

Short Communication

Effect of antimycotic agents on the activity of aspartyl proteinases secreted by *Candida albicans*

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The inhibitory effect of human immunodeficiency virus (HIV) proteinase inhibitors amprenavir and saquinavir and antifungal agents terbinafine, ketoconazole, amphotericin B and ciclopiroxolamine on aspartyl proteinases (Saps) secreted by *Candida albicans* was tested in an *in vitro* spectrophotometric assay. As expected, both HIV proteinase inhibitors showed a significant inhibitory effect on Sap activity, which was comparable to that of the classical aspartyl proteinase inhibitor pepstatin A ($P < 0.001$). Antifungal drugs such as ketoconazole, terbinafine and amphotericin B had no, or only minor, inhibitory effects on proteolytic activity. In contrast, a significant reduction in Sap activity could be demonstrated during treatment with the antifungal agent ciclopiroxolamine ($P < 0.001$). These results point to a multiple effect of this antimycotic agent and might explain the reduced adherence of *C. albicans* to human epithelial cells at subinhibitory doses.

In the last decade, it has been demonstrated that secreted aspartyl proteinases (Saps) are important virulence factors for several types of *Candida albicans* infections and that inhibition of these proteinases have a protective effect for the host (De Bernardis *et al.*, 2001; Hube & Naglik, 2001). Based on the observation that antifungal drugs may have broad modes of action, we questioned whether certain antifungal components may also influence the activity of Saps, which, in turn, may enhance the antifungal activity of a particular drug. For example, Wu *et al.* (1999) showed that the natural antimicrobial agent lysozyme not only showed a candidacidal effect at higher concentrations but also decreased the extracellular concentration of Saps significantly without affecting cell growth or viability of *C. albicans*. In addition, lysozyme also directly caused degradation of purified Sap protein (Wu *et al.*, 1999). Other studies showed that certain inhibitors designed to inhibit human immunodeficiency virus (HIV) proteinase also had a direct effect on the activity of Saps (Korting *et al.*, 1999; Cassone *et al.*, 1999; Borg-von Zepelin *et al.*, 1999). To investigate whether recently designed HIV proteinase inhibitors, such as amprenavir, or antifungal agents, such as members of the allylamines, azoles, polyenes or pyridone antimycotic components, inhibit Sap activity, we measured the effects of selected HIV inhibitors and antimycotic drugs on the proteolytic activity of *C. albicans* using a spectrophotometric assay.

The inhibitory effect of the antimycotic agents and the HIV proteinase inhibitors on Sap activity was analysed using proteinase-containing culture supernatants from three *C. albicans* strains (hereafter named strains 1–3) isolated from the oral mucosa of three HIV-infected patients. Results were compared to the inhibition of pepstatin A. Isolates were identified as *C. albicans* by their colony morphologies, their ability to form germ tubes and their biochemical patterns, as assigned using the ABI system ATB 32 C (bioMérieux). The HIV proteinase inhibitor saquinavir was kindly provided by Roche; amprenavir was obtained from GlaxoSmithKline and pepstatin A was obtained from Sigma. Terbinafine (Novartis), ketoconazole (Janssen–Cilag), amphotericin B (Bristol Myers Squibb) and ciclopiroxolamine (Aventis) were obtained as reagent-grade powders from their respective manufacturers. Each *C. albicans* strain was grown in Sabouraud/glucose broth (Difco) in an incubator (Heraeus) for 48 h at 27 °C. The induction of *C. albicans* Saps was performed as described previously (Korting *et al.*, 1999). Briefly, 100 µl of *C. albicans* suspension was added to 10 ml Remold medium [2 % glucose, 0.1 % KH₂PO₄, 0.5 % MgSO₄, 1.25 ml 100× sterile filtered minimum essential medium vitamins (Sigma) and 1 % BSA]. The mixture was incubated for 7 days at 27 °C in a shaker at 150 r.p.m. Thereafter, titres (c.f.u.) were determined and the yeast cells were removed by centrifugation at 1500 g for 30 min. Supernatants were adjusted to pH 6.5 with NaOH to limit auto-degradation and frozen at –20 °C after filter sterilization (500 ml Stericup, pore size 0.22 µm, Millipore) to give the final crude enzyme preparation.

Abbreviations: HIV, human immunodeficiency virus; LSD, least significance difference; Sap, secreted aspartyl proteinase; TCA, trichloroacetic acid.

Stock solutions were prepared for amprenavir, saquinavir and pepstatin A by dissolving in absolute methanol at a concentration of 1.0 µM for saquinavir and 100 µM for pepstatin A and amprenavir. Amprenavir and saquinavir were diluted with 0.2 M sodium citrate/HCl buffer (pH 4.5) (Merck) to 1.0, 0.2 and 0.1 µM; pepstatin A was diluted with sodium citrate/HCl buffer to 0.5, 0.75 and 1.0 µM. Terbinafine was diluted in distilled water to 100 µM. Ketoconazole and amphotericin B were diluted in dimethyl formamide (Sigma) to 1 µM. Ciclopiroxolamine was diluted in dimethyl formamide to 100 µM. Dilutions were 0.5, 1 and 2 µM for terbinafine and ciclopiroxolamine, 0.2, 0.5 and 1 µM for amphotericin B and 0.5, 0.75 and 1 µM for ketoconazole.

