Characterizing the Physiochemical Properties of Copper Chelating Agents: an Effort Towards Understanding their Antifungal Activity

by

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Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Chemistry in the Graduate School of Duke University

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ABSTRACT
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Abstract

An increase in drug-resistant infections and the paucity of new antibiotics present a major world health problem. Cryptococcus neoformans (C. neoformans) is an opportunistic fungal pathogen responsible for life-threatening infections in immunocompromised individuals and occasionally in those with no known immune impairment. The Franz lab recently identified several copper (Cu) chelators containing O, S and O, N donor atoms that exhibit Cu-dependent antifungal activity against C. neoformans. Interestingly, the O, O analogs of these chelators do not exhibit anti-fungal activity. Here, using UV-visible spectroscopy the Cu(I) binding properties of these ligands are determined. The lipophilic properties of these ligands and their bis-Cu(II) complexes were also determined using the traditional shake flask method. Lipophilicity and binding studies indicate that ligands exhibiting Cu-dependent antifungal activity are able to bind Cu(II) with a binding affinity, Log $K'_{CuL2}$, greater than 19 and they form hydrophobic bis-Cu(II) complexes. Further, inductively coupled plasma-mass spectrometry (ICP-MS) was used to analyze total metal content of C. neoformans fungal cells treated with these ligands and Cu(II). This analysis revealed that the ligands displaying antifungal activity increased Cu, zinc (Zn), and iron (Fe) levels in the fungal cells dramatically compared to the ligand or Cu only treatment. Lastly, a new group of linear and cyclic thiohydroxamic acids (O,S donor atoms) was screened for their effect on C. neoformans’ growth,
in the presence and absence of Cu(II). These studies indicate that cyclic thiohydroxamic acids are able to elicit Cu-dependent antifungal activity opening the possibility of a new class of metallo-antifungals. Further initial attempts were made to understand the Cu(II) binding properties of these thiohydroxamic acids using calcein fluorescence competition assays. The results from this work suggest that small molecules, capable of binding Cu(II) to form hydrophobic complexes, can deliver Cu to fungal cells altering not only their Cu but also intracellular Zn and Fe levels. This hypothesis about Cu delivery agents sets the stage for future work in genome-wide approaches to probe how alteration in metal levels affects different biochemical pathways to induce Cu-dependent antifungal activity.
Dedication

I dedicate this thesis to all my family, friends, teachers, and colleagues who have helped me in my graduate career so far. This thesis is specially dedicated to my mother, who always encouraged and motivated me to pursue my dreams even when they seemed impossible.
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List of Abbreviations

Atox1: Antioxidant1; metallochaperone that delivers Cu to ATP7A & ATP7B
ATP: Adenosine triphosphate
ATPase: Adenosine triphosphatase
ATP7A: Cu-transporting ATPase1
ATP7B: Cu-transporting ATPase2
BCA: Bicinchoninate Acid Ion
BCS: Bathocuproine Disulfate
Ccc2: Cu(II)-transporting P-type ATPase
CFU: Colony forming units
CMT1/CMT2: Cu metallothionein 1 and 2
CQ: Clioquinol
Ctr1/Ctr4: Cu-transporting protein 1 and 4
Dfp: Deferiprone
DMSO: Dimethyl sulfoxide
EDTA: Ethylenediaminetetraacetic Acid
Fe-S: Iron Sulfur
8HQ: 8-hydroxyquinoline
ICP-MS: Inductively Coupled Plasma-Mass Spectrometry
Log P: Partition Coefficient
MIC: Minimum Inhibitory Concentration
NAT: Native
NEO: Neomycin
OH⁻: Hydroxyl Ion
OH: Hydroxyl Radical
PBS: Phosphate Buffer Saline
PyO: Pyridinol-n-oxide
PyS: Pyrithione
SC Media: Synthetic Complete Media
ThDfp: Thiodeferriprone
ThM: Thiomaltol
UV: Ultra-violet
UV-Visible: Ultra-Violet Visible
WT: Wild Type
YPFG Media: Yeast Protein Ethanol Glycerol Media
1. Introduction

1.1 Copper’s Role in the Innate Immune Response

An increase in drug-resistant microbial infections and the scarcity of drugs in the development pipeline is a considerable worldwide health threat. Therefore, efforts are being made to discover new drugs capable of overcoming microbial drug resistance. During microbial infections, pathogens encounter many different host-derived antimicrobial effectors in macrophages, including low iron, zinc, and calcium; low pH; release of hydrolytic enzymes; and reactive oxygen and nitrogen species (Figure 1). Limiting nutrient availability is one of the ways hosts combat pathogens, so trace elements, like zinc (Zn), iron (Fe), and calcium (Ca); are withdrawn from microbial pathogens. Unlike the other trace metals, the host concentrates copper, Cu (reaching Cu concentration in the high micromolar range) in the proximity of invading pathogens as a way to take advantage of Cu’s toxicity and elicit antimicrobial response. Recent X-ray microprobe analysis of peritoneal macrophages shows that upon infection with Mycobacterium tuberculosis, the concentration of Cu is dramatically increased. This claim is supported by the increased expression of the Cu(I) import protein, Ctr1, along with the increased partitioning of the Cu(I) pump protein, ATP7A, to phagolysosome compartments enabling the loading of these compartments. The precise mechanism of Cu’s antimicrobial activity is not known currently; possible modes of Cu’s biocidal behavior include generation of hydroxyl radicals via...
Fenton chemistry and/or disruption of exposed Fe-S clusters.\textsuperscript{5} It has been suggested that the acidic pH inside the phagolysosome of the macrophage further enhances the Cu associated toxicity as at lower pH Cu becomes more labile and its reactivity with reactive oxygen and nitrogen species increases.\textsuperscript{3} On the other hand, Cu’s role in virulence of pathogens has been established for some microbes, including \textit{Mycobacterium tuberculosis}, \textit{Pseudomonas Aeruginosa}, and \textit{Cryptococcus Neoformans (C. neoformans)}; virulence is greatly attenuated by mutations in Cu transporters, which pathogens use to efflux Cu to counter the outburst of Cu’s biocidal effect in the macrophages.\textsuperscript{2a, 4}
Figure 1: Model of Copper-mediated Killing of Bacteria and Fungi by Activated Macrophages.\textsuperscript{2b}

Pathogens, \textit{C. neoformans}, are phagocytosed by macrophages, where they are exposed to high Cu; low Zn and Fe; low pH; and reactive oxygen and nitrogen species. Increased Cu is delivered to phagolysosome via increased expression of Cu(I) transporter, Ctr1, along with greater partitioning of Cu(I) transporter, ATP7A to their membrane. Cu’s biocidal activity is speculated to occur through generation of hydroxyl radical via Fenton chemistry and/or disruption of Fe-S clusters in proteins.

Inspired by the innate immune system’s use of Cu as a biocide and the need for new antimicrobials, the Franz lab has started a new effort to identify small molecules that work synergistically with biological metals to kill pathogens. Further, it was recently shown that Cu synergistically enhances antibiotic activity of campreomycin against \textit{Mycobacterium tuberculosis};\textsuperscript{6} similarly, Cu complexation to dithiocarbazate and salicylidamine Schiff base ligands improved their antibacterial activity.\textsuperscript{6-7} Moreover, the Cu(II) complexes of bis(thiosemicarbazone) have been shown to have antimicrobial activity against many multi-drug resistant strains of the \textit{Neisseria gonorrhoeae}.\textsuperscript{8}
1.2 Copper Homeostasis in C. neoformans

The air-borne fungal pathogen *C. neoformans* was selected for initial antifungal testing because of the unique Cu biology of *C. neoformans*. Besides, *C. neoformans* causes cryptococcusis in both immune-deficient and immune-competent patients causing thousands of deaths from lethal meningitis annually.\(^2a\) Figure 2 shows some of the many of Cu-containing proteins in *C. neoformans*.\(^9\) Cu is also required for Fe acquisition, as iron uptake occurs via a high affinity Fe uptake oxidase-permease complex that contains Cu. Moreover, for *C. neoformans*’ antioxidant defense mechanism it needs superoxide dismutase, which is another Cu containing protein. Like host cells, *C. neoformans* also have high affinity Cu(I) permeases on the plasma membrane, which facilitate Cu delivery to the fungal cell. Intracellular Cu(I) is bound to various Cu chaperones and putative ligands, which through protein-protein or protein-ligand interactions, deliver Cu to distinct cellular compartments and proteins thereby minimizing labile Cu ions. For instance, cystosolic chaperone proteins, Atx1 delivers Cu from the Ctr1/Ctr4 to the Cu-transporting ATPase called Ccc2. Ccc2 then delivers this Cu to proteins of the secretory pathway like laccase. Laccase is essential for melanin production that protects the fungal cell from radicals produced by host cells. These examples show that Cu-dependent processes are critical for the pathogen, so Cu acquisition is necessary for infection.\(^{9a}\)
While \textit{C. neoformans} has mechanisms for acquiring Cu, it also has the cellular machinery for Cu detoxification in case of excess Cu as is common during infection settings. Cysteine rich metallothioneins Mt1 and Mt2 bind Cu(I) and help in Cu detoxification.\textsuperscript{2a} Interestingly, both Cu deficiency or excess are regulated by a single metallo-sensing transcription factor (Cuf1) pointing to the delicate balance of Cu within this pathogen.\textsuperscript{9b} This dual need for Cu utilization as well as Cu detoxification prompts the question of which pathway (Cu acquisition or Cu detoxification) is more important for the pathogen’s survival and virulence.

