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Melanization of *Cryptococcus neoformans* and *Histoplasma capsulatum* Reduces Their Susceptibilities to Amphotericin B and Caspofungin

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The fungal pathogens *Cryptococcus neoformans* and *Histoplasma capsulatum* produce melanin-like pigments in the presence of L-dopa in vitro and during mammalian infection. We investigated whether melanization affected the susceptibilities of the fungi to amphotericin B, caspofungin, fluconazole, itraconazole, or flucytosine (5FC). Using the standard macrodilution MIC protocol (the M27A protocol) of the National Committee for Clinical Laboratory Standards for yeast, we found no difference in the susceptibilities of melanized and nonmelanized *C. neoformans* and *H. capsulatum* isolates. Killing assays demonstrated that melanization reduced the susceptibilities of both fungi to amphotericin B and caspofungin. Laccase-deficient *C. neoformans* cells grown with L-dopa were significantly more susceptible than congenic melanin-producing yeast to killing by amphotericin B or caspofungin. Preincubation of amphotericin B or caspofungin with melanins decreased their antifungal activities. Elemental analysis of melanins incubated with amphotericin B or caspofungin revealed an alteration in the C:N ratios of the melanins, which indicated binding of these drugs by the melanins. In contrast, incubation of fluconazole, itraconazole, or 5FC with melanins did not significantly affect the antifungal efficacies of the drugs or the chemical composition of the melanins. The results suggest a potential explanation for the inefficacy of caspofungin against *C. neoformans* in vivo, despite activity in vitro. Furthermore, the results indicate that fungal melanins protect *C. neoformans* and *H. capsulatum* from the activities of amphotericin B and caspofungin and that this protection is not demonstrable by standard broth macrodilution assays.

Cryptococcus neoformans and *Histoplasma capsulatum* are relatively frequent causes of serious fungal infections in immunocompromised patients. The prevalence in the United States of cryptococcal meningoencephalitis in patients with AIDS is estimated to be 5 to 10% (6, 22). Disseminated histoplasmosis occurs in approximately 5% of patients with AIDS residing in areas of the United States where histoplasmosis is endemic (12). Patients with AIDS complicated by cryptococcosis or histoplasmosis often respond poorly to treatment and require lifelong maintenance therapy since current antifungal agents seldom eradicate these fungal pathogens in the setting of severe immune suppression (11, 38).

The ability to produce melanin-like pigments in vitro was associated with virulence in *C. neoformans* (4, 17, 19), and melanin synthesis occurs during human infection (28). *H. capsulatum* has also recently been shown to produce melanin in vitro and in vivo (25a). Melanins are complex polymers that are ubiquitous in nature (14) and have been associated with diverse biological effects ranging from camouflage to energy transformation to drug binding (14, 15, 20). In *C. neoformans*, melanin protects the fungus against damage caused by UV light, oxidants, extremes in temperature, and macrophages in vitro (for a review, see reference 5). Additionally, there is evidence that melanin affects the susceptibility of *C. neoformans* to certain compounds, such as amphotericin B, trifluoroperazine, and microbicidal peptides (7, 34, 35). Therefore, we evaluated the effects of melanin synthesis on the susceptibilities of *C. neoformans* and *H. capsulatum* to currently available systemic antifungals.

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MATERIALS AND METHODS

Chemicals. Glucose, $MgSO_4$, and KH_2PO_4 were purchased from J. T. Baker Inc. (Philipsburg, N.J.). Glycine, NaCl, RPMI 1640 medium (with L-glutamine and without sodium bicarbonate), vitamin B_1, synthetic melanin (prepared by the oxidation of tyrosine with hydrogen peroxide), morpholinepropanesulfonic acid (MOPS), and L-dopa were purchased from Sigma Chemical Co. (Cleveland, Ohio). Amphotericin B was purchased from Bristol-Myers Squibb (New York, N.Y.), fluconazole was purchased from Pfizer (New York, N.Y.), flucytosine (5FC) was purchased from Roche (Nutley, N.J.), itraconazole was purchased from Janssen (Spring House, Pa.), and caspofungin was purchased from Merck (Whitehouse Station, N.J.). Caspofungin was used in these experiments, even though it has limited clinical efficacy against these pathogens, to establish proof of principle for the effect of melanin on antifungal drugs.

