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Cryptococcus neoformans Resistance to Echinocandins: (1,3)β-Glucan Synthase Activity Is Sensitive to Echinocandins

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(1,3)β-D-Glucan synthase (EC 2.4.1.34. UDP-glucose: 1,3-β-D-glucan 3-β-glucosyltransferase) uses UDP-glucose as substrate and catalyzes the polymerization of glucose ([1,3]-β-linkages) to form the major carbohydrate component of the fungal cell wall. We have optimized in vitro assay conditions for (1,3)β-glucan synthase activity from *Cryptococcus neoformans*. Cells lysed in 50 mM Tris, pH 7.75, containing 20% glycerol, 2 mM NaF, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM $MgCl_2$, 0.1% protease and phosphatase inhibitor cocktails, and 60 μM GTPγS produced maximum specific activity in vitro. We tested in vitro *C. neoformans* (1,3)β-glucan synthase activity against the (1,3)β-glucan synthase inhibitors, caspofungin and cilofungin, and have determined that (1,3)β-glucan synthase activity is very sensitive (apparent K_i of 0.17 ± 0.02 μM and 22 ± 5.7 μM, respectively) to these echinocandins. Taken together with high MICs for *C. neoformans* (caspofungin MIC, 16 μg/ml; cilofungin MIC, 64 μg/ml), our results indicate that *C. neoformans* is resistant to caspofungin and cilofungin by a mechanism(s) unrelated to (1,3)β-glucan synthase resistance.

Cryptococcus neoformans is an encapsulated pathogenic yeast that causes pulmonary infections and fatal meningoencephalitis in humans. Disseminated cryptococcosis is an important disease primarily associated with individuals whose cellular immunity has been compromised by viral infection, suppression due to tissue transplantation, or antineoplastic chemotherapy (8, 11, 23, 36). An estimated 7 to 10% of AIDS patients acquire cryptococcosis during the course of infection with human immunodeficiency virus (20, 27, 39). Cryptococcosis is a leading cause of death among immunocompromised patients, and cryptococcal pneumonia is the next most frequent manifestation of cryptococcosis in AIDS patients and occurs as a primary infection in approximately 4% of cases (39).

Amphotericin B or azoles are recommended for treatment of cryptococcosis (35), either alone or in combination with 5-flucytosine. Flucytosine targets pyrimidine biosynthesis and is not used as sole therapy because strains frequently become resistant (45). Though they are the current standard, these drugs are not ideal for treating fungal infections in humans. Amphotericin B binds both fungal and human sterols, which results in toxic side affects. The azoles, while more tolerated than amphotericin B, are fungistatic and not fungicidal, requiring lifelong therapy to prevent infection relapse (11, 46). In addition, fluconazole-resistant strains of *C. neoformans* are emerging (32), and long-term treatment with fluconazole is likely to hasten this trend.

The cell wall of *C. neoformans* is primarily composed of three major types of glucan polymers, and electron microscopy studies using gold-labeled antibodies against (1,3)β-linkages confirms the presence of (1,3)β-linked glucan in the cell wall (12). In most fungi, compounds that inhibit (1,3)β-glucan synthase activity lead to fungal death; furthermore, deletions in the gene(s) encoding the FKS subunit(s) of glucan synthase are also lethal. Taken together, these and other results indicate that the synthesis of (1,3)β-glucan is essential for fungal growth in vitro and in vivo (9, 10, 19, 25, 26, 34, 42). (1,3)β-Glucan synthase activity is ubiquitous among fungi, including *C. neoformans*, and is a complex of at least two proteins, a *rho-1* GTPase regulatory protein and Fksp, which has been shown by our laboratory to bind the substrate, UDP-glucose (37), and is likely the catalytic subunit. The enzyme synthesizes (1,3)β-linked glucan using UDP-glucose (UDP-glc) as substrate and vectorially synthesizes nascent glucan through the plasma membrane. In *C. neoformans* the FKS subunit is present in single copy and has been shown to be essential for growth (42).