\textbf{Figure 2: Copper Homeostasis in \textit{C. neoformans}}\textsuperscript{9}

Cu(I) transporters, Ctr1 and Ctr4, deliver Cu to the cell from extracellular space. Cu is then transferred intracellularly through chaperone proteins (like Atx1) to various Cu requiring proteins. Cu requiring proteins include, antioxidant defense protein, Sod1; Cu detoxification proteins, Mt1 and Mt2, and laccase. Laccase is required for synthesizing melanin needed for fungal defense system. Transcription factor Cuf1 regulates expression of Ctr1 and Crt4 along with Mt1 and Mt2.
1.3 Statement of Objectives

Cu’s critical role in *C. neoformans*’ virulence prompted Marian Helsel, a former group member to screen a group of metal binding agents with and without supplemental Cu against *C. neoformans* to test the influence of Cu on their antifungal activity. These studies revealed that bi-dentate Cu-chelators, like 8-hydroxyquinoline (8HQ), pyrithione (PyS) and thiomaltol (ThM) inhibited growth in the presence of Cu (Table 1). Intriguingly, Maltol (Mal) and Pyridine N-oxide (PyO), analogues of ThM and PyS that have two oxygen donor atoms instead of one oxygen and one sulfur, did not show any antifungal activity, and neither did deferiprone (Dfp) and thiodeferriprone (ThDfp) (Figure 3 & Table 1). My overarching objective is to understand the antifungal mechanism of action of these ligands to aid in the discovery of new Cu-based antifungals. The goals of this thesis were to determine the physiochemical properties of both the biologically active and inactive ligands as a crucial step in elucidating their mechanism of action and establishing structure-function relationships that will inform future development of Cu-based antimicrobials (Figure 3 & Table 1).

The fate of Cu once the Cu complexes of these ligands enters cells is unknown; however, given the reductive nature of the intracellular environment it is likely that Cu(II) bound to these ligands is reduced. Therefore, the ability of these ligands to bind Cu(I) and/or Cu(II) was probed, along with how tightly...
these ligands bind Cu in either oxidation state. The pH specific characterization of Cu binding is important as the pH varies across different cellular compartments. Also, C. neoformans’ antifungal growth assays were performed in media with pH 4.5. Hence, conditional Cu(I) binding constants of these ligands were determined at physiological relevant pH 7.4 and compared to the previously determined Cu(II) binding constants of these ligands; further preliminary data on Cu(II) binding at pH 4.5 is also shown.

The lipophilicity of a drug influences its metabolism, bioaccumulation, and toxicity. Therefore, **lipophilicity of the ligands and their [Cu\textsuperscript{II}L\textsubscript{2}] complexes were determined in order to gauge how the ligand and Cu influence each other’s propensity to partition in aqueous or hydrophobic environments.**

In order to understand the pharmacological response of these ligands, it is important to consider how these ligands interact with potential intracellular Cu proteins. The reducing intracellular environment will likely reduce [Cu\textsuperscript{II}L\textsubscript{2}] complexes, inducing competition for Cu(I) between the ligand and various intracellular Cu(I) binding proteins (“Cu sinks”). **To test this hypothesis, selected ligands, acting as mimics of Cu sinks, were tested for their ability to extract Cu(I) from our ligands.**

Earlier studies in the Franz lab have suggested that the ligands displaying Cu-dependent antifungal activity against C. neoformans are able to alter the metal homeostasis within fungal cells. As an attempt to understand their
antifungal mechanism of action, metal content analysis of these fungal cells was done upon treatment with and without these ligands in the presence and absence of exogenous Cu.

Initial screen of compounds against *C. neoformans* informed us that the O, S donor atom containing ligands (PyS and ThM) might be a promising class of Cu-based antifungal against *C. neoformans*. Therefore, another set of O, S donor atom containing ligands called thiohydroxamic acids was screened for their Cu-dependent antifungal activity against *C. neoformans*. 
<table>
<thead>
<tr>
<th>O, S Ligands</th>
<th>O, N Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure" /></td>
<td><img src="image2.png" alt="Structure" /></td>
</tr>
<tr>
<td>Pyritine (PyS)</td>
<td>8-Hydroxyquinoline (8HQ)</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure" /></td>
<td><img src="image4.png" alt="Structure" /></td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure" /></td>
<td><img src="image6.png" alt="Structure" /></td>
</tr>
</tbody>
</table>

**Figure 3: Copper Chelators Investigated in this Study**

Blue atoms show metal binding groups in the ligands. Ligands shown as yellow showed Cu-dependent antifungal activity against *C. neoformans*, whereas white boxes indicate no antifungal activity against *C. neoformans*, and green box indicates antifungal activity independent of Cu.
Table 1: Antifungal Activity of the Ligands in the Absence and Presence of 1 mM CuSO₄ (these studies were done by Marian Helsel and published earlier)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>0 mM CuSO₄</th>
<th>1 mM CuSO₄</th>
<th>Ligand</th>
<th>0 mM CuSO₄</th>
<th>1 mM CuSO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>8HQ</td>
<td>&gt;100</td>
<td>6</td>
<td>CQ</td>
<td>50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>PyS</td>
<td>0.8</td>
<td>3</td>
<td>PyO</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>ThM</td>
<td>&gt;100</td>
<td>25</td>
<td>Mal</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>ThDfp</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>Dfp</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Color Code:
- MIC > 100 µM with and without 1mM CuSO₄ indicates no antifungal activity (white);
- MIC < 100 µM in the presence 1mM CuSO₄ & MIC > 100 µM in the absence of 1mM CuSO₄ indicates Cu-dependent antifungal activity (yellow);
- MIC < 100 µM and MIC < 100 µM in the presence of 0 mM and 1 mM CuSO₄, respectively indicate Cu-dependent antifungal activity (yellow);
- MIC < 100 µM in the absence of 0 mM CuSO₄ & MIC > 100 µM in the presence 1mM CuSO₄ indicates Cu-independent antifungal activity (green).
2. Cu Binding and Lipophilicity Studies of Select Ligands

2.1 Introduction and Significance

As discussed in Chapter 1, the Franz lab has recently identified some small molecular chelators, pyrithione (PyS), thiomaltol (ThM), and 8-hydroquinoline (8HQ) as displaying Cu-dependent antifungal activity against fungal pathogen \textit{C. neoformans} (Figure 3, Table 1). In this same work, Helsel also showed that these ligands are able to provide bio-available Cu to \textit{C. neoformans} mutants lacking the Cu(I) transporter proteins, Ctr1 and Ctr4. These results indicate that using small molecules to deliver Cu to pathogens is a promising strategy to fight microbial infections. In order to employ this strategy of using small molecules as Cu delivery agents to fight microbial infections, we need to develop a deeper understanding of the physical and chemical properties of these small molecules.

It was interesting to note from the work of Helsel that PyS and ThM, which contain O, S donor atoms, display Cu-dependent antifungal activity, whereas the O, O analogues of these ligands did not display any antifungal activity. Interestingly, another O, S containing ligand, thiodeferriprone (ThDfp) did not show any antifungal activity; similarly, its O, O analogue, deferiprone (Dfp), did not show any antifungal activity. Among the O, N donor atom containing ligands, only 8HQ showed Cu-dependent antifungal activity whereas clioquinol (CQ) did...
not display any Cu dependence to its antifungal activity. Therefore, the identity of the donor atoms in the metal binding reagents cannot be the sole determinant of Cu-dependent antifungal activity. Other factors like whether they preferentially bind Cu(I) or Cu(II) might be more important determinants of this Cu-dependent nature of antifungal behavior. Here, we used UV-visible spectroscopy to characterize Cu(I) binding of these select ligands and also determine Cu(I) binding constants of the these ligands.

Besides Cu binding, lipophilicity of the ligands and their respective bis-Cu(II) complexes seems like an important determinant of their biological activity. The ligands of interest have been used for various applications aside from antifungals, antidiabetic agents or as radiopharmaceutical reagents.\textsuperscript{11} Interestingly, Chaves et al. have shown that among deferiprone, maltol and thiomaltol, the insulin mimetic behavior of the bis-Zn(II) complexes of these chelators increased with increasing lipophilic nature of the ligands.\textsuperscript{11b} Encouraged by these literature findings and our observations, we determined the lipophilicity of our select ligands and their bis-Cu(II) complexes (Figure 3) using a traditional shake flask method to understand their differential antifungal activity. Lastly, we explored using UV-visible spectroscopy how the bis-Cu(II) complexes of these ligands interact with cellular mimics of Cu(I) binding proteins.
2.2 Results

2.2.1 Cu(I) Binding Studies

Anaerobic UV-vis spectroscopy was done to confirm Cu(I) binding to our ligands. No spectral shifts were observed in the UV-visible spectra of O, O ligands taken with and without Cu(I) (Figure 4), indicating no Cu(I) binding. However, with O, S ligands (PyS, ThM, ThDfp) the $\lambda_{\text{max}}$ of the ligand in solution by itself shifts upon addition of Cu(I), suggesting a binding interaction. For instance, PyS has $\lambda_{\text{max}}$ at 343 nm and 278 nm, but with the addition of Cu(I), $\lambda_{\text{max}}$ changes to 306 nm (Figure 5). The UV-visible spectrum of 100 µM 8HQ with 50 µM Cu(I) or Cu(II) shows $\lambda_{\text{max}}$ at 375 nm (Figure 6) indicating that under both Cu(I) and Cu(II) conditions the same species is formed. Ascorbic acid was added to the solution of 100 µM 8HQ and 50 µM Cu(I); even in this reducing environment, the 375 nm band was observed, indicating that 8HQ is unable to bind Cu(I). Furthermore, CQ’s Cu(I) binding ability could not be monitored at pH 7.4 as addition of Cu(I) to a solution of CQ leads to formation of precipitate.
Figure 4: UV-visible Spectroscopy of O, O Ligands in the Presence and Absence of Cu(I) in an Anaerobic Environment.

Conditions: A solution of 100 µM Ligand with and without 50 µM ([Cu(CH₃CN)₄]PF₆) was prepared in universal buffer, pH 7.4 with 0.5% DMSO.
Figure 5: UV-visible Spectroscopy of O, S ligands in the Presence and Absence of Cu(I) in an Anaerobic Environment.

