The human immunodeficiency virus (HIV) protease inhibitor indinavir directly affects the opportunistic fungal pathogen Cryptococcus neoformans

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Abstract

Highly active antiretroviral therapy (HAART), that includes human immunodeficiency virus (HIV) protease inhibitors (PIs), has been remarkably efficacious including against some opportunistic infections. In this report we investigated the effect(s) of the PI indinavir on protease activity by Cryptococcus neoformans, an opportunistic fungal pathogen responsible for recurrent meningoencephalitis in AIDS patients. Indinavir was also tested for potential effects on other parameters, such as fungal viability, growth ability and susceptibility to immune effector cells. It was found that indinavir impaired cryptococcal protease activity in a time- and dose-dependent fashion. The phenomenon was similarly detectable in ATCC/laboratory strains and clinical isolates. C. neoformans growth rate was also significantly reduced upon exposure to indinavir, while fungal viability was not affected and mitochondrial toxicity not detected. Furthermore, as assessed by an in vitro infection model, indinavir significantly and consistently augmented C. neoformans susceptibility to microglial cell-mediated phagocytosis and killing. Overall, by providing the first evidence that indinavir directly affects C. neoformans, these data add new in vitro insights on the wide-spectrum efficacy of PIs, further arguing for the clinical relevance of HAART against opportunistic infections in AIDS.

Keywords: Cryptococcus neoformans; Proteases; Protease inhibitors; Microglial cells; Phagocytosis; Antimicrobial effects

1. Introduction

Highly active antiretroviral therapy (HAART) that includes human immunodeficiency virus (HIV) protease inhibitors (PIs), has been remarkably efficacious against viral replication, AIDS progression, and mortality [1,2]. Moreover, it has been demonstrated that HAART has beneficial effects on some opportunistic infections by fungal pathogens, known to be major causes of morbidity and mortality in AIDS subjects [3,4]. The effect of HAART therapy on the prognosis of HIV infected subjects is attributed to immune reconstitution by a fall in HIV viremia, increase in CD4 cells, reconstitution of lymphoproliferative responses, normalisation of antimicrobial and effector functions by polymorphonuclear leukocytes [5–9]. Nevertheless, initial evidence demonstrates that two PIs, indinavir and ritonavir, commonly used in HAART, also exert a direct inhibitory effect on AIDS-related opportunistic pathogens [10,11]. In particular, Candida albicans in vitro growth and experimental pathogenicity are profoundly reduced by PIs. The phenomenon is associated with a direct effect of such drugs on the
production of \textit{C. albicans} secretory aspartyl proteases, whose involvement in pathogenicity has been partially elucidated [11–15].

\textit{Cryptococcus neoformans} is also able to produce proteases [16–21], that are expected to favour assimilation of nitrogen from proteinaceous sources and have been proposed as potential virulence factor(s), contributing to host tissue invasion and colonisation by the pathogen. In line with this latter hypothesis, evidence exists that \textit{C. neoformans} is able to alter its phenotype by passages in mice [22] and, particularly, its protease production during a three-year-long persistence in an AIDS patient experiencing recurrent cryptococcal meningoencephalitis [23].

Because of its marked neurotropism [24], \textit{C. neoformans} has been investigated for resistance/susceptibility to microglial cells, the professional phagocytes of the central nervous system. Such studies are greatly hampered by difficulties in isolating sufficient cell numbers and sufficiently pure populations of microglial cell. An in vitro established microglial cell-line BV2 [25] is available and retains the morphological, phenotypical and functional properties described for freshly isolated microglial cells [26]. In particular, BV-2 cells exert constitutive and IFN\(\gamma\)-enhanced anticryptococcal effects via fungal ingestion and nitric oxide production [27–29]. On the other hand, cryptococcal capsule formation and melanization have been described as escape mechanisms by which \textit{C. neoformans} overcomes host defences and particularly microglial cells [30–33]. Thus, by means of an in vitro model, some biomolecular events involved in \textit{C. neoformans}–microglial cell interaction have been investigated as prototype of the complex series of events occurring in vivo, at the cerebral level, during \textit{C. neoformans} infection.

In this report, we have used an in vitro study to determine whether the PI indinavir may exert any direct effect on \textit{C. neoformans}, and/or alter its resistance to microglial effector cells. We show that both effects occur and the implications are discussed.

2. Materials and methods

2.1. Reagents

Indinavir was provided by Dr. Paola Viscardi (Merck Sharp & Dohme Italia SpA, Rome, Italy). Pepstatin A was purchased from SIGMA (Saint Louis, MO). Both drugs, indinavir and pepstatin A, were dissolved in DMSO (SIGMA, San Louis, MO), stock solutions were kept at \(-20^\circ\)C. Azoalbumin was purchased from SIGMA (St. Louis, MO). Proteinase K was purchased from Boehringer Mannheim Corp. (Indianapolis, Ind).

