Canavanine Resistance in Cryptococcus neoformans

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All of the isolates of Cryptococcus neoformans var. gattii which we tested were resistant to ≥3.5 mM canavanine. All of the serotype D isolates and 28% of the serotype A isolates of C. neoformans var. neoformans tested were susceptible to ≥18 μM canavanine, whereas the remaining 72% of the serotype A isolates were as resistant as the C. neoformans gattii isolates. In the naturally resistant isolates, the mechanism of resistance appeared to be decomposition of canavanine to a nontoxic product. However, in a resistant mutant derived from a naturally susceptible isolate, the mechanism of resistance was an impaired uptake system for canavanine. The toxic effect of canavanine in Cryptococcus results from the incorporation of canavanine into the protein component that is essential for the synthesis of proteins and RNAs.

L-Canavanine is a guanidinoxy structural analog of L-arginine [H₂N-C(=NH)-NH-O-CH₂-CH₂-CH(NH₂)COOH] which is synthesized by various legumes as a secondary metabolite (7). Although the biochemical and physiological activity of this compound is not fully understood, its biological effect and mode of action as a growth inhibitor have been well established (21).

Canavanine is incorporated into proteins by arginyl-tRNA synthetase, which is able to charge canavanine (2, 17, 23). As a result, canavanyl proteins are produced (5, 12). These proteins have different tertiary and quaternary structures that alter the biological properties of the original arginine-containing proteins. Substitution of arginine by canavanine has been observed in many groups of proteins. Canavanine functions as a metabolic inhibitor in nearly every enzyme that contains arginine (20). Often, RNA metabolism (16, 29), DNA metabolism (16, 28), and overall protein production are affected (22). Thus, canavanine has a highly toxic effect on a broad spectrum of organisms.

Although susceptibility to canavanine has been studied in various yeasts and filamentous fungi (21), the species Cryptococcus neoformans was not included in previous investigations. In this study, we examined both the susceptibility and the mechanism of resistance to canavanine in the two varieties of Cryptococcus neoformans, Cryptococcus neoformans var. neoformans and Cryptococcus neoformans var. gattii.

MATERIALS AND METHODS

Isolates, media, and growth conditions. A total of 179 isolates, including isolates belonging to serotypes A and D of Cryptococcus neoformans var. neoformans and isolates belonging to serotypes B and C of Cryptococcus neoformans var. gattii, were used to screen for canavanine susceptibility. These cultures were maintained on malt extract agar slants. We used strains B-3501 (serotype D) and NIH 191 (serotype C) for an in-depth study of the mechanisms that affect susceptibility and resistance to canavanine. In addition to these two isolates, strains NIH 271 and NIH 372 (serotype A) were used for an L-canavanine uptake experiment.

The medium used to screen for canavanine susceptibility contained (per liter) 10 g of glucose, 1 g of asparagine, 3 g of KH₂PO₄, 1 g of MgSO₄ · 7H₂O, 50 μl of a thiamine vitamin solution (Abbott Laboratories, North Chicago, Ill.), 30 mg of L-canavanine sulfate (Sigma Chemical Co., St. Louis, Mo.), and 20 g of agar. L-Canavanine sulfate was filter sterilized and added to the agar medium at a concentration of 110 μM (30 mg/liter) just prior to preparation of the plates. The liquid growth medium for cells used for various assays was the glucose-ammonium broth described by Polacheck and Kwong-Chung (19); this medium contained (per liter) 20 g of glucose, 3 g of ammonium sulfate, 1 g of MgSO₄ · 7H₂O, 1 g of KH₂PO₄, and 50 μl of the thiamine vitamin solution. In some experiments, the broth medium was supplemented with 110 μM arginine or 110 μM L-canavanine. The pH of all media was adjusted to 6.5. Cultures were incubated on a shaker at 25°C, and the cells were harvested at the exponential growth phase (18).

Screening for canavanine susceptibility. Cells were grown on malt extract agar slants for 48 h, harvested in phosphate-buffered saline, and diluted to obtain 5 × 10⁵ to 1 × 10⁶ cells per ml. The cell suspensions were plated (0.2 ml per plate) on malt extract agar (controls) and canavanine agar plates in duplicate. The plates were examined after 4 days of incubation at 30°C.