Caspofungin is a member of the echinocandin class of (1,3)β-glucan synthase inhibitors that is effective against many species of fungi and is safe, effective, and well-tolerated in humans (21). However, *C. neoformans* is resistant both in vitro and in vivo to caspofungin and other echinocandins (1, 4, 21, 22). This is puzzling, given that the gene encoding the (1,3)β-glucan synthase Fks subunit is essential for *C. neoformans* growth and that (1,3)β-glucans are found in the cell wall. It is not understood why *C. neoformans* is resistant to echinocandins, but resistance is likely due to common microbial drug resistance mechanisms involving one (or more) of the following possibilities: (i) the target itself, (1,3)β-glucan synthase, is resistant to caspofungin; (ii) caspofungin is excluded from cells and the target by, for example, ATP-binding cassette (ABC) transporters or other mechanisms; or (iii) caspofungin is degraded either extra- and/or intracellularly.

There is a report in the literature of an in vitro (1,3)β-glucan synthase assay using *C. neoformans* crude microsomal preparations that revealed that the *C. neoformans* H99 strain (and other strains) had detectable in vitro glucan synthase activity (42). Importantly, these authors did not test the effect of caspofungin on enzyme activity (42). However, this assay was not

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optimized for in vitro assay conditions and parameters such as pH optimum, substrate concentration, requirement for metal divalent ions, buffer type and ionic strength, and linear product formation with protein and time. Unfortunately, suboptimal in vitro conditions are likely to obfuscate the effect of inhibitors on enzyme activity.

The long-term goal of our work is to determine why *C. neoformans* is not sensitive to echinocandins so that new inhibitors can be designed. In this paper, we begin to elucidate the specific mechanism(s) of resistance by first determining the sensitivity of *C. neoformans* (1,3)β-glucan synthase activity to caspofungin and another echinocandin, cilofungin. As a first step, we have determined the optimal in vitro conditions for (1,3)β-glucan synthase activity from *C. neoformans*. Using these optimal in vitro conditions for the assay, we show that *C. neoformans* (1,3)β-glucan synthase is indeed very sensitive to caspofungin and cilofungin. Therefore, *C. neoformans* is resistant to echinocandins by a mechanism(s) other than the resistance of (1,3)β-glucan synthase activity. Studies comparing MICs and the apparent K_i [$K_{i(\mathrm{app})}$] of other fungi confirm this conclusion.

MATERIALS AND METHODS

Chemicals. Yeast extract, Bacto peptone, Sabouraud medium, yeast nitrogen base, and agar were purchased from Difco (Detroit, MI). RPMI 1640 tissue culture medium (with glutamine, without bicarbonate, and with pH indicator) was purchased from Gibco (Rockville, MD). Caspofungin was a gift from Merck and Co. (Rahway, NJ); cilofungin was a gift from Eli Lilly and Company (Indianapolis, IN). UDP-[^{14}C]glucose was purchased from MP Biomedicals (Irvine, CA). Laminarinase (from mollusk), α-amylase (type II-A), protease and phosphatase inhibitor cocktails, and all other chemicals were of reagent grade from Sigma-Aldrich, Inc. (St. Louis, MO).

Culture conditions. *C. neoformans* H99 strain was obtained from Jennifer Lodge of St. Louis University School of Medicine, St. Louis, Mo. Working stocks of H99 cells were prepared by inoculating cells onto agar-solidified PYG (0.5% [wt/vol] peptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] glucose) medium. The slants were incubated at 37°C for 3 to 4 days and stored at −80°C until used. Frozen slants were thawed at room temperature, flooded with 2 ml of sterile water, and used to inoculate 50 ml of YPAD (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] glucose, 0.01% [wt/vol] adenine hemisulfate) medium. Fifty-milliliter cultures in 250-ml Erlenmeyer flasks were grown with shaking (140 rpm) at 35°C for 12 to 16 h. The cell density of each 50-ml culture was determined using a hemocytometer, and cells were used to inoculate 1-liter cultures of YPAD in 2-liter Erlenmeyer flasks to an initial concentration of 1×10^6 cells/ml. One-liter cultures were incubated with shaking (140 rpm) at 35°C for 24 to 30 h. Cells were harvested by centrifugation ($3{,}000 \times g$ for 10 min at 4°C), washed three times by centrifugation with ice-cold water, quick-frozen on dry ice, and stored at −80°C until used.

