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3 # VORICONAZOLE INHIBITS MELANIZATION IN
4 # *Cryptococcus neoformans*
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24 **ABSTRACT**

25 Voriconazole is a triazole antifungal drug that inhibits ergosterol synthesis and has broad activity
26 against yeast and moulds. While studying the interaction of voriconazole and *Cryptococcus*
27 *neoformans*, we noted that cells grown in the presence of sub-inhibitory concentrations of
28 voriconazole reduced melanin pigmentation. We investigated this effect systematically by
29 assessing melanin production in the presence of voriconazole, amphotericin B, caspofungin,
30 itraconazole, and fluconazole. Only voriconazole impeded the formation of melanin at sub-
31 inhibitory concentrations. Voriconazole did not affect the autopolymerization of L-Dopa and 0.5
32 MIC of voriconazole did affect gene expression of *C. neoformans*. However, voriconazole
33 inhibited the capacity of laccase to catalyze the formation of melanin. Hence, voriconazole
34 affects melanization in *C. neoformans* by interacting directly with laccase, which may increase
35 the efficacy of this potent antifungal against certain pigmented fungi.

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37 **INTRODUCTION**

38 Voriconazole, a synthetic derivative of fluconazole, is a broad-spectrum triazole
39 antifungal that inhibits cytochrome P450-dependent 14α-lanosterol demethylation, which is a
40 critical step in fungal cell membrane ergosterol synthesis. We have previously shown that
41 voriconazole is highly active against melanized and non-melanized *Cryptococcus neoformans*,
42 an important human pathogenic fungus, in vitro (12) and during experimental infection (4).
43 Melanins are negatively charged, hydrophobic pigments of high molecular weight that are formed
44 by the oxidative polymerization of phenolic and/or indolic compounds (15) and the pigments are
45 found in all biological kingdoms (2). Melanin synthesis occurs in *C. neoformans*, dimorphic fungi,
46 and diverse moulds and has been associated with virulence for the human pathogenic fungi
47 *Cryptococcus neoformans*, *Aspergillus* species, *Exophiala [Wangiella] dermatitidis* and *Sporothrix*
48 *schenckii* [reviewed in (7)]. In *C. neoformans,* pigment production protects the fungus against
49 diverse insults, including oxidants, elevated temperature, amphotericin B, caspofungin, microbicidal
50 peptides, enzymatic degradation, and macrophages in vitro [reviewed in (7)]. In our studies with
51 voriconazole on *C. neoformans*, we noted that the drug appeared to affect *C. neoformans*
52 melanization and we therefore investigated this phenomenon by assessing the impact of
53 voriconazole, fluconazole, itraconazole, caspofungin, and amphotericin B on melanin production.
54 Additionally, we analyzed the effect of sub-inhibitory voriconazole on gene expression.
55

56 **MATERIALS AND METHODS**

57 **Antifungal drugs, *C. neoformans* and melanization.** Voriconazole and fluconazole were
58 provided by Pfizer (Sandwich, England). We purchased Amphotericin B from Gibco (Invitrogen
59 Corp., Carlsbad, CA), itraconazole from Janssen (Spring House, PA), and caspofungin from

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60 Merck (Whitehouse Station, NJ). Although caspofungin has limited clinical efficacy in
61 cryptococcosis, it has activity against *C. neoformans* in vitro (1) and was used in these
62 experiments to establish proof of principle for the effect of this drug class on melanin production.
63 *C. neoformans* serotype D strain 24067 from the American Type Culture Collection (Rockville,
64 MD) was selected for these studies since it was used in our prior melanin and cellular
65 morphology studies (8, 10, 12). Cultures inoculated with 5×10^4 cells *C. neoformans* yeast cells
66 were grown either in 50 mL of a chemically defined minimal medium [15 mM glucose, 10 mM
67 $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine and 3.0 μM vitamin B_1] or on minimal medium agar
68 [minimal medium plus 2% agar] with 1 mM L-Dopa (Sigma, St. Luis, MO) as substrate for
69 melanization at 30°C. Liquid cultures were shaken at 150 rpm. MICs for *C. neoformans* were
70 determined by us previously (11, 12): 0.015 μg/ml for voriconazole, 0.125 μg/ml for amphotericin
71 B, 1 μg/ml for fluconazole, < 0.625 μg/ml for itraconazole, and 8 μg/ml for caspofungin. To
72 determine whether these antifungal drugs could impact melanization of *C. neoformans* at sub-
73 inhibitory concentrations, these compounds were added at various concentrations to a maximum
74 concentration of 0.5 MIC to the minimal medium with L-Dopa. The cultures were wrapped in foil
75 to avert autopolymerization of L-Dopa and examined daily for growth and melanin production.
76 **Growth studies.** *C. neoformans* strain 24067 was grown in L-Dopa minimal media in the
77 absence and presence of voriconazole or fluconazole at 30°C. Both antifungal drugs were added
78 at various concentrations to a maximun concentration of 0.5 MIC. The initial inoculum was $5 \times$
79 10^4 cells in 50 ml media for each concentration.
80 **Isolation of melanin from *C. neoformans* after incubation with antifungal drugs.** A density
81 of 5×10^4 *C. neoformans* 24067 yeast cells were grown in 50 mL of minimal medium
82 supplemented with 1 mM L-Dopa without or with voriconazole (0.25 or 0.5 MIC) or fluconazole

