HIV protease inhibitors attenuate adherence of Candida albicans to epithelial cells in vitro

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Abstract

Oropharyngeal candidiasis is one of the first and most commonly reported opportunistic infections of untreated AIDS patients. With the introduction of the new antiviral HAART therapy, including HIV protease inhibitors, this mucocutaneous infection is nowadays only rarely observed in treated patients. It was recently shown that HIV protease inhibitors have a direct attenuating effect on Candida albicans secreted aspartic proteinases (Saps), an investigation prompted by the fact that both Sap and HIV protease belong to the superfamily of aspartic proteinases and by the observation that mucocutaneous infections sometimes resolve even in the absence of an immunological improvement of the host. As these Saps are important fungal virulence factors and play a key role in adhesion to human epithelial cells we tried to assess the effect of the HIV protease inhibitors Ritonavir, Indinavir and Saquinavir on fungal adhesion to these cells. The effect on phagocytosis by polymorphonuclear leukocytes was also assessed. Ritonavir was found to be the most potent inhibitor of fungal adhesion. A dose-dependent inhibition of adhesion to epithelial cells was found already at 0.8 μM and was significant at 4 μM or higher, at 500 μM the inhibition was about 55%. Indinavir and Saquinavir inhibited significantly at 4 μM or 20 μM, respectively; at 500 μM the inhibition was 30% or 50%. In contrast, no protease inhibitor was able to modulate phagocytosis of Candida by polymorphonuclear leukocytes. In conclusion, inhibition of Saps by HIV protease inhibitors may directly help to ease the resolution of mucosal candidiasis. In future, derivatives of HIV protease inhibitors, being more specific for the fungal Saps, may form an alternative in the treatment of mucosal candidiasis insensitive to currently available antimycotics. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Candida albicans, a pleomorphic yeast, is part of the normal human oro-gastrointestinal flora and is a major cause of the increasing number of opportunistic fungal infections in immunocompromised patients [1,2]. Oropharyngeal candidiasis (OPC), primarily caused by C. albicans, may represent the first manifestation of human immuno-deficiency viral (HIV) infection. The presence of OPC is a predictor of poor outcome in HIV-infected persons [3]. As HIV disease progresses (and immunosuppression wors-
C. albicans [9]. The majority of Sap proteins are secreted by those C. albicans cells that directly adhere to the epithelial surface [10]. A dominant Sap isoenzyme in vitro and possibly also in vivo is Sap 2 [9,11]. C. albicans Saps and HIV aspartic proteinases are enzymes which belong to the same broad aspartic proteinase superfamily.

Since the introduction of new antiretroviral agents, especially HIV protease inhibitors, OPC is less often observed in AIDS patients [12–14]. Considering this effect of HIV protease inhibitors we intended to assess the adherence of C. albicans to human epithelial cells in the presence of various concentrations of three HIV protease inhibitors, namely Ritonavir, Indinavir and Saquinavir. We also evaluated their influence on phagocytosis of C. albicans by polymorphonuclear leukocytes.

2. Materials and methods

2.1. C. albicans cultivation

C. albicans CBS 5982 (Central Bureau voor Schimmelcultures, Baarn, The Netherlands) was used throughout the study. C. albicans was initially grown on Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, UK) plates for 24 h and then transferred into RPMI 1640 medium (Hyclone, Cramlington, UK) without any supplements. This cell suspension was used as stock solution and was kept for 1 week at 4°C. All experiments were performed under sterile conditions.

2.2. HIV protease inhibitors

Three HIV protease inhibitors, namely Ritonavir (Abbott, Chicago, IL, USA), Indinavir (Merck, Rahway, NJ, USA) and Saquinavir (Roche, Welwyn Garden City, UK), were used for this study. They were prepared as follows: Ritonavir was dissolved in methanol at a concentration of 40 mM. Indinavir and Saquinavir were dissolved in double-distilled water at concentrations of 20 mM and 2 mM, respectively. These solutions were used as stock solutions and were kept at −70°C.

