Influence of Indinavir on Virulence and Growth of *Cryptococcus neoformans*

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Indinavir selectively inhibited production of some virulence factors of *Cryptococcus neoformans*, such as urease and protease, but not melanin and phospholipase; moreover, it interfered with capsule formation. These effects led to increased susceptibility of *C. neoformans* to intracellular killing by natural effector cells. Prolonged incubation with indinavir resulted in inhibition of fungal growth. Indinavir can attenuate the virulence of the fungus, thus augmenting its susceptibility to the antimicrobial activity of natural effector cells. The reduction in cryptococcal infections in human immunodeficiency virus–positive patients might also be related to the antifungal activity of highly active antiretroviral therapy.

The advent of highly active antiretroviral therapy (HAART) with combination regimens including protease inhibitors (PIs) has led to dramatic reduction in AIDS-related morbidity and mortality as a result of improved cellular immune function and suppression of HIV replication [1]. Several studies have demonstrated the beneficial effects of PIs on the incidence and outcome of opportunistic infections, such as candidiasis [2], cryptococcosis [3], and microsporidiosis [4]. Although, in most cases, these clinical benefits were attributed to immune reconstitution, improvements in treatment of opportunistic infections have been demonstrated even in the absence of immunological recovery [4]. These observations led to the hypothesis that HIV PIs have a direct effect on microorganisms, an assertion that is supported by the fact that HAART directly inhibits the *Candida albicans* proteinase that enables adherence to host tissue [2].

*Cryptococcus neoformans* is an opportunistic fungus that has a marked predilection for the central nervous system and causes disease in immunocompromised hosts, including patients with AIDS. After the introduction of HAART, the drastic reduction in HIV-related cryptococcosis observed was apparently correlated with PI-mediated immune reconstitution [3]. Hence, a possible effect against the fungus should be considered even though, to date, no evidence of a direct effect of PIs on *C. neoformans* exists. The aim of the present study was to determine whether HIV PIs, indinavir in particular, exert a direct effect on growth and virulence factors of *C. neoformans*.

**Methods.** RPMI 1640 medium with glutamine and fetal calf serum was obtained from Gibco BRL. Indinavir was obtained from Antonio Cassone (Istituto Superiore di Sanità, Rome, Italy). A thinly encapsulated strain of *C. neoformans* variant *neoformans* serotype A (Royal Netherlands Academy of Arts and Sciences number 6995; National Institutes of Health number 37) was obtained from Central Bureau Schimmel Cultures.

Mononuclear cells were first separated by density gradient centrifugation from blood of healthy donors. Monocytes (i.e., peripheral blood mononuclear cells [PBMCs]) were harvested after plastic adherence, as described elsewhere [5]. The pellet containing granulocytes (i.e., polymorphonuclear leukocytes [PMNLs]) and erythrocytes was added with hypotonic saline to lyse the erythrocytes. PMNLs were collected by centrifugation, counted, and adjusted to the desired concentration. The purity of the isolated PMNLs was >98%, as determined by Giemsa staining.

To study the effect of indinavir on growth of *C. neoformans*, the yeast (107 cells/mL) was incubated in RPMI 1640 medium with or without graded doses of indinavir for various times. After washing, the yeast cells were tested for virulence factors and growth. After subculture in Sabouraud-dextrose agar (Sigma) for 72 h and incubation at 28°C, growth was measured by counting colony-forming units.

Determination of urease activity was performed by use of BBL urea slants (Becton Dickinson). Detection of production of phospholipase and protease was performed essentially as described by Polak [6], and the activity was expressed as described by Price et al. [7]. Determination and quantification of the production of melanin was performed as described by Franzot et al. [8].

To evaluate capsule synthesis, *C. neoformans* was stained with India ink and examined by use of an Axioskop 2 microscope integrated into AxioVision 3.0 and equipped with AxioCam (all from Zeiss). The distance from the edge of the capsule to the...
Table 1. Effect of different doses of indinavir on virulence factors of Cryptococcus neoformans.