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83 (0.25 or 0.5 MIC) at 30°C for 7 days. Liquid cultures were shaken at 150 rpm. On day 7,
84 melanized *C. neoformans* cells were treated with enzymes, denaturant and hot acid results in the
85 isolation of purified melanin in the shape and size of the parental melanized cryptococcal cell,
86 and these particles are referred to as melanin 'ghosts' (15). Briefly, *C. neoformans* from the
87 subcultures of cells grown for 10 days and transferred to fresh medium with or without L-dopa
88 for 36 h were collected by centrifugation at 2010 *g* for 30 min, washed with PBS and suspended
89 in 1·0 M sorbitol/0·1 M sodium citrate (pH 5.5). Cell-wall-lysing enzymes (from *Trichoderma*
90 *harzianum*; Sigma) were added at 10 mg ml^{-1} and the suspensions were incubated at 30 °C
91 overnight. The resulting protoplasts were collected by centrifugation, washed with PBS and
92 treated with 1 mg proteinase K ml^{-1} (Roche Laboratories) made up in a reaction buffer (10 mM
93 Tris, 1 mM $CaCl_2$ and 0·5 % SDS; pH 7·8) at 37 °C overnight. The debris was collected, washed
94 with PBS and then boiled in 6 M HCl for 1 h. If particles remained, they were collected, washed
95 in PBS and lyophilized. Finally, the amount of melanin produced by yeast cells after incubation
96 with drugs was quantitated by dry weight measurement.

97 **Autopolymerization.** To determine whether voriconazole directly interacted with L-Dopa to
98 impede melanization, this drug was incubated with L-Dopa in minimal medium and exposed to
99 ambient light to catalyze the autopolymerization of the phenolic compound to melanin.
100 Voriconazole at concentrations 0.0075, 0.015, or 0.03 µg/ml was incubated in Erlenmeyer flasks
101 with 25 ml of minimal media supplemented with 1mM L-Dopa at 30°C with shaking at 150 rpm.
102 A flask without drug was utilized as a control.

103 **Gene expression.** *Cryptococcus neoformans* yeasts were grown in minimal medium with L-
104 Dopa in triplicates alone or with 0.0625 µg/ml of voriconazole for 3 days. Approximately 2 ×
105 10^9 to 6 ×10^9 cells were suspended in 5 ml of PBS and then homogenized with 0.5-mm-diameter

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106 zirconium-silica glass beads (Biospec, Bartlesville, OK) by using a glass bead beater (Biospec)
107 for 4 min to ensure complete lysis. Cell debris was removed by centrifugation at 3,900 x *g* for 10
108 min at room temperature. Isolation of high quality *Cryptococcus neoformans* RNA was
109 performed using the Ambion Kit (Ambion, Austin, TX) according to the manufacturer's
110 instructions. At the Microarray Facility at the Genome Sequencing Center of the Washington
111 University in St. Louis, the RNA was hybridized to a microarray containing all the currently
112 predicted genes in serotype D *C.neoformans*[http://genome.wustl.edu/activity/ma/cneoformans/].
113 The slides were scanned immediately after hybridization on a ScanArray Express HT Scanner
114 (Perkin Elmer, Wellesley, MA) to detect Cy3 and Cy5 fluorescence. The laser power was kept
115 constant and photomultiplier tube values were set for optimal intensity with minimal
116 background. Gridding and analysis of images were performed with ScanArray software Express
117 V2.0 (Perkin Elmer) and the intensity values were imported into GeneSpring 7.3 software
118 (Agilent, Redwood city, CA). A Lowess curve was fit to the log-intensity versus log-ratio plot
119 and 20.0% of the data was used to calculate the Lowess fit at each point. This curve was used to
120 adjust the control value for each measurement and mean signal to Lowess adjusted controlled
121 ratios are calculated. Cross-chip averages were derived from the antilog of the mean of the
122 natural log ratios across the 2 microarrays.
123 **Real-time RT-PCR for *LAC1* gene expression.** *C. neoformans* 24067 yeast cells were grown in
124 minimal medium agar plates supplemented with 1 mM L-Dopa without or with voriconazole (0.25
125 or 0.5 MIC) or fluconazole (0.25 or 0.5 MIC) at 30°C for 7 days. Plates were covered with
126 aluminum foil to prevent autopolymerization. After incubation, *LAC1* gene expression was
127 analyzed by qRT-PCR. Briefly, cells were collected and washed, then, RNA was isolated
128 according to the RNeasy^{R} kit protocol (Qiagen). For real time RT-PCR detection of *LAC1*

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129 transcripts, 10 µg of total RNA was treated with DNase at 37°C for 1 h, precipitated with
130 ethanol, and suspended in 100 µl of nuclease-free water. cDNA synthesis was carried out from
131 equal amounts of RNA in a cyclic BioRad MyCycler (BioRad) using reagents from Invitrogen
132 according to the manufacturers instructions. The expression of the *LAC1* gene was examined via
133 RT-PCR with the primers LAC1a (CCAGCGAGGAGCCTTTGTGAATGT) and LAC1b
134 (GCCGTGCAGGTGGTAAGGATGG). For an internal mRNA control, we used primers specific
135 for the *ACT1* gene of *C. neoformans* ACT1a (GCCCTTGCTCCTTCTTCTAT) and ACT1b
136 (GACGATTGAGGGACCAGACT). To confirm that similar concentrations of cDNA were
137 achieved, signals of *ACT1* PCR were compared. *LAC1* transcript levels were determined and
138 quantitatively assessed using a Bio-Rad iQ icycler and the Cycler iQ software, respectively. The
139 cycling conditions used were 95°C for 5 min, and 40 cycles of 95°C for 15 s, 55°C for 30 s, and
140 72°C for 30 s. Next, the samples were cooled to 55°C, and a melting curve for temperatures
141 between 55 and 95°C with 0.5°C increments was recorded. Real-time expression measurements
142 were normalized against expression of the reference gene *ACT1*. Relative RNA levels were
143 calculated using the $\Delta\Delta$ C_t method; all primers resulted in amplification efficiencies of at least
144 95%.
145 **Laccase assays.** A quantitative laccase assay using the oxidation of 2,2′-Azino-bis[3-
146 ethylbenzothiazoline-6-sulfonic acid] (ABTS, Sigma) as substrate was performed with *C.*
147 *neoformans* yeast cells, *C. neoformans* cytoplasmic extractions, and a commercially available
148 recombinant laccase from *Rhus vernificera* (Sigma). For intact cells, yeasts were grown in
149 asparagine medium [1 g/liter asparagine, 10 mM sodium phosphate (pH 6.5), 0.25 g/liter $MgSO_4$,
150 10 µM $CuSO_4$] with glucose (1.5 g/liter) for 72 h at 30°C. The cells were collected by
151 centrifugation, washed with with phosphate-buffered saline (PBS), and transferred into