Studies were carried out using bovine haemoglobin (Sigma) as substrate (Korting *et al.*, 1999). Test tubes were each filled with 750 µl 0.2 M sodium citrate/HCl buffer, 750 µl fresh substrate solution (1% substrate in 0.2 M sodium citrate/HCl buffer), 250 µl each sample and 250 µl amprenavir, saquinavir, pepstatin A, terbinafine, ketoconazole, amphotericin B or ciclopiroxolamine. Control experiments included assays without the addition of antifungal agents or inhibitors. Control experiments also included assays with dimethyl formamide or sodium citrate/HCl buffer alone without addition of antimycotics or proteinase inhibitors. Test reactions were incubated at 37 °C for 60 min (T60) in a shaker. The reaction was linear with time for up to 60 min. Three triplicate reactions were used for each experiment. Reactions were stopped with 500 µl trichloroacetic acid (TCA) and stored on ice. For each reaction mixture, an additional control was prepared by adding all ingredients plus 20% TCA simultaneously prior to incubation (T0). After the addition of TCA, all specimens were centrifuged at 3000 g for 30 min at 4 °C. A 160 µl sample of each cleared supernatant was then added to 40 µl dye reagent (Coomassie brilliant blue G-250, Bio-Rad). Peptides produced by proteolytic activity are not precipitated by TCA and bind to the dye. The amount of peptides in the supernatant can therefore be measured in a spectrophotometer (MR 4000, Dynatech) as a shift in the maximum absorbance value of the dye (measured at a wavelength of 595 nm) and correlated with proteolytic activity. Activity was calculated as the change in absorbance value using the following formula: sample (T60) – control (T0). One unit of activity was defined as an increase in 0.100 per 60 min at 595 nm. Activities were calculated for 1 l of Remold medium at a yeast density of 10^8 cells ml⁻¹. The least significance difference (LSD) test was used to determine differences between means. *P* values of < 0.05 were considered to be statistically significant.

Sap activity of the three *C. albicans* strains was inhibited by all proteinase inhibitors. The results of the present study confirmed data published previously for pepstatin A and saquinavir (Korting *et al.*, 1999). Pepstatin A, at concentrations ranging from 0.5 to 1.0 µM, inhibited Sap by approximately 18–78% (*P* < 0.001). Saquinavir, at concentrations ranging from 0.1 to 1.0 µM, inhibited Sap by approximately 15–79% (*P* < 0.001). Amprenavir was tested at the same concentrations and inhibited Sap by approximately 28–84%

(*P* < 0.001). Statistical analysis of Sap activity with and without a proteinase inhibitor showed a high degree of significance for each of the three agents (Fig. 1). Sap activity of the three *C. albicans* strains was not significantly inhibited by ketoconazole, terbinafine and amphotericin. In contrast, in the presence of ciclopiroxolamine, a significant inhibition (*P* < 0.001) was seen and ranged from 40 to 86% (Fig. 1).

The present study confirms the data published previously of Sap inhibition by pepstatin A and saquinavir (Korting *et al.*, 1999; Borg-von Zepelin *et al.*, 1999). In addition, a marked and highly significant inhibition of Sap activity by amprenavir could be demonstrated for the first time. These results provide further evidence for a direct effect of HIV proteinase inhibitors on one of the most relevant virulence attributes of *C. albicans*. In addition, we also observed that treatment of *C. albicans* proteinases with the hydroxypyridone antimycotic agent ciclopiroxolamine caused reduced proteolytic activity. Effects were not dose related. A possible explanation for this observation might be an interference of the antifungal agent/proteinase inhibitor at higher concentrations. One possible function of Sap activity is the attachment of *C. albicans* to epithelial cells and invasion into deeper tissue (Ollert *et al.*, 1993; Watts *et al.*, 1998; Schaller *et al.*, 1999). Braga *et al.* (1992) investigated the effects of subinhibitory concentrations of ciclopirox on the adherence of *C. albicans* to human buccal and vaginal epithelial cells. These authors found a significant reduction in adhesion at concentrations from 1/2

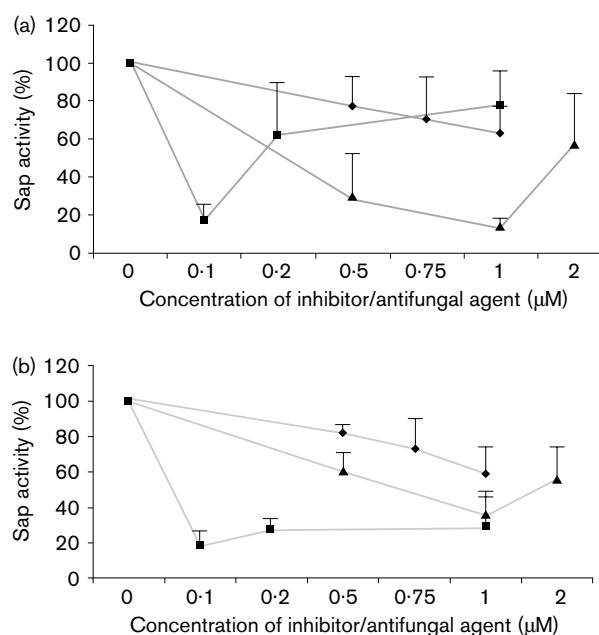


Fig. 1. Effects of pepstatin A (◆), amprenavir (■) and ciclopiroxolamine (▲) on Sap activity of *C. albicans* strain 1 (a) and 2 (b). Each point represents the mean \pm SD for three triplicate determinations. Differences in Sap activity between untreated and inhibitor-treated samples were highly significant (*P* < 0.001), as determined by the LSD test.

to 1/16 of MIC₅₀ and explained this effect by a reduced intracellular uptake of essential substrates and ions necessary for the ability of *C. albicans* to express its adherence mechanisms. Our study suggests that ciclopiroxolamine also directly effects the activity of Saps of *C. albicans*, which, in turn, may cause reduced adherence *in vivo*.

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