Identification of Multiple Cryptococcal Fungicidal Drug Targets by Combined Gene Dosing and Drug Affinity Responsive Target Stability Screening

Yoon-Dong Park, Wei Sun, Antonio Salas, Avan Antia, Cindy Carvajal, Amy Wang, Xin Xu, Zhaojin Meng, Ming Zhou, Gregory J. Tawa, Jean Dehdashti, Wei Zheng, Christina M. Henderson, Adrian M. Zelazny, Peter R. Williamson

Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA; National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, Maryland, USA; Protein Characterization Laboratory, Cancer Research Technology Program, Frederick National Laboratory for Cancer Research, Frederick, Maryland, USA; Microbiology Service, Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland, USA; Section of Infectious Diseases, Department of Medicine, University of Illinois at Chicago College of Medicine, Chicago, Illinois, USA

ABSTRACT Cryptococcus neoformans is a pathogenic fungus that is responsible for up to half a million cases of meningitis globally, especially in immunocompromised individuals. Common fungistatic drugs, such as fluconazole, are less toxic for patients but have low efficacy for initial therapy of the disease. Effective therapy against the disease is provided by the fungicidal drug amphotericin B; however, due to its high toxicity and the difficulty in administering its intravenous formulation, it is imperative to find new therapies targeting the fungus. The antiparasitic drug bithionol has been recently identified as having potential fungicidal activity. In this study, we used a combined gene dosing and drug affinity responsive target stability (GD-DARTS) screen as well as protein modeling to identify a common drug binding site of bithionol within multiple NAD-dependent dehydrogenase drug targets. This combination genetic and proteomic method thus provides a powerful method for identifying novel fungicidal drug targets for further development.

IMPORTANCE Cryptococcosis is a neglected fungal meningitis that causes approximately half a million deaths annually. The most effective antifungal agent, amphotericin B, was developed in the 1950s, and no effective medicine has been developed for this disease since that time. A key aspect of amphotericin B’s effectiveness is thought to be because of its ability to kill the fungus (fungicidal activity), rather than just stop or slow its growth. The present study utilized a recently identified fungicidal agent, bithionol, to identify potential fungicidal drug targets that can be used in developing modern fungicidal agents. A combined protein and genetic analysis approach was used to identify a class of enzymes, dehydrogenases, that the fungus uses to maintain homeostasis with regard to sugar nutrients. Similarities in the drug target site were found that resulted in simultaneous inhibition and killing of the fungus by bithionol. These studies thus identify a common, multitarget site for antifungal development.

Received 14 June 2016 Accepted 16 June 2016 Published 2 August 2016

C ypto c o ccal meningitis, caused by the fungus Cryptococcus neoformans, results in approximately 600,000 deaths from 1,000,000 infections annually (1, 2). The disease is associated predominately with immunosuppressed individuals, such as those infected with HIV or immunosuppressed with transplant conditioning or cancer chemotherapy, but it can also occur in previously healthy individuals as well (3, 4). In countries with a high prevalence of HIV/AIDS, such as those in sub-Saharan Africa, Cryptococcus neoformans is one of the most common causes of meningitis (1). Administration of intravenous amphotericin B and flucytosine is the standard therapy regimen for cryptococcal meningitis patients (5); however, due to the renal toxicity and lack of oral formulations of amphotericin B and hematological toxicity of flucytosine, novel antifungal drugs are sorely needed. Fluconazole is an important orally absorbed, nontoxic drug useful for prophylaxis and follow-up treatment after amphotericin B induction, but administration in the acute setting is associated with poor efficacy and a 90% mortality rate (6). The fungicidal activity of amphotericin B is thought to be critical for its efficacy in the acute setting, with the rate of fungal clearance in the cerebrospinal fluid (early fungicidal activity [EFA]) being an important discriminator between ineffective fungistatic therapies such as fluconazole and more effective fungicidal therapies such as amphotericin B (6). Thus, identifying new drug targets, especially those associated with fungicidal activity, has become a priority (7).

Previously, the parasitic drug bithionol was demonstrated to...
have fungicidal properties based on a high-throughput drug-repurposing screen of 1,280 pharmacologically active compounds against Cryptococcus neoformans (8). Bithionol is a diphenolic compound that, prior to the advent of praziquantel, was used extensively as an anthelmintic agent against pulmonary paragonimiasis for both individual and mass treatment in areas where paragonimiasis is endemic (9). The drug is well tolerated in humans and reaches reported blood levels of up to 140 μg/ml, much higher than the fungicidal concentrations, which are in the low microgram per milliliter range (10). In addition, relevant for neurological infections, the drug has been used to treat cerebral helminthic infections, such as central nervous system (CNS) paragonomus; in one report, 24 patients were treated with bithionol and cures were reported for 22 when bithionol doses of 40 to 50 mg/kg of body weight/day were used (11). Some work has been performed regarding mechanisms of action related to mammalian toxicity at high concentrations. In mammalian tissues at higher doses, bithionol acts to slow rapidly growing cells, such as ovarian cells, and appears to target the NF-κB and mitogen-activated protein kinase signaling pathways (12). In addition, bithionol has been used to model allosteric binding of GTP to glutamate dehydrogenase in crystallographic studies (13). However, fungicidal mechanisms of bithionol that could inform the design of novel agents remain poorly understood.

While bithionol may not be an optimal chemical moiety for modern use because of its potentially DNA-reactive phenolic groups (14), identification of target enzymes of a potentially effective and relatively nontoxic drug may prove valuable for future drug development (7). In the present study, we utilized the method of drug affinity responsive target stability (DARTS) screening, whereby protein lysates are incubated in the presence or absence of drug, partially digested with protease, and drug-protected active site peptides are identified by differential mobility on SDS-PAGE gels followed by mass spectroscopy (15). This method was combined with a gene dosing strategy, whereby a whole-genomic overexpression library of the yeast Saccharomyces cerevisiae was used to identify genes whose overexpression resulted in drug resistance (gene dosing and drug affinity responsive target stability [GD-DARTS]). This method identified 75 protein targets via DARTS and 9 genes after overexpression. Combining these two modalities identified NAD-dependent enzymes whose combined inhibition was associated with potent fungicidal activity.