152 asparagine medium without glucose for 36h at 30°C. The strains were collected by
153 centrifugation, washed, and diluted to 1×10^8 cells/ml in PBS with or without voriconazole. A
154 final concentration of 1 mM ABTS was achieved by adding 100 µl of 10 mM ABTS to 900 µl of
155 a yeast cell suspension. After incubation at 30°C for 2 h, the cells were removed with
156 centrifugation and the absorbance readings of the solutions were measured at 420 nm. A yeast
157 cell suspension without ABTS was used as a baseline. Commercially produced laccase from
158 *Rhus vernificera* [activity, 50 U per mg of solid] was used as a positive control at 1 unit in 1 mL
159 of PBS. For cytoplasmic extracts, yeast cells were collected, suspended in 0.1 M Na_2HPO_4 with
160 protease inhibitor, and treated for 6 min in a bead beater at 2-min intervals alternating with 5 min
161 on ice. Supernatants were separated from cellular debris by centrifugation and used in place of
162 the yeast cell suspensions in the ABTS assay. This assay was also used with commercial laccase
163 incubated with voriconazole using various concentrations of either compound.
164 **Phagocytosis assays.** J774.16 is a well-characterized murine macrophage-like cell line that has
165 been extensively used to study *C. neoformans*-macrophage interactions. The J774.16 cells were
166 maintained at –80°C prior to use and were prepared for the phagocytosis assays as described
167 previously (12). A density of 5×10^4 *C. neoformans* 24067 yeast cells were grown in 25 mL of
168 minimal medium supplemented with 1 mM L-Dopa without or with voriconazole (0.25 or 0.5
169 MIC) or fluconazole (0.25 or 0.5 MIC) at 30°C for 7 days. Liquid cultures were shaken at 150 rpm.
170 On days 3, 5 and 7, an aliquot was collected, and washed three times in PBS. Cells were added to
171 the J774.16 monolayer in a macrophage/yeast ratio of 1:1. The plates were incubated for 2 h at
172 37°C with 10 µg of monoclonal antibody (MAb) 18B7/ml. MAb 18B7 binds to cryptococcal
173 glucuronoxylomannan, the major component of the fungal capsule. The monolayer was washed
174 three times with PBS to remove non-adherent cells, fixed with cold methanol, and stained with

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175 Giemsa (Sigma). The phagocytic index is the number of internalized yeast cells per number of
176 macrophages per field. Internalized cells were differentiated from attached cells by their presence
177 in a well-defined phagocytic vacuole. These measurements were determined by light microscopy
178 using an Axiovert 200 M inverted microscope (Carl Zeiss MicroImaging, NY) at a magnification
179 of 400X. For each experiment, three wells were examined, and the numbers of ingested
180 cryptococcal cells and macrophages in three fields were counted with approximately 100
181 macrophages per field.

182 **Statistical analysis.** All data were subjected to statistical analysis using Origin 7.0 (Origin Lab
183 Corp., Northampton, MA). *P* values were calculated by Student's *t* test or analysis of variance
184 depending on the data. *P* values of <0.05 were considered significant.

185

186 **RESULTS**

187 **Voriconazole inhibits melanization at sub-inhibitory concentrations.** *C. neoformans*
188 melanization was significantly reduced and visibly delayed at ≥ 0.125 MIC of voriconazole (Fig.
189 1). In contrast, the addition of ≤ 0.5 MIC of amphotericin B, caspofungin, fluconazole, or
190 itraconazole to *C. neoformans* cultures did not visibly affect melanization. Inhibition of
191 melanization occurred in a similar manner in both liquid and solid medium. The growth rate of
192 *C. neoformans* was not affected by the incubation in sub-inhibitory concentrations of
193 voriconazole or fluconazole (Fig. 2). L-Dopa polymerization was not impeded by the presence
194 of voriconazole at drug concentrations of up to 2X MIC for *C. neoformans*. By the third day of
195 incubation, small black particles were visible in the flasks with and without antifungal drug and
196 the particle density increased similarly in all the flasks over a two week period.

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197 To confirm the results obtained by the plating assay, the percent inhibition in
198 melanization of cryptococcal cells was determined by dry weight (Table 1). The amount of
199 melanin produced by fungal cells co-incubated in minimal medium with L-Dopa and
200 voriconazole or fluconazole was isolated, quantified and compared relative to yeast cells grown
201 in minimal medium with L-Dopa. *C. neoformans* melanization was significantly inhibited by
202 voriconazole. Melanin production was reduced 82.1 and 94.5% after co-incubation with 0.25 and
203 0.5 MIC of voriconazole, respectively. However, fluconazole did not prevent melanization at
204 sub-inhibitory concentrations.

205 **Voriconazole does not affect global gene regulation at sub-inhibitory concentrations.** Three
206 independent replicate experiments were carried out to generate the data set. After normalization
207 of the data using lowess smoothing, there were no genes identified with at least a two-fold
208 upward or downward change, which is considered to be the cutoff for significance with the
209 microarray system used. Hence, although the concentration of voriconazole was sufficient to
210 inhibit melanization, there was no evidence that the phenotype was transcriptionally based.