Conditions: A solution of 100 µM Ligand with and without 50 µM ([Cu(CH₃CN)₄]PF₆) was prepared in universal buffer, pH 7.4 with 0.5% DMSO.
**Figure 6: UV-visible Spectroscopy of 8HQ in the Presence and Absence of Cu(I) in an Anaerobic Environment**

Conditions: A solution of 100 µM 8HQ with 50 µM ([Cu(CH₃CN)₄]PF₆) or 50 µM CuSO₄ was prepared in universal buffer pH 7.4 with 0.5% DMSO; A solution of 100 µM 8HQ with 50 µM ([Cu(CH₃CN)₄]PF₆) was prepared in universal buffer pH 7.4 with 1mM Ascorbic Acid

The O, S ligands’ Cu(I) conditional binding affinities, $K'_{[Cu(L₂)]^+}$ (Equation 1) were determined using competition assays with the Cu(I) chelator, bichinchonic acid (BCA) (Figure 7 and 8; Equation 2) in universal buffer pH 7.4 as detailed in the materials and methods section. In Figure 8, as ThDfp is titrated into a solution of [Cu(BCA)₂]³⁻, the 562 nm band decreases indicating competition for Cu(I) between the ligand and BCS. The equilibrium constant $K_{ex}$ describes this competition. $K_{ex}$ was calculated using Equation 3; with the conditional formation constant at pH 7.4, $\beta₂', [Cu(BCA)₂]³⁻$ known,¹² the $K'_{[Cu(L₂)]^+}$ was calculated
For these binding constant determinations, 1:2 Cu:ligand stoichiometry was based on direct titration of ligand into Cu(I).\textsuperscript{10} The relative Cu(I) binding affinities of the three O, S ligands are similar (Log $K'_\text{[CuL_2]} \sim 16$) (Table 2).

\[ Cu^+ + 2L \xrightarrow{K_{CuL_2}} [Cu^L_2]^{1-} \] (Equation 1)

\[ [Cu^l(BCA)_2]^{3-} + 2L \xrightarrow{K_{ex}} [Cu^L_2]^{1-} + 2BCA \] (Equation 2)

\[ K_{ex} = \frac{[CuL_2][BCA]^2}{[Cu^l][L]^2} = \frac{K_{CuL_2}}{\beta_2^l} \] (Equation 3)

\[ K_{CuL_2} = K_{ex}\beta_2^l \] (Equation 4)

\[ Cu^l + 2BCA^{2-} \xrightarrow{\beta_2^l=K_1K_2} [Cu^l(BCA)_2]^{3-} \] (Equation 5)

Figure 7: Cu(I) Chelator, Bichinchonic Acid (BCA)

Bichinchonic Acid, BCA forms a 1:2 Cu(I):ligand complex with $\log \beta_2^l = 17.2 ([Cu^l(BCA)_2]^{3-}; \lambda_{max}=562 \text{ nm}, \varepsilon=7900 \text{ M}^{-1} \text{ cm}^{-1})$.\textsuperscript{13} Atoms indicated in blue bind Cu(I).
Figure 8: UV-visible Spectrum for Monitoring Competition of ThDfp and BCA for Cu(I) to Determine a Conditional Cu(I) Binding Constant for ThDfp in an Anaerobic Environment.

Conditions: A) A solution of 110 µM BCA and 50 µM ([Cu(CH₃CN)₄]PF₆) was prepared in 0.5% DMSO, universal buffer pH 7.4 (red spectrum, top) at 25 °C. Thiodeferiprone was added in equivalents of 0, 0.25, 0.5, 1, 2, 5, 10, and 20 relative to Cu(I) concentration. After addition of each ligand addition, the solution was allowed to equilibrate (until no further spectral changes were observed) before the next addition of ligand. B) Concentration of [Cu'(BCA)₂]³⁻ plotted versus the ratio of Thiodeferiprone to Cu(I)

Table 2: Conditional Cu(I) Binding Constants of O, S ligands at pH 7.4 as Determined by Cu(I) Competition with BCA using Specfit

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Log $K'_{[CuL]^{1-}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PyS</td>
<td>16.04±0.14</td>
</tr>
<tr>
<td>ThM</td>
<td>16.12±0.04</td>
</tr>
<tr>
<td>ThDfp</td>
<td>15.89±0.61</td>
</tr>
</tbody>
</table>
2.2.2 Lipophilicity Studies of the Ligands and their Bis-Cu(II) Complexes

The partition coefficient, $P$, is a measure of a compound’s concentration in two immiscible phases at equilibrium; therefore, it is used for predicting how compounds partition between membranes and aqueous solution. The $P$ values for the ligands in Figure 3 and their Cu$^{II}L_2$ complex were measured by partitioning these compounds between n-octanol and 0.01 M Hepes buffer, pH 7.4 using the traditional shake flask method to separate the two phases followed by UV-visible spectroscopy to determine compound concentrations in each phase. Positive Log $P$ value indicates that ligand or [Cu$L_2$] has a higher concentration in the octanol phase relative to the aqueous phase (hydrophobic). Conversely, a negative Log $P$ value indicates the reverse (hydrophilicity). Ligand binding Cu(II) changes the lipophilicity of these complexes relative to the ligand (Table 3). For instance, PyS and PyO’s lipophilicity increases upon forming the [Cu$^{II}L_2$] complex while the other ligands’ lipophilicity decreases with Cu(II) complexation. O, S ligands’ [Cu$^{II}L_2$] complexes are more hydrophobic than their counterpart O, O ligands. Replacement of O with S in ThDfp makes it more hydrophobic relative to Dfp; this increase in lipophilicity of the ligand with the replacement of oxygen with sulphur is only observed with ThDfp but not with PyS and ThM.
Table 3: Partition Coefficients of the Ligands and their Respective Bis-Cu(II) Complexes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$\text{Log } P_L$</th>
<th>$\text{Log } P_{[\text{Cu}^{II}\text{L}_2]}$</th>
<th>Ligand</th>
<th>$\text{Log } P_L$</th>
<th>$\text{Log } P_{[\text{Cu}^{II}\text{L}_2]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8HQ</td>
<td>2.15±0.09</td>
<td>1.65±0.06</td>
<td>CQ</td>
<td>3.64$^#$,a</td>
<td>#</td>
</tr>
<tr>
<td>PyS</td>
<td>-1.81±0.04</td>
<td>1.64±0.08</td>
<td>PyO</td>
<td>-1.77±0.01</td>
<td>-0.30±0.01</td>
</tr>
<tr>
<td>ThM</td>
<td>1.379±0.006</td>
<td>0.98±0.03$^*$</td>
<td>Maltol</td>
<td>0.206±0.001</td>
<td>-0.080±0.01</td>
</tr>
<tr>
<td>ThDfp</td>
<td>0.14±0.03</td>
<td>-0.16±0.04$^*$</td>
<td>Dfp</td>
<td>0.774±0.001</td>
<td>-1.20±0.01</td>
</tr>
</tbody>
</table>

* Determined by Marian Helsel
# Couldn’t be experimentally determined as both CQ and $[\text{Cu}^{II}(\text{CQ})_2]$ complex precipitated out of the aqueous layer; CQ’s Log P is a predicted number from Chem Draw

Conditions: aqueous phase: 0.01 M Hepes, pH 7.4; organic phase, n-octanol; for $[\text{Cu}^{II}\text{L}_2]$ calculations: 200 µM Ligands and 100 µM CuSO$_4$; for ligand calculations: 200 µM Ligand

2.2.3 Kinetic Study of Ligands with a High Affinity Cu(I) Chelator

A kinetic study was conducted to determine how fast Cu is transferred from the ligands to the high affinity Cu(I) chelator bathocuproic disulfonic acid (BCS) (Figure 9) in Synthetic Complete Media (SC Media) (Equation 6). BCS is commonly used as a mimic for intracellular high affinity Cu(I) proteins like Ctr1, SOD1, and Atx1 (Table 4); BCS is a useful choice as mimic of these Cu(I) protein because the affinities of many these Cu(I) proteins have been determined by competition with BCS indicating that these Cu(I) proteins and BCS’s affinity for Cu(I) are comparable.$^{12, 14}$ SC Media is selected as the C. neoformans’ growth assays are conducted in this media. In each experiment, 100 µM ligand and
50 µM CuSO₄ were added to SC Media to form the [Cu⁺²L₂] complex; this solution was allowed to equilibrate until no further spectral changes were observed. Next, 500 µM BCS was added to [Cu⁺²L₂] solution, and UV-visible spectra were taken at regular intervals (Figure 10A). The exchange rate of Cu from the ligands to BCS is monitored by the increase in the [Cu⁺(BCS)₂]³⁻ band at 483 nm (Figure 10B). With no exogenous ligand added to SC Media, the rate of formation of [Cu⁺(BCS)₂]³⁻ is similar to Cu exchange rate from Maltol to BCS (Figure 10B, C). ThDfp is able to completely transfer its Cu(II) to BCS as Cu(I) within the first few minutes of the reaction, while PyO and 8HQ are the slowest at exchanging their Cu with BCS (Figure 10B, C).

\[
[Cu^{II}L_2] + 2(BCS)^{2-} \rightarrow [Cu^I(BCS)_2]^{3-} + 2L^- \quad \text{(Equation 6)}
\]

Figure 9: Bathocupronic Disulfonic Acid, a Cu(I) Specific Chelator

Bathocupronic Disulfonic Acid (BCS) forms 1:2 Cu(I):ligand complex with log \( \beta_2 = 19.8 \) ([Cu(BCS)₂]³⁻, \( \lambda_{\text{max}} = 483 \text{ nm}, \epsilon = 13500 \text{ M}^{-1} \text{ cm}^{-1} \)). Atoms indicated in blue bind Cu(I).
Table 4: Intracellular Cu(I) Binding Proteins of *Saccharomyces Cerevisiae* Yeast with Sub-femtomolar Dissociation constants

<table>
<thead>
<tr>
<th>Cu(I)-binding proteins <em>Saccharomyces Cerevisiae</em></th>
<th>Dissociation Constant $K_d^{12,14}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atx1</td>
<td>$7.3 \times 10^{-19}$ M$^{-1}$</td>
</tr>
<tr>
<td>Ctr1</td>
<td>$4.7 \times 10^{-19}$ M$^{-4}$</td>
</tr>
<tr>
<td>SOD1</td>
<td>$5 \times 10^{-21}$ M$^{-1}$</td>
</tr>
</tbody>
</table>