RESULTS

Drug affinity responsive target stability. The overall scheme used to identify bithionol drug targets is shown in Fig. 1. The DARTS method (Fig. 1A) utilizes fungal cell extracts incubated in the presence or absence of saturating concentrations of drug, followed by proteolysis. Drug binding peptides are essentially “protected” against proteolysis, and after gel electrophoresis they have higher mobilities due to reduced rates of proteolysis compared to identical reactions in the absence of drug. The identified set of proteins derived from the retarded peptides was then compared to a gene dosing screen whereby genes were identified whose overexpression resulted in resistance to the given drug (Fig. 1B). For these studies, we utilized the genetically tractable fungus Saccharomyces cerevisiae. We first tested drug sensitivity against S. cerevisiae.
BY4741 and Cryptococcus neoformans H99, which were both sensitive to bithionol at micromolar concentrations, with increased sensitivity of the S. cerevisiae strain (Fig. 2A). In addition, we tested 10 cryptococcal strains which demonstrated low-micromolar fungicidal inhibition, even for strains demonstrating high-level resistance to other antifungals, such as fluconazole (Table 1). To identify potential bithionol-interacting proteins, we utilized DARTS against S. cerevisiae BY4741 as well as C. neoformans H99 protein extracts (Fig. 2B). Seventy-five candidate proteins that potentially interacted with bithionol were identified from the two yeast species (see Tables S2 and S3 in the supplemental material). Interestingly, a predominance of dehydrogenases (8), including NADH dehydrogenases, were identified.

Identification of candidate bithionol fungicidal target genes.

Because of a large number of candidate bithionol-interacting proteins identified through DARTS, a complementary gene dosing strategy was utilized to identify genes whose products conveyed resistance to bithionol. An overexpression genomic library containing 4,500 genes was transformed into the sensitive S. cerevisiae strain and screened in the presence of increasing amounts of bithionol. Using replica plating, we obtained bithionol-resistant S. cerevisiae colonies (Fig. 2C). Plasmids were recovered from re-
sistant colonies, and genes were identified through sequencing. Because the average size of the inserts in the overexpression library was 10 kb and inserts contained multiple genes, single-gene overexpression constructs were introduced into \textit{S. cerevisiae} to identify and confirm the gene that conveyed the resistance phenotype. Using this method, 9 genes were identified whose overexpression conferred resistance on plate assays with bithionol compared to the wild type (WT) (Fig. 2D). A summary of the corresponding functions of the protein products of the overexpressed genes is shown in Table 2. Of the 9 genes, three shared a common putative NAD-dependent dehydrogenase activity: malate dehydrogenase (\textit{MDH3}), glutamate dehydrogenase (\textit{GDH1}), and 6-phosphogluconate dehydrogenase (\textit{GND1}) (Fig. 3A), and two (\textit{GDH1} and \textit{GND1}) were also identified in the DARTS screen, suggesting a class effect against these enzymes. In addition, yeast strains overexpressing the gene \textit{PNC1}, \textit{TFB6}, or \textit{Sec6} also conferred resistance to bithionol compared to WT (Fig. 3B). Two additional genes, \textit{VMA22} and \textit{FYV4}, conferred increased resistance only at high concentrations of 50 \textmu M, while they had similar sensitivities to the wild type at low concentrations of 0.3 \textmu M (Fig. 3C). On the other hand, one gene, \textit{AAR2}, conferred resistance only at low concentrations (0.3 and 1.0 \textmu M), with similar sensitivities at high concentrations (10 and 50 \textmu M) (Fig. 3D). These latter activities suggest indirect roles in bithionol resistance.

\textbf{Validation of NAD-dependent dehydrogenases as targets of bithionol.} The three gene products sharing a common putative NAD-dependent dehydrogenase activity, \textit{Mdh3}, \textit{Gdh1}, and \textit{Gnd1}, from the gene dosing assay were selected for further evaluation. All these genes are expressed under no-glucose conditions (see Fig. S1 in the supplemental material). RNA interference (RNAi) strains of \textit{MDH3}, \textit{GDH1}, and \textit{GND1} were constructed to examine the effect on drug resistance to gene dosing reduction and to confirm their role in \textit{C. neoformans}. As shown in Fig. 4, all knockdown strains exhibited increased sensitivity against bithionol compared to WT strains containing empty vector alone.

To further confirm NAD-dependent dehydrogenases as a direct target of bithionol, we selected the representative enzyme malate dehydrogenase (\textit{Mdh3}) and expressed and purified recombinant \textit{Mdh3} in \textit{S. cerevisiae} as a fusion protein with the affinity tag maltose binding protein (16). Recombinant cryptococcal proteins were not active when expressed in \textit{Escherichia coli} (data not shown). Recombinant enzyme expressed in \textit{S. cerevisiae} or cryptococcal extract was active both by a commercial assay (Fig. 5A; see also Fig. S2 in the supplemental material) and using constituent substrates (Fig. 5B), and the cryptococcal enzyme was found to require NAD for reduction of malate. In addition, bithionol inhibited \textit{Mdh3} activity in a Michaelis-Menten-type manner, dis-

\begin{table}
\centering
\caption{Putative bithionol target genes of \textit{S. cerevisiae} based on the gene dosing methodology.} 
\begin{tabular}{lll}
\hline
Gene name & Systematic name & Description \\
\hline
\textit{MDH3} & YDL078c & Peroxisomal malate dehydrogenase \\
\textit{GDH1} & YOR375c & NADP\textsuperscript{+}-dependent glutamate dehydrogenase \\
\textit{GND1} & YHR183w & 6-Phosphogluconate dehydrogenase \\
\textit{PNC1} & YGL037c & Nicotinamidase that converts nicotinamide to nicotinic acid; part of the NAD\textsuperscript{+} salvage pathway \\
\textit{SEC6} & YIL068C & Essential 88-kDa subunit of the exocyst complex \\
\textit{TFB6} & YOR352w & Subunit of TFIH complex; facilitates dissociation of the S12p helices from TFIH \\
\textit{AAR2} & YBL074C & Component of the U5 snRNP complex; required for splicing of U3 precursors \\
\textit{FYV4} & YHR059w & Protein of unknown function; required for survival upon exposure to K1 killer toxin \\
\textit{VMA22} & YHR060 & Protein that is required for vacuolar H\textsuperscript{+}-ATPase (V-ATPase) function \\
\hline
\end{tabular}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Fungicidal assay results for \textit{S. cerevisiae} overexpression strains exposed to increasing concentrations of bithionol. The indicated overexpressed strains and the WT BY4741 strain were used, and assays to determine CFU were performed in triplicate. *, \textit{P} < 0.05; **, \textit{P} < 0.01; ***, \textit{P} < 0.001; ****, \textit{P} < 0.0001.}
\end{figure}
playing noncompetitive inhibition of both malate and NAD with $K_i$ inhibitory constants of 3.0 M for malate and 18.45 M for NAD (Fig. 5C and D). These findings suggest that bithionol acts as an allosteric inhibitor of both malate and NAD for Mdh3. Inhibition of multiple dehydrogenase targets likely contributes to the potent fungicidal activity of bithionol.