Isolation of a resistant mutant from a susceptible isolate. To isolate and determine the prevalence of resistant mutants that arose spontaneously, inocula containing 10⁵ to 10⁶ cells of naturally susceptible isolates were spread onto the surfaces of canavanine agar plates. One of the resistant mutants, strain B-3501-R, was chosen for further study.

Uptake measurements. Cellular uptake of L-arginine and L-canavanine was measured with various isolates that were either resistant or susceptible to canavanine. The resistant isolates used were strain NIH 191 (serotype C), strain NIH 271 (serotype A), and mutant B-3501-R (isolated from susceptible wild-type strain B-3501 [serotype D]). The susceptible isolates used were strains B-3501 and NIH 372 (serotype A). The cells were grown in glucose-asparagine broth (400 ml) and were harvested on an A₆₀₀ of 0.8. After being washed once with glucose-ammonium broth, the cells were suspended in 50 ml of the same medium. A 35-ml portion of the cell suspension was washed three times in cold distilled water and centrifuged; the pellet was then dried in a 75°C oven to determine the dry weight. To 5 ml of cell suspension, 0.02% sodium azide was added. These cells were used as a control for the uptake experiments. To* Corresponding author.
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468
different portions of the remaining cell suspension and to the azide control we added D-\[guanido\]l-arginine (final concentration, 0.5 μCi/ml; 44.5 mCi/mmol; Schwarz/Mann, Orangeburg, N.Y.) or L-\[guanido\]l-arginine (final concentration, 0.5 μCi/ml; 54.3 mCi/mmol; Amersham Corp., Arlington Heights, Ill.). The uptake experiments were carried out by using the filtration technique described by Polacheck et al. (18).

**Determination of protein synthesis.** The experimental conditions used for growth, harvesting, and reactions for strains B-3501 and NIH 191 were the same as the conditions described above for the uptake test. Each reaction mixture contained 0.1 μCi of D-\[U-14C\]leucine (339 mCi/mmol; Amersham) per ml and 10 μg of unlabeled L-leucine per ml as a carrier with or without 2 mM nonradioactive canavanine. Samples (1.0 ml) were removed at intervals and divided into two portions. One portion was kept for 30 min in cold 10% trichloroacetic acid (TCA) containing 100 μg of unlabeled leucine per ml and then washed through filters with the same solution, described previously (19); 2 ml of 0.5 N NaOH was added to the other portion, and the proteins were extracted by incubating the preparation for 15 min at 100°C. The amount of protein was estimated by the method of Lowry et al. (4).

**Nucleic acid synthesis.** Strain B-3501 and NIH 191 cells were grown, harvested, and reacted with chemicals by using the conditions described above for the uptake experiments. Each reaction mixture contained 0.5 μCi of D-\[U-14C\]adenine (276 mCi/mmol; Amersham) per ml with or without 2 mM nonradioactive canavanine. The method used to measure nucleic acid synthesis was the method of McMahon and Blaschko (15), modified as described below. Samples (1 ml) were removed at appropriate intervals and were added to equal volumes of cold 10% TCA. After incubation for 30 min at 0°C, the cells were washed, and the pellet was heated at 90°C for 30 min in 5% TCA in order to hydrolyze the nucleic acids. The samples were then filtered through type GF/A filters and washed twice with 5 ml of 5% TCA. The radioactivity in the filtrate, which represented the incorporation of adenine into both RNA and DNA, was measured.

The incorporation of adenine into DNA was measured after the treatments described below. Samples (1 ml) were mixed with 4 ml of 0.5 N NaOH and incubated at 37°C for 40 min; then 0.6 ml of 50% TCA was added to each sample. Next, the samples were washed on filters (18), and the radioactivity on each filter was counted. This radioactivity represented the incorporation of radiolabeled adenine into DNA. The amount of adenine incorporated into RNA was calculated by subtracting the amount of radioactivity in the DNA from the total radioactivity incorporated into nucleic acids.

**Preparation of cell-free extracts.** Cells grown in 400 ml of glucose-asparagine broth were harvested at an A600 of 0.8, washed, centrifuged, and broken by the glass bead method described previously (19). A cell-free extract was prepared by separating the supernatant from the cell debris by centrifugation at 105,000 × g for 45 min at 4°C. The extract was then concentrated at 4°C in a pressure cell (Amicon Corp., Danvers, Mass.) equipped with a type YM-10 membrane. After 10-fold concentration, the solution was brought up to the original volume by diluting it with 25 mM phosphate buffer (pH 7.0). The concentration procedure was repeated twice, and the final volume was adjusted to about 0.5 ml/g of cells used.