*$K_d$ is defined as shown with M indicating Cu while P shows protein*

\[
K_d = \frac{[M]^a[P]^b}{[M_aP_b]},
\]

\[
M_aP_b \overset{K_d}{\leftrightarrow} aM + bP
\]
Figure 10: UV-visible Spectrum Illustrating the Transfer of Cu(II) from Bis-Cu(II)-complexes of the Ligands to BCS as Cu(I) in SC Media

A) Conditions: 100 µM PyS, 50 µM CuSO₄ followed by addition of 500 µM BCS; after BCS addition, UV-Visible spectra were taken every 3 min for 200 min and then a final spectra taken after 1109 min. B) Rate of Formation of [Cu'(BCS)₂]³⁻ over time as Cu transfers from either PyS, ThM, ThDfp, 8HQ, Dfp, Maltol, and PyO to BCS C) Percentage exchange of Cu from the ligands to BCS at 10 min and 30 min after addition of excess BCS to the [Cu''L₂] solution

<table>
<thead>
<tr>
<th>Ligand</th>
<th>% of [Cu'(BCS)₂]³⁻ formed relative to the initial [Cu²⁺]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min</td>
</tr>
<tr>
<td>ThDfp</td>
<td>107</td>
</tr>
<tr>
<td>ThM</td>
<td>43.4</td>
</tr>
<tr>
<td>PyS</td>
<td>14.0</td>
</tr>
<tr>
<td>Dfp</td>
<td>14.4</td>
</tr>
<tr>
<td>Maltol</td>
<td>11.6</td>
</tr>
<tr>
<td>No ligand</td>
<td>10.2</td>
</tr>
<tr>
<td>PyO</td>
<td>9.00</td>
</tr>
<tr>
<td>8HQ</td>
<td>9.20</td>
</tr>
</tbody>
</table>

2.3 Discussion

2.3.1 Cu(I) Binding Studies

UV-visible spectroscopy studies confirm that the soft O,S ligands are able bind to Cu(I) while the harder O, N ligands do not bind Cu(I) (Figure 4, 5). The O, N containing ligand, 8HQ, is unable to bind Cu(I), but it binds Cu(II) (Figure 6). Interestingly the presence of 8HQ causes Cu(I) disproportion into Cu(II) and Cu since 8HQ cannot stabilize Cu(I) through a binding interaction (Figure 6; equation 7). This disproportionated Cu(II) then binds to 8HQ explaining why the [Cu''L₂] is
observed during spectroscopy of 8HQ and Cu(I) (Equation 8). The observation that the [Cu^{II}L_{2}] complex concentration in 100 µM 8HQ and 50 µM Cu(I) solution corresponds to only 25 µM bis-Cu(II)-complex of 8HQ further substantiates the idea of Cu(I) disproportionation facilitated by 8HQ. 8HQ’s inability to bind Cu(I) suggests that the ligands are able to show Cu-dependent antifungal activity independent of their ability to bind Cu(I).

\[ 2Cu^{I} \rightarrow Cu^{II} + Cu^{0} \] (Equation 7)

\[ Cu^{II} + 2(8HQ) \rightarrow [Cu(8HQ)]_{2} \] (Equation 8)

The O, S ligands’ conditional Cu(I) binding constants were determined at pH 7.4 with all O, S ligands showing similar Cu(I) binding affinity (Log \( K'_{[CuL_{2}]} \approx 16 \), Table 2). To fully understand the influence of redox state on antifungal activity, the literature reported Cu(II) binding affinities of the ligands were also compared (Table 5).\(^{10, 15} \) The reduction potentials of the Cu(II)/Cu(I)-ligand system for the O, S ligands were calculated using the methodology shown in Figure 11 and Nernst equation (equation 10), where \( E^0_{(Cu, aq)} = 0.153 \) V versus NHE was utilized.\(^{16} \) The Cu(II)/Cu(I)-ligand reduction potentials of PyS, ThM, and ThDfp were calculated as 103 mV, 107 mV, and 109 mV, respectively. These reduction potentials were then compared to the reduction potentials of biological relevant reducing agents, glutathione and ascorbic acid, at pH 7.4 (\( E^0_{\text{gl}} = -230 \) mV and \( E^0_{\text{asc}} = 58 \) mV versus NHE).\(^{17} \) The comparison of the reduction potentials of glutathione and ascorbic acid with the
reduction potential of bis-Cu(II) complexes of O, S ligands shows that these bis-Cu(II) complexes can be reduced intracellularly.

Table 5: Literature Reported Conditional Cu(II) binding Constants of Ligands at pH 7.4

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Log $K'_{[Cu^{II}L_2]}$</th>
<th>Ligands</th>
<th>Log $K'_{[Cu^{II}L_2]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PyS</td>
<td>20.61±0.09$^{10}$</td>
<td>PyO</td>
<td>14.08±0.08$^{10}$</td>
</tr>
<tr>
<td>ThM</td>
<td>19.75±0.12$^{10}$</td>
<td>Maltol</td>
<td>12.72±0.06$^{10}$</td>
</tr>
<tr>
<td>ThDfp</td>
<td>19.78±0.20$^{10}$</td>
<td>Dfp</td>
<td>15.31±0.29$^{10}$</td>
</tr>
<tr>
<td>8HQ</td>
<td>22.9±0.1$^{15}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 11: Schematic for Calculating Cu(II)/Cu(I)-ligand System Reduction Potential**

$$E_0^{0 (CuL_2)} = E_0^{0 (Cu,aq)} - \frac{2.303RT}{nF} \log \frac{K_{[Cu^{II}L_2]}}{K_{[Cu^{II}L_2]}^{1-}} \quad \text{(Equation 10)}$$
Table 6: Calculated Reduction Potentials of Cu(II)/Cu(I)-ligand System

<table>
<thead>
<tr>
<th>Ligands</th>
<th>$E^0_{(CuL_2)}$ (mV vs. N.H.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PyS</td>
<td>-117</td>
</tr>
<tr>
<td>ThM</td>
<td>-76</td>
</tr>
<tr>
<td>ThDfp</td>
<td>-65</td>
</tr>
</tbody>
</table>

The weaker affinity of O, O ligands for Cu(II) relative to their O, S counterpart suggests a possible reason for their lack of Cu-dependent antifungal activity (Table 5). Differences in O, S ligands’ antifungal activity cannot be attributed solely to differences in their Cu(I) and Cu(II) binding abilities at pH 7.4. For instance, the ThDfp’s Cu(I) and Cu(II) binding constants are comparable to that of the other O, S ligands, PyS and ThM, but ThDfp does not show any Cu-dependent antifungal activity, while the other two (PyS and ThM) do. These results suggest that other factors may be determinants of antifungal activity, particularly how the Cu(II) and Cu(I) binding affinity changes with pH. It is important to understand how the relative affinities of these ligands change at pH 4.5 since the antifungal growth assays against *C. neoformans* are done in SC Media, pH 4.5. Some preliminary UV-visible spectroscopy direct titrations of CuSO$_4$ into ThM ligand were conducted, and the preliminary data (not shown here) from this titration was used to calculate an approximate binding constant (Log $K'_{[CuL_2]}$ $\sim$13) of ThM with Cu(II) at pH 4.5. This conditional binding constant is much lower compared to the Log $K'_{[CuL_2]}$ $\sim$20 at pH 7.4 This decrease in Cu(II)
affinity is not surprising considering the pKₐ of ThM is about 8.2 suggesting that at a lower pH (i.e. 4.5) there exists presumably more protonated form of ThM and hence less Cu(II) binding is observed. Currently, Cu(II) direct titrations with PyS and ThDfp are underway; as ThDfp’s pKₐ is 9.45, it is anticipated that similar reductions in ThDfp’s Cu(II) affinity at pH 4.5 will be observed as those of ThM.¹¹c The Cu(II) direct titrations with PyS at pH 4.5 to determine conditional binding constants have been particularly challenging as the pKₐ of PyS is reported to be 4.5.¹¹a Thus, it is predicted that PyS’s affinity for Cu(II) at pH 4.5 would remain relatively unchanged whereas the other two O, S ligands’ (ThDfp and ThM) affinity for Cu(II) will decrease at this reduced pH.

2.3.2 Partition Coefficient Studies

Using the traditional shake flask method, the partition coefficients of ligands and their [Cu(II)L₂] complexes were successfully determined (Table 3). Ligand partition coefficients have been previously determined;¹¹a, ¹¹b, ¹⁵, ¹⁸ however, the inconsistencies in reported values prompted us to reexamine them. For instance, empirically determined Log P of ThM at pH 7.4 is reported as both 0.54 and 1.36,¹¹b, ¹⁸ whereas computationally determined log P values for ThM are 0.40 (ACD Chem Spider) and 0.62 (ChemDraw). This work is the first report of the partition coefficient of the [Cu(II)L₂] of ThM, PyS, ThDfp, Maltol, PyO, and Dfp. Interestingly, ligands demonstrating Cu-dependent antifungal activity form
hydrophobic Cu(II)-complexes (log P > 0 for [Cu\(^{II}\)L\(_2\)] of PyS, 8HQ and ThM) while the ligands lacking antifungal activity form hydrophilic Cu(II)-complexes. Therefore, these results suggest that ligands’ ability to form hydrophobic [Cu\(^{II}\)L\(_2\)] complexes allows them to move across the fungal cell membrane and exert their antifungal activity, but ligands lacking antifungal activity are unable to cross the membranes as they form hydrophilic Cu(II)-complexes. Furthermore, sulfur replacement with oxygen makes ThDfp more hydrophobic than its O, O counterpart ligands possibly due to the sulfur being less electronegative than oxygen and hence it forms weaker hydrogen bonding network with its aqueous environment. The bis-Cu(II) complexes of the O, S ligands are also more hydrophobic than their counterpart O, O ligands. PyS and ThM do not show the trend of increasing hydrophobicity with the replacement of oxygen donor atoms with sulfur.