Bithionol binds to a conserved fungal NAD-dependent dehydrogenase binding pocket. The finding of multiple NAD dehydrogenases as bithionol targets by GD-DARTS suggested a common fungal bithionol binding site within this class of enzymes. For each of these Cryptococcus target sequences, a BLAST search was run against Protein Data Bank (PDB) structural parameters, and the protein structures with the highest sequence homologies for each of the Cryptococcus targets were identified and used as templates for homology modeling of the bithionol binding pocket, as described in Materials and Methods. The modeled C. neoformans protein Mdh3, which demonstrated inhibition of the recombinant cryptococcal enzyme, is shown in Fig. 6A. A close-up image of the putative fungal binding site with bithionol (yellow) and NAD and malate (green) is shown complexed with Mdh3 (Fig. 6B and C). Other dehydrogenases identified in the DARTS screen were also modeled for bithionol binding, including glutamate dehydrogenase, D-lactate dehydrogenase, dihydrolipoyl dehydrogenase, aldehyde dehydrogenase, D-arabinitol 2-dehydrogenase, and NADH dehydrogenase, and are shown in Fig. S3 in the supplemental material. The best docked configuration of bithionol within each of the binding pockets was then used as a starting point for binding energy calculations.

The calculated binding energies of bithionol are given in Table 3, with lower values denoting higher binding energies. The binding energy of bithionol to Mdh3 was predicted to be $-20$ kcal/mol, far larger in magnitude than the binding energy of bithionol to Gdh1 ($-11$ kcal/mol). These results suggest that bithionol binds to the first 5 proteins, including Mdh3 and Gdh1,
but does not bind strongly to the last three. The major interactions of bithionol with the Mdh3 binding pocket are shown in Fig. 6B. Important to this binding appears to be the bridging hydrogen bonding interaction between HIS-199, bithionol hydroxyl, and MET-250. In addition, ARG-175 makes electrostatic contacts with the aromatic chlorines on bithionol. Otherwise, in Gdh1, one strong hydrogen bond exists between bithionol and LYS-138 and one C-H bond exists between bithionol and VAL-441. Bithionol is thus predicted to be a less potent inhibitor of Gdh1 than it is of Mdh3, probably because it only has one classic hydrogen bond to Gdh1, whereas in the case of Mdh3 it has two. Taken together, these results suggest that bithionol is predicted to be a broad-spectrum NAD-dependent dehydrogenase inhibitor, with more potent inhibition of Mdh3 than of Gdh1.

Reduced levels of active bithionol in mice and humans limit the suitability of bithionol as a pharmaceutical for use against *C. neoformans*. Because of high published serum levels of bithio-

---

**TABLE 3** Absolute binding energies of putative bithionol target dehydrogenases

| Protein                          | Accession no.   | Bithionol binding energy (kcal/mol) |
|----------------------------------|-----------------|-------------------------------------|
| Lactate dehydrogenase            | CNAG_02644      | −32                                 |
| Dihydrolipoyl dehydrogenase      | CNAG_07004      | −31                                 |
| Malate dehydrogenase             | CNAG_03225      | −20                                 |
| Aldehyde dehydrogenase           | CNAG_02377      | −19                                 |
| Glutamate dehydrogenase          | CNAG_01577      | −9                                  |
| Succinate-semialdehyde dehydrogenase | CNAG_01027 | 22                                  |
| Arabinol 2-dehydrogenase         | CNAG_02925      | 29                                  |
| NADH dehydrogenase               | CNAG_00788      | 32                                  |

\(^a\) The magnitude of the binding energy is related to the compound’s potency.

\(^b\) Accession numbers are from the Broad Institute’s *Cryptococcus neoformans* var. *grubii* 1999 database.

---

FIG 6 The binding environment of bithionol within the Mdh3 core region. (A and B) Ribbon (A) and stick (B) figures of the MDH-bithionol complex, with some of the contact residues highlighted. In panel B, the predicted binding energies (in kilocalories per mole) at the bithionol binding sites (yellow) are shown. (C) A closer view of the Mdh3 binding pocket structure. Bithionol is docked to the modeled *C. neoformans* Mdh3 protein. The bithionol binding sites (yellow) and NAD and malate (green) molecules complexed with Mdh3 are indicated. Residues within 5 Å of bithionol are labeled and defined as the binding pocket.
nol (up to 30× the level of effective fungicidal activity) and use of the drug in central nervous system infections, we investigated the suitability of the compound bithionol itself in mammalian infections. As shown in Fig. S4 in the supplemental material, treatment with a single dose of 50 mg/kg in mice resulted in peak drug levels in plasma of approximately 13.2 μg/ml, with an area under the concentration-time curve (AUC) up to the last sample point (AUCClast) of 139 μg/ml, an AUC extrapolated to infinity (AUCINF) of 144 μg/ml, and an area under the drug concentration moment curve up to the last sample time point (AUMClast) of 861 μg/ml. In the brain, there was a peak drug concentration of 1.0 μg/ml, AUCClast of 139 μg/ml, AUCINF of 11.3 μg/ml, and AUMClast of 68.9 μg/ml. Single dosing with 10 mg/kg resulted in peak plasma drug levels of approximately 4.3 μg/ml, with an AUCClast of 58.3 μg/ml, AUCINF of 62.6 μg/ml, and an AUMClast of 395 μg/ml; in this dose group, in the brain there was a peak concentration of 0.1 μg/ml, AUCClast of 2.4 μg/ml, and AUMClast of 20.0 μg/ml. Furthermore, in a patient with refractory Cryptococcus neoformans meningitis after receiving treatment with amphotericin B (Ambisome), dosing with bithionol at 50 mg/kg/day for 3 days resulted in trough plasma drug levels of 36.3, 46.3, and 44.9 μg/ml on days 1, 2, and 3, respectively, and cerebrospinal fluid (CSF) drug levels on day 3 1.12 μg/ml, with a simultaneous drug level of 46.2 μg/ml in the plasma, suggesting comparable levels in humans and in mice. No side effects were identified in the patient after 3 days of therapy. However, it is important to note that these findings showed that bithionol reaches significantly lower concentrations in humans than those reported in the literature (10). Further studies were conducted in mice in an intravenous model of C. neoformans, to determine clearance of the organism from the brain in order to compare the efficacy of bithionol to that of amphotericin B or fluconazole. As shown in Fig. S5 in the supplemental material, bithionol at 100 mg/kg was found not to be effective compared to the vehicle alone, with deaths occurring almost simultaneously. These data also suggest that the low levels achievable in blood and CSF in both mice and humans may limit the suitability of bithionol itself as an effective anticytotoxic agent, despite its favorable fungicidal and side effect profiles.