2.2. Cryptococcus neoformans

The following \textit{C. neoformans} strains were employed: two encapsulated ATCC strains, 11240 and H99, the acapsulated ATCC strain Cap 67 and two clinical isolates 1526 and 1782, obtained from a relapsing case of meningoencephalitis, as detailed elsewhere [23]. \textit{C. neoformans} cultures were maintained in Sabouraud dextrose agar (Oxoid Hampshire, England) by biweekly passages. The plates were kept at room temperature.

2.3. Microglial cell line

The BV-2 microglial cell line, established as previously described [25], was maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10\% heat-inactivated fetal bovine serum (FBS) (Defined Hyclone, Logan, Utah), gentamicin (50 \(\mu\)g/ml) and \(L\)-glutamine (2 mM). Cells were detached by vigorous shaking biweekly and fresh cultures were started at a concentration of \(5 \times 10^5\)/ml.

2.4. Experimental protocol

\textit{Cryptococcus neoformans} yeast cells were suspended in protease induction medium (glycine 13 mM, thiamine 3 \(\mu\)M, KH\(_2\)PO\(_4\) 4 mM, MgSO\(_4\) 10 mM, glucose 15 mM and bovine serum albumin 0.2\%; at pH 5) in the presence of indinavir or pepstatin A, at doses and times indicated in the text, and then incubated at 37 \(^\circ\)C. Controls consisted of yeast cells incubated with DMSO. At different times, cell-free supernatants were assayed for protease activity, while cell pellets were evaluated for viability, growth ability or susceptibility to immune effector cells as detailed below.

2.5. Extracellular protease activity

\textit{Cryptococcus neoformans} yeast cells (\(10^8\)/ml in protease induction medium, 1 ml/well in 24 well plates purchased from Becton–Dickinson NY, USA) were exposed to indinavir or pepstatin A for 24, 48, 72, 96 or 168 h at 37 \(^\circ\)C. Then, cell-free supernatants were assayed for proteolytic activity according to a previously established procedure [34]. Briefly, azoalbumin, used as substrate at 0.3\%, was thermo-equilibrated to 37 \(^\circ\)C and the reaction was started by addition of test supernatants. A standard curve was established using protease K in a range between 312 and 10,000 ng/ml. Aliquots of reaction mixture were removed at 40 min and immediately added to trichloroacetic acid 10\% (w/v) to precipitate proteins. The samples were mixed, kept on ice for 30 min and centrifuged at 10,000 \(g\) for 15min; the supernatant was then mixed with NaOH 15M and the \(A_{440}\) was measured on a UV/visible-light spectrophotometer (Sunrise, Tecan, Salzburg, Austria).
2.6. Mitochondrial membrane potential (ΔΨ)

The method has been described in detail elsewhere [35,36]. Briefly, C. neoformans yeast cells (10⁶/ml in protease induction medium, 1 ml/well in 24 well plates) were exposed or not to indinavir or pepstatin A for different incubation times; then, cells were stained with the ΔΨ-sensitive probe JC-1 (2.5 μg/ml) in RPMI 1640 plus 10% FBS, for 10 min at room temperature, in the dark. Cytofluorimetric analysis was performed using a FACSscan cytometer (Becton–Dickinson, San José, CA) equipped with an argon-ion laser tunes at 488 nm. Green fluorescence from JC-1 monomers was detected through the standard band-pass filter centered at 520 ± 10 nm and orange fluorescence from propidium iodide and JC-1 aggregates though the long pass filter at 575 ± 10 nm. A standard cytogram based on the measurement of side-angle scatter versus forward-angle scatter was designed to eliminate cellular debris and aggregates.

2.7. Growth ability

Cryptococcus neoformans yeast cells (10⁵/ml in protease induction medium, 200 μl/well in 96 well plates purchased from Becton–Dickinson NY, USA) were exposed or not to indinavir or pepstatin A and then incubated at 37 °C. Following different times of incubation, serial dilutions were made in saline solution and plated (triplicate samples) on Sabouraud Dextrose Agar. The colonies were counted after 48–72 h of incubation at room temperature. Results were expressed as colony-forming units (CFU)/ml.