(1,3)β-Glucan synthase assays. *C. neoformans* H99 cells were disrupted by shaking on ice (~300 rpm) with equal parts frozen cells and various buffers containing 10 μM GTPγS and 8 g of acid-washed glass beads (425 to 600 microns) (Sigma-Aldrich, Inc.) for 2 h. Crude lysates were separated from the beads by aspiration, and after centrifugation ($1{,}000 \times g$ for 10 min at 4°C), the low-speed supernatants were used as enzyme sources. The protein concentrations of supernatants were determined using bovine serum albumin, fraction V, as the standard (5).

Enzyme reactions were performed in 96-well V-bottom microtiter plates (Dynatech Laboratories, Alexandria, VA); each reaction mixture contained 0.5 mM UDP-[^{14}C]glucose (~60,000 cpm per assay), 50 μg of α-amylase, various additions, and low-speed supernatant (~40 to 120 μg of protein) in a final volume of 26 μl. Reactions were started by adding cell lysate to ice-cold reaction mixtures, incubated at 25°C for various times, and stopped by the addition of 50 μl 5% (wt/vol) trichloroacetic acid (TCA). Reaction products were filtered through glass fiber filters (Printed Filtermat A; Perkin-Elmer Life Sciences, Boston, MA), which had been previously washed with 1% (wt/vol) tetra-sodium pyrophosphate and 5% (wt/vol) TCA using a Milliblot-D (Millipore Corporation, Bedford, MA) apparatus, and washed twice with water (40). Filters were dried in a vacuum oven, wrapped in Saran wrap, and exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA). The amount of radioactive glucan bound to filters was quantitated with a Molecular Dynamics PhosphorImager SI. Pixels were converted to nmol of glucose incorporated into TCA-insoluble product; the number of cpm per pixel was determined by exposing a reaction mixture to a phosphor screen and determining the number of cpm in an identical mixture using a liquid scintillation counter. Units of (1,3)β-glucan synthase activity are defined as nmol of glucose incorporated into TCA-insoluble glucan per minute of incubation at 25°C.

Enzyme kinetics. The $K_{m(\mathrm{app})}$ was determined for *C. neoformans* glucan synthase by varying the amount of substrate, UDP-glc (final concentration of 0.3 to 4 mM) and calculating the amount of product formed at various times. The $K_{m(\mathrm{app})}$ was calculated using ChemSW Enzyme Kinetics Pro (version 2.36) by SynexChem (Fairfield, CA). To determine the effects of caspofungin and cilofungin on *C. neoformans* glucan synthase activity, cells were lysed as described above, and enzyme activity was assayed in the presence of various concentrations of substrate and inhibitor. For *Saccharomyces cerevisiae* BJ2168 (ATCC 208277), *Neurospora crassa* (74-OR8-1a), and *Aspergillus fumigatus* (ATCC 16424), $K_{i(\mathrm{app})}$ values were determined essentially as previously described (9, 40, 48). Enzyme kinetic data were analyzed using ChemSW Enzyme Kinetics Pro (version 2.36) by SynexChem (Fairfield, CA) for MS Windows 2000. Enzyme inhibitor data were tested against 12 different kinetics models, and the best fit was reported.

MIC of caspofungin. Antifungal assays were performed according to the CLSI (formerly NCCLS; Wayne, PA) M27A method, and the amount of growth after incubation was compared to no-drug controls. MICs for each organism were determined by testing a range of caspofungin concentrations in a 96-well microtiter broth assay. Cells were inoculated into RPMI 1640 medium (yeast nitrogen base medium for *S. cerevisiae*) to a final concentration of 1×10^4 cells/ml (*A. fumigatus*, *N. crassa*, and *S. cerevisiae*) or 2.5×10^3 cells/ml (*C. neoformans*), incubated for 24 h (72 h for *C. neoformans*) at 35°C, and examined visually for growth. The MIC was defined as the lowest concentration of drug that showed absence of growth compared to no-drug control.