211 **Voriconazole affects the melanin regulator gene *LAC1* expression at sub-inhibitory**
212 **concentrations.** To explore changes in the expression of *LAC1* gene of *C. neoformans*, that is
213 required for melanin production and full virulence, *q*RT-PCR was performed on RNA extracted
214 from cryptococcal cells grown in the absence or in the presence of sub-MIC of voriconazole and
215 fluconazole (Fig. 3). Our results showed that co-incubation with voriconazole significantly
216 reduced *LAC1* gene expression. Voriconazole decreased *LAC1* gene expression by
217 approximately 40% when compared with control cells. However, *LAC1* was not affected by
218 similar sub-MIC of fluconazole.

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219 **Voriconazole directly inhibits laccase activity.** Voriconazole reduces the capacity of laccase to
220 oxidize ABTS (Fig. 4). When 0.5 MIC of voriconazole was added to suspensions of cryptococci
221 or to cytoplasmic extracts of the fungus, voriconazole dramatically inhibited the activity of the
222 fungal laccases. In order to reproducibly examine these interactions, a commercial standardized
223 recombinant laccase from *Rhus vernificera* was used to demonstrate that voriconazole inhibited
224 the activity in a dose dependent fashion (Fig. 5A). Similarly, reducing laccase in the presence of
225 a constant concentration of voriconazole resulted in a dose dependent reduction in activity (Fig.
226 5B).
227 **Reduction in *C. neoformans* cells melanization by voriconazole alters phagocytosis by**
228 **J774.16 cells.** We investigated whether co-incubation of voriconazole with cryptococci in
229 minimal media supplemented with L-dopa altered the phagocytic activity by macrophage-like
230 cells. Significantly more cryptococcal cells grown in the presence of the sub-MIC of
231 voriconazole were phagocytosed by J774.16 cells compared with cells grown in the absence of
232 antifungal drug or in the presence of sub-inhibitory concentrations of fluconazole (Fig. 6).
233 Voriconazole reduced melanization by fungal cells in culture and increased the phagocytosis of
234 yeast cells by J774.16 cells. As found in previous studies in our laboratory, exposure of
235 *C. neoformans* to sub-inhibitory concentrations of fluconazole also enhanced phagocytosis by
236 macrophages.
237
238 **DISCUSSION**
239 Voriconazole significantly impacts the ability of *C. neoformans* to produce melanin, which
240 makes the yeast cells more susceptible to host effector cells. There are various mechanisms by
241 which voriconazole could interfere with melanization. First, voriconazole could interfere with

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242 protein trafficking as a side effect of its action on fungal sterols with consequent disruption of
243 secretion pathways. In this regard, melanin synthesizing enzymes are often located in the cell
244 wall (5, 17) and the proper expression involves transport to the outside of the cell– possibly in
245 secretory vesicles. Voriconazole may function in a manner analogous to glyphosate, which has
246 been shown to inhibit melanin formation, probably through interference with polymerization of
247 laccase-oxidized precursors (9). The structure of voriconazole differs from fluconazole, its
248 parent structure, by the addition of a methyl group and fluorine, which appears to be sufficient to
249 alter the compounds ability to engage laccase. Interestingly, miconazole, a related imidazole,
250 has been reported to interfere with the production of melanin in melanoma cells by inhibiting
251 tyrosinase in a dose dependent fashion, beginning at a concentration of 30 μM (6). Although the
252 cryptococcal microarray failed to show any global differences upon challenge with sub-
253 inhibitory amounts of voriconazole, the more sensitive qRT-PCR revealed that voriconazole
254 reduced the expression of the *LAC1* gene. In addition to its role in *C. neoformans* melanization,
255 laccase protects the fungus from toxic metabolites produced by macrophage via its iron oxidase
256 activity (3). Hence, if voriconazole binds laccase then the capacity of the enzyme to regulate iron
257 oxidation could be impacted as well. Previous to this study, there was no evidence of any
258 antifungal drug directly inhibiting fungal melanin synthesizing enzymes. However, voriconazole
259 has been shown to be effective against fungi at sub-therapeutic concentrations by inhibiting
260 conidiation in *Aspergillus* species (14).

261 Demonstrating that voriconazole interferes with melanization suggests the exciting
262 possibility that this drug may retain antimicrobial activity even if the targeted microbe develops
263 resistance by mutations of the sterol synthetic pathway and/or by selection of enhanced efflux
264 mechanism. This attribute would not be detected by in vitro susceptibility tests since these are

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265 standardized in conditions where fungi are not ordinarily melanized. Hence, it is conceivable
266 that voriconazole would be active in vivo against fungi for which it has minimal or no in vitro
267 activity since reduction in melanin production would translate into reduced virulence that in turn
268 would allow increased clearance by host immune mechanisms. In this regard, Serena *et al.*
269 recently demonstrated that voriconazole reduces fungal burden and enhances survival in a
270 murine model of cryptococcal Central Nervous System (CNS) infection (10). Human brain
271 contains various phenolic compounds, such as norepinephrine, 3, 4-dihydroxyhenylacetic acid,
272 homovanillic acid, 5-hydroxyindolacetic acid, serotonin, and dopamine, all of which can serve as
273 substrates for the *C neoformans* laccase. In addition to interfering with the production of
274 ergosterol, our study shows that voriconazole can suppress laccase production, which could
275 reduce the ability of *C. neoformans* yeast cells to utilize phenolic compounds as substrates for
276 melanin production in the brain and other tissues further crippling the organism's capacity to
277 cause disease.
278

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288 **FIGURE LEGENDS**

289 **Figure 1.** *C. neoformans* yeast were grown on minimal medium 1 mM L-Dopa agar plates for 10
290 days at 30°C with or without the addition of antifungal drugs. (**A**) Growth of *C. neoformans* in the
291 absence of antifungal drugs demonstrating dark pigmentation of the colonies and polymerization of
292 the L-Dopa in the agar surrounding the colonies due to the secretion of laccase into the medium. *C.*
293 *neoformans* grown with voriconazole at **(B)** 0.125, **(C)** 0.25 and **(D)** 0.5 MIC showing increasingly
294 lower amounts of melanin formation in the colonies or within the agar. There was no significant
295 reduction of melanin production with 0.5 MIC of **(E)** amphotericin B, **(F)** caspofungin, **(G)**
296 fluconazole, or **(H)** itraconazole. Plates were done in triplicate. This experiment was done twice,
297 with similar results each time.