2.8. Anticytococcal activity

Cryptococcus neoformans yeast cells (10⁵/ml in protease induction medium, 100 μl/well in 96 well plates) were exposed or not to indinavir or pepstatin A and then incubated at 37 °C for different times. Then, BV-2 cells were added (10⁶/ml, 100 μl/well in RPMI complete medium). After additional 24 h of incubation, the plates were vigorously shaken, and Triton X-100 (0.1% final concentration) was added to the wells. According to previously described protocols [28], serial dilutions from each well were then made in saline and plated (triplicate samples) on Sabouraud dextrose agar. The number of CFU were determined after 48 or 72 h of incubation at room temperature. Control cultures consisted of C. neoformans incubated in protease induction medium without effector cells. The results were expressed as a percentage of anticytococcal activity according to the following formula:

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\text{% anticytococcal activity} = \frac{\text{CFU experimental group}}{\text{CFU control cultures}} \times 100.
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2.9. Phagocytosis assay

Cryptococcus neoformans yeast cells (10⁷/ml in protease induction medium) were plated in 24 well plates (0.5 ml/well) in the presence of indinavir or pepstatin A and then incubated at 37 °C for different times. Then, BV-2 cells were added (10⁶/ml in RPMI complete medium, 0.5 ml/well). After 1 h of incubation, the excess of C. neoformans was removed by centrifugation of cell suspension on a Fycoll cushion at 300g for 10 min, according to previously described protocols [28]. The cells at the interface were recovered, washed and used for cytospin preparations. C. neoformans uptake was directly evaluated in Azure-stained cytospin preparations. A minimum of 200 cells were scored and any BV2 microglial cells containing one or more particles were counted as phagocytic.

2.10. Statistical analysis

Results in figures are the mean ± SD of three independent experiments or representative data from of 3–6 experiments. Statistical analysis was performed by the Student’s t test.

3. Results

3.1. Protease activity by different C. neoformans strains

In the first series of experiments, both ATCC C. neoformans strains and clinical isolates were assessed for protease activity by the azoalbumin hydrolyze assay [34]. Accordingly, three ATCC strains (11240, H99 and Cap67) and two clinical isolates (1526 and 1782) were incubated at 37 °C in protease induction medium and aliquots of supernatants were removed at different time-points up to 168 h. The azoalbumin hydrolysis assay was performed and results were expressed as OD₄₅₀ and pg/ml according to a standard curve. As shown in Fig. 1, all the strains exhibited protease activity at time 72 h (Fig. 1) and similar trends were observed at later times (data not shown). In particular, 11240 and H99 yeast cells produced the highest levels of activity with respect to the others, the acapsular CAP67 produced significantly lower activity than the capsulated strains (p < 0.05: CAP67 vs 11240; p < 0.05: CAP67 vs H99). Moreover, when comparing the two clinical isolates, it was found that 1526 produced protease levels significantly lower than 1782 (p < 0.05), such a difference was consistently observed and closely correlated with data previously established by means of a different protease activity assay [23].
3.2. Effect of indinavir on protease activity by C. neoformans

In order to evaluate the effects of indinavir on protease activity by C. neoformans, H99 was chosen for pilot experiments, where 10^8 yeast cells/ml were exposed to indinavir (10 μM) or pepstatin A (100 μg/ml), that had been added as positive control, for 72 h; then, supernatants were harvested and tested in the azoalbumin hydrolysis assay. As shown in Fig. 2, protease activity significantly dropped in indinavir-treated yeast cells with respect control cells. Pepstatin A was also highly effective, by producing approximately an eightfold reduction in the levels of protease activity. Kinetic experiments revealed that H99 yeast cells exposed to indinavir or pepstatin A were inhibited in protease levels at all the time-points tested, with the most relevant effect after 48–72 h of treatment (data not shown).

3.3. Effect of indinavir on C. neoformans mitochondrial membrane potential and viability

The effect of indinavir on mitochondrial activity of C. neoformans was investigated, according to a well-established method [35,36]. Briefly, C. neoformans H99 control cells or cells exposed to indinavir (10 μM) for 72 h were stained with the ΔΨ-sensitive probe JC-1 and then evaluated for mitochondrial membrane potential (ΔΨ). Fig. 3 shows no appreciable differences in terms of ΔΨ variation between control and indinavir-treated cells, ΔΨ remaining consistently high in all the groups. As shown, minimal amounts of dead or suffering cells (low ΔΨ) were also detectable in both cases. When in parallel groups, viability was evaluated by the MTT assay or propidium iodine flow cytometry analysis, little or no differences were observed between groups, irrespective also of the times of treatment (data not shown).
3.4. Effect of indinavir on C. neoformans growth ability

To establish whether the indinavir might have any effect on C. neoformans growth potential, we performed experiments in which H99 yeast cells were assayed for growth ability by a CFU assay, following 24, 48 and 72 h exposure to indinavir. In parallel groups, pepstatin A was added. As depicted in Fig. 4, the CFU in H99 yeast cells exposed to indinavir were significantly lower than CFU in control cultures, the inhibitory effects were evident at 48 and 72 h. Pepstatin A produced a similar trend at both time-points.