Product characterization. The product of reactions mixtures was characterized as (1,3)β-glucan as previously described (16), except that the volume of enzyme reaction mixtures was increased 10-fold and contained 0.5 mM [^{14}C]glucose (~600,000 counts/assay), 500 μg of α-amylase, and 1,800 to 2,000 μg of cell protein. Microcentrifuge tubes were siliconized and used to perform the reactions, started by the addition of lysate to ice-cold reaction mixtures. Reaction mixtures were incubated at 25°C for 60 min, stopped by boiling for 10 min, and cooled on ice. Reaction mixtures were centrifuged ($16{,}000 \times g$ for 30 min at 4°C); pellets were washed by centrifugation once with ice-cold 95% (vol/vol) ethanol and twice with ice-cold water and incubated overnight at 37°C in 200 μl of 50 mM acetate buffer, pH 5.4, containing the following: (i) 2 mg of α-amylase, (ii) 2 mg of bovine serum albumin (BSA), (iii) 2 mg of laminarinase [(1,3)β-glucanase], and (iv) buffer only. Reactions were then stopped by the addition of 50 μl of 50% (vol/vol) acetic acid, and the amounts of radioactive (1,3)β-glucan remaining were determined using the filter method described previously (14).

RESULTS

***C. neoformans* cell lysis.** Various methods of lysing H99 cells were attempted, including the use of a mini bead-beater with glass and/or zirconium beads, breaking the cells in early log growth phase (cell walls are less resilient in early log phase), grinding cells under liquid nitrogen, vortexing with glass beads, and forming protoplasts. The majority of these methods resulted in <20% cell disruption (as viewed by phase contrast microscopy) and/or in lysates with little or no (1,3)β-glucan synthase activity (results not shown). The optimal method for lysing H99 cells that resulted in maximum cell disruption and preserved enzyme activity was shaking equal parts cells and lysis buffer on ice (~300 rpm) with acid-washed glass beads (four times the volume of cells) in Erlenmeyer flasks for 2 h.

(1,3)β-Glucan synthase activity from *C. neoformans*. (i) Optimal buffer and pH. *C. neoformans* cells were lysed as described in Materials and Methods (and above) in 50 mM Tris, MOPS (morpholinepropanesulfonic acid) HEPES, Tricine, or potassium phosphate buffers over their pK_a ranges. Low-speed supernatants were obtained as enzyme sources, and the (1,3)β-glucan synthase activity of each supernatant was determined as

TABLE 1. Concentrations of various additions to lysis buffer for optimal *C. neoformans* (1,3)β-glucan synthase activity[a]

Addition	Concn tested	Optimal concn
$MgCl_2$ (mM)	1–10	5
$CaCl_2$ (mM)	1–10	7
NaF (mM)	1–5	2
Phosphatase inhibitor (% [vol/vol])	0.01–1	0.1
Glycerol (% [vol/vol])	10–50	20
Sucrose (M)	0.1–1.0	0.5
PMSF (mM)	0.05–1	0.1
DTT (mM)	1–100	1
Protease inhibitor (% [vol/vol])	0.01–1	0.1
BSA (mg/ml)	1–5	
EDTA (mM)	1–100	50
EGTA (mM)	1–100	20
GTPγS (μM)	5–200	60

[a] H99 cells were lysed as described in Materials and Methods, and the optimal concentration of each addition to the lysis buffer resulting in maximal activity was determined as described in Materials and Methods and the text.

described in Materials and Methods. The pH optimum for each buffer was broad, with Tris at pH 7.75 showing the highest specific activity (results not shown). Varying the Tris buffer (pH 7.75) concentration from 25 to 200 mM produced maximal enzyme activity at a concentration of 50 mM (results not shown).

(ii) Buffer additions. After determining that 50 mM Tris at pH 7.75 was optimal, we tested singly the effects of various additions, each at a range of concentrations, on enzyme activity. The addition that produced the greatest increase in enzyme activity was included in the buffer to test each subsequent addition. When sucrose (0.1 to 1.0 M) and glycerol (10% to 50%) were tested individually, 20% glycerol resulted in maximal activity, as did 500 mM sucrose (not shown). Twenty percent glycerol increased enzyme activity to a greater extent than sucrose (500 mM); hence, 20% glycerol was included to test the effect of other additions.

