans*. Our findings suggest that ritonavir was effective in the inhibition of hyphal growth therefore explaining in part the reduction of oral candidoses prevalence. The mechanism of PI action in controlling virulence factors associated with hyphal formation and growth is not known and requires further investigations.

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RESUMO

Efeito da droga anti-retroviral HIV-1 no crescimento de hifas de *C. albicans* monitoradas pelo sistema "Bio-Cell Tracer"

O declínio na incidência de candidose orofaríngea e infecções oportunistas associadas a infecção pelo HIV tem sido atribuído a introdução da terapia antiretroviral combinada (HAART). Infecção por *C. albicans* envolve aderência e colonização da mucosa superficial. Durante este processo leveduras são capazes de transformar-se na forma de hifas e penetrar nos tecidos mais profundos. Usando o sistema "Bio-Cell Tracer", o crescimento de hifas de *C. albicans* foi observado dinamicamente a nível celular. Ritonavir, inibidor de protease do HIV, foi efetivo na inibição do crescimento de hifas com media de 0.8 µm/min.O presente estudo demonstrou o efeito *in vitro* de um agente anti-retroviral HIV sobre o crescimento de hifas de *C. albicans.*

Palavras-chave: *Candida*, hifa, inibidor de protease

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