298 **Figure 2.** Growth curve of *C. neoformans* with subinhibitory concentrations of voriconazole or
299 fluconazole used in this study.

300 **Figure 3.** Voriconazole affects *C. neoformans LAC1* gene expression at sub-inhibitory
301 concentrations. * denotes $P < 0.05$ in comparison with control or fluconazole groups. This
302 experiment was done twice, with similar results.

303 **Figure 4.** Oxidation of ABTS by *C. neoformans* laccase from intact cells or from supernatants
304 of yeast cell extracts is suppressed by voriconazole. Recombinant laccase from *Rhus vernificera*
305 incubated with ABTS represents the positive control and the negative control is ABTS alone.
306 The experiment was done twice and similar results were obtained.

307 **Figure 5.** The oxidation of ABTS by laccase is reduced by the presence of voriconazole. **(A)**
308 Increasing concentrations of voriconazole in the presence of a constant amount of laccase. #
309 represents $P < 0.05$ * indicates $P < 0.001$ in comparison to the activity of laccase in the absence
310 of voriconazole. **(B)** Decreasing concentrations of laccase result in the presence of a constant

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311 amount of voriconazole. ** indicates $P < 0.001$ in comparison to the activity of the highest
312 concentration of laccase in the absence of voriconazole. The experiments were done twice with
313 similar results.

314 **Figure 6.** Voriconazole reduces *C. neoformans* 24067 melanization and alters yeasts
315 phagocytosis by J774.16 cells. Bars are the averages of three wells, and brackets denote standard
316 deviations. * denotes $P < 0.001$ in comparison with control group. # represents $P < 0.05$ in
317 comparison of voriconazole group with fluconazole group. This experiment was done twice, with
318 similar results.

319

320 **Table 1.** Inhibition of *C. neoformans* 24067 melanin production by voriconazole.

Drug	MIC	Dry Weight (g)	% melanin inhibition[†]
Voriconazole	0.25	0.0302	82.1
	0.5	0.0092	94.5
Fluconazole	0.25	0.1641	2.7
	0.5	0.1394	17.3

321

322 [†] The percentage of melanin inhibition was determined by comparing dry weights obtained from
323 50 ml cultures of *C. neoformans* cells grown in the presence of L-Dopa and drugs relative to
324 yeast cells grown in the presence of L-Dopa only.