***C. neoformans* and *H. capsulatum*.** *C. neoformans* strain 24067 (serotype D) and *H. capsulatum* strain G217B were obtained from the American Type Culture Collection (Manassas, Va.). *C. neoformans* H99 (serotype A) was obtained from J. Perfect (Durham, N.C.). A pair of congenic strains, 2E-TUC (laccase positive) and 2E-TU (laccase deficient), were gifts from P. R. Williamson (Chicago, Ill.) (32). *H. capsulatum* strain CIB 1980 was a gift from A. Restrepo (Medellín, Colombia). Melanization was induced by growing yeast cells on defined minimal medium agar plates (15 mM glucose, 10 mM $MgSO_4$, 29.4 KH_2PO_4, 13 mM glycine, 3.0 μM vitamin B_1, 2% agar; Difco) with the addition of 1 mM L-dopa for 10 days. Nonmelanized controls were obtained by growing yeast cells on defined minimal medium agar plates without L-dopa for 10 days. *C. neoformans* and *H. capsulatum* cells were grown at 30 and 37°C, respectively. Growth of *C. neoformans* strain 2E-TU in agar with L-dopa resulted in albino colonies.

MIC determination. MICs were determined by the standardized protocol (the M27A protocol) for yeasts developed by the National Committee for Clinical Laboratory Standards (NCCLS) (24). Briefly, melanized or nonmelanized yeast cells were suspended in sterile normal saline and diluted to a concentration of 2×10^6 cells/ml. Cell counts were determined with a hemacytometer. The suspensions were diluted 1:1,000 in RPMI 1640 medium with L-glutamine, without bicarbonate, and buffered to pH 7.0 with 0.165 M MOPS. Polystyrene tubes containing 0.1-ml aliquots of an antifungal at 10 times the final drug concentra-

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TABLE 1. MICs for *C. neoformans* and *H. capsulatum* strains

Drug	MIC (μg/ml)					
	C. neoformans H99		*C. neoformans* 24067		*H. capsulatum* CIB 1980	
	With L-dopa	Without L-dopa	With L-dopa	Without L-dopa	With L-dopa	Without L-dopa
Amphotericin B	0.125	0.125	0.1875	0.125	0.125	0.25
Fluconazole	1	1	1	1	0.5	0.5
Itraconazole	0.625	<0.625	<0.625	<0.625	<0.625	<0.625
5FC	2	2	2	3	0.125	0.1875
Caspofungin	8	8	8	8	<0.125	<0.125

tion were inoculated with 0.9 ml of the diluted suspensions. Final drug concentrations ranged from 0.0625 to 4 μg/ml for amphotericin B, 0.125 to 16 μg/ml for fluconazole and 5FC, 0.0625 to 4 μg/ml for itraconazole, and 0.125 to 64 μg/ml for caspofungin. The MICs were recorded after incubation at 35°C (*C. neoformans* strains 24067 and H99) or 37°C (*H. capsulatum* strain CIB 1980) for 72 h. The MICs of amphotericin B and caspofungin were defined as the lowest concentrations at which there was an absence of growth. The MICs of fluconazole, itraconazole, and 5FC were defined as the lowest drug concentrations which achieved 80% growth inhibition compared to the growth of the drug-free control.

Killing assay. Yeast cells were suspended in sterile normal saline at a density of 2.2×10^3 cells/ml. Cell counts were determined with a hemacytometer. Microcentrifuge tubes containing 0.1-ml aliquots of an antifungal at 10 times the final concentration were inoculated with 0.9 ml of the yeast suspensions. For *C. neoformans*, final drug concentrations ranged from 0.125 to 0.5 μg/ml for amphotericin B, 16 to 64 μg/ml for caspofungin, 0.25 to 4 μg/ml for 5FC and fluconazole, and 0.125 to 0.5 μg/ml for itraconazole. For *H. capsulatum*, final drug concentrations ranged from 0.125 to 0.5 μg/ml for amphotericin B, 2 to 8 μg/ml for caspofungin, 0.25 to 4 μg/ml for 5FC and fluconazole, and 0.125 to 0.5 μg/ml for itraconazole. After incubation at 35°C (*C. neoformans*) or 37°C (*H. capsulatum*) for 2 h, aliquots were plated on Sabouraud dextrose agar (*C. neoformans*) or brain heart infusion agar (*H. capsulatum*) to determine their viabilities, as measured by determination of the numbers of CFU. The rate of survival was compared to that of fungal cells incubated in phosphate-buffered saline (PBS).