2.3. Epithelial cell culture

The human vaginal epithelial cancer cell line HeLa S3 (American Type Culture Collection, Rockville, MD, USA) was cultivated in Ham’s F12 medium (PAA, Linz, Austria) containing 10% fetal calf serum (FCS; Boehringer, Ingelheim, Germany) and l-glutamine (HyClone, Cramlington, UK) in cell culture flasks (Falcon, 75 cm²; Costar, Cambridge, UK). The cells were incubated at 37°C (5% CO₂, 95% humidity). For the adherence assay the cells were prepared as follows: the medium was poured off and the cells were washed once with phosphate buffered saline (PBS). For the detachment of the cells, they were incubated at 37°C (5% CO₂, 95% humidity) for 7 min in Ham’s/EDTA containing 50% Ham’s, 5% FCS, 50% PBS and 5 mM EDTA. Then the cells were rigorously shaken causing cell detachment. The HeLa S3 cells were mixed 1:1. C. albicans without HIV protease inhibitors in the absence or presence of 5% methanol served as control. All preparations were incubated at 37°C (without CO₂) for 1 h. The wells of the microtiter plate containing adherent HeLa S3 cells were washed twice with PBS and then incubated with 100 µl of C. albicans/HIV protease inhibitor solution. Subsequently, the plate was incubated at 37°C (with 5% CO₂) for 30 min. Thereafter, all wells of the microtiter plate were washed twice with PBS to remove non-adherent yeasts. Liquid SDA (100 µl, 6.5%, 40°C) was added to each well. Finally, the microtiter plate was incubated at 30°C for 16 h. This incubation was performed both to enable an easy quantitation of adherent colonies the next day (under microscopic control at a magnification of 40×), and to restrict the number of adherent organisms to those which were able to adhere and replicate on the surface of the epithelial cells. Incubation at 37°C was comparable but the colonies were larger and confluent and thus more difficult to count. The colony forming units (CFU) derived from samples lacking HIV protease inhibitors were set at 100%.

2.4. Adherence assay

C. albicans, diluted in RPMI 1640 medium without any supplements, was prepared in Ham’s (containing 10% FCS and l-glutamine) at a concentration of 2 × 10⁶ cells ml⁻¹. The HIV protease inhibitors Ritonavir, Indinavir and Saquinavir, diluted as described above, were also prepared in Ham’s (containing 10% FCS and l-glutamine) at different concentrations. C. albicans (final concentration 10⁴ cells ml⁻¹) and HIV protease inhibitors (final concentrations 500, 100, 20, 4 and 0.8 µM) were mixed 1:1. C. albicans without HIV protease inhibitors in the absence or presence of 5% methanol served as control. All preparations were incubated at 37°C (without CO₂) for 1 h. The wells of the microtiter plate containing adherent HeLa S3 cells were washed twice with PBS and then incubated with 100 µl of C. albicans/HIV protease inhibitor solution. Subsequently, the plate was incubated at 37°C (with 5% CO₂) for 30 min. Thereafter, all wells of the microtiter plate were washed twice with PBS to remove non-adherent yeasts. Liquid SDA (100 µl, 6.5%, 40°C) was added to each well. Finally, the microtiter plate was incubated at 30°C for 16 h. This incubation was performed both to enable an easy quantitation of adherent colonies the next day (under microscopic control at a magnification of 40×), and to restrict the number of adherent organisms to those which were able to adhere and replicate on the surface of the epithelial cells. Incubation at 37°C was comparable but the colonies were larger and confluent and thus more difficult to count. The colony forming units (CFU) derived from samples lacking HIV protease inhibitors were set at 100%.

2.5. Labelling and opsonization of C. albicans

C. albicans CBS 5982 was initially grown on SDA plates for 24 h and then transferred into 15-ml Falcon tubes containing 10 ml PBS and centrifuged at 300×g for 5 min. The pellet was diluted in PBS to a concentration of 2 × 10⁷ cells ml⁻¹. Fluorescein isothiocyanate isomer I (FITC, 10 µM; Sigma, St. Louis, MO, USA) was added to
this *C. albicans* suspension. The suspension was mixed, shielded from light by aluminum foil and shaken for 30 min at room temperature. After centrifugation at $300 \times g$ for 5 min the supernatant was poured off and the cells were washed three times with PBS (to obtain a colorless supernatant) and then diluted in white Hanks’ balanced salt solution (HBSS, BioWhitaker, Verviers, Belgium). This *C. albicans* suspension was kept on ice until use.

For opsonization FITC-labeled *C. albicans* were incubated with 5% normal human serum for 30 min at 37°C on a vertical rotator. The cells were washed three times with PBS and then centrifuged at $5000 \times g$ for 5 min. The pellet was diluted in HBSS to a concentration of $2 \times 10^7$ cells ml$^{-1}$.

### 2.6. Isolation of polymorphonuclear leukocytes

Polymorphonuclear leukocytes (PMNLs) were isolated according to established procedures [15]. After Ficoll (Pharmacia Biotech, Uppsala, Sweden) treatment the bottom layer of granulocytes and erythrocytes was diluted in ice-cold HBSS and centrifuged at $400 \times g$ (4°C) for 10 min. The pellet containing PMNLs and erythrocytes was suspended in 0.8% dextran and left for 30 min at room temperature to allow the erythrocytes to sediment. The leukocyte-rich supernatant was removed, centrifuged and washed in HBSS. Erythrocytes still remaining in the pellet were removed by two cycles of hypotonic lysis with ice-cold distilled water. The PMNLs were washed with PBS, resuspended in HBSS at a final concentration of $10^6$ cells ml$^{-1}$ and kept on ice until use.