**DISCUSSION**

The present study was prompted by the identification of bithionol as a potential fungicidal agent in a large repurposing screen (17) and by multiple reports for humans suggesting high serum drug levels and low toxicity (11), as well as also bithionol’s potential utility in treating CNS infections (18). The further studies described here confirmed bithionol as a potentially useful antifungal agent against Cryptococcus neoformans due to its antifungal effects, suggesting its utility in identifying the antifungal target of this agent, for which few data are available regarding its activity in fungi. This was particularly important for bithionol because the presence of multiple phenolic groups in the bithionol molecule as well the poor blood and brain drug concentration levels determined using modern assay methodologies have suggested that the compound itself would require significant synthetic modifications to improve bioavailability and reduce potential DNA interactions to reduce oncogenic and teratogenic potentials.

DARTS is a recently developed method for identifying potential drug targets, and it is particularly useful for compounds that have lower affinities with binding constants in the micromolar range (15). Gene dosing strategies have also been useful for identifying potential drug targets and have typically utilized either drug-induced or systematic mutations to increase drug susceptibility (19, 20). However, overexpression (rather than gene reduction) strategies can provide a similar set of candidate drug targets, but identification of resistant colonies on plates is technically more facile than identifying hypersensitive ones. In the present study, we identified 75 candidate bithionol targets, which were reduced to 2, by using the gene dosing strategy, and we identified a third target due to its similar enzymatic process. Thus, the combination strategy of GD-DARTS proved to be an efficient method of obtaining fungicidal drug targets in the present studies. The first gene, MDH3, is a gene that codes for malate dehydrogenase, which is involved in the glycoxylate pathway of yeast and catalyzes the oxidation of malate to oxaloacetate (21). GDH1 codes for glutamate dehydrogenase 1, an enzyme that catalyzes the synthesis of glutamate from ammonia and α-ketoglutarate (22). GND1 codes for 6-phosphogluconate dehydrogenase, which catalyzes the oxidative decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO2, with concomitant reduction of NAD to NADH (23). Further modeling studies identified additional structurally conserved NAD dehydrogenases that were likely bithionol targets. Lactate dehydrogenase (LDH) catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+. Replicating cells rely on increased glycolysis, resulting in increased lactate production instead of aerobic respiration in the mitochondria, even under oxygen-sufficient conditions (24). Dihydrolipoamide dehydrogenase (DLD) is a flavo-protein enzyme that oxidizes dihydrolipoamide to lipoamide. DLD is a mitochondrial enzyme that plays a vital role in energy metabolism in eukaryotes, converting dihydrolipoic acid and NAD+ into lipoic acid and NADH (25). Aldehyde dehydrogenases (EC 1.2.1.3) are a group of enzymes that catalyze the oxidation (dehydrogenation) of aldehydes (26). The active site of the aldehyde dehydrogenase enzyme is largely conserved throughout the different classes of the enzyme. The active site binds to one molecule of an aldehyde and an NAD+ that functions as a cofactor. A cysteine and a glutamate interact with the aldehyde substrate.

Allosteric inhibition was found to be important for bithionol inhibition of fungal dehydrogenases, exemplified by inhibition of the class representative, Mdh3. Modeling studies, based on crystallographic studies of the mammalian Gdh1 (13, 27), identified a shared bithionol binding site, offering an explanation for its potent fungicidal activity. Previous studies in other pathogens, such as Trichomonas vaginalis, have suggested that bithionol inhibits the production of volatile thiols from l-methionine (28), which could be due to inhibition of enzymes such as glutamate dehydrogenases that have been linked to methionine metabolism (29). Bithionol has also been demonstrated to inhibit respiration of intact trophozoites of Entamoeba histolytica (30). Since maintenance of NAD/NADH ratios is important for efficient respiration (31), it is plausible that inhibition of multiple NAD-dependent enzymes involved in alternative substrate acquisition, such as lactate dehydrogenase, could result in pathogen respiratory perturbations.

Other potential indirect targets were identified in the gene dosing study and may also play a role in bithionol fungicidal activities, but they were not further characterized in recombinant enzyme studies as they were not suggested to be targets based on the modeling studies. For example, PNC1 is a gene that codes for nicoti-
namidase, a protein that converts nicotinamide into nicotinic acid. Because nicotinamidase is the first enzyme involved in the NAD+ salvage pathway, which functions to produce NAD+, these results suggest that bithionol could hinder many interacting pathways involved with dehydrogenases by also inhibiting the synthesis of NAD+. In addition, inhibition of potential virulence factors may potentiate bithionol’s effects, such as FVY4, which codes for a mitochondrial protein implicated in resistance to K1 killer toxin, which binds to the cell wall and allows it to form pores (33). Genes conveying resistance to bithionol include TFB6, which codes for a subunit involved in the formation of the TFIIH complex and is needed to form the essential RNA polymerase II preinitiation complex (34). More specifically, Tfb6 phosphorylates Sd2, which is another TFIIH subunit with helicase activity, and allows the two subunits to form a heterodimer that dissociates from the TFIIH (34). As an indirect mechanism, overexpression of the TFB6 gene could allow increased transcription of multiple genes necessary to counteract bithionol’s activity. Such an indirect mechanism would explain their absence in the DART screen. In the same way, the AAR2 gene codes for a protein that is a component of the U5 of the snRNP complex and is integral to the splicing of U3 precursors (35). The increased resistance of the AAR2 mutant and TFIIH overexpression strains could prove protective against fungicidal activities of bithionol. Furthermore, Sec6 was found in the gene dosing study; this gene codes for an 88-kDa subunit of the exocyst complex which is essential for the function of the complex. The Sec6 protein is important in directing post-Golgi complex secretory vesicles to appropriate locations on the cell membrane for exocytosis (36) and is important in cryptococcal pathogenesis (37). The overexpression of this gene could aid in resistance by potentially increasing the ability of cells to remove bithionol through exocytosis. On the other hand, VMA22 is a gene that codes for an endoplasmic reticulum protein required for synthesis of vacuolar proton-transporting ATPase, which is required for creating the acidic environment of lysosomes and other organelles (38, 39). The overexpression of the VMA22 gene could aid in providing an environment for lysosomes, endosomes, and other organelles to maintain an acidic internal environment when exposed to bithionol.