Melanin ghosts. The term "melanin ghosts" refers to the melanin structures isolated from melanized *C. neoformans*. The ghosts were obtained from melanized *C. neoformans* cells as described previously (31). Briefly, melanized cryptococcal cells were serially treated with 10 mg of cell wall-lysing enzymes (from *Trichoderma harzianum*; Sigma) per ml, 4.0 M guanidine thiocyanate, and 1.0 mg of proteinase K per ml, extracted three times with chloroform, and boiled in 6.0 M HCl for 1 h. The resulting black debris was then dialyzed against distilled H_2O (dH_2O) for 10 days. Nonmelanized cryptococcal cells that are subjected to this protocol are completely solubilized.

Binding of antifungal drugs to melanin. Two milliliters each of stock solutions of fluconazole (5 mg/ml in dH_2O), amphotericin B (0.25 mg/ml in dimethyl sulfoxide), and caspofungin (5 mg/ml in dimethyl sulfoxide) was incubated with cryptococcal melanin (7.7×10^9 particles/ml) and synthetic melanin (20 mg) for 2 h at 30°C. Itraconazole and 5FC (5 mg/ml in dH_2O) were incubated with synthetic melanin. The melanins were removed by centrifugation. The supernatants of the amphotericin B and caspofungin solutions were used in the killing assay with *C. neoformans* strain 24067 and *H. capsulatum* strain CIB 1980. The supernatants of the fluconazole, itraconazole, and 5FC solutions were used for MIC determinations with *C. neoformans* strain 24067 and *H. capsulatum* strain CIB 1980. The melanin pellets from the incubated solutions were washed extensively with dH_2O and lyophilized with a Flexi-Dry microprocessor (FTS Systems, Inc., Stone Ridge, N.Y.). As controls, cryptococcal melanin and synthetic melanin in dH_2O were lyophilized. Quantitative elemental analysis of the melanins was performed by Quantitative Technologies Inc. (Whitehouse, N.J.). Briefly, the melanin samples were converted into gases by combustion, and the product gases were separated under steady-state conditions. The percentage of each element was measured as a function of thermal conductivity. Elemental ratios were calculated by dividing the percentage of each element measured by its respective atomic weight.

Statistics. Microsoft Excel 97 software was used to determine the standard deviations, standard errors of the means, and *P* values (Student's *t* test).

RESULTS

MICs. Table 1 summarizes the in vitro susceptibilities of melanized versus nonmelanized cells of *C. neoformans* strains 24067 and H99 and *H. capsulatum* strain CIB 1980 to amphotericin B, caspofungin, fluconazole, itraconazole, and 5FC. The MICs were within the range of values previously reported for these fungi (9, 16, 18, 21, 36), except for the relatively low MIC of caspofungin for *H. capsulatum* (16, 18). There were no significant differences in the MICs for melanized and nonmelanized cells of the same strain for any of the drugs tested. For *H. capsulatum*, an extended incubation time for MIC testing has been described (29, 36). Even though there was sufficient growth to determine the MICs at 72 h, we also measured the MICs for *H. capsulatum* at 96 to 144 h. These tests also showed no differences in the MICs for the melanized and the nonmelanized cells (data not shown).

In an attempt to determine whether the inability to demonstrate differences between melanized and nonmelanized cells was due to the absence of substrate for further melanin formation in the melanized cells and their progeny (J. Nosanchuk, A. L. Rosas, and A. Casadevall, Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. F13, 1999), we added L-dopa to the RPMI medium. However, this resulted in the formation of black precipitates when the antifungals were added to the medium, whether or not yeast cells were present. We also tried a modification of the NCCLS protocol using yeast nitrogen base medium buffered at a pH of 7.0 instead of RPMI medium (10, 37). Unfortunately, this modification was not successful since precipitates also formed when antifungals and L-dopa were added to this medium in the presence or absence of cells. The precipitates could be a complex of drug and L-dopa or could represent the induction of auto-oxidation of L-dopa by the antifungals. Other precursors, such as epinephrine, caused similar precipitates. Hence, we were unable to modify the assay to investigate this question.