### 2.7. Phagocytosis assay

Opsonized and non-opsonized *C. albicans* (500 μl, final concentration $10^7$ cells ml$^{-1}$) were mixed with PMNLs (500 μl, final concentration $5 \times 10^5$ cells ml$^{-1}$) and incubated at 37°C for 30 min. After addition of 10 μl gentian violet solution (2 g crystal violet (Merck, Darmstadt, Germany) in 100 ml ethanol diluted with 8 g ammonium oxalate (Merck) to 1 l distilled water) to quench the fluorescence of extracellular (i.e. free and attached yeast cells [16]), the suspension was incubated for 5 min on ice then centrifuged at $400 \times g$ for 1 min. The pellet was diluted in 800 μl FACS buffer (4% formalin in PBS) and the suspension was incubated in the dark for 1 h on ice. Finally, the fluorescence intensity, as a measure of phagocytosis of *C. albicans* by PMNLs, was assessed using a FACSScan (Becton Dickinson, Heidelberg, Germany).

### 2.8. Statistics

Statistical significance was determined using Student’s *t*-test analysis. All comparisons were two-sided and a *P* value of less than 0.05 was considered significant.

### 3. Results

#### 3.1. Effect of HIV protease inhibitors on *C. albicans* adherence

The influence of HIV protease inhibitors (Ritonavir, Indinavir and Saquinavir) on the adherence of *C. albicans* CBS 5982 to the epithelial cell line HeLa S3 was tested using SDA as adherence medium. The number of *C. albicans* CFU was assessed under the microscope (Fig. 1). The CFU derived from samples lacking HIV protease inhibitors were set at 100%. Ritonavir was found to be the most potent inhibitor of *C. albicans* adherence. A dose-dependent inhibition of adhesion to epithelial cells was found already at 0.8 μM and was significant at 4 μM or higher, at 500 μM the inhibition was about 55% (Fig. 2a). Saquinavir inhibited significantly at 20 μM and at 500 μM the inhibition was 50% (Fig. 2b). Indinavir inhibited significantly at 4 μM but no further augmentation of adhesion was achieved using higher concentrations. The maximum inhibition at 500 μM was approximately 30% (Fig. 2c).

#### 3.2. Effect of HIV protease inhibitors on *C. albicans* phagocytosis by PMNLs

*C. albicans* CBS 5982 was labelled with FITC and then opsonized (Fig. 3) or not opsonized (Fig. 4) and treated with Ritonavir or Indinavir at final concentrations of 500 μM or 250 μM. Neither of the two protease inhibitors was able to modulate phagocytosis of *C. albicans* CBS 5982 by PMNLs as measured by FACS.

### 4. Discussion

The introduction of new anti-HIV drugs of the protease inhibitor type has attracted great interest. Although they
are not a cure, they can significantly inhibit the viral protease enzyme thereby reducing the viral load and improving the quality of life for HIV-infected patients [17]. Another important observation was the decreased occurrence of OPC predominantly in HIV protease inhibitor-treated patients [12–14]. It was speculated that a direct elimination of candidial Saps may have a supportive role, as it has been earlier shown that inhibition of Sap activity by treatment with the specific proteinase inhibitor pepstatin A resulted in reduced adherence and virulence [18]. Pepstatin-like drugs, however, are not used clinically because of their metabolism in the liver and rapid clearance from blood [18].

This led to recent studies showing that HIV protease inhibitors have indeed a direct attenuating effect on C. albicans Saps in vitro [19–22] and in vivo [22]. The rank order of Sap inhibition was Ritonavir > Indinavir > Saquinavir [19]. Interestingly, although Sap isoenzymes are important for pathogenesis of candidiasis, and in particular involved in adhesion [23–27], the effect of HIV protease inhibitors on human epithelial cells in vitro has not been evaluated so far.

On Vero cells of monkey origin Ritonavir was the strongest inhibitor [28]. Ritonavir inhibited Candida adherence to Vero cells significantly at 25 µM, the endpoint of adherence inhibition caused by Saquinavir was seen at a fourfold higher concentration [28]. The data obtained here, using the more representative human in vitro model, correlated with these earlier results: Ritonavir was the strongest drug, inhibiting significantly at 4 µM and at 500 µM the inhibition was about 55%. However, at higher concentrations the (undesirable) inhibition of pepsin is also more pronounced with Ritonavir when compared to the other HIV protease inhibitors [19].

