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, 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.

The inhibitory effect of the antifungal 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 Sap was suppressed by adding 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.
Stock solutions were prepared for amprenavir, saquinavir and pepstatin A by dissolving in absolute methanol at a concentration of 1.0 M 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 1.0 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 antifungics 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 absorbance value was measured at 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 10⁸ 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.

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 ± SD for three triplicate determinations. Differences in Sap activity between untreated and inhibitor-treated samples were highly significant (P < 0.0001), as determined by the LSD test.
to 1/16 of MIC<sub>50</sub> and explained this effect by a reduced intracellular uptake of essential substrates and ions necessary for the ability of <i>C. albicans</i> to express its adherence mechanisms. Our study suggests that ciclopiroxolamine also directly affects the activity of Saps of <i>C. albicans</i>, which, in turn, may cause reduced adherence in vivo.

References