325

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Figure 1

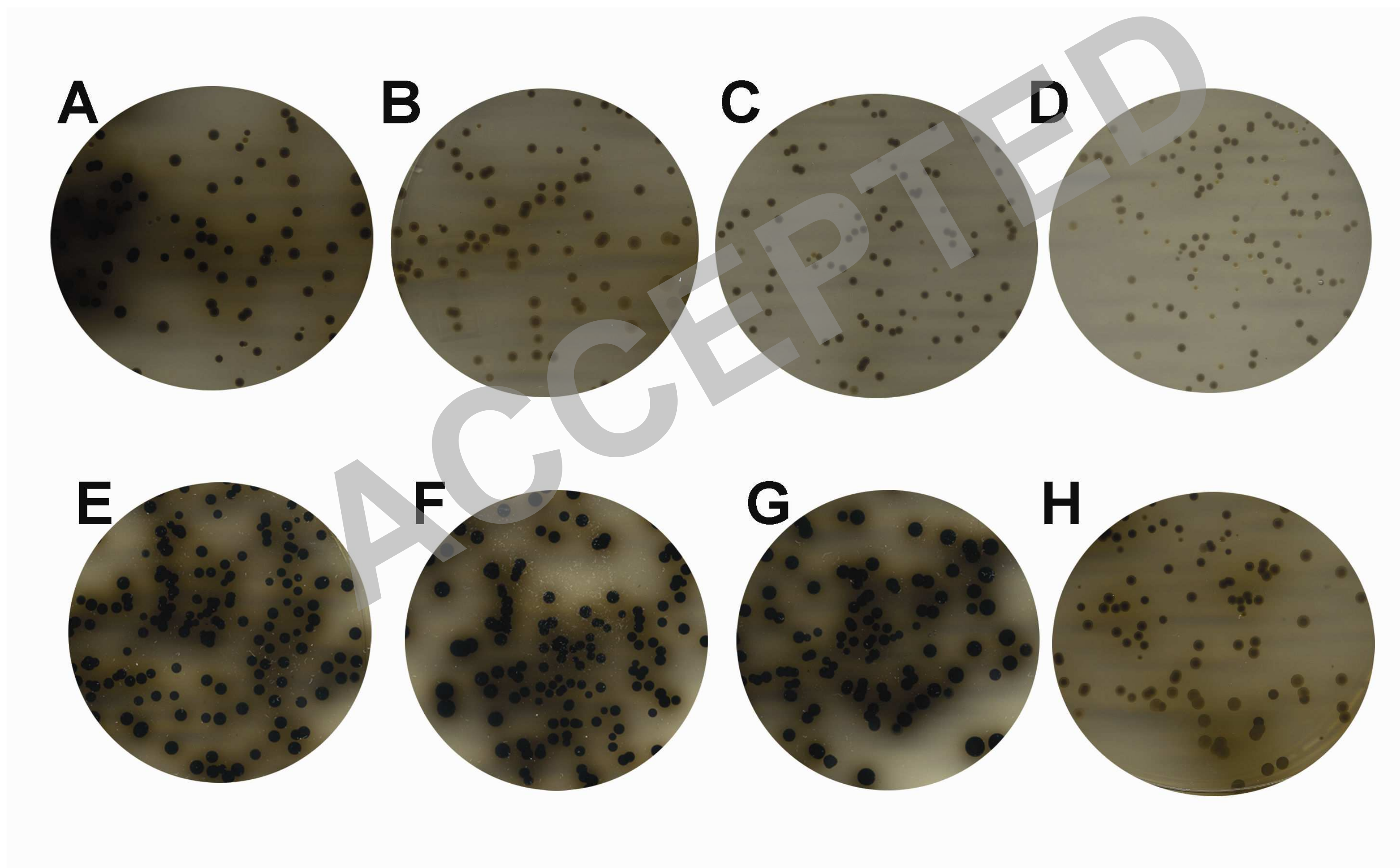

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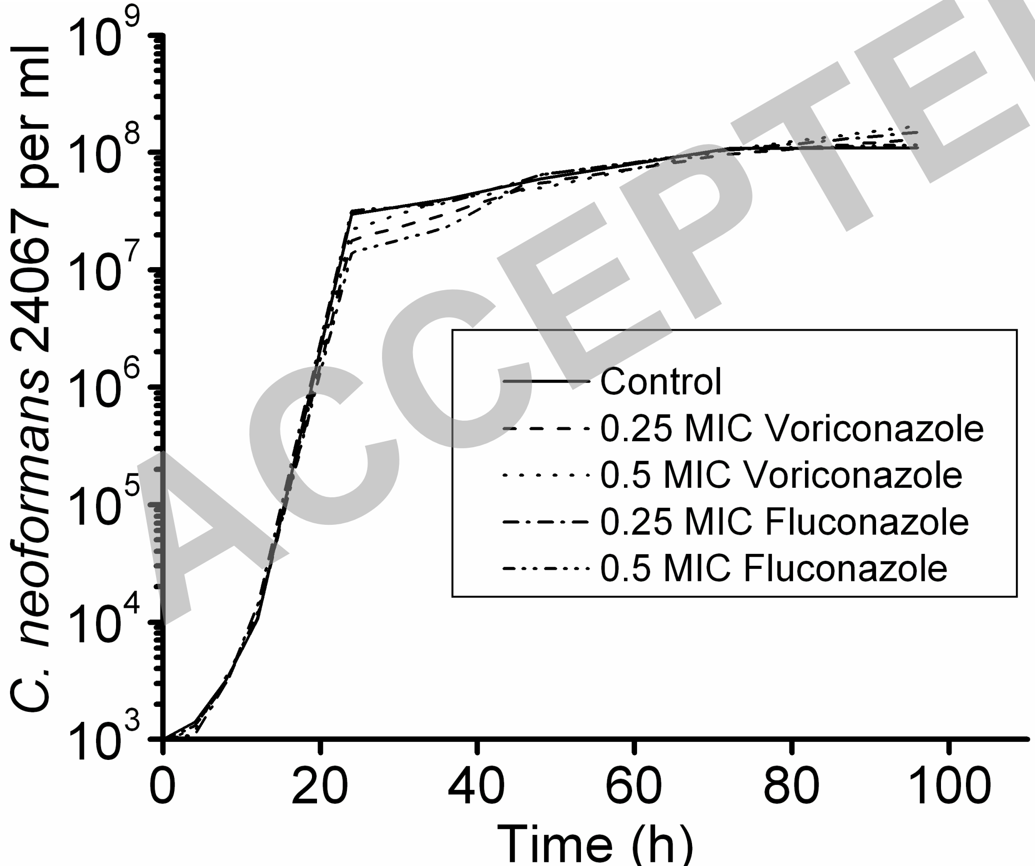
Figure 2



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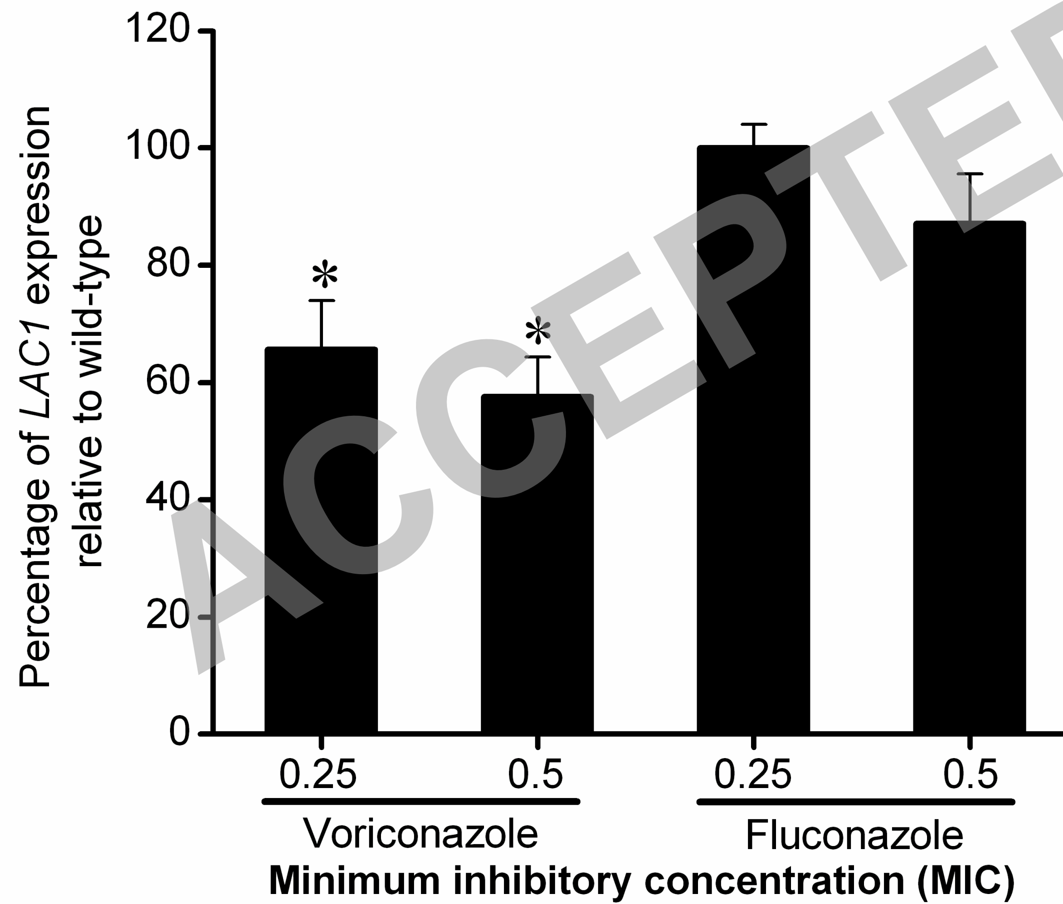