To establish the possible dose-dependency of the phenomenon, H99 yeast cells were exposed to different doses of indinavir or pepstatin A for 72 h and then fungal growth was evaluated. Fig. 5 (upper panel) shows that indinavir inhibited the growth of H99 in a

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**Fig. 3.** Mitochondrial activity of C. neoformans H99 exposed or not to indinavir. Yeast cells (10^7/ml) were incubated in protease induction medium without or with indinavir (10 μM) for 72 h. Then, cells were washed and stained with JC-1 probe to assess mitochondrial membrane potential by flow cytometry, as detailed in Section 2. Numbers indicate the percentage of cells with depolarized mitochondria. Data from a representative experiment are shown.

**Fig. 4.** Growth ability of C. neoformans H99 exposed or not to indinavir. Yeast cells (10^5/ml) were incubated in protease induction medium, in the presence of indinavir (10 μM) or pepstatin A (100 μg/ml) for the indicated times. Then, cells were washed and assessed for growth ability by CFU assay. Results shown are the mean ± SD of triplicate samples from a representative experiment. *p < 0.01 (treated groups vs controls).

**Fig. 5.** Dose-dependent inhibitory effects of indinavir on C. neoformans growth ability. C. neoformans yeast cells (10^5/ml), ATCC strain H99 and clinical isolate 1782, were exposed to indinavir or pepstatin A at the indicated doses for 72 h. Then, cells were washed and assessed for growth ability by CFU assay. Results shown are the mean ± SD of triplicate samples from a representative experiment. *p < 0.01 (treated groups vs controls).
strictly dose-dependent manner and a similar trend was observed in yeast cells exposed to pepstatin A.

Furthermore, the clinical isolate 1782 was also assessed for susceptibility to indinavir. As shown in Fig. 5 (lower panel), the growth ability of 1782 was significantly inhibited upon exposure to the drug; the phenomenon was strictly dose-dependent reaching about 75% CFU reduction at 50 µM dose. As expected, similar results were observed in parallel groups with pepstatin A. Since 10 µM gave the most reliable results, that dose was used hereafter as the most convenient condition.

3.5. Effect of indinavir on susceptibility of C. neoformans to immune effector cells

We evaluated the effects of indinavir on cryptococcal susceptibility to immune effector cells. Thus, H99 yeast cells were pre-exposed to indinavir (10 µM) or pepstatin A (100 µg/ml) for 72 h and then employed in the phagocytosis assay. The murine microglial cell line BV2 was chosen as prototype of immune effector cell. Fig. 6 (upper panel) shows data from the 1 h phagocytosis assay. H99 control cells were ingested by 5.8% of the microglial cells, while yeast cells pre-exposed to indinavir or pepstatin A were phagocytosed to a higher extent (9.5% and 14.4%, respectively). Moreover, Fig. 6 (lower panel) shows the results obtained when assaying C. neoformans, pre-exposed or not to indinavir, for susceptibility to microglia-mediated anticryptococcal activity. It was found that indinavir-pretreated yeast cells were growth inhibited by BV2 cells significantly more than controls (35% versus 18%, p < 0.01), while pepstatin A had intermediate effects. In parallel groups, morphology studies revealed appreciable differences between groups. As depicted in Fig. 7, C. neoformans yeast cells that had been pre-treated with indinavir and then exposed to BV2 microglial cells for 3 h appeared phagocytosed, damaged and partially or fully digested (right panel). Control C. neoformans exposed to BV2 cells, though being ingested, still retained a well conserved morphology (left panel).