**Analytical methods.** (i) Arginase assay. The arginase assay was performed by using the method of Ruegg and Russell (27), except that we used 3 μCi of radiolabeled arginine per ml.

(ii) Internal pool of arginine. An internal pool of arginine was extracted from washed, exponentially growing cells by incubating the cells in cold 5% TCA for 30 min. The extract was then lyophilized, and the amount of arginine was determined with a model 126 data analyzer (Beckman Instruments, Inc., Fullerton, Calif.) by using the appropriate standard.

(iii) Arginyl-tRNA synthetase assay. Arginyl-tRNA synthetase levels were determined by measuring the attachment of radiolabeled arginine or canavanine to tRNA, using the procedure described by Chrispeels et al. (4) with some modifications. Each reaction mixture (1.0 ml) contained 50 μmol of Tris hydrochloride buffer (pH 7.5), 5 μmol of MgCl2, 1 μmol of ATP (sodium salt), 3 mg of tRNA Escherichia coli W (type XXI; Sigma), 15 μmol of KCl, 0.5 μmol of EDTA, 5 μmol of 2-mercaptoethanol, 3 nmol of L-\[guanido\]l-arginine (1.0 μCi/ml) or 5 nmol of D-\[guanido\]l-arginine (2 μCi/ml), and a limited amount of cell-free extract. The mixture was incubated at 37°C for 15 min. Samples (0.5 ml) were removed at 2-min intervals, placed into ice-cold 10% TCA containing 1 mg of unlabeled amino acid per ml, and incubated for 1 h. Each sample was then filtered through a cellulose acetate filter and washed four times with the same solution. The filters were dried, and the radioactivity was determined with a scintillation counter as described previously (18). To estimate the K<sub>m</sub> values for arginine or canavanine, the substrate concentration used ranged from 5 to 5 × 10<sup>3</sup> nmol/ml; the incubation time was 10 min. A correction for nonspecific absorption of radioactive material was made by subtracting the values obtained with a reaction mixture lacking ATP.

(iv) Canavanine degradation. Canavanine degradation was measured in both strain B-3501 and strain NIH 191 as described below. A cell-free extract (1 mg of protein per ml) was mixed with a solution containing 25 mM phosphate buffer (pH 7.0), 2 mM MgSO<sub>4</sub>, and 1 mM L-canavanine in a final volume of 200 μl, and the preparation was incubated for up to 10 h at 37°C. To stop the reaction, the mixture was heated at 100°C for 5 min. The reaction mixture was concentrated in a pressure cell by using the method described above. The dilution-concentration step was repeated three times by using 0.2 N sodium citrate buffer (pH 3.25). The filtrate was then lyophilized and suspended in the same buffer. The canavanine was separated by the Beckman simple-column method, using W-3 microparticle resin, in a Beckman model 119 CL amino acid analyzer, as described in the Beckman model 119CL application notes. The amount of canavanine was estimated by using a Beckman model 126 data analyzer and the appropriate standard. To block the enzyme activity in the controls, we either heated the reaction mixture or added 0.1% potassium persulfate prior to the addition of canavanine. The activity was assayed by using potassium persulfate or to proteolytic enzymes, such as pronase. In some experiments, 1 μCi of L-\[guanido\]l-arginine per ml was used as the substrate. The degradation product was separated by using thin-layer chromatography on Silica Gel 60 (E. Merck, Darmstadt, Federal Republic of Germany) and two-dimensional development. For the solvents, we used a solution containing n-butanol, acetone, ammonia, and water (37:37:19:7), followed by phenol-ethanol-water (35:10:2.5). The standards used were canalin, homoserine, ornithine, urea, guanidoacetic acid, guanidinosuccinic acid, homogargin, α-aminobutyric acid, α-ureido-L-homoserine, and creatine (9, 10, 22, 24–26).
RESULTS

Canavanine susceptibility in Cryptococcus neoformans. A total of 179 Cryptococcus neoformans var. gattii and Cryptococcus neoformans var. neoformans isolates from clinical or natural sources were tested for susceptibility to canavanine. All 43 serotype B isolates and 17 serotype C isolates (Cryptococcus neoformans var. gattii) were resistant to canavanine at concentrations of at least 3.5 mM. All of the serotype D isolates tested (Cryptococcus neoformans var. neoformans) were susceptible to canavanine at concentrations at or below 18 μM. There were two types of serotype A isolates (Cryptococcus neoformans var. neoformans); 23 of 83 isolates were susceptible to canavanine at a concentration of 18 μM, whereas the remaining 60 were resistant to concentrations up to 3.5 mM. The frequency of drug-resistant mutants in a susceptible serotype D isolate (strain B-3501) was 1.8 mutants per 10^8 cells.