### 2.3.3 Kinetic Study of Ligands with a High Affinity Cu(I) Chelator

The BCS transfer experiments show the exchange of Cu from the ligands to BCS is fastest for ThDfp and slowest for PyO and 8HQ. These different exchange rates indicate differences in the reactivity of these ligands with BCS that serves as a mimic of cellular Cu sink (Figure 10). In the absence of additional ligands, the rate of [Cu'(BCS)\(_2\)]\(^{3-}\) formation is comparable to Maltol's Cu transfer rate (Figure 10 C). In pH 4.5 SC Media, both Cu\(^+\) and H\(^+\) are competing with BCS (pK\(_{N,a}\) = 5.6) thereby slowing the formation of
The exchange of Cu(II) from the O, S ligands to BCS as Cu(I) is faster relative to O, O ligands as the free O, S ligands can possibly undergo oxidation to form disulfides thereby facilitating the reduction of [Cu(II)L₂] complex followed by ligand exchange with BCS (Route 1, Figure 12). In our system, this disulfide-facilitated reduction of bis-Cu(II) complexes of O, S ligands can be enabled by not only the free O, S ligands but also by the presence of cysteine in SC Media. Such disulfide-facilitated reduction of Cu(II)-complexes in the presence of a Cu(I) sink has been observed before for thiols like cysteine and glutathione.¹⁹

On the other hand, the thiol lacking O, N and O, O ligands cannot undergo oxidation to form the disulfides. Furthermore, the O, N and O, O ligands are unable to bind Cu(I), so direct reduction of bis-Cu(II) complex of these ligands to bis-Cu(I) complex is not possible. Hence, it is plausible that Cu exchange from these ligands to BCS occurs via route 2 (Figure 12). Route 2 involves [Cu(II)L₂] undergoing ligand dissociation followed by Cu(II) ion reducing to become Cu(I), which then binds to BCS. Among the O, O and O, N ligands, the exchange rate is fastest for Dfp and slowest for 8HQ; however, this trend in exchange rate does not correlate with Cu(II) binding affinities at pH 7.4 as 8HQ has the highest binding affinity while maltol has the lowest affinity (Figure 11). This discrepancy is possibly due to the Cu(II) binding affinities of O, O and O, N ligands changing at pH 4.5 of SC Media. Similarly, the differential exchange rates observed for

[Cu(I)(BCS)₂]³⁻.¹³


O, S ligands are also possibly due to Cu(I)/Cu(II) binding affinities changing at lower pH. Overall, these ligand Cu exchange rates with BCS suggest that intracellular Cu(I) proteins can acquire Cu(I) from the O, S ligands faster than the O, O and O, N ligands.

Figure 12: Possible Routes for Reductive Exchange of Cu(II) from the ligands to Cu(I) sink, BCS
2.4 Conclusions & Future Work

Here, I have successfully demonstrated that the O, S ligands bind Cu(I) while the other ligands are unable to stabilize Cu(I) at physiologically relevant pH 7.4. Similarity in O, S ligands’ Cu(I) binding affinities at pH 7.4 suggests that ThDfp’s lack of antifungal behavior is not related to differences in its Cu(I) affinity compared to the other O,S ligands at pH 7.4. The lack of correlation between ligands’ Cu exchange rates with BCS and their Cu(I)/Cu(II) binding affinities hints that ligands’ Cu(I)/Cu(II) binding affinities are altered with pH change. Therefore, in the future, we will determine how the Cu(I)/Cu(II) affinity of these ligands changes at pH 4.5 using direct metal titration into the ligands; these studies will inform the design of future Cu-based antifungals.

Antifungal ligands are able to form hydrophobic Cu(II)-complexes while ligands lacking antifungal activity form hydrophilic Cu(II)-complexes (Figure 14), indicating the lipophilicity of Cu(II)-complexes as an important determinant of
antifungal activity. Previously, addition of a methyl group to picolonic acid (bidentate O, N ligand) has been shown to increase its Zn-complex’s lipophilicity. Therefore, in the future we will test the antifungal activity and lipophilic behavior of new ThDfp derivatives with additional CH₃ groups that can potentially increase the lipophilicity of [CuIIₗ₂] complexes (Figure 14).

![Figure 14: Relation Between Lipophilicity of Bis-Cu(II) Complexes of Ligands and their Antifungal Activity](image)

Antifungal activity is indicated by MIC (minimum inhibitory concentration) in µM. Dfp, PyO, ThDfp, and Mal all have MICs greater than 100 µM in presence of 1 mM CuSO₄, but they are graphically represented as just 100 µM. Lipophilicity of this bis-Cu(II) complexes is shown by Log P values at pH 7.4
This chapter's focus was to understand the chemical properties enabling PyS, 8HQ, and ThM to have Cu-dependent antifungal activity. This characterization, however, does not completely illuminate the mechanism by which these ligands perturb the delicate balance of metals. For instance, PyS despite being hydrophilic still shows antifungal activity in the absence of Cu, which brings forth the question: how is PyS gaining entry into the fungal cell. A possible explanation is that PyS is using the Cu present in the media to form its Cu(II) complex that enables the delivery of PyS to the cell. Alternatively, PyS could possibly forming its dimer in SC Media by forming a disulfide bond using the thione moiety; the disulfide presumably being more hydrophobic than the monomer PyS could facilitate its delivery intracellularly. Therefore, it is intriguing to know if PyS itself alters intracellular metal levels since PyS by itself is antifungal. Hence, in the next chapter, using inductively coupled plasma mass
spectrometry (ICP-MS) metal content analysis of these fungal cells treated with the ligands was performed to see how biologically inactive and active ligands alter Cu levels.

These metal content analysis studies, however, do not clarify why these ligands and their delivered Cu cargo are fungicidal or whether the different molecules hit the same or different biochemical pathways. For instance, does PyS and the combination of PyS and Cu treatment have different or similar biochemical targets? Further, it is not clear why PyS in the presence of Cu shows higher antifungal activity (MIC is 3 µM) than 8HQ (MIC is 6 µM) though the lipophilic character of their bis-Cu(II) complexes is identical (Figure 14). Therefore, in the future genome-wide approaches (DNA microarrays) will be used to monitor gene expression levels upon exposure to the ligand or its bis-Cu(II) complex ([Cu^{II}L_2]). We will use Saccharomyces cerevisiae instead of C. neoformans as an experimental model to study these ligands' mechanism of action because of its well-characterized genetics, physiology and Cu homeostasis machinery and ease of availability of its microarrays. Before the microarray experiments are done, the antifungal activity of these ligands in the presence or absence of Cu(II) against S. cerevisiae will be investigated. The microarray analysis will identify genes and pathways affected by these molecules in synergy with Cu, informing our studies of C. neoformans and other pathogens. Importantly, PyS and ThM are all amenable to chemical modification enabling
preparation of prochelators that are conditionally activated by macrophage’s oxidative burst. Understanding similarities and/or differences in biological outcomes of these ligands will guide the further development of these agents as targeted antimicrobial agents.

2.5 Materials and Methods

2.5.1 Materials and Instrumentation

All reagents and solvents were purchased from Sigma Aldrich and used as received unless otherwise stated. Marian Helsel graciously provided 100 µM stock solutions of ThM and ThDfp in dimethylysulfoxide (DMSO). For aerobic experiments, UV-Visible (UV-Vis) spectroscopy was done using Varian Cary 50 UV-VIS spectrophotometer while for anaerobic experiments, SI Photonics (Tuscon, Arizona) 420 Fiber Optic CCD Array UV-Visible spectrophotometer was used at 25°C in a 1 cm pathlength quartz cell. In the anaerobic experiments, the cuvette was placed inside MBraun Uni-Lab Nitrogen filled glovebox and connected via fiber optic cables to the spectrophotometer. An Accument Dual/Channel pH Ion Meter equipped with an Orion Ross pH electrode filled with 3M NaCl solution was used for all pH measurements. Synthetic complete media, SC Media (SC, MP biochemist) was used as received.
2.5.2 UV-Vis Spectroscopy of Bis-Cu(I) Complexes of Ligands in an Anaerobic Environment and Determination of Bis-Cu(I) Complex Binding Constant

These experiments were done in collaboration with Marian Helsel. For Cu(I) binding studies, reaction buffers were prepared in thoroughly deoxygenated water (Milli-Q water) and stored in an anaerobic glove box (O₂ < 0.5 ppm). The Cu(I) stock solution was prepared by dissolving tetrakis(acetonitrile)copper(I) hexafluorophosphate ([Cu(CH₃CN)₄]PF₆) (Sigma) in deoxygenated acetonitrile. The Cu(I) stock solution's concentration in the glove box was determined by titrating aliquots of Cu(I) stock solution into a solution of excess bicinchoninic acid (BCA) in deoxygenated 0.01 M Hepes buffer, pH 7.4, while monitoring the absorbance of [Cu'(BCA)₂]³⁻ at 562 nm (ε = 7900 M⁻¹ cm⁻¹). The Cu(I) binding studies were done in Britton-Robinson/universal buffer (0.04 M H₃BO₄, 0.04 M H₃PO₄, and 0.04 M CH₃COOH) at pH 7.4.

Marian Helsel synthesized Thiomaltol and Thiodeferiprone. All ligands were prepared and stored anaerobically in the glove box by dissolving each of the ligands in deoxygenated DMSO (100 mM ligand stocks). For determining the formation of Cu(I) complexes of the ligands, UV-visible spectra were taken of the solution of 100 µM ligands with and without additional 50 µM ([Cu(CH₃CN)₄]PF₆) in universal buffer, pH 7.4 with 5% DMSO.