Killing assay. Melanized cells were significantly less susceptible to amphotericin B or caspofungin than nonmelanized cells (Fig. 1 and 2). The differences were statistically significant for all concentrations tested with *C. neoformans* (Fig. 1). Melanized cells of *H. capsulatum* were significantly protected against amphotericin B compared to the susceptibilities of nonmelanized cells at concentrations <0.25 and <0.5 μg/ml for strains CIB 1980 and G217B, respectively. Melanized *C. neoformans* cells were also less susceptible than nonmelanized cells to caspofungin at all concentrations tested, except for strain 24067 with caspofungin at 64 μg/ml (Fig. 2). For *H.*

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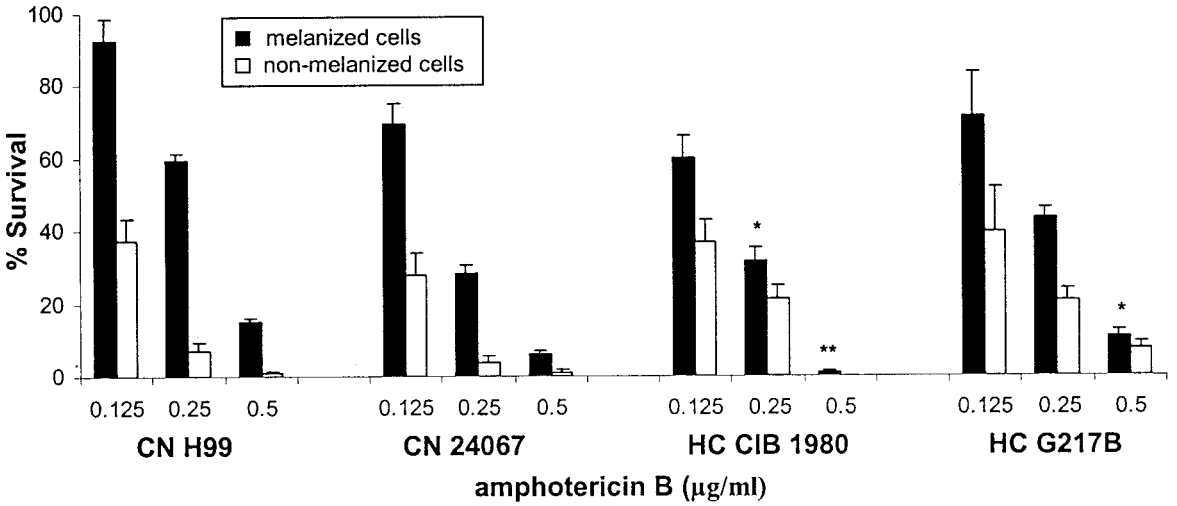

FIG. 1. Amphotericin B killing assay. The rates of survival of melanized versus nonmelanized yeast cells (*C. neoformans* strains H99 [CN H99] and 24067 [CN 24067] and *H. capsulatum* strains CIB 1980 [HC CIB 1980] and G217B [HC G217B]) after exposure to various concentrations of amphotericin B for 2 h were compared to those of fungal cells incubated in PBS. Values are averages ± standard errors of the means for four measurements. *P* values were calculated by comparing nonmelaninzed and melanized cells. *P* values were <0.01 unless noted otherwise (*, $P = 0.06$; **, $P = 0.1$) (Student's *t* test). Similar results were obtained in two separate experiments.

capsulatum, significant differences in rates of survival between melanized and nonmelanized cells were demonstrated at concentrations >2 and >4 μg/ml for strains CIB 1980 and G217B, respectively. With fluconazole, itraconazole, and 5FC, there were no significant differences in rates of survival for melanized versus nonmelanized cells (data not shown).