Mechanism of canavanine toxicity. The inhibition of cryptococcal growth by canavanine stems from the effect of this compound on protein synthesis (Fig. 1). As measured by the incorporation of [14C]leucine, 2 mM canavanine very efficiently inhibited protein synthesis in susceptible strain B-3501. There was a total block of protein synthesis during the first 30 min, and even after 3.5 h the level of incorporation of leucine into protein was only about 15% of the level observed in the control. In resistant isolate NIH 191, incorporation of [14C]leucine was not affected by the drug (Fig. 2).

Compared with inhibition of protein synthesis, inhibition of nucleic acid synthesis by canavanine in the susceptible isolate was less effective. Canavanine slightly decreased the rate of adenine incorporation into RNA (Fig. 3). The incorporation of adenine into DNA was relatively resistant to the effects of canavanine and was not inhibited for at least the first 40 min, after which the rate of incorporation slowly decreased (Fig. 4). In resistant isolate NIH 191, the rate of adenine incorporation into RNA, as well as DNA, was not affected by canavanine (data not shown).

Cell-free extracts of Cryptococcus neoformans, which contained arginyl-tRNA synthetase, charged canavanine, although the rate was slightly less than the rate observed with arginine. The arginyl-tRNA synthetases from the resistant isolate and the susceptible isolate had similar K_m values for L-arginine and L-canavanine (see below). As a result, radiolabeled canavanine was found in proteins isolated from cryptococcal cells that were exposed to L-[guanidoxy-14C]canavanine. After TCA precipitation and total hydrolysis with 6 N HCl as described by Rosenthal et al. (25), the neutralized hydrolysate was placed on an amino acid analyzer column; the canavanine fraction was then separated, and the radioactivity was measured. A total of 49.9 nmol of [14C]canavanine was found in 1 mg of protein in the susceptible isolate, but not in the resistant isolate.

Possible mechanisms of canavanine resistance. The following four possible mechanisms for resistance to canavanine in Cryptococcus neoformans have been suggested previously (21): (i) the existence of an impaired uptake system, which

![FIG. 1. Protein synthesis by canavanine-susceptible strain B-3501. Symbols: ▲, synthesis in the absence of canavanine; ●, synthesis in the presence of 2 mM L-canavanine.](image1)

![FIG. 2. Protein synthesis by canavanine-resistant strain NIH 191. Symbols: △, synthesis in the absence of canavanine; ○, synthesis in the presence of 2 mM L-canavanine.](image2)

![FIG. 3. RNA synthesis in the presence of L-canavanine by strain B-3501. Symbols: ▲, synthesis in the absence of canavanine; ●, synthesis in the presence of 2 mM L-canavanine.](image3)
permits no accumulation of canavanine; (ii) the presence of arginyl-tRNA synthetase, which discriminates arginine from canavanine; (iii) excessive production of intracellular arginine sufficient to counteract the inhibitory effect of canavanine; and (iv) decomposition of canavanine to a nontoxic compound.

(i) Canavanine uptake. Figure 5 shows various uptake rates for susceptible and resistant isolates. Of the five isolates tested, resistant isolate NIH 191 accumulated radioactive canavanine at the highest rate. The uptake of the drug by susceptible isolate NIH 372 (Cryptococcus neoformans var. neoformans serotype A) was less efficient than the uptake observed in strain NIH 191, but the rate of uptake was much higher than in susceptible isolate B-3501 (serotype D). Resistant isolate NIH 271 (serotype A) accumulated the drug at a slower rate than strain NIH 191, but at a higher rate than strain B-3501. The only isolate that failed to accumulate the drug was spontaneously resistant mutant B-3501-R, which was derived from strain B-3501. When two resistant isolates of Cryptococcus neoformans var. gattii serotype B were tested, the result was similar to the result obtained with strain NIH 191 (data not shown). These findings indicate that the natural resistance to canavanine observed in Cryptococcus neoformans did not result from impaired uptake, whereas the resistance in the mutant derived from a susceptible isolate did.