The conditional Cu(I) binding constants of PyS, ThM, and ThDfp were determined by competition equilibrium titrations. The equilibrium titrations were
monitored spectrophotometrically from 300 nm to 800 nm, and the resulting spectra were then analyzed using SpecFit/32 Version 3.0.35 Global Analysis Fitting Software. In order to provide wavelength dependent molar absorptivities values for light-absorbing species in solution, reference spectra for the ligand alone, bis-Cu(I) complex of the ligand, indicator alone, and bis-Cu(I) indicator complex were acquired. The association constants of the 1:2 [CuL₂]⁺ were determined in universal buffer, pH 7.4 with 5% DMSO. Some of these ligands can form 1:1 Cu(I): ligand complexes,¹⁰ but by using excess of ligand it was ensured only the 1:2 Cu(II):ligand complex forms.

A 2 mL solution of 125 µM BCA and 50 µM ([Cu(CH₃CN)₄]PF₆) were titrated under anaerobic conditions with at least 20 equivalents of O, S ligands. After each aliquot addition, the solution was allowed to equilibrate until no further spectral changes were observed. The decrease in 562 nm band [Cu(BCA)₂]³⁻ after addition of aliquots of ligand indicated an exchange of Cu(I) from BCA to ligand (Equation 13). Equation 18 was used to determine the concentration of [CuL₂]⁺. The free BCA and ligand concentrations were determined by using their respective mass balance equations (Equation 19 & 20). Calculations for determining binding constants were fit to the model shown in Table 7 using SpecFit software and confirmed by hand-calculations done according to Equation 15. All titrations were done in triplicate, and the binding affinities and standard deviations as determined by SpecFit were then averaged.
\[ Cu^I + 2L \xrightarrow{K_{CuL_2}} [Cu^I L_2]^+ \] (Equation 11)

\[ [Cu^I(BCA)_2]^3^- + 2L \xrightarrow{K_{ex}} [Cu^I L_2] + 2BCA \] (Equation 13)

\[ K_{ex} = \frac{K_{CuL_2}}{\beta_2'} = \frac{[CuL_2][BCA]^2}{[Cu][L]^2} \] (Equation 14)

\[ K_{CuL_2} = K_{ex}\beta_2' \] (Equation 15)

\[ Cu^I + 2BCA^{2-} \xrightarrow{\beta_2'' = K_1K_2} [Cu^I (BCA)_2]^3^- \] (Equation 16)

\[ \beta_2'' = K_1K_2 = \frac{[Cu(BCA)_2]}{[Cu][BCA]^2} \] (Equation 17)

\[ [Cu^I L_2] = [(Cu(BCA)_2)]_l - [(Cu(BCA)_2)]_n \] (Equation 18)

\[ [(BCA)]_{total} = 2[(Cu(BCA)_2)]_n + [(BCA)]_{free} \] (Equation 19)

\[ L_{total} = 2[Cu^I L_2] + [(L)]_{free} \] (Equation 20)

**Table 7: Model Used for Competition Between Ligands and BCA for Cu(I) in Universal Buffer, pH 7.4 with 5% DMSO**

<table>
<thead>
<tr>
<th>Species</th>
<th>Log β</th>
<th>Cu</th>
<th>BCA</th>
<th>Ligand</th>
<th>H</th>
<th>Refinement</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCA</td>
<td>-</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>Constant</td>
</tr>
<tr>
<td>L</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>Constant</td>
</tr>
<tr>
<td>[Cu'(BCA)_2]^3^-</td>
<td>17.2^13</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>Constant</td>
</tr>
<tr>
<td>[Cu^I L_2]^+</td>
<td>-</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>Refined</td>
</tr>
<tr>
<td>Cu</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Constant</td>
</tr>
</tbody>
</table>
Figure 16: UV-visible Spectrum of Competition Between Thiomaltol and BCA for Cu(I) at pH 7.4

A) A solution of 110 μM BCA and 50 μM ([Cu(CH$_3$CN)$_4$]PF$_6$) were prepared in universal buffer pH 7.4 with 0.5% DMSO, at 25 °C (red spectrum, top). Thiomaltol was added in equivalents of 0, 0.25, 0.5, 1, 2, 5, 10, and 20 relative to [Cu$^+$]. After addition of each addition, the solution was allowed to equilibrate (until no further spectral changes were observed) before the next addition of ligand. B) Concentration of [Cu$^+$](BCA)$_2$Cu$^{3+}$ plotted versus the ratio of Thiomaltol to Cu(I)
Figure 17: UV-visible Spectrum of Competition Between Pyrithione and BCA for Cu(I) at pH 7.4

A) A solution of 110 µM BCA and 50 µM ([Cu(CH3CN)4]PF6) was prepared in universal buffer pH 7.4 with 0.5% DMSO at 25 °C (red spectrum, top). Pyrithione was added in equivalents of 0, 0.25, 0.5, 1, 2, 5, 10, and 20 relative to [Cu+]2. After addition of each equivalent, the solution was allowed to equilibrate (until no further spectral changes were observed) before the next addition of ligand was added B) Concentration of [Cu^+\textit{(BCA)}_2]^3- plotted versus the ratio of Pyrithione to Cu(I)

2.5.3 Shake-flask Method for Determination of Partition Coefficient of [Cu^{II}(Mal)_2], [Cu^{II}(PyO)_2], [Cu^{II}(ThDfp)_2], & [Cu^{II}(Dfp)_2]

For these partition coefficient experiments, a minimum of three parallel experiments was done. A 4 mL solution of [CuL_2] was prepared in 0.01 M Hepes, pH 7.4 having 100 µM CuSO_4 and 200 µM of ligand; a UV-vis spectrum of this solution was taken to determine initial concentration of [CuL_2]. The 4 mL bis-Cu(II) complex solution in 0.1 M Hepes, pH 7.4 was added to 4 mL of n-octanol in a 15 mL centrifuge tube. The resulting solution was then shaken for 3 h using a 360° rotation device. Next, the above solution was centrifuged for 3
min at 5000 rpm to separate the n-octanol and aqueous phases. After shaking and centrifuging, a 1.5 mL aliquot of the aqueous phase and organic phase, respectively were carefully removed and the concentration of the \([\text{Cu}^{II}\text{L}_2]\) in both phases was then determined using UV-visible spectroscopy. The partition coefficient was determined as shown below:

\[
\text{Partition Coefficient, } P = \frac{[\text{CuL}_2]_{\text{octanol}}}{[\text{CuL}_2]_{\text{aqueous}}} \quad (\text{Equation 21}).
\]

**2.5.4 Shake-flask Method for Determination of Partition Coefficient of \([\text{Cu}(8\text{HQ})_2], [\text{Cu}(\text{PyS})_2], \text{& } [\text{Cu}(\text{ThM})_2]\)**

For these partition coefficient experiments, a minimum of three parallel experiments was done. A 4 mL solution of 100 µM CuSO\(_4\) and 200 µM of ligand in n-octanol was prepared using 100 mM CuSO\(_4\) stock solution in deionized water and 100mM ligand stock solution in DMSO; an initial spectra of this n-octanol solution was taken to determine the concentration of the bis-Cu(II) complex. The 4mL bis-Cu(II) complex in n-octanol was added to a solution of 4 mL of 0.01 M Hepes, pH 7.4 in a 15 mL centrifuge tube. The resulting solution was then shaken for 3 h using a 360° rotation device. Next, the above solution was centrifuged for 3 min at 5000 rpm to separate the n-octanol and aqueous phases. After shaking and centrifuging, a 1.5 mL aliquot of the n-octanol and aqueous phase, respectively were carefully removed and the
concentration of the $[\text{Cu}^{II}L_2]$ in each phase was then determined using UV-visible spectroscopy (Table 1). The partition coefficient was determined as shown below:

$$\text{Partition Coefficient, } P = \frac{[\text{CuL}_2]_{\text{octanol}}}{[\text{CuL}_2]_{\text{aqueous}}} \quad \text{(Equation 22)}.$$  

2.5.5 Shake-flask Method for Determination of Partition Coefficient: 8HQ, ThDfp, ThM, & Maltol

At least three parallel shake flask experiments were done for each of the ligand. A 4 mL solution of 200 µM of ligand in n-octanol was prepared using 100 mM stock of ligand in DMSO; the initial concentration of the ligands in the n-octanol was determined using UV-visible absorption spectroscopy. 4 mL of 0.01 M Hepes, pH 7.4 was added to a 15 mL centrifuge tube, and then 4 mL octanol solution containing 200 µM ligand was then added to the Hepes solution. The resulting solution was then shaken for 3 h using a 360° rotation device to prevent emulsion formation. Next, the above solution was centrifuged for 3 min at 5000 rpm to separate the n-octanol and aqueous phases. After shaking and centrifuging, a 1.5 mL aliquot from both the n-octanol and aqueous phase was carefully removed. UV-visible spectrum of each phase was taken and the concentration determined using the extinction coefficients in Table 1. The partition coefficient was determined as shown below:

$$\text{Partition Coefficient, } P = \frac{[L]_{\text{octanol}}}{[L]_{\text{aqueous}}} \quad \text{(Equation 23)}.$$
2.5.6 Calculation of Extinction Coefficients by UV-visible Spectroscopy

In order to determine the concentration of ligand and CuL₂ in each of the phases in the partition coefficient experiments, the extinction coefficient of each of the ligands and their respective [Cu\(^{II}\)L₂] concentration was determined. Stock solutions, 100 mM, of each of the ligands indicated in Table 1 were prepared in DMSO and stored at -20 °C. A 100 mM CuSO₄ stock solution in deionized H₂O was used for extinction coefficient determinations in 0.01 M HEPES, pH 7.4 while 100 mM CuCl₂ stock solution in acetonitrile was used for extinction coefficient determinations in n-octonal. For each extinction coefficient determination at least three replicate runs were made. Solutions of ligands with concentrations ranging between 10-100 µM in either 0.01 M Hepes, or n-octanol were prepared, and their UV-visible spectra were recorded after equilibration. The absorbance at the λ\(_{\text{max}}\) of each ligand was recorded and plotted versus ligand concentration. The gradient of linear fit of this plot was used to determine the extinction coefficient (Table 8). For [Cu\(^{II}\)L₂], different solutions were prepared with varying concentrations of [Cu\(^{II}\)L₂] (concentration range: 10 µM to 80 µM of Cu(II); 20 µM to 160 µM of ligand) in either 0.01 M Hepes, or n-octanol. Likewise, a UV-Vis spectrum of each of the bis-Cu(II) complex solutions was taken after the solution has equilibrated indicated by no further spectral changes. As before, the
absorbance at $\lambda_{\text{max}}$ for each $[\text{Cu}^{\text{II}}\text{L}_2]$ was recorded and plotted versus bis-Cu(II) complex concentration. The gradient of linear fit of this plot was used to determine the extinction coefficient (Table 8). Sample data set for calculating extinction coefficient of the bis-Cu(II) complex of PyO in 0.01 M Hepes, pH 7.4 is shown in Figure 18.