To further validate these findings, we investigated the effects of antifungal agents on laccase-deficient *C. neoformans* strain 2E-TU, which does not melanize in the presence of L-dopa. The MICs, determined by the NCCLS M27A protocol, for melanized and nonmelanized cells of strains 2E-TU and 2E-TUC were similar (Table 2). However, melanized *C. neoformans* strain 2E-TUC was significantly less susceptible to killing by either antifungal than strain 2E-TU (laccase-deficient) incubated with L-dopa or either strain grown without L-dopa ($P < 0.001$ for all concentrations tested) (Fig. 3). This demonstrates that the protective effect seen in cells grown in the presence of L-dopa occurs only if the yeast cells produce melanin.

Binding of antifungals to melanin. To evaluate whether the results of the killing assays were due to binding of the drugs to

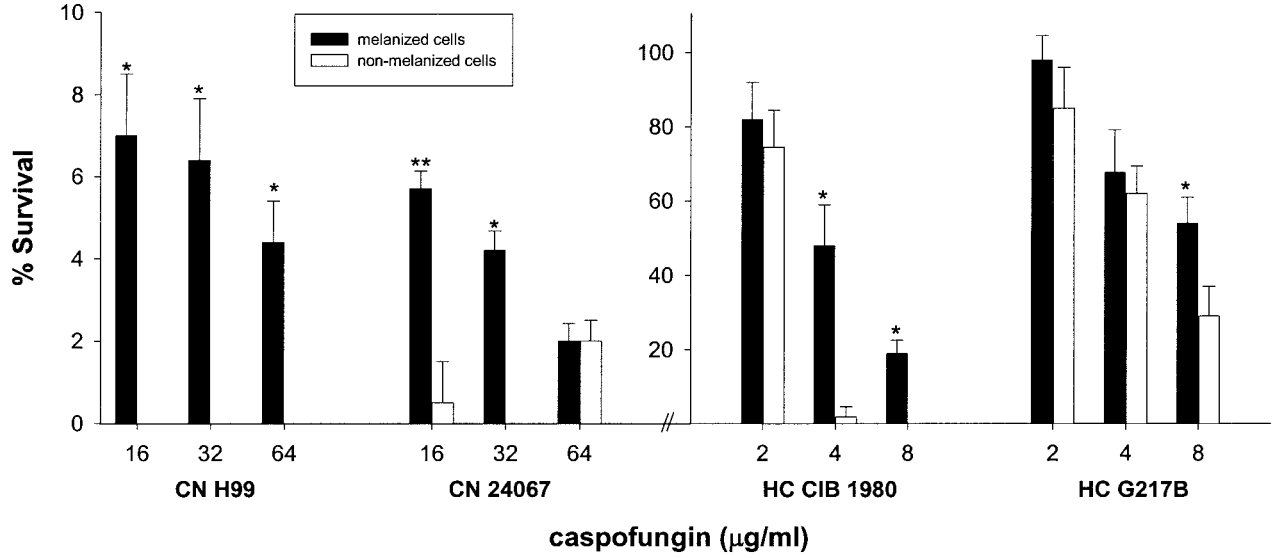

FIG. 2. Caspofungin killing assay. The rates of survival of melanized versus nonmelanized yeast cells (*C. neoformans* strains H99 [CN H99] and 24067 [CN 24067] and *H. capsulatum* strains CIB 1980 [HC CIB 1980] and G217B [HC G217B]) after exposure to various concentrations of caspofungin for 2 h were compared those of fungal cells incubated in PBS. Values are averages ± standard errors of the means for four measurements. *P* values were calculated by comparing nonmelaninzed and melanized cells. *, $P < 0.001$; **, $P < 0.05$ (Student's *t* test). Similar results were obtained in two separate experiments.

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TABLE 2. MICs for *C. neoformans* 2E-TU and 2E-TUC

Drug	MIC (μg/ml)			
	C. neoformans 2E-TU		*C. neoformans* 2E-TUC	
	With L-dopa	Without L-dopa	With L-dopa	Without L-dopa
Amphotericin B	0.25	0.125	0.25	0.25
Caspofungin	16	16	16	16

melanin, we incubated the antifungals with melanin before we performed the killing assays or determined the MICs. Incubation of amphotericin B or caspofungin with either *C. neoformans* or synthetic melanin prior to use significantly reduced the efficacies of the drugs compared to their efficacies in assays in which the antifungals were not preincubated with melanin (Fig. 4). The MICs of fluconazole, itraconazole, and 5FC determined after incubation with melanin were unchanged (data not shown).