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<tr>
<th>Treatment, dose</th>
<th>Virulence factor, duration of incubation</th>
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<tr>
<td></td>
<td>Urease 24 h</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
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<tr>
<td>Indinavir</td>
<td></td>
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<tr>
<td>10 μmol/L</td>
<td>+</td>
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<td>25 μmol/L</td>
<td>-</td>
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**NOTE.** Urease production was determined after inoculation of C. neoformans, untreated or treated with indinavir for 24 or 48 h, onto urea agar slants. Determination of phospholipase and protease activity was performed by use of a plate method. In brief, C. neoformans, untreated or treated for 24 or 48 h with indinavir, was incubated at 37°C on bovine serum albumin (protease) or egg yolk (phospholipase) agar plates. After 7 days, the diameter of the colony (a) and the colony plus the precipitation zone (b) was recorded. Melanin production by C. neoformans, untreated or treated for 24 or 48 h with indinavir, is evidenced by the organisms’ ability to form a brown-to-black pigment. +, Production of urease or melanin; −, no production of urease or melanin.

a Protease and phospholipase activity were expressed as \( P_z = \alpha + b \) (a and b are defined above in the note), and the parameters of classification of activity were as follows: strong (\( P_z < 0.5 \)), moderate (\( 0.50 \leq P_z < 0.70 \)), weak (\( P_z < 0.70 \)), or negative (\( P_z = 1 \)).

cell was measured with the help of a grid. The capsule size of 20 cells was measured, and the mean value was determined.

To evaluate the antimicrobial activity, PMNLs and PBMCs were mixed with C. neoformans (10⁶ cells/mL), either untreated or pretreated with indinavir, at an effector-to-target ratio of 10:1. Killing activity was expressed as described elsewhere [9]. Granule extracts were isolated from PMNLs and PBMCs, and their anticryptococcal activity was tested by adding them to 10⁷ C. neoformans cells/mL, either untreated or pretreated with indinavir, as described by Lehrer et al. [10].

Production of superoxide anion (O₂⁻) by PMNLs or PBMCs in response to C. neoformans, either untreated or pretreated with indinavir, was determined by use of superoxide dismutase (SOD)–inhibitable cytochrome C reduction, as described elsewhere [9]. In selected experiments, catalase and SOD were also used (both from Sigma).

**Figure 1.** Effect of different doses of indinavir on capsule size of Cryptococcus neoformans. Capsule size was measured in micrometers. *P < .05, treated vs. untreated C. neoformans.
Figure 2.  

A. Effect of treatment with different doses of indinavir on growth of *Cryptococcus neoformans*.  
B. Effect of treatment of with different doses of indinavir on antifungal activity, superoxide production, and granule extract killing by peripheral blood mononuclear cells and polymorphonuclear leukocytes. *P < .05, treated vs. untreated *C. neoformans.*

Data are reported as the mean ± SD of replicate experiments. Data were evaluated by use of one-way analysis of variance. Post hoc comparisons were done with Bonferroni’s test. A value of *P < .05* was considered to be statistically significant.

**Results.** The first step in the experiment was to analyze the virulence factors: urease, protease, phospholipase, and melanin. To this end, either 10 or 25 μmol/L indinavir was added to *C. neoformans* for 24 and 48 h. The results show that indinavir selectively affected protease and urease activity but not phospholipase and melanin activity. Urease activity was inhibited and was undetectable after 48 h of treatment with either 10 or 25 μmol/L indinavir. A decrease in protease activity was de-
ected after incubation for 24 h with either dose (10 or 25 μmol/L), as shown in table 1. The capsule is considered to be the major virulence factor of *C. neoformans*, and we observed that, at the dose of 25 μmol/L, indinavir is able to inhibit capsule formation within 48 h. Moreover, prolonged incubation (72 h) resulted in a more evident effect (figure 1).