\[
y = 0.0071x + 0.0113 \\
R^2 = 0.99398
\]

Figure 18: Plot of Absorbance of Bis-Cu(II) Complex of PyO at 300 nm Versus Concentration of Complex in 0.01 M Hepes, pH 7.4
Table 8: Extinction Coefficient of Ligands and their Bis-Cu(II) Complex in 0.01 M Hepes, pH 7.4 and n-octanol

<table>
<thead>
<tr>
<th>Ligand</th>
<th>ε (M⁻¹ cm⁻¹), [CuII₂L₂] 0.01 M Hepes, pH 7.4</th>
<th>ε (M⁻¹ cm⁻¹), L 0.01 M Hepes, pH 7.4</th>
<th>ε (M⁻¹ cm⁻¹), [CuII₂L₂] n-Octanol</th>
<th>ε (M⁻¹ cm⁻¹), L n-Octanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltol</td>
<td>13900 (λ_max=306 nm)</td>
<td>9200 (λ_max=274 nm)</td>
<td>3600 (λ_max=331 nm)</td>
<td>7400 (λ_max=279 nm)</td>
</tr>
<tr>
<td>7400 (λ_max=300 nm)</td>
<td>4300 (λ_max=307 nm)</td>
<td>9000 (λ_max=308 nm)</td>
<td>4500 (λ_max=307 nm)</td>
<td></td>
</tr>
<tr>
<td>Dfp</td>
<td>20400 (λ_max=299 nm)</td>
<td>14300 (λ_max=279 nm)</td>
<td>8600 (λ_max=314 nm)</td>
<td>15100 (λ_max=314 nm)</td>
</tr>
<tr>
<td>CQ</td>
<td>#</td>
<td>#</td>
<td>4010 (λ_max=350 nm)</td>
<td>2320 (λ_max=329 nm)</td>
</tr>
<tr>
<td>5900 (λ_max=372 nm)</td>
<td>3800 (λ_max=306 nm)</td>
<td>3500 (λ_max=394 nm)</td>
<td>2500 (λ_max=317 nm)</td>
<td></td>
</tr>
<tr>
<td>11900 (λ_max=320 nm)</td>
<td>4000 (λ_max=333 nm)</td>
<td>12200 (λ_max=327 nm)</td>
<td>4500 (λ_max=353 nm)</td>
<td></td>
</tr>
<tr>
<td>14400* (λ_max=384 nm)</td>
<td>15700 (λ_max=357 nm)</td>
<td>17500 (λ_max=393 nm)</td>
<td>16600* (λ_max=360 nm)</td>
<td></td>
</tr>
<tr>
<td>ThM</td>
<td>26600* (λ_max=320 nm)</td>
<td>22600 (λ_max=330 nm)</td>
<td>25800 (λ_max=367 nm)</td>
<td>26900* (λ_max=350 nm)</td>
</tr>
<tr>
<td>ThDfp</td>
<td>26600* (λ_max=320 nm)</td>
<td>22600 (λ_max=330 nm)</td>
<td>25800 (λ_max=367 nm)</td>
<td>26900* (λ_max=350 nm)</td>
</tr>
</tbody>
</table>

*Determined by Marian Helsel. #[Cu(PyO)₂]²⁺ precipitates out of the aqueous solution.

2.5.7 BCS Transfer Kinetic Experiments in SC Media

100 mM CuSO₄ stock solution was prepared by dissolving CuSO₄.5H₂O in deionized water, and this stock solution was standardized with 0.0500 M EDTA to a murexide endpoint in ammonia buffer. Synthetic Complete Media (SC Media) was prepared by dissolving 2 g of SC Media Amino Acid mixture, 20 g Dextrose, and 6.7 g of yeast nitrogen base without amino acids in 1 L of water;
this solution was autoclaved for 40 min. The pH of the SC Media was determined to be 4.5. Marian Helsel synthesized Thiomaltol and Thioleferiprone, and 100 mM stock solutions of Thiomaltol and Thioleferiprone in DMSO were prepared and used for all experiments. For the BCS transfer kinetic experiments, 100 mM or 50 mM stock solutions of ligands (both O,S ligands, O, N ligands, and 8HQ) in DMSO, 25 mM CuSO₄ in deionized H₂O, and 50 mM BCS in deionized H₂O were prepared and diluted in SC Media at the concentrations needed for the experiment. An initial UV-visible spectrum was taken of 100 µM Ligand and then another spectra was taken after 50 µM CuSO₄ was added to the ligand solution; this solution was allowed to equilibrate until no further spectra changes were observed. BCS, 500 µM, was added to the above bis-Cu(II) complex solution, and UV-visible spectra were taken at regular intervals over the course of several hours as indicated in the figure legends. As a control experiment, 250 µM BCS was added to the SC Media solution and UV-visible spectra were taken over time to detect how much Cu(I)/Cu(II) was in SC Media. As a further control, 50 µM CuSO₄ and 0 µM ligand was added to SC Media followed by addition of 500 µM BCS; as before UV-visible spectra were taken at regular intervals of time to monitor how quickly [Cu'(BCS)₂]³⁻ complex forms in SC Media.
Figure 19: UV-visible Spectrum as Cu(II) Transfers from Bis-Cu(II) Complex of ThDfp to Cu(I)-chelator, BCS, as Cu(I)

Conditions: 100 µM ThDfp, 50 µM CuSO₄ followed by addition of 500 µM BCS in SC Media. After BCS addition, UV-Visible spectra were taken every 2 min for 60 min, every 5 min from 60-120 min, 20 min from 120-180 min, & then a final spectrum was taken after 240 min.

Figure 20: UV-visible Spectrum as Cu(II) Transfers from Bis-Cu(II) Complex of ThM to Cu(I)-chelator, BCS, as Cu(I)

Conditions: 100 µM ThDfp, 50 µM CuSO₄ followed by addition of 500 µM BCS in SC Media. After BCS addition, UV-Visible spectra were taken every 2 min for 60 min.
Figure 21: UV-visible Spectrum as Cu Transfers from Bis-Cu(II) Complex of Dfp to Cu(I) Chelator, BCS, as Cu(I)

Conditions: 100 µM Dfp, 50 µM CuSO₄ followed by addition of 500 µM BCS in SC Media, pH 7.4. After BCS addition, UV-Visible spectra were taken every 2 min for first 60 min; every 5 min for next 60 min; every 20 min for next 60 min; every 40 min for every 220 min; and finally a spectrum after 900 min.
Figure 22: UV-visible Spectrum as Cu(II) transfers from Bis-Cu(II) Complex of 8HQ to Cu(I) Chelator, BCS, as Cu(I)

Conditions: 100 µM 8HQ, 50 µM CuSO₄ followed by addition of 500 µM BCS in SC Media, pH 7.4. After BCS addition, UV-Visible spectra were taken every 2 min for first 60 min; every 5 min for next 60 min; every 20 min for next 60 min; and every 40 min for every 220 minutes, and finally a spectra after 960 min.
Figure 23: UV-visible Spectrum as Cu(II) Transfers from Bis-Cu(II) Complex of Maltol to Cu(I) Chelator, BCS, as Cu(I)

Conditions: 100 µM Maltol, 50 µM CuSO₄ followed by addition of 500 µM BCS in SC Media, pH 7.4. After BCS addition, UV-Visible spectra were taken every 2 min for the first 60 min, every 5 min for the next 60 min, every 20 min for the next 60 min; and every 40 min for the 220 min.
Figure 24: UV-visible Spectrum as Cu(II) Transfers from Bis-Cu(II) Complex of PyO to Cu(I) chelator, BCS, as Cu(I)

Conditions: 100 µM Maltol, 50 µM CuSO₄ followed by addition of 500 µM BCS in SC Media, pH 7.4; After BCS addition, UV-Visible spectra were taken every 2 min for the first 60 min; every 5 min for the next 60 min, every 20 min for the next 60 min, and every 40 min for the next 220 min.
Figure 25: UV-visible Spectrum as Bis-Cu(I) Complex of BCS forms in SC Media

Conditions: 50 µM CuSO₄ and 0 µM of ligand followed by addition of 500 µM BCS in SC Media, pH 7.4. After BCS addition, UV-Visible spectra were taken every 2 min for the first 60 min, every 5 min for the next 60 min; every 20 min for the next 60 min; and every 40 min for the every 220 min; and a final spectra taken after 1068 min.
Figure 26: UV-visible Spectrum as bis-Cu(I) Complex of BCS Forms in SC Media Without Any Exogenous Ligand or Cu

Only 0.2 µM Cu⁺ was detected in SC Media over the course of 110 min in SC Media. Conditions: 250 µM BCS in SC Media; UV-Visible spectra were taken every 5 min.
3. Metal Content Analysis Studies of *C. neoformans*

3.1 Background and Significance

In an initial screen, a former group member tested various metal binding agents with and without supplemental CuSO₄ against *C. neoformans* to test their antifungal activity. In this screen (Table 1), the bidentate chelators, PyS, 8HQ, and ThM, were identified to show copper dependent anti-fungal activity against *C. neoformans*. On the other hand, the O, O analogues of PyS and ThM, PyO and Mal, did not show any copper-dependent antifungal activity. Surprisingly, structurally very similar bidentate chelator, ThDfp, and its O, O analogue, Dfp, did not show any Cu dependent antifungal activity. Many of these bidentate ligands showing Cu-dependent antifungal activity are known as ionophores meaning that they are able to form lipophilic metal complexes which enable their transport across the cell-membranes allowing delivery of metals intracellularly. Further, recent studies using genetic analysis as well as atomic absorption spectroscopy have shown that PyS increases intracellular Zn and Cu in the yeast *Sachromycess cerevisae*.²²ᵃ Similarly, in mammalian cells the Cu(II) complex of 8HQ has been shown to increase intracellular Cu levels relative to cells treated with only 8HQ or Cu.²⁴ Inspired by these literature precedents and the Franz lab’s work on the Cu-dependent antifungal activity of PyS, ThM and 8HQ, we wondered how these ligands were manipulating intracellular levels of essential metal nutrients, Cu, Zn, and Fe. Also, we were interested in seeing how the
ligands lacking Cu-dependent antifungal activity were affecting the metal levels of the fungal cells. Inductively coupled mass-spectrometry (ICP-MS) was used to determine the total metal content of the fungal cells after 1 hr treatment with or without CuSO$_4$ and/or ligand including a control treatment with no CuSO$_4$ or ligand treatment.