4. Discussion

Here, we provide evidence that the viral PI indinavir has multiple effects on C. neoformans. In particular, by means of a previously established azoalbumin hydrolysis assay [34], this report shows that C. neoformans yeast cells exposed to indinavir exhibit significantly and consistently reduced protease activity with respect to control yeast cells. The phenomenon is time- and dose-dependent and similar to what observed when employing pepstatin A, as prototypal inhibitor of proteases [37]. Thus, the present data imply a direct inhibitory effect of indinavir on C. neoformans protease(s). Such a conclusion is in line with a previous report showing the efficacy of HIV PIs on protease activity by another fungal pathogen, C. albicans [10]. Notoriously, C. albicans possesses numerous genes that encode for secretory aspartyl proteases, whose role in the pathogenesis of candidiasis has been partially established [12–15]. Cas-
Cassone et al. [10] provide an elegant demonstration of PI effects against *C. albicans* proteases both at molecular and biological levels. Western blot analysis and functional data on enzymatic activity document a decrease in protease production by *Candida* yeast cells in vitro exposed to indinavir or ritonavir. It is worth noting that the indinavir dose-range effective against *C. albicans* [10] or *C. neoformans* [present study] is within the μmol order, while nmol doses are enough to impair retroviral proteases [38]; this suggests that indinavir has lower affinity for fungal than viral proteases. In this respect, it is known that, as other HIV PIs, indinavir contains a synthetic analogue of the phenylalanne-proline sequence, present gag and gag-pol retroviral protein precursors and known to be cleaved by the proteases during viral cycle [38]. Thus, by preventing such retroviral protein cleavage, indinavir impairs maturation and thereby infectivity of nascent virions. Preliminary experiments indicate that, similarly to that shown for *C. albicans* [10], not only protease production but also *C. neoformans* protease activity is directly affected by indinavir, since cryptococcal culture supernatants exposed to indinavir (10 μM for 1 h at 37 °C) and then tested for protease activity showed a reduction by 60% to 75% with respect to controls (data not shown).

Taken together, previous [10] and present data argue for the wide-spectrum activity of HIV PIs, being them effective also on microorganisms phylo-genetically distant from HIV, such as *Candida* and *Cryptococcus*. Not only *C. neoformans* ATCC H99 strain but also the clinical isolate 1782 is susceptible to indinavir inhibitory effects and similar trends have been observed with other laboratory as well as clinical isolates, including an acapsular strain (data not shown). This implies that what we observed is not restricted to a given laboratory strain but rather is a generalised phenomenon. In contrast to what has been shown in pancreatic and hepatic cells [39], indinavir does not affect mitochondrial membrane potential in *C. neoformans*. *C. neoformans* susceptibility to indinavir involves fungal growth as assessed by a CFU assay. Since it is proposed that *C. neoformans* produces proteases, as biochemical device(s) for nutritional recruitment [16,19], we suggest that such a pathway is critically disturbed upon exposure to the PI indinavir.

By a murine model of experimental meningoencephalitis, it has been demonstrated that the brain phagocytic compartment is an important defence barrier against *C. neoformans* [27,30–32]. Accordingly, in vitro studies reveal that microglial cells are endowed with direct antifungal activity and their efficacy may further increase, under appropriate conditions [27,28,33]. Here, we provide evidence that *C. neoformans* is more effectively ingested and growth inhibited by microglial cells once it has been exposed to indinavir. In particular, the rate of phagocytosis at early times (1 h) doubles and the levels of microglial cell-mediated antifungal activity (measured in a 24 h assay) are also significantly higher against indinavir-treated than against control yeast cells; accordingly, the morphology of intracellular cryptococci appears altered (already after 3 h) in the indinavir-treated groups but not in the untreated controls, further arguing for the importance of a rapid ingestion to achieve an effective intracellular growth inhibition.

Whether these in vitro data will have an in vivo counterpart, we may envisage that cryptococcal pathogenic potential is indeed affected by indinavir. Only an in depth investigation employing in vivo experimental models might provide direct insights into this issue. Encouraging information comes from previous studies [38] indicating that indinavir concentrations detectable in patients undergoing HAART therapy are, at least at the plasma level, within the dose-range known to be experimentally effective against fungal pathogens either in vitro (present study and previous reports [10,21]) or in vivo [10]. Certainly, when considering pathogens with peculiar tropism(s), tissue distribution should also be carefully considered, and particularly diffusion through the blood-brain barrier in the case of *C. neoformans*. In this respect, Haas et al. [40] have shown that the cerebrospinal fluid-to-plasma ratio for indinavir is 14%; yet, greater than 90% of the drug in cerebrospinal fluid is unbound, whereas only 42% is free in plasma. Thus although valuable pharmacokinetics data have been made available as yet, the therapeutic efficacy of indinavir remains to be established.

Overall, although aware of the intrinsic limitations of an in vitro model, we provide some insights on the multiple effects of indinavir. The most direct implication concerns the dual relevance of indinavir, since not only is fungal growth directly impaired but also the efficacy of brain defence(s) is enhanced once *C. neoformans* is exposed to the drug. Furthermore, our data support the hypothesis that protease activity by *C. neoformans* is a critical virulence factor, through which the pathogen counteracts host defences particularly brain macrophage-mediated phagocytosis and intracellular killing. Studies are in progress to identify the gene(s) coding for protease activity in *C. neoformans* and their potential susceptibility to PIs.

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