Cells that were grown in the absence or presence of either arginine or canavanine showed the same uptake kinetics for canavanine (data not shown), indicating that the canavanine uptake system in Cryptococcus neoformans was not inducible. Canavanine was a very effective inhibitor of arginine uptake. The $K_v$ value for canavanine was 42 $\mu$M, compared with the arginine $K_m$ value of 15 $\mu$M. A 10:1 ratio of canavanine to arginine caused 82% inhibition of the initial velocity of arginine uptake. This finding suggests that the same transport system is involved in the uptake of both arginine and canavanine. Amino acids, including glutamic acid, ornithine, citrulline, asparagine, and argininosuccinic acid, were tested for their ability to reverse inhibition by canavanine (18 $\mu$M) in susceptible isolates, and none of these compounds showed any reversing capability. Arginine (1 mg/ml) was the only effective agent in counteracting the inhibitory effect of canavanine.

(ii) Arginyl-tRNA synthetase. Arginyl-tRNA synthetases from resistant and susceptible isolates showed a marked affinity for arginine and canavanine. In resistant isolate NIH 191, the $K_m$ values of the arginyl-tRNA synthetase for arginine and canavanine were $4.8 \times 10^{-5}$ and $1.2 \times 10^{-4}$ M, respectively; in susceptible isolate B-3501, these $K_m$ values were $5.5 \times 10^{-5}$ and $3.5 \times 10^{-4}$ M, respectively. These findings indicate that while the enzyme has slightly higher affinity for arginine in both isolates, it is not the major mechanism for resistance.

(iii) Intracellular pool of arginine. Since excessive amounts of intracellular arginine can counteract the inhibitory effect of canavanine, the intracellular levels of arginine in susceptible strain B-3501 and resistant strain NIH 191 were measured. Strain B-3501 had considerably more free arginine within the cells (25.7 nmol/mg [dry weight]) than strain NIH 191 (7.7 nmol/mg [dry weight]).

(iv) Decomposition of canavanine to a nontoxic compound. Cell-free extracts of canavanine-resistant and -susceptible isolates were incubated with canavanine for 24 h. Table 1 shows the kinetics of canavanine degradation during a 10-h incubation. Throughout the 10-h incubation, the reaction was linear, and 60% of the drug was degraded by the cell-free extract from a resistant isolate. On the other hand, the cell-free extract from a susceptible isolate did not cleave a significant amount of canavanine for up to 24 h of incubation.
Canavanine degradation appears to involve an enzyme since the activity can be blocked by heat, potassium persulfate, or a proteolytic enzyme. The enzyme involved in canavanine degradation in Cryptococcus neoformans is not known at present. However, because the susceptible and resistant isolates had similar arginine activities with arginine as a substrate, we concluded that arginase was not the enzyme involved. A cell-free extract of a canavanine-resistant isolate releasing 26.9 µmol of urea per h from arginine, while a cell-free extract of a susceptible isolate released 20.1 µmol/h per mg of protein. The degradation product separated by Silica Gel 60 thin-layer chromatography produced a single spot with both solvent systems used. The Rf values (0.03 in the first solvent and 0.066 in the second) did not correspond to any of 10 standards that are known to be canavanine metabolites (9, 10, 22, 23, 25, 26).

**DISCUSSION**

Resistance of Cryptococcus neoformans isolates to canavanine has been a useful criterion for differentiating the two varieties of the species (13). Natural resistance to canavanine is not known in other yeasts (21). The isolates of Saccharomyces cerevisiae that are known to be resistant all resulted from a mutation in the uptake system (21). Cryptococcus neoformans is unique in that more than 65% of the isolates are naturally resistant to the drug, and their resistance is not due to an impaired uptake system. Spontaneous mutants that were resistant to canavanine were readily isolated from susceptible isolates at a rate of 1.8 mutants per 10^6 cells. In one such mutant, strain B-3501-R, it appears that resistance was the result of a mutation in a nuclear gene (unpublished data) that controls the uptake of the drug (Fig. 5).

The mode of resistance to canavanine in Cryptococcus neoformans was attributed to a canavanine-detoxifying system that degrades canavanine into a presumably nontoxic compound. Canavanine-detoxifying systems that degrade canavanine have been reported previously in liver tissue (11), bruchid beetles (2), leguminous plants (24), and bacteria (9, 10). In each case, arginase-mediated cleavage is the first step in the process of detoxification. The intermediate or final degradation products in these systems include canaline, guanidine, homoserine, ureidohomoserine, and ammonia.