The results of the elemental analysis of the melanins incubated with the antifungals are shown in Table 3. The analysis was performed twice with melanins from different experiments, with similar results obtained in each experiment. Incubation of melanin with amphotericin B or caspofungin resulted in alterations of the C:N ratios of the melanins, which is consistent with the fact these drugs had bound to the melanins. In contrast, incubation of melanin with the azoles or 5FC did not alter the melanin C:N ratios. Additionally, analysis of the melanins revealed no fluorine after incubation with either fluconazole or 5FC and no chloride after incubation with itraconazole (data not shown). These results are consistent with the notion that caspofungin and amphotericin B bind to melanin, whereas fluconazole, itraconazole, and 5FC do not.

DISCUSSION

The prognosis for AIDS patients with cryptococcosis or histoplasmosis is poor, despite therapy (13, 30). The problem is that the currently available antifungal agents seldom eradicate infection in the setting of severe immune suppression. In a small study carried out several years ago, we suggested that melanization of certain fungal pathogens may affect the activities of antifungal drugs (5). Here we have revisited that question and demonstrate that melanization of *C. neoformans* and *H. capsulatum* reduces their susceptibilities to amphotericin B and caspofungin. However, melanin did not protect these organisms against fluconazole, itraconazole, or 5FC.

We were unable to demonstrate the protective effect of melanization against these antifungal drugs using the NCCLS standard broth macrodilution protocol for assessing the susceptibilities of yeast to antifungals. This may have resulted from the absence of substrates for melanization in the medium used in the NCCLS protocol. Even though we started with melanized cells for MIC determinations, replicating yeast cells were unable to further synthesize melanin. Growth of melanized *C. neoformans* in the absence of substrates results in large defects in the melanin layer of the parent cells after budding and an absence of melanin in the daughter cells (Nosanchuk et al., Abstr. 99th Gen Meet. Am. Soc. Microbiol.). Hence, the NCCLS assay does not actually compare melanized and nonmelanized cells. We attempted to bypass this problem by adding L-dopa to RPMI, but this resulted in the formation of a black precipitate that precluded use of this modification to the assay. The NCCLS methodology has previously been shown to have inherent problems with measuring the susceptibility of *C. neoformans*, including a long incubation period, suboptimal growth, and a narrow range of MICs (33). Furthermore, the MICs obtained by the NCCLS protocol are not predictive of the clinical outcomes of *C. neoformans* infec-

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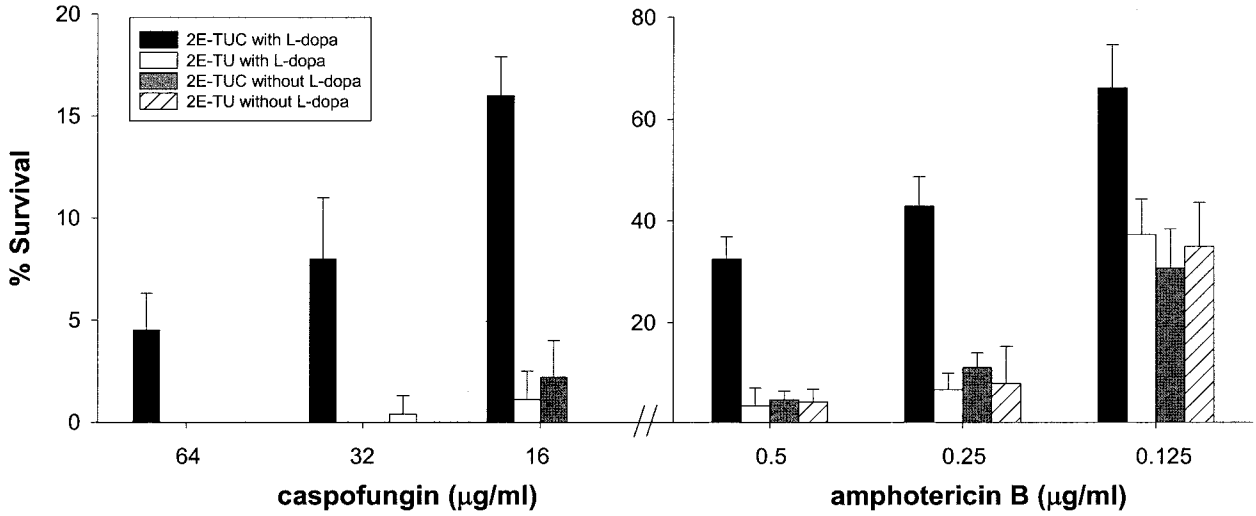