Next, the effects of EDTA (ethylenediamine tetra-acetic acid) and EGTA [ethyleneglycol-bis-(-β-amino-ethyl ether)-*N*,*N*′-tetra-acetic acid] on enzyme activity were tested. Both were found to stimulate enzyme activity, with EDTA stimulating activity to a greater extent than EGTA, and maximal activity was found when 50 mM EDTA was added (not shown). The effects of NaF, phenylmethylsulfonyl fluoride (PMSF), and dithiothreitol (DTT) were then tested, and enzyme activity was maximal when 2 mM NaF, 0.1 mM PMSF, and 1 mM DTT were added to lysis buffers (not shown).

Additional compounds were tested for their effects on enzyme activity in lysis/assay buffer that contained the following: 50 mM Tris, pH 7.75, 10 μM GTPγS, 2 mM NaF, 0.1 mM PMSF, and 1 mM DTT. Buffer containing up to 5 mM $MgCl_2$ increased enzyme activity threefold, while buffer containing up to 7 mM $CaCl_2$ only doubled the enzyme activity (results not shown). We tested the effects on enzyme activity of the following: EDTA plus an excess amount of $MgCl_2$, the optimal concentration of EDTA alone, and the optimal concentration $MgCl_2$ alone. We found that $MgCl_2$ alone increased enzyme activity to a greater extent than EDTA alone or EDTA plus excessive amounts of $MgCl_2$ (results not shown). Thus, for subsequent tests, the buffer contained 5 mM MgCl. A slight increase in enzyme activity was observed after the addition of BSA to the buffer. However, BSA interfered with the protein quantification of cell lysates and was not included. We tested the effect of adding GTPγS to the buffer and found 60 μM to result in maximal enzyme activity (not shown). Protease and phosphatase inhibitor cocktails were each tested individually from 0.01% to 1% and in combination. A combination of both cocktails at 0.1% increased enzyme activity to a greater extent than each cocktail individually (results not shown).

Based on these results, we found the optimal buffer that resulted in maximal (1,3)β-glucan synthase activity from *C. neoformans* to be the following: 50 mM Tris, pH 7.75, 20% (vol/vol) glycerol, 2 mM NaF, 1 mM DTT, 0.1 mM PMSF, 5 mM $MgCl_2$, 0.1% (vol/vol) protease inhibitor cocktail, 0.1% (vol/vol) phosphatase inhibitor cocktail, and 60 μM GTPγS. These results are summarized in Table 1.

(iii) Properties of (1,3)β-glucan synthase activity from *C. neoformans*. Other in vitro properties of enzyme activity were determined in order to further characterize the (1,3)β-glucan synthase assay from *C. neoformans*. The acid-insoluble radioactive material synthesized using 1,000-g supernatants from bead-beaten cells as the enzyme source was hydrolyzed by (1,3)β-glucanase as described in Materials and Methods; the results indicated that the product formed by reaction mixtures was indeed (1,3)β-linked glucan.

We determined that the in vitro enzyme reaction was linear as judged by product formation, with protein from 25 to 240 μg per assay (60-min incubation) and time for at least 60 min of incubation at 25°C (results not presented). Frozen cell lysates stored at −80°C and thawed retained 100% of the initial enzyme activity for greater than 25 weeks (results not shown). (1,3)β-Glucan synthase activity from low-speed supernatants had a specific activity of 0.045 units mg protein^{-1} (results not shown). A summary of the in vitro properties of (1,3)β-glucan synthase activity is shown in Table 2.

Enzyme kinetics. The $K_{m(\mathrm{app})}$ of (1,3)β-glucan synthase activity with respect to UDP-glucose was 0.21 ± 0.09 mM, and the $V_{\max(\mathrm{app})}$ was 0.01 ± 0.0007 nmol min^{-1}. To test the effect of caspofungin and cilofungin on *C. neoformans* (1,3)β-glucan synthase activity, cells were lysed, and enzyme activity was assayed in the presence of various concentrations of caspofungin or cilofungin at various substrate concentrations as described in Materials and Methods (Fig. 1). Plots of 1/*v* intercept versus *I* and 1/slope versus *I* (where *v* is velocity and *I* is inhibitor) were linear, and caspofungin and cilofungin inhibited *C. neoformans* (1,3)β-glucan synthase activity noncompetitively, with a $K_{i(\mathrm{app})}$ of 0.17 ± 0.02 μM and 22 ± 5.7 μM, respectively. The $K_{i(\mathrm{app})}$ values determined are similar to if not