Given that indinavir affected the virulence of *C. neoformans*, we tested whether it could influence fungal growth as well. The results reported in figure 2A show that indinavir did not inhibit fungal growth until 48 h of incubation, but a significant decrease was observed after this time.

We questioned whether treatment with indinavir renders the fungus more susceptible to natural effector cells. For this purpose, *C. neoformans* was exposed to indinavir for 48 h, a time frame that guaranteed fungal survival. The results reported in figure 2B show that both PMNLs and PBMCs killed indinavir-treated *C. neoformans* more efficiently than they killed untreated *C. neoformans*. This phenomenon correlated with improved production of O₂⁻ by both cell types (figure 2B). The neutralization of O₂⁻ by the addition of 80 μg/mL catalase and 8 μg/mL SOD produced a significant reduction in killing. Moreover, the antimicrobial capacity of granule extracts from PMNLs killed the indinavir-treated fungus more efficiently, suggesting that intracellular mechanisms are involved (figure 2B).

**Discussion.** The results reported here show that (1) indinavir directly affects selected virulence factors of *C. neoformans* (i.e., urease and protease) and capsule formation, whereas production of melanin and phospholipase is unaffected; (2) prior incubation with indinavir increases the susceptibility of *C. neoformans* to intracellular killing by natural effector cells; and (3) prolonged incubation with indinavir results in inhibition of fungal growth. Of note, the concentrations of indinavir used in the present study are similar to those found in the blood of patients receiving HAART regimens [11], strongly supporting the likelihood that indinavir has a beneficial effect against *C. neoformans* in vivo.

*C. neoformans* proteases have been considered to be virulence factors able to interfere with host defense mechanisms by affecting the integrity of host proteins, thereby promoting tissue invasion [12]: hence, the inhibition of protease secretion mediated by indinavir could restrain dissemination of *C. neoformans*. Given that indinavir affects secretion of aspartic protease in *C. albicans* infection [2], it is conceivable that a similar mechanism takes place in *C. neoformans* infection.

The augmented susceptibility of indinavir-treated *C. neoformans* to intracellular killing by immune cells could be ascribed, in part, to inhibition of urease activity, given that the role of urease in promoting survival of the fungus within mammalian hosts has been described elsewhere [13]. This phenomenon is consistent with the observed enhancement of the cytotoxic activity of PMNL granule extracts against indinavir-treated *C. neoformans*. The increased respiratory burst correlates with increased antifungal activity, suggesting that both oxidative and nonoxidative mechanisms participate in the killing; moreover, inhibition of oxidative mechanisms by SOD and catalase results in a partial reduction of anticyptococcal activity. Given that capsule size is important in influencing anticyptococcal function of phagocytic cells, impairment of capsule formation could contribute to increased susceptibility to killing and to increased production of O₂⁻, which is considered to be a cryptococcidial mechanism for phagocytic cells [14]. This effect could be particularly relevant in vivo because, apart from its antiphagocytic activity, the *C. neoformans* capsule is also endowed with immunosuppressive properties [15].

Finally, inhibition of growth was observed after prolonged exposure of *C. neoformans* to indinavir, confirming a direct effect exerted on the fungus. The complex mechanisms triggered by indinavir leading to a decrease in virulence and inhibition of fungal growth remain largely unknown; however, it seems logical to speculate that temporal events possibly begin with inhibition of fungal proteolytic potential, followed by suppression of capsule formation as an indirect and late effect, and eventually lead to the inhibition of fungal growth.

In conclusion, these data provide evidence of previously unknown effects of indinavir, including the attenuation of important virulence factors of *C. neoformans* that render the fungus more susceptible to intracellular killing by natural effector cells and the inhibition of *C. neoformans* growth during prolonged incubation with the drug. Thus, the beneficial effect of HIV PIs on cryptococcosis is not due solely to elevation of the CD4 cell count and improvement in immune function but is associated with direct inhibitory effects on selected virulence factors of the fungus.

**Acknowledgment**

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**References**

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