Saquinavir inhibited C. albicans adherence slightly more weakly than Ritonavir but more strongly than Indinavir. In addition, and in contrast to the other two drugs, Saquinavir not only exhibits anti-Sap, but also, at higher concentrations, fungicidal activities in vitro [19]. A comparable inhibition of adhesion was ascertained on Vero cells using Saquinavir at a concentration of 200 µM, whereas Indinavir, which was able to inhibit fungal adhesion to human epithelial cells, had no effect on C. albicans adherence to Vero cells [28]. To explain this discrepancy it is important to note that both assays do not only differ in the cell type used. Due to the amplification of adherent cells by overnight culture on the epithelial cell layer, our adhesion assay works well with 2 × 10⁶ cells ml⁻¹ whereas the less laborious previous assay [28] is run with 2 × 10⁶ cells ml⁻¹ and these different concentrations may in part account for the difference.

More importantly, Indinavir has been shown to preferably inhibit Sap 2 [19,28] and has virtually no effect on Sap 1, in contrast to the other two protease inhibitors investigated [28]. This may explain why Indinavir has a good anti-Candida effect in the rat vaginitis model [22], where
Sap 2 is the main acting proteinase [27], and suggests that in our assay adherence may be more dependent on Sap 1 and less dependent on Sap 2.

A different involvement of the Sap isoenzymes may also be responsible for the absent influence of HIV protease inhibitors on phagocytosis. In this respect it is important to note that Saps 4–6 play a dominant role during phagocytosis as a SAP 4–6 deletion mutant was more effectively killed in vitro after contact with peritoneal macrophages than the wild-type strain [29]. In addition, the SAP 4–6 deletion mutant is also less virulent in vivo, as the alanine aminotransferase activity, as a measure for liver damage, is significantly reduced [30], further supporting the importance of Saps 4–6. Sap isoenzymes 4–6, however, have been found to be unaffected by HIV protease inhibitors in vitro [28], which may explain the absent influence in our in vitro phagocytosis model.

A main question which has to be addressed is whether concentrations of \(4 \text{ M} \) or \(20 \text{ M} \) can actually be reached in vivo. This appears to be very likely, as, first, the maximum systemic concentration \((C_{\text{max}})\) listed by Flexner [31] is between 8 and 11 \(\mu\text{g ml}^{-1}\) Indinavir or Ritonavir, equaling 12 \(\mu\text{M}\) and 15 \(\mu\text{M}\), respectively. Second, local concentrations may reach somewhat higher concentrations than systemic ones. Saquinavir has a \(C_{\text{max}}\) of only 0.3 \(\mu\text{M}\) [31] but may have a particular antifungal role when applied locally, especially considering its fungicidal activity. Such a local in vivo application has been recently suggested by

Fig. 3. Phagocytosis of opsonized \textit{C. albicans} by PMNLs in the presence of Ritonavir and Indinavir. \textit{C. albicans} was opsonized with 5\% normal human serum. The fluorescence intensity was assessed using FACS (FACScan; Becton Dickinson, Heidelberg, Germany). Phagocytosis of opsonized \textit{C. albicans} in the absence of HIV protease inhibitors was used as control (a). Ritonavir at final concentrations of 500 \(\mu\text{M}\) (b) and 250 \(\mu\text{M}\) (c), and Indinavir at the same concentrations (500 \(\mu\text{M}\) (d), 250 \(\mu\text{M}\) (e)) were tested.
Cassone and co-workers who showed that in a rat vaginitis model of mucosal candidiasis both Indinavir and Saquinavir directly inhibited Sap activity in vivo [22].

In conclusion, HIV protease inhibitors were found to attenuate adhesion of \textit{C. albicans} to epithelial cells in vitro, but were not able to modulate phagocytosis of \textit{Candida} by PMNLs. The inhibition of Saps by HIV protease inhibitors may directly help to ease the resolution of mucosal candidiasis. Its treatment with currently available antifungals is cumbersome with reduction in susceptibility and appearance of resistance [32]. In patients with itraconazole- or fluconazole-resistant mucocutaneous candidiasis, the treatment of choice consists of amphotericin B as oral suspension or parenteral preparation [33] which is, however, accompanied by a higher toxicity. In future, derivatives of HIV protease inhibitors, being more specific for the fungal Saps, and preferably covering all Sap iso-enzymes, may thus form an alternative in the treatment of mucosal candidiasis insensitive to currently available antifungals.

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