It was disappointing that bithionol itself was not a more effective anticryptococcal agent. We felt that it was important to include negative data concerning this drug for CNS cryptococcosis, both to prevent inadvertent clinical use and to address recent concerns that negative data are sometimes omitted despite their potentially sizeable scientific impact in generating new hypotheses (40). Serum and brain drug levels of approximately 40 and 1 µg/ml, respectively, corresponded to levels that produced only a 50% fungicidal activity in our assay, suggesting that ineffective absorption and transport through the blood-brain barrier was a strong contributor to this inefficacy in the mouse model. Previous assays estimated serum drug levels of 140 µg/ml in humans, but the methods for these assays were not described (10); however, these assays, conducted in the early 1960s, likely used an absorptive method alone after chromatography, rather than high-resolution chromatography and mass spectroscopy, which were used in the present studies. Further reductions through protein binding or problems in cellular diffusion could have further reduced effective concentrations. Removing potentially chromatin-damaging phenolic groups (41) and increasing solubility may improve the effectiveness of the agent. It is encouraging that a compound with only a single phenolic group, GW5074, was also found to bind to a similar site of mammalian enzymes (13). We did not test a pulmonary model of cryptococcosis, because there is less need for an anticytaspoccal drug for pulmonary cryptococcosis, for which azole therapy is preferred (42).

In summary, the combination GD-DARTS screen yielded a powerful method of identifying potential drug targets. This method, combined with modeling studies, identified multiple NAD-dependent dehydrogenases as potential fungicidal drug targets of the model antiparasitic agent, bithionol. Further studies identifying similar NAD-dependent dehydrogenase binding compounds are likely to identify useful fungicidal candidates.

MATERIALS AND METHODS

Strains and growth media. Saccharomyces cerevisiae strain BY4741 and Cryptococcus neoformans strain H99 (ATCC 208821; a kind gift of J. Perfect) were used in this study. H99-FOA was the recipient strain for the expression of RNAi constructs. Cryptococcal strains were grown in YPD (2% glucose, 1% yeast extract, 2% Bacto peptone) or asparagine salts with or without 2% glucose (as indicated) and 1 g/liter asparagine, 10 mM sodium phosphate (pH 6.5), and 0.25 g/liter MgSO4. S. cerevisiae strains were grown in SD-leaf medium (drop-out mix synthetic minus leucine; catalog c13012203; U.S. Biological) with or without bithionol.

Patient participation and study drug. Bithionol was a generous gift of the Centers for Disease Control and Prevention under single-patient FDA investigational new drug number 118,453.

The National Institute of Allergy and Infectious Diseases (NIAID) Institutional Review Board (IRB) approved the study under NIAID protocol 931-0106. The subject provided written informed consent after obtaining National Institutes of Health (NIH) bioethics consultation.

Bithionol fungicidal assays. S. cerevisiae and C. neoformans cells were grown on YPD agar at 30°C for 48 h. Cells were counted and diluted with phosphate-buffered saline (PBS) to a final concentration of 1,000 cells/ml. Aliquots of 400 µl of each cell solution were placed into microcentrifuge tubes, and concentrations of 0.3, 1, 10, and 50 µM bithionol were added. The bithionol was diluted with dimethyl sulfoxide (DMSO) to achieve its corresponding concentrations, and DMSO to 1% was additionally added to the samples without bithionol as a control. The samples were grown overnight at 30°C with shaking. After incubation, 200-µl aliquots of the suspensions were plated on YPD and incubated for 48 h at 30°C. Colonies were counted on each plate, and CFU were determined. Independent experiments were performed in triplicate.

Determination of bithionol concentrations in female Webster mouse plasma and brain samples. Plasma samples were placed in a 96-well plate, and preweighed frozen brain samples were placed in a 48-well plate. Samples were stored at −80°C until analysis. An ultrahigh-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method was developed to determine bithionol concentrations in plasma and brain samples. Mass spectrometric analysis was performed on a Waters Xevo TQ-S triple-quadrupole instrument using electrospray ionization in negative mode with selected reaction monitoring (SRM). The SRM for bithionol was 352.9/160.9 and 352.9/194.9 at a collision energy of 24 V. The separation was performed on an Acquity BEH C8 column (50 by 2.1 mm, 1.7 µm) using a Waters Acquity UPLC system with a 0.6-ml/min flow rate. The column temperature was maintained at 60°C. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The UPLC gradient method was 5% B (0 to 0.1 min), 5% B to 60% B (0.1 to 0.5 min), 60% B to 95% B (0.5 to 1.5 min), 95% B (1.5 to 2.2 min), and 5% B (2.2 to 2.3 min). The total run time was 2.5 min. The brain sample was homogenized with 3 volumes of water. The calibration standards (10.0 to 20,000 ng/ml) were prepared in the control blank mouse plasma and brain homogenates. Ten microliters of plasma or 40 µl of brain homogenate was mixed with 200 µl of internal standard in acetonitrile to precipitate proteins in a 96-well
plate. A 1.0-μl volume of the supernatant was injected for the UPLC-MS/MS analysis.

Animal efficacy studies. Virulence studies were conducted using an adaptation of a previously described intravenous mouse meningocencephalitis model (43) with 10 ND4 mice for each group and using cryptococcal strain ATCC 208821. Briefly, mice were challenged with 10⁴ CFU of C. neoformans (strain H99), and 3 days later therapy was started with the indicated therapy, a daily intraperitoneal injection of amphotericin B or vehicle alone in an equivalent volume; daily bithionol by oral gavage, or fluconazole by oral gavage (100 μl) twice daily. All experimental procedures involving animals were conducted under guidelines of the National Institutes of Health and protocols approved by the Institutional Animal Care Committees (IAUC) of the Intramural NIH/NIAID. Statistical comparison of survival times was made by using a log-rank comparison within GraphPad Prism 5.

Drug affinity responsive target stability. DARTS was performed by following a previously described method (15). Briefly, S. cerevisiae strain BY4741 or Cryptococcus neoformans (strain H99) cells were lysed with M-PER (Peirce Chemical) supplemented with protease and phosphatase inhibitors. After centrifugation at 16,000 × g for 20 min, the protein concentration in the supernatant was quantified with a bicinchoninic acid assay kit (Peirce Protein Biology). Proteins at 5 mg/ml were treated with 110 μM bithionol or with DMSO at 4°C overnight. The samples were treated with 0.04 mg/ml pronase (catalog number P6911; Sigma-Aldrich) for 30 min at room temperature. The digestion was stopped by adding SDS-PAGE sample loading buffer and boiling the sample at 70°C for 10 min. The samples were separated on a 10% bis-Tris gel and visualized by silver staining.