FIG. 3. Killing assay with *C. neoformans* congenic strains 2E-TUC (laccase positive) and 2E-TU (laccase deficient). Yeast cells were grown with or without L-dopa for 10 days and then exposed to various concentrations of amphotericin B or caspofungin for 2 h, and the rates of killing were compared to those for fungal cells incubated in PBS. Values are averages ± standard errors of the means for three measurements. Melanized 2E-TUC yeast cells were significantly ($P < 0.001$) less susceptible to killing under each condition examined.

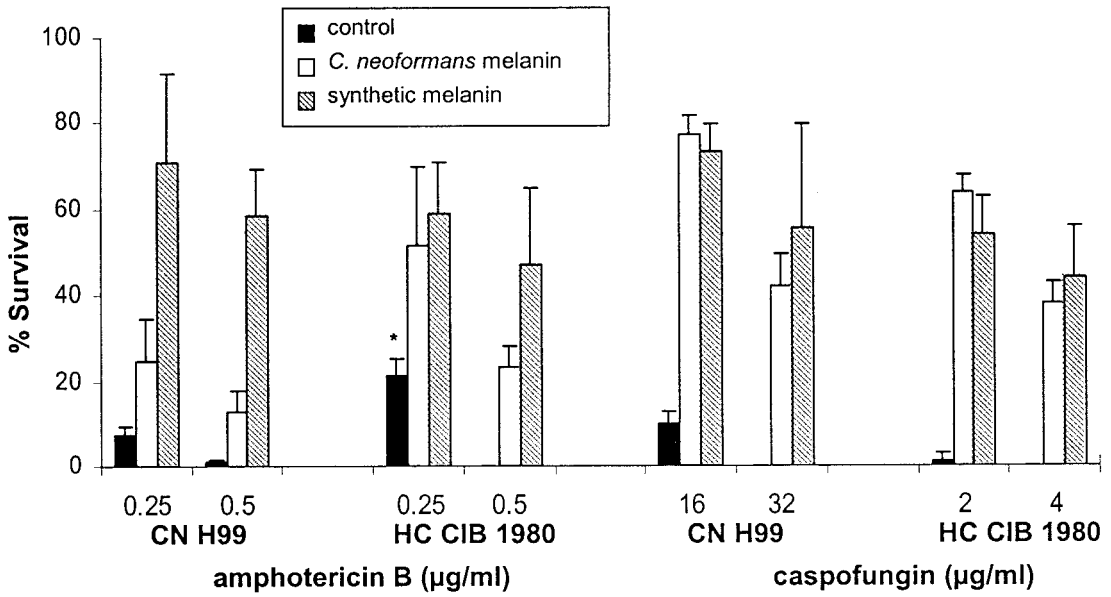

FIG. 4. Killing assay with melanins. The rates of survival of nonmelanized yeast cells (*C. neoformans* strain H99 [CN H99] and *H. capsulatum* strain CIB 1980 [HC CIB 1980]) exposed to amphotericin B or caspofungin with or without preincubation of the antifungals with *C. neoformans* or synthetic melanin were compared. Values are averages ± standard errors of the means for four measurements. *P* values refer to the difference between the rates of survival for nonmelanized yeast cells after preincubation of the antifungals with *C. neoformans* or synthetic melanin and those for cells not preincubated with synthetic melanin (control). All *P* values were <0.001 unless noted otherwise (*, $P < 0.01$) (Student's *t* test).

tions in patients with AIDS (37). This lack of correlation between MIC and outcome could reflect melanization of the fungus in vivo (28). Our findings that the NCCLS protocol cannot be adapted to measure susceptibility to *C. neoformans* in the presence of laccase substrates indicates yet another limitation of this method.