TABLE 2. Summary of in vitro properties of *C. neoformans* (1,3)β-glucan synthase activity

Property	Value
Linear with time (min)	0~60
Linear with protein (μg)	25~240
Specific activity (nmol min^{-1} mg^{-1})	0.045
Lysate activity after freeze/thaw (%)	100
100% Lysate activity, stored at −80°C (weeks)	>25
(1,3)β-glucan in product (%)	>75
$K_{m(\mathrm{app})}$ (mM)	0.21 ± 0.09
$V_{\max(\mathrm{app})}$ (nmol min^{-1})	0.01 ± 0.0007

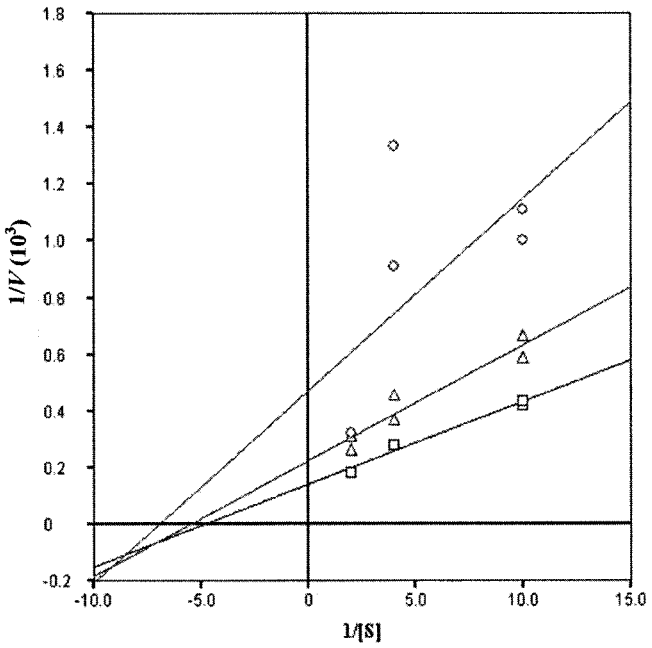

FIG. 1. Caspofungin inhibits (1,3)β-glucan synthase activity. Cells were lysed, and (1,3)β-glucan synthase activity was assayed as described in Materials and Methods. Reaction mixtures contained the indicated UDP-[^{14}C]glucose concentrations (~60,000 cpm per assay), 200 μg of cell protein, and 2.5 μl of 100% dimethyl sulfoxide containing the following final concentrations of caspofungin: 0 μM (□), 0.05 μM (△), and 0.25 μM (○). The amounts of glucan formed after incubation at 25°C for 20 min and resulting velocities were determined as described in Materials and Methods. *V*, initial velocity; *S*, substrate.

lower than those of other fungi, indicating that enzyme activity is sensitive to caspofungin and to cilofungin.

Correlation between MIC and $K_{i(app)}$. We compared the caspofungin MIC and the $K_{i(app)}$ values for *C. neoformans* with those of *A. fumigatus*, *S. cerevisiae*, and *N. crassa.* It is important to note that, in each case, we used assay conditions optimum for each fungus (9, 40, 48). We reasoned that if resistance to caspofungin were due solely to the resistance of the enzyme activity, then we would observe a strong positive correlation between MIC and $K_{i(app)}$. However, as shown by the data presented in Table 3, no such correlation existed. For example, (1,3)β-glucan synthase from *N. crassa* showed a 100-fold higher $K_{i(app)}$ than the glucan synthase of *C. neoformans*, yet *N. crassa* growth is at least fourfold more sensitive to the drug than is *C. neoformans*. Importantly, *A. fumigatus* is ~100-fold more sensitive in vitro to caspofungin (by MIC) and is sensitive in vivo (15, 30), but its glucan synthase activity has a 20-fold higher $K_{i(app)}$ than that of *C. neoformans*. Taken together, these results strongly show that *C. neoformans* (1,3)β-glucan synthase activity is very sensitive to caspofungin in vitro.