The excised gel bands were destained in a 1:1 mixture of K₃Fe(CN)₆/FeCl₃ (30 mM) and Na₂S₂O₅ (100 mM), dehydrated in acetonitrile, reduced with dithiothreitil (50 mM), alkylated by iodoacetamide (120 mM), and digested with sequencing-grade trypsin overnight at 37°C. The digested peptides were extracted with 1% formic acid and subjected to MS/MS analysis using an Agilent 1100 nanoflow LC system coupled online with tandem MS to search the genome database (www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html) or the Saccharomyces Genome Database (by using SEQUEST). Positive hits were reevaluated by using the Scaffold program (Proteome Softwares, Inc., Portland, OR), and hits that showed ≥95% probability in Scaffold were considered significant hits.

Overexpression library and screening of yeast colonies resistant to bithionol. An S. cerevisiae overexpression library was constructed using the Yeast Genome Tiling Collection assay’s Ready Pooled DNA (YSC5103; Thermo Scientific), which contains 4,500 transformed S. cerevisiae colonies. Resistant yeast colonies were identified based on persistent growth on plates containing increasing amounts of bithionol in comparison to that on plates without drug by using the replica plating method. A total of 9,000 transformants were cultured on SD-Leu medium with or without 10 μM bithionol at 30°C for 2 days. Nine drug-resistant S. cerevisiae colonies were recovered from plates containing 10 μM bithionol. After plasmid recovery from resistant transformants, plasmid sequences were verified, linearized with SceI, and transformed into C. neoformans H99 MATa ura5 (H99-FOA) cells by using electroporation by standard methods (46). An H99 MATa ura5 strain transformed with a pORA-KUTAP plasmid without the RNAi construct served as a control for UR5 expression for in vivo studies.

Expression of MDH3-MBP and GDH1-MBP recombinant proteins. For Mdh3-MBP and Gdh1-MBP fusion protein constructs, cryptococcal genes MDH3, GDH1, and MBP were amplified by PCR (using primer sets listed in Table S1 in the supplemental material) and cut with BamHI and HindIII for MDH3 and GDH1 or with HindIII and PstI for MBP restriction digestion. The fragments were inserted into the pH125 vector and transformed into bacterial cells by electroporation. Indicated plasmids from Escherichia coli were transformed into S. cerevisiae cells as described above. Recombinant enzyme isolation was performed as previously described with a slight modification (16). Briefly, after seeding a 50-ml culture of S. cerevisiae in SD-Leu medium, cells were inoculated into 1 liter of YPD broth with shaking at 30°C until mid-log phase (optical density at 600 nm of 0.4 to 0.6). Cells were centrifuged and washed twice in column buffer (20 mM Tris-Cl [pH 7.4], 0.2 M NaCl, 1 mM EDTA, 10 mM sodium phosphate buffer (pH 7.5) with or without 10 μM bithionol, and mixed with 15% glycerol, and resuspended to an optical density at 600 nm of 1.0 at 4°C. The samples were young cell cultures were used in the colorimetric assays. Cells at the end of log phase (optical density at 600 nm of 0.4) were used for enzyme activity assays.

Expression of iMDH3, iGDH1, and iGND1 C. neoformans mutant strains. The cryptococcal shuttle vector pORA-KUTAP, containing the UR5 transactivation marker, was used to effect RNAi suppression of MDH3, GDH1, and GND1 following the method of Liu et al. (45), with modification by replacement with a 500-bp fragment of intron I of LAC1 for the intervening region between the sense and antisense strands (producing the iMDH3, iGDH1, and iGND1 RNAi mutant constructs, respectively). First, pORA-KUTAP, containing sequence of the EF-1α terminator, was digested with EcoRI and ligated simultaneously to a mixture of a Xhol-digested PCR-amplified LAC1 intron fragment from H99 (using the primers listed in Table S1 in the supplemental material) and a second Xhol-EcoRI-digested PCR-amplified fragment of the MDH3, GDH1, or GND1 open reading frame, respectively (using primers listed in Table S1) to produce pORA-iMDH3, pORA-iGDH1, and pORA-iGND1, respectively. The plasmids were recovered, sequences were verified, linearized with SceI, and transformed into C. neoformans H99 MATa ura5 (H99-FOA) cells by using electroporation by standard methods (46). An H99 MATa ura5 strain transformed with a pORA-KUTAP plasmid without the RNAi construct served as a control for UR5 expression for in vivo studies.

Enzyme activity assays and RT-PCR assays. To measure enzyme activity, enzyme assays were performed according to the manufacturer’s protocol (catalog number E-124 for malate dehydrogenase and catalog number E-123 for glutamate dehydrogenase; Biochemical Research and Clinical Application, University at Buffalo). Briefly, assays were performed by addition of 50 ng protein to 50 μl of assay solution with or without 10 μM bithionol, and mixtures were incubated at 37°C for 30 min. The assay was stopped by addition of 60 μl of 3% acetic acid. Enzyme activity was determined by measuring the reduction of 2-(p-iodoophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to formazan, which exhibits an absorption maximum at 492 nm, with control mixtures containing reagents alone. Reverse transcription (RT) was performed on DNase-treated RNA using the iScript kit (Bio-Rad Laboratories), according to the manufacturer’s protocol. PCRs were set up using iQ SyberGreen SuperMix (Bio-Rad Laboratories), according to the manufacturer’s protocol. Quantitative RT-PCR was performed using a Bio-Rad iCycler (MiyiQ2).

Enzyme kinetics. The malate dehydrogenase enzyme concentration was adjusted to 100 μg/ml, and the amount of enzyme in each reaction mixture was adjusted to yield optimal steady-state velocities. All solutions were made immediately prior to use, and materials were obtained from Sigma-Aldrich. Enzyme assays were performed by monitoring reduced coenzyme production at 340 nm using a Molecular Devices FlexStation3. For the kinetic assays, reaction mixtures were prepared in triplicate in 0.1 M sodium phosphate buffer (pH 7.5) with or without 10 μM bithionol. When the malate concentration was varied, the concentration of NAD+ was adjusted to yield optimal steady-state velocities. All solutions were made immediately prior to use, and materials were obtained from Sigma-Aldrich. Enzyme assays were performed by monitoring reduced coenzyme production at 340 nm using a Molecular Devices FlexStation3.
was 0.2 mM. When the NAD concentration was varied, a concentration of 2 mM maltate was utilized.

**Modeling studies and predicted binding energy calculations.** For each of the *Cryptococcus* target sequences, a BLAST (47) search was run against the Protein Data Bank (PDB) (48), and protein structures with the highest sequence homologies to each of the *Cryptococcus* targets were identified and used as templates for homology modeling. The template structures are given in the Table S4 in the supplemental material. Structural models for each of the *Cryptococcus* target sequences were generated using the homology model application within the Molecular Operating Environment (MOE) program with default parameters. The binding pockets in the modeled structures were identified by aligning to the template structure. Locations of the template ligands relative to the modeled structures thereby identified model structure binding pockets.