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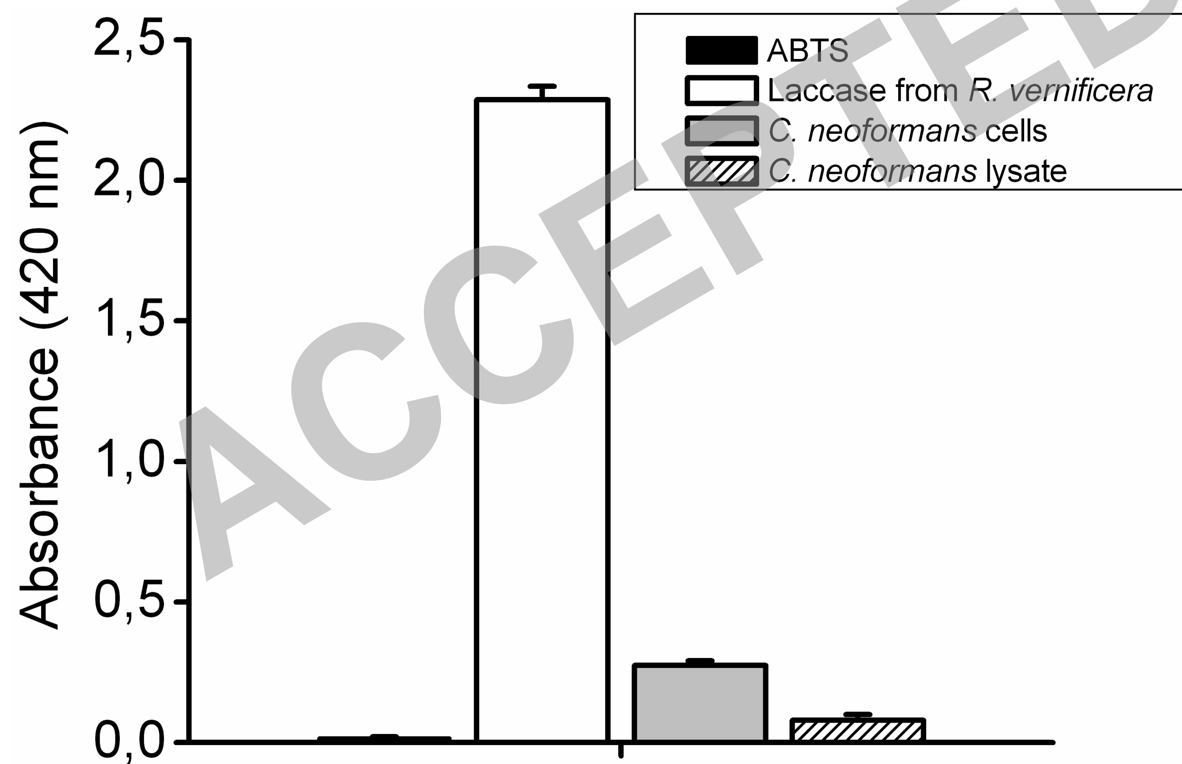