DISCUSSION

C. neoformans is a pathogenic yeast that causes pulmonary infections and fatal meningoencephalitis in humans and other animals. There has been a dramatic increase in the incidence of cryptococcosis during the last 20 years that mirrors the increase in the numbers of human immunodeficiency virus infections and other immunocompromising conditions. While the advent of highly active antiretroviral therapy has dramatically reduced the incidence of cryptococcosis in AIDS patients, cryptococcosis remains important in developing countries and in other populations of immunocompromised patients. The new echinocandin class of (1,3)β-glucan synthase inhibitors, caspofungin and now anidulafungin and micafungin, has proven successful in treating systemic mycoses caused by many fungi. Unfortunately, *C. neoformans* is resistant both in vitro and in vivo to these drugs (7). This is not understood. The possibility exists that (1,3)β-glucan is not structurally important, and thus glucan synthase inhibitors would not have an effect on the organism. One salient observation argues strongly against this idea, namely, that the gene encoding FKS (likely the catalytic subunit of glucan synthase activity) is present in single copy and is essential (42). Determining why *C. neoformans* is resistant to caspofungin will invite the possibility of designing new drugs that circumvent resistance.

Optimizing (1,3)β-glucan synthase assay conditions is a very important first step in determining the mechanism of resistance; suboptimal assay conditions may obscure the effects of enzyme inhibitors. Reports in the literature exist for in vitro (1,3)β-glucan synthase activity from several fungal organisms (13, 16, 28, 29, 40, 41, 43, 48) as well as from *C. neoformans* (42); however, optimal in vitro assay conditions have not been previously reported for *C. neoformans*. We have determined the optimal assay conditions for in vitro (1,3)β-glucan synthase activity from *C. neoformans* by systematically testing compounds that have been reported to stimulate or stabilize (1,3)β-glucan synthase activity in vitro (10, 13, 24, 29, 41). We have found the optimum lysis/assay buffer to be 50 mM Tris, pH 7.75, containing 20% glycerol, 2 mM NaF, 1 mM DTT, 0.1 mM PMSF, 5 mM $MgCl_2$, 0.1% protease and phosphatase inhibitor cocktails, and 60 μM GTPγS.

Importantly, we have determined a number of in vitro properties of *C. neoformans* (1,3)β-glucan synthase (Table 2). Critically, we have determined that the enzyme activity is indeed very sensitive to caspofungin and to cilofungin [$K_{i(app)}$ 0.17 ± 0.02 μM and 22 ± 5.7 μM, respectively]. Together, the high MIC and significantly low $K_{i(app)}$ show that resistance is not due to (1,3)β-glucan synthase's being resistant to caspofungin but that another mechanism(s) is involved. This notion is reinforced by the report that echinocandin sensitivity is enhanced when either calcineurin inhibitors (e.g., tacolimus) are used in combination with caspofungin or the tacolimus-binding protein FKBP12 gene is deleted (6).

We have begun testing the involvement and contribution of multidrug resistance pumps (2, 31, 33, 47), melanin (17, 18,

TABLE 3. MICs and $K_{i(app)}$ values of caspofungin for *C. neoformans*, *A. fumigatus*, *S. cerevisiae*, and *N. crassa*[a]

Organism	$K_{i(app)}$ (μM)	MIC (μg/ml)
C. neoformans	0.17 ± 0.02	16
C. neoformans (cilofungin)	22 ± 5.7	64
A. fumigatus	4.1 ± 0.5	0.13
S. cerevisiae	6.7 ± 2.2	0.13
N. crassa	22 ± 3.4	4

[a] Values were determined as described in Materials and Methods. *C. neoformans* was also tested against cilofungin, as indicated.

44), and degradation pathways (3, 38, 49) to resistance. Once the specific mechanism is discovered, new drugs to treat cryptococcosis may be designed that bypass the resistance of *C. neoformans*. This is of great medical significance, as *C. neoformans* is resistant to echinocandins, and cryptococcosis is a leading cause of death in immunocompromised individuals.

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