Killing assays demonstrated that melanization of *C. neoformans* and *H. capsulatum* protected the yeast cells from amphotericin B and caspofungin. No benefit for melanin formation was detected when fluconazole or itraconazole was used. However, neither azole resulted in significant killing, which is consistent with the fungistatic properties of these drugs. We also did not detect significant killing when we tested the potentially fungicidal drug 5FC in our killing assay. The protective effect of melanin against amphotericin B and caspofungin, but not against the azoles or 5FC, can be explained by the results of the binding assays performed after incubation of the antifungals with synthetic or *C. neoformans* melanin and the subsequent analyses of these melanins. Preincubation of amphotericin B and caspofungin with melanin reduced the fungicidal activities of the drugs, with the synthetic melanin having a greater effect. The two melanins have significant differences in their surface areas and epitope distributions (27), charges (24), and C:N ratios (31). Therefore, the interactions of the two melanins with the antifungals may be different. In our assays we based the quantity of melanin used for the experiments on the amount necessary for quantitative elemental analysis, rather than attempting to have equivalent surface areas. Therefore, the significant finding is that preincubation with melanin reduced the efficacies of amphotericin B and caspofungin. Chemical analysis indicated that both synthetic and *C. neoformans* melanins bound to amphotericin B and caspofungin, as indicated by a change in the C:N ratios of the melanin exposed to these drugs. However, there was no change in the melanin C:N ratio or the F or Cl content after exposure to either azole or 5FC, consistent with no binding by these drugs.

In addition to intercalating into fungal cell membranes and presumably causing pore formation, amphotericin B is a potent immunomodulator (for a review, see reference 25). Thus, binding of amphotericin B by melanin may also have a significant impact on the course of fungal infection by abating the effect of the drug on diverse host factors, such as T-cell and macrophage activation. Melanins are also immunologically active molecules with notable anti-inflammatory effects (2, 23). Consequently, melanins down regulate cytokines as well as decrease host cell stimulation by binding to amphotericin B. These factors may contribute to the inability of amphotericin B to eradicate *C. neoformans* and *H. capsulatum* infections in im-

TABLE 3. Elemental analysis of melanins

Sample	C:N ratio
Control ghosts	8:1
Amphotericin B + ghosts	11.5:1
Caspofungin + ghosts	10:1
Fluconazole + ghosts	8:1
Itraconazole + ghosts	8:1
5FC + ghosts	8:1
Control synthetic melanin	9:1
Amphotericin B + synthetic melanin	10:1
Caspofungin + synthetic melanin	10:1
Fluconazole + synthetic melanin	9:1
Itraconazole + synthetic melanin	9:1
5FC + synthetic melanin	9:1

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munocompromised individuals. The antifungal effects of caspofungin are attributed to the inhibition of synthesis of 1,3-β-D-glucan linkages in fungal cell walls (3). For *C. neoformans*, caspofungin may also affect 1,6-β-D-glucan synthesis (8). Our findings suggest that drug binding by melanin may also represent a mechanism for the lack of efficacy of caspofungin in vivo for cryptococcosis (1) and histoplasmosis (16).

Melanization is essential for virulence in *C. neoformans*, and melanin is synthesized during human infection (28). Melanization also occurs in mammalian tissues during *H. capsulatum* infection (25a). Here, we show that melanization decreases the susceptibilities of *C. neoformans* and *H. capsulatum* to amphotericin B and caspofungin. With standard in vitro susceptibility testing, this effect was not appreciated, and thus, these tests may underestimate the resistance of these organisms to amphotericin B or caspofungin in vivo. Since amphotericin B is considered the "gold standard" antifungal for the treatment of infections caused by *C. neoformans* or *H. capsulatum*, this finding is particularly important when the relative levels of effectiveness of antifungal agents are compared. Standard macrodilution susceptibility testing may also underestimate the activities of amphotericin B and caspofungin against other fungal organisms that produce melanin, such as *Aspergillus* spp., *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, and *Wangiella dermatitidis*. These results suggest that drugs that inhibit fungal melanization, as has recently been reported for glyphosate (26), could be synergistic with amphotericin B and caspofungin. These findings should be considered in the design of future susceptibility testing protocols and in drug development.

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