Cryptococcal target-bithionol complex binding energies were minimized to a 0.1 RMSD (root mean square deviation) by using the MMFF94 force field (49) (within MOE). The binding energy of bithionol to the protein was calculated as follows: \( \Delta E_{\text{bind}} = E_{\text{complex}} - E_{\text{protein}} - E_{\text{ligand}} \). The total energy of the minimized systems was calculated and labeled as \( E_{\text{complex}} \). Bithionol was then removed from each of the target proteins, and the target proteins were then minimized (without bithionol); these energies were calculated and labeled as \( E_{\text{protein}} \). Bithionol itself was minimized (starting with its protein-bound structure). The energy of minimized bithionol binding energy was labeled as \( E_{\text{ligand}} \), as described in reference 47.

**MIC determinations.** Fluconazole MICs were determined via the Etest (AB Biodisk). Etests were carried out as described by the manufacturer and were performed on SD agar (1X YNB without amino acids, 2% glucose).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01073-16/-/DCSupplemental.

- Figure S1, TIF file, 0.1 MB.
- Figure S2, TIF file, 0.1 MB.
- Figure S3, TIF file, 0.9 MB.
- Figure S4, TIF file, 0.4 MB.
- Figure S5, TIF file, 0.2 MB.
- Table S1, DOCX file, 0.02 MB.
- Table S2, DOCX file, 0.02 MB.
- Table S3, DOCX file, 0.01 MB.
- Table S4, DOCX file, 0.01 MB.

**ACKNOWLEDGMENT**

This research was supported in part by the Intramural Research Program of the NIH, NIAID.

**FUNDING INFORMATION**

This work, including the efforts of Peter R Williamson, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (AI001123). This work, including the efforts of Peter R Williamson, was funded by HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) (AI001124).

**REFERENCES**

1. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS 23:525–530. http://dx.doi.org/10.1097/QAD.0b013e3282322ffac.
2. Sloan DJ, Parris V. 2014. Cryptococcal meningitis: epidemiology and therapeutic options. Clin Epidemiol 6:169–182. http://dx.doi.org/10.2147/CLEP.S38850.
3. Panackal AA, Williamson PR. 2015. Fungal infections of the central nervous system. Continuum (Minneapolis) 21:1662–1678. http://dx.doi.org/10.1212/CNS.00000000000003CLEP.S38850.
4. Pyrgos V, Seitz AE, Steiner CA, Prevots DR, Williamson PR. 2013. Epidemiology of cryptococcal meningitis in the US: 1997–2009. PLoS One 8:e56269. http://dx.doi.org/10.1371/journal.pone.0056269.
5. Day JN, Chau TT, Wolbers M, Mai PP, Dung NT, Mai NH, Phu NH, Nghia HD, Phong ND, Thai QQ, Thi le H, Chuong LV, Sinh DX, Duong VA, Hoang TN, Diep PT, Campbell JJ, Sieu TP, Baker SG, Chau NV, Hien TT, Laloo DG, Farrar JJ. 2013. Combination antifungal therapy for cryptococcal meningitis. N Engl J Med 368:1291–1302. http://dx.doi.org/10.1056/NEJMoa1110404.
6. Bicanic T, McInties G, Wood R, Hayes M, Rebe K, Bekker LG, Harrison T. 2007. Fungal burden, early fungical activity, and outcome in cryptococcal meningitis in antiretroviral-naïve or antiretroviral-experienced patients treated with amphotericin B or flucytosine. Clin Infect Dis 45: 76–80. http://dx.doi.org/10.1086/518607.
7. Rabjohns JL, Park YD, Dehdashi J, Sun W, Henderson C, Zelazny A, Metallo SJ, Zheng W, Williamson PR. 2014. A high-throughput screening assay for fungicidal compounds against Cryptococcus neoformans. J Biol Screen 19:270–277. http://dx.doi.org/10.1177/10870511346847.
8. Dehdashi SJ, Abbott J, Nguyen DT, McKew JC, Williamson PR, Zheng W. 2013. A high-throughput screening assay for assessing the viability of Cryptococcus neoformans under nutrient starvation conditions. Anal Bioanal Chem 405:6823–6829. http://dx.doi.org/10.1007/s00216-013-7134-4.
9. Yokogawa M. 1984. Experimental chemotherapy of paragonimiasis. A review. Arzneimittelforschung 34:1193–1196.
10. Yokogawa M, Yoshimura H, Sano M, Okura T, Tsuji M, Takizawa A, Harada Y, Kihata M, Iwasaki M, Hirose H. 1981. Chemotherapy of paragonimiasis with bithionol. II. Clinical observation on the treatment of bithionol. Jpn J Parasi tol 10:317–327.
11. Oh SJ. 1967. Bithionol treatment in cerebral paragonimiasis. Am J Trop Med Hyg 16:585–590.
12. Ayyagari VN, Brard L. 2014. Bithionol inhibits ovarian cancer cell growth in vitro: studies on mechanism(s) of action. BMC Cancer 14:61. http://dx.doi.org/10.1186/1471-2407-14-61.
13. Li M, Smith CJ, Walker MT, Smith TJ. 2009. Novel inhibitors complexed with glutamate dehydrogenase: allosteric regulation by control of protein dynamics. J Biol Chem 284:22988–23000. http://dx.doi.org/10.1074/jbc.M109.020222.
14. Attia SM. 2010. Deleterious effects of reactive metabolites. Oxid Med Cell Longev 3:238–253. http://dx.doi.org/10.4162/oxmed.2010.132.
15. Lomenick B, Hao R, Jonai N, Chin RM, Aghajan M, Warburton S, Wang J, Wu RP, Gomez F, Loo JA, Wohlschlegel JA, Vondriska TM, Pelletier J, Hershchman HR, Clardy J, Clarke CF, Huang J. 2009. Target identification using drug affinity responsive target stability (DARTS). Proc Natl Acad Sci U S A 106:21964–21989. http://dx.doi.org/10.1073/pnas.0910040106.
16. Riggs P. 2001. Expression and purification of maltose-binding protein fusions. Curr Protoc Mol Biol Chapter 16:Unit16.6. http://dx.doi.org/10.1002/0471142727.mb1606s28.
17. Sun W, Park YD, Sugui JA, Fothergill A, Southall N, Shinn P, McKew JC, Kwon-Chung KJ, Zheng W, Williamson PR. 2013. Rapid identification of antifungal compounds against Exserohilum rostratum using high throughput drug repurposing screens. PLoS One 8:e70506. http://dx.doi.org/10.1371/journal.pone.0070506.
18. Diamond RD, Bennett JE. 1974. Prognostic factors in cryptococcal meningitis. A study in 111 cases. Ann Intern Med 80:176–181. http://dx.doi.org/10.1053/j.1644-5346.s1974.132.13246.
19. Enfield KS, Stewart GI, Pikor LA, Alvarez CE, Lam WL, Chari R. 2011. MicroRNA gene dosage alterations and drug response in lung cancer. J Biomed Biotechnol 2011:474632. http://dx.doi.org/10.1155/2011/474632.
20. Leprohon P, Fernandez-Prada C, Gazanion É, Monte-Neto R, Ouellette M. 2015. Drug resistance analysis by next generation sequencing in Leishmania. Int J Parasitol Drugs Resist 5:26–35. http://dx.doi.org/10.1016/j.ipjdr.2014.09.005.
21. Stefan JS, McAlister-Henn L. 1992. Isolation and characterization of the yeast encoding the MDH3 isozyme of malate dehydrogenase. J Biol Chem 267:24708–24715.
22. Levine PH, Diamond RD, Reisser JL. 1975. Antibodies to Epstein-Barr virus in patients with cryptococcosis. J Clin Microbiol 1:363–365.
23. We H, Wang Y, Liu W, Zhou C-Z. 2007. Crystal structure of Saccharomyces cerevisiae 6-phosphogluconate dehydrogenase Gnd1. BMC Struct Biol 7:38. http://dx.doi.org/10.1186/1472-6807-7-38.
24. Warburg O. 1956. On the origin of cancer cells. Science 123:309–314. http://dx.doi.org/10.1126/science.123.3191.309.