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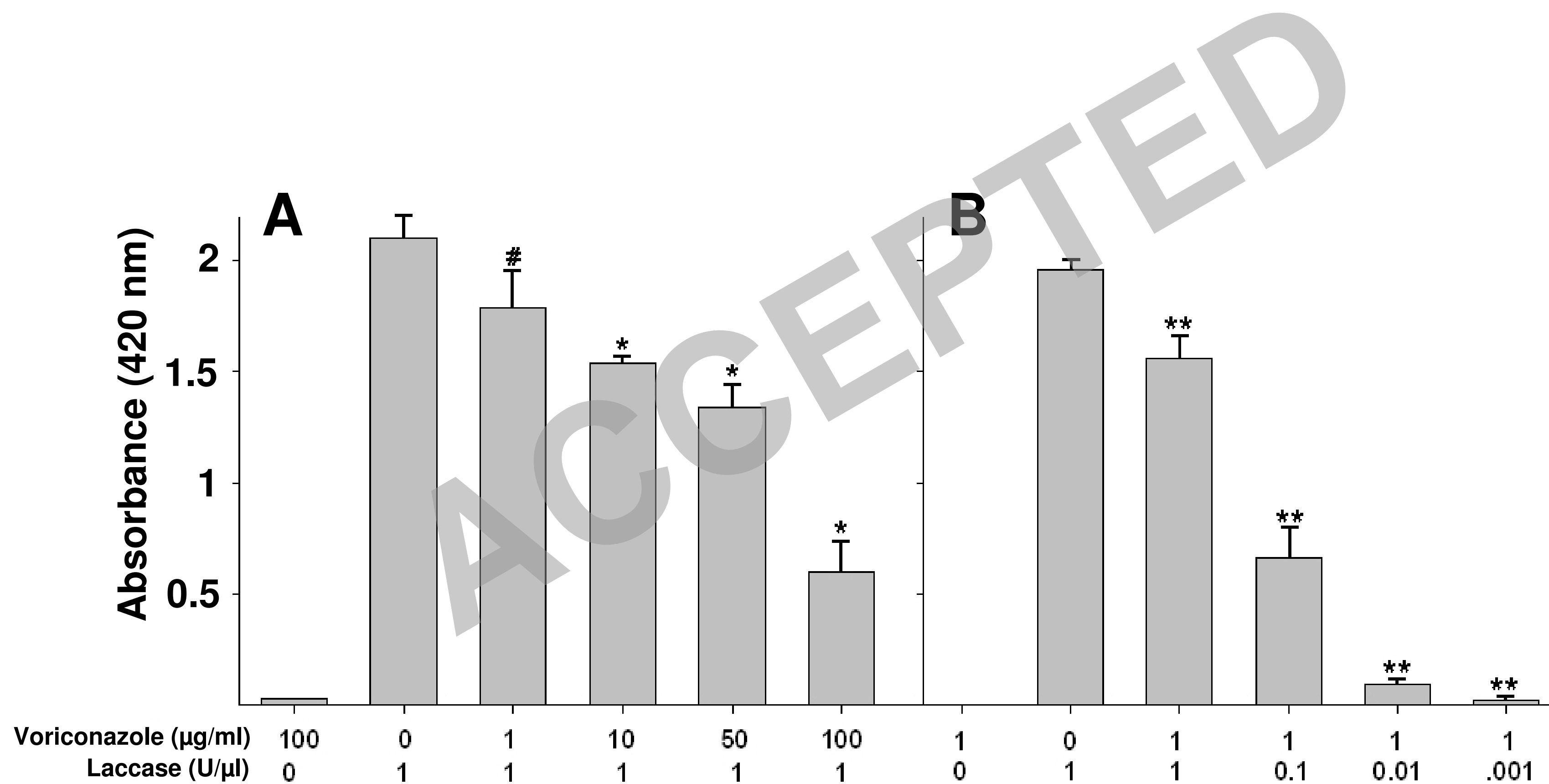

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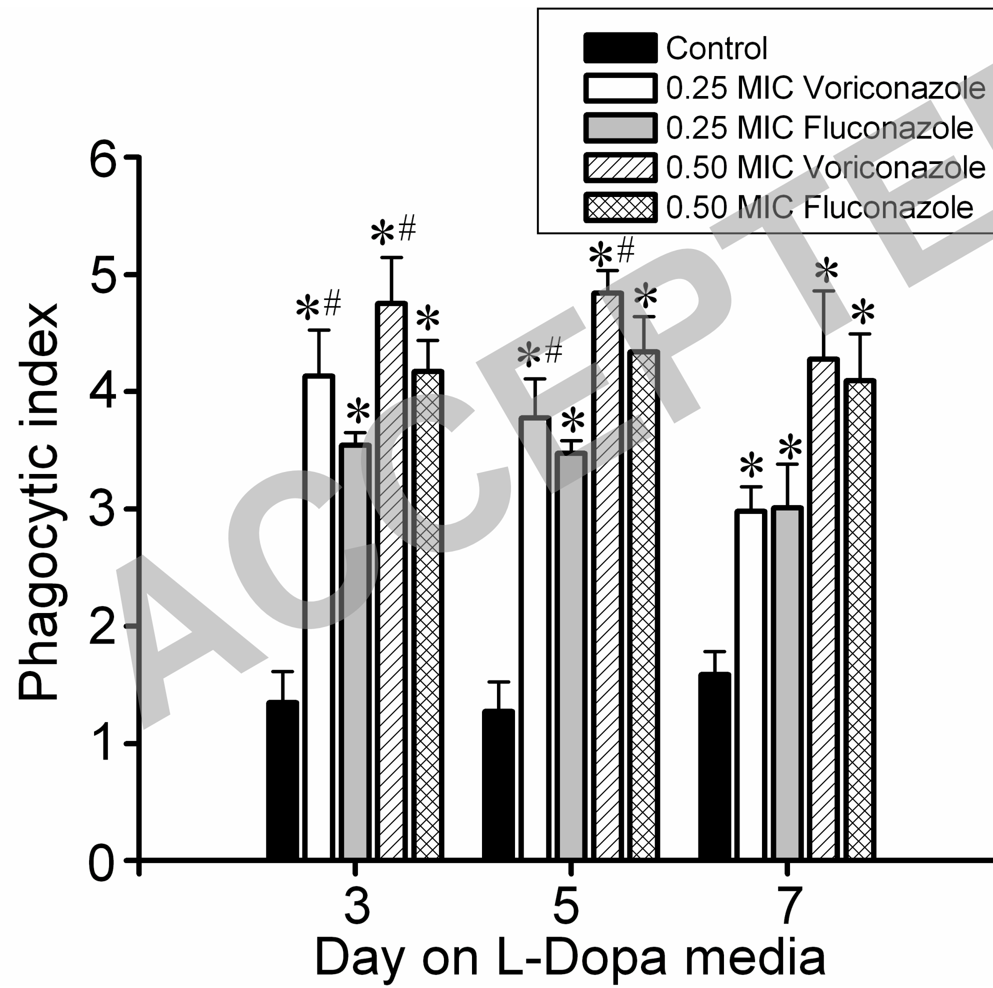
Figure 6



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