In Cryptococcus neoformans, however, arginase activity was found in both canavanine-resistant and canavanine-susceptible isolates. This activity is in contrast to the degradation system, which was found only in the resistant isolates of bacteria. Furthermore, canaline was nontoxic to both susceptible and resistant isolates of Cryptococcus neoformans (unpublished data). On the other hand, 0.5 µM homoserine was toxic to isolates that were susceptible to canavanine, while isolates that were resistant to canavanine tolerated more than 5 µM homoserine (unpublished data).

These results, in addition to the fact that the degraded product is not related to any of the known canavanine metabolites, suggest that the degradation of canavanine in Cryptococcus neoformans is different from the degradation of canavanine reported previously. We did not attempt to detect the possible products of canavanine decarboxylation or deamination. However, the degradation product produced a blue color with the ninhydrin reagent, indicating that it contains both free amino and free carboxyl groups. Final conclusions can be drawn only after chemical identification of the product.

The toxic effect of canavanine on the susceptible isolates of Cryptococcus neoformans is similar to the toxic effect observed in other organisms. It is based mainly on the production of canavanyl proteins. Arginyl-tRNA synthetase showed a marked affinity for canavanine. The K_m values for canavanine in the resistant and susceptible isolates (1.2 x 10^{-6} and 3.5 x 10^{-6}M) are very close to the values reported by Mitra and Meher (17) for the E. coli enzyme (4 x 10^{-4} to 6 x 10^{-4}M). Although the enzyme was not purified, its inability to discriminate clearly between arginine and its analog was proven by the formation of canavanyl proteins, which was determined by the incorporation of [14C]canavanine into proteins. Total hydrolysis of canavanyl proteins revealed radiolabeled canavanine. The precise effect of substituting canavanine for arginine on the catalytic and regulatory properties of the resulting proteins has not been determined. However, Attias et al. (3) have demonstrated that canavanine incorporation inhibits the activity of alkaline phosphatase. Canavanine can serve as the substrate for nearly every enzyme that preferentially utilizes arginine and thereby effectively inhibits the enzymes of arginine metabolism found in many organisms (21). In Cryptococcus neoformans, substitution of arginine is not restricted to a limited group of proteins, but it also affects nucleic acid and protein metabolism (Fig. 1 through 4). As shown in Fig. 1, while canavanine had a marked and immediate effect on protein synthesis in a susceptible isolate, it allowed RNA synthesis at a decreased rate for about 1 h before complete inhibition occurred (Fig. 3). In its synthesis of DNA, Cryptococcus neoformans is relatively resistant to canavanine and shows no response to the drug for at least 40 min (Fig. 4). The kinetics of inhibition of macromolecule synthesis is slightly different from the kinetics observed in algae (16) and bacteria (17, 28, 29), in which RNA synthesis is severely inhibited while protein synthesis still occurs. It may be that macromolecule production is prevented by a defective key protein that is associated with translation or protein synthesis.

The production of canavanyl proteins can influence DNA metabolism directly through disrupted formation of proteins involved in DNA replication. On the other hand, canavanine can have an indirect influence by substituting for arginine in histones, which could decrease the basicity of histone molecules and affect their interaction with the nucleic acid. Such a phenomenon has been reported in HeLa cells (1) and cultured mouse and hamster cells (8).

Cryptococcus neoformans has a transport system for arginine that can be inhibited by canavanine and vice versa, indicating that the uptake of both arginine and canavanine is brought about by the same transport system. In view of the evidence shown in Fig. 5, we ruled out alteration of the uptake system as a possible explanation for natural resistance to the drug.

Overproduction of arginine due to loss of a control mechanism reportedly accounts for the resistance to canavanine in the fungus Coniochaeta velutina (30). Since the intracel-
lular level of arginine in a canavanine-resistant isolate was no higher than the level found in a susceptible isolate, it appears that this mechanism does not exist in Cryptococcus neoformans var. gattii NIH 191.

In both resistant and susceptible isolates, the arginyl-tRNA synthetase showed similar affinity for canavanine. These data indicate that the enzyme does not preferentially charge arginine over canavanine as a way of escaping canavanine toxicity.

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LITERATURE CITED