25. Carothers DJ, Pons G, Patel MS. 1989. Dihydrolipoamide dehydrogenase: functional similarities and divergent evolution of the pyridine nucleotide-disulfide oxidoreductases. Arch Biochem Biophys 268:409–425. http://dx.doi.org/10.1016/0003-9861(89)90309-3.

26. Marchitti SA, Brocker C, Stagos D, Vasil'iou Y. 2008. Non-P450 alde- hyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. Expert Opin Drug Metab Toxicol 4:697–720. http://dx.doi.org/10.1517/17425255.4.6.697.

27. Li M, Allen A, Smith TJ. 2007. High throughput screening reveals several new classes of dopamine dehydrogenase inhibitors. Biochemistry 46:15089–15102. http://dx.doi.org/10.1021/bi7018783.

28. Thong KW, Coombs GH, Sanderson BE. 1987. t-Methionine catabolism in trichomonads. Mol Biochem Parasitol 23:223–231. http://dx.doi.org/10.1016/0166-6851(87)90029-6.

29. Wang XY, Luo JP, Chen R, Zha XQ, Wang H. 2014. The effects of daily supplementation of Dendrobium huoshanense polysaccharide on ethanol-induced subacute liver injury in mice by proteomic analysis. Food Funct 5:2020–2035. http://dx.doi.org/10.1039/c5fo00629e.

30. Takeuchi T, Kobayashi S, Kawasaki H. 1984. Entamoeba histolytica: inhibition in vitro by bithionol of respiratory activity and growth. Exp Parasitol 58:1–7. http://dx.doi.org/10.1016/0014-4894(84)90015-8.

31. Babot M, Birch A, Labarbuta P, Galkin A. 2009. Sec6-dependent sorting of fungal extracellular exosomes and laccase of Cryptococcus neoformans. Mol Microbiol 71:1165–1176. http://dx.doi.org/10.1111/j.1365-2958.2008.06588.x.

32. Finbow ME, Harrison MA. 1997. The vacuolar H+–ATPase: a universal proton pump of eukaryotes. Biochem J 324:697–712. http://dx.doi.org/10.1042/bj3240697.

33. Graham LA, Hill KJ, Stevens TH. 1998. Assembly of the yeast vacuolar H+–ATPase occurs in the endoplasmic reticulum and requires a Vma12p/Vma22p assembly complex. J Cell Biol 142:39–49. http://dx.doi.org/10.1083/jcb.142.1.39.

34. Unger JM, Barlow WE, Ramsey SD, LeBlanc M, Blanke CD, Hershman DL. 10 March 2016. The scientific impact of positive and negative phase 3 cancer clinical trials. JAMA Oncol http://dx.doi.org/10.1001/jamaoncol.2015.6487.

35. Chen H, Eastmond DA. 1995. Synergistic increase in chromosomal breakage within the euchromatin induced by an interaction of the benzene metabolites phenol and hydroquinone in mice. Carcinogenesis 16:1963–1969. http://dx.doi.org/10.1093/carcin/16.6.1963.

36. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS, Larsen RA, Lortholary O, Nguyen MH, Pappas PG, Powderly WG, Singh N, Sobel JD, Sorrell TC. 2010. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of America. Clin Infect Dis 50:291–322. http://dx.doi.org/10.1086/649858.

37. Graybill JR, Mitchell L, Levine HB. 1978. Treatment of experimental murine cryptococcosis: a comparison of miconazole and amphotericin B. Antimicrob Agents Chemother 15:277–283. http://dx.doi.org/10.1128/AAC.13.2.277.

38. Kawai S, Hashimoto W, Murata K. 2010. Transformation of Saccharomyces cerevisiae and other fungi. Bioeng Bugs 1:395–403. http://dx.doi.org/10.4161/bbug.1.6.13257.

39. Liu H, Cottrell TR, Pierini LM, Goldman WE, Doering TL. 2002. RNA interference in the pathogenic fungus Cryptococcus neoformans. Genetics 160:463–470.

40. Erickson T, Liu L, Gueyikian A, Zhu X, Gibbons J, Williamson PR. 2005. Multiple virulence factors of Cryptococcus neoformans are dependent on VPH1. Mol Microbiol 42:1121–1131. http://dx.doi.org/10.1046/j.1365-2958.2001.02712.x.

41. Needelman SB, Wunsch CD. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. J Mol Biol 48:443–453. http://dx.doi.org/10.1016/0022-2836(70)90057-4.

42. Berman HM, Westbrook J, Feng Z, Neuwander L, Bellott D, Thankenhia A, Nakamura H, Holbrook SJ, Moyano Y, Nakamura Y, et al. 2000. The Protein Data Bank. Nucleic Acids Res 28:235–242. http://dx.doi.org/10.1093/nar/28.1.235.

43. Halgren TA. 1996. Merck molecular force field. I. Basis, form, scope, parameterization, and performance of MMFF94. J Comput Chem 17:490–519. http://dx.doi.org/10.1002/(SICI)1096-987X(199604)17:5<490::AID-JCC1>3.0.CO;2-P.