3.2 Results and Discussion

The Cu, Zn, and Fe levels in C. neoformans fungal cells increase approximately 3.5, 2, and 3.3 fold respectively upon treatment with 10 µM CuSO$_4$ and 0 µM ligand as compared to the positive control of no Cu and no ligand treatment (Figure 27, 28, and 29). The cells’ total intracellular levels of Cu, Zn, and Fe remain relatively unchanged when treated with 10 µM PyS compared to when the fungal cells were treated with neither CuSO$_4$ nor ligand (Figure 27, 28, and 29). However, the treatment of the fungal cells with both 10 µM PyS and 10 µM CuSO$_4$ led to an approximately 2700 fold increase in Cu relative to the untreated cell. The increase in Cu levels when treated with both PyS and CuSO$_4$ is also marked when compared to the cells treated with either 10 µM PyS (about 2000 fold increase) or 10 µM CuSO$_4$ (about 750 fold increase) (Figure 27). Similarly, the total Zn and Fe levels increased approximately 75 fold when the fungal cells were treated with both 10 µM PyS and 10 µM CuSO$_4$ as compared to the control treatment (no CuSO$_4$ and no ligand) (Figure 26 and 27). When evaluating the influence of PyS and CuSO$_4$ treatment on intracellular metal
levels, it is more appropriate to compare the PyS and Cu treatment to 10 µM CuSO₄ treatment instead of the no Cu and no ligand control. Upon comparing the 10 µM PyS and 10 µM CuSO₄ treatment to 10 µM CuSO₄ condition, it is observed that the Cu, Zn, and Fe levels increased approximately 760, 35, and 24 fold respectively (Figure 27, 28, 29). Therefore, both comparisons reveal that the Cu and PyS treatment triggers a metal imbalance within the fungal cells much greater than the metal imbalance caused either by just PyS or Cu alone. This differential response of PyS and Cu combination compared to PyS alone in altering cellular metal levels was surprising as PyS by itself as well as its combination with Cu show an antifungal response against C. neoformans. This difference might allude to the difference in the antifungal mechanism of action of just PyS by itself compared to its Cu(II) complex; perhaps, the PyS antifungal mechanism relates to its ability to form a disulfide, and this property allows it to potentially disrupt the disulfide bonds in proteins. Therefore, future work needs to be done to determine if the fungal cellular cysteine containing proteins are modified by PyS. It would also be intriguing to see how the disulfide form of PyS is altering the intracellular levels of Zn, Fe, and Cu.

On the other hand, the O, O analogue of PyS, PyO, does not alter the Cu, Zn, and Fe iron levels compared to the positive control (no Cu and no Ligand) (Figure 27, 28, and 29). The addition of Cu along with 10 µM PyO increases the Cu and Fe levels two fold relative to the no treatment control; however, the Cu,
Zn, and Fe levels of 10 µM Cu and 10 µM PyO treatment are very similar to those of just the 10 µM CuSO₄ treatment. Therefore, the metal content analysis of just PyO or PyO and Cu treated cells lends support to our hypothesis that this ligand is unable to transport Cu across the fungal membrane as it forms a hydrophilic bis-Cu(II) complex and hence is unable to alter intracellular metal levels.

In comparison to the no treatment control, the treatment with 10 µM Mal increases Cu levels by approximately 2.5 fold, whereas the Fe levels remain relatively unchanged and the Zn levels decrease 10 fold. Upon addition of Mal with Cu, the total Cu levels remain comparable to the 10 µM CuSO₄ condition; whereas both Fe and Zn levels decrease. On the other hand, the O, S analogue ThM increases Cu levels 40 fold compared to the basal level; the Zn and Fe levels with 10 µM ThM only increase by 2 fold compared to the no treatment. The co-treatment of 10 µM Cu and 10 µM ThM increases Cu levels to 165 fold relative to the no treatment control and 45 fold relative to 10 µM Cu treatment. However, the co-treatment of Cu with ThM increases Zn levels 11 fold, while the Fe levels increase 23 fold relative to the no treatment. The increase in Zn and Fe levels with both Cu and ThM are much larger in magnitude compared to the increase in Zn and Fe levels seen with only ThM. Both ThM treatments with and without Cu cause an increase in intracellular Cu levels (165 and 45 fold respectively, relative to the no treatment control). On the other hand, the
antifungal activity assays show that ThM alone is unable to elicit antifungal activity, whereas the addition of Cu to ThM allows it to exhibit antifungal activity. This result suggests that ThM alone is unable to create a sufficient metal imbalance to enable fungal killing while the combination of Cu and ThM causes a metal imbalance that the fungi is unable to tolerate, so this combination elicits antifungal activity.

The 10 µM ThDfp treatment of fungal cells results in decreased Cu, Zn, and Fe as compared to the no treatment control. The combination of 10 µM ThDfp and 10 µM CuSO₄ results in approximately 5 fold increase in Cu levels relative to the no treatment control, but comparing the Cu levels of ThDfp and Cu combined treatment to the 10 µM Cu treatment reveals no difference. However, the combined Cu and ThDfp treatment just like the ThDfp treatment results in a decrease in Zn and Fe levels as compared to the no treatment control. On the other hand, the O, O analogue of ThDfp, Dfp, doesn’t alter the Cu, Zn, and Fe levels of the cell compared to the no treatment control. Similarly, the co-treatment of Cu and Dfp leaves the Cu levels unchanged compared to Cu alone treatment, but the Zn and Fe levels decrease relative to the Cu alone treatment. The metal content studies of fungal cells treated with Dfp and ThDfp in combination with Cu reveal that these ligands are unable to increase intracellular Cu suggesting as to why they are unable to elicit Cu-dependent antifungal activity. These results also lend support to our hypothesis that Dfp and ThDfp form hydrophilic bis-Cu(II)
complexes, which are unable to move across fungal cell membranes, so these ligands are unable to increase intracellular Cu content.

The fungal cell treatment with 10 µM 8HQ results in a decrease of Cu and Zn levels, whereas the Fe levels relatively unchanged compared to the no treatment control. Not surprisingly, the combination of 10 µM Cu and 10 µM 8HQ combination leads to an approximately 400 fold greater accumulation of Cu compared to the no treatment control; this 400 fold increase in Cu levels upon treatment with Cu and 8HQ becomes less stark if the Cu levels are compared to 10 µM Cu treatment as this comparison only reveals 100 fold increase (Figure 27). Similarly, 10 µM Cu and 10 µM 8HQ treatment enhances the Zn and Fe levels approximately 5 and 10 fold respectively in comparison to the Cu alone treatment (Figure 28 and 29). The metal content studies indicate that the combination of Cu and 8HQ elicits antifungal activity against C. neoformans as this combination is able to create a intracellular metal imbalance by increasing levels of Cu, Zn, and Fe, which is not created by 8HQ alone. The boost in Cu levels with the combination treatment of Cu and 8HQ also substantiates our hypothesis that the lipophilic bis-Cu(II) complexes of 8HQ are more membrane permeant, thereby facilitating the delivery of Cu transport across fungal membranes and causing Cu accumulation.
Figure 27: Cu Content Analysis of C. neoformans by ICP-MS

Plot showing amount of Cu detected by ICP-MS analysis of C. neoformans wild type cells treated with or without 10 µM ligand in the presence and absence of 10 µM CuSO₄; data are mean ± S.E.M for n≥4 samples for all conditions.
Figure 28: Fe Content Analysis of *C. neoformans* by ICP-MS

Plot showing amount of Fe detected by ICP-MS analysis of *C. neoformans* wild type cells treated with or without 10 µM ligand in the presence and absence of 10 µM CuSO₄; data are mean ± S.E.M for n≥4 samples for all conditions
Figure 29: Zn Content Analysis of *C. neoformans* by ICP-MS

Plot showing amount of Zn detected by ICP-MS analysis of *C. neoformans* wild type cells treated with or without 10 µM ligand in the presence and absence of 10 µM CuSO₄; data are mean ± S.E.M for n ≥ 4 samples for all conditions.
3.3 Summary

Overall, these metal content analysis studies of fungal cells treated with or without Cu and/or ligands reveals the following:

1) Ligands that are able to elicit antifungal activity in the presence of exogenous Cu are increasing not only the intracellular Cu levels, but they are also causing increase in Zn and Fe levels. Ligands like PyS, 8HQ, and ThM all show Cu dependent antifungal activity (Table 1) and all these ligands in combination with Cu result in increases in Cu, Zn, and Fe levels. The increase in intracellular levels of Zn and Fe with PyS, 8HQ, or ThM co-treatment with Cu can not be attributed to these ligands binding to Zn and Fe in the media and bringing it intracellularly; if these ligands were just binding Zn and Fe from the media and bringing it intracellularly, then the control experiments with just the ligands (PyS, ThM, and 8HQ) would have shown dramatic increase in Zn and Fe levels. Therefore, we hypothesize that increase in Zn and Fe levels observed with Cu and ligand treatment (PyS, 8HQ, and ThM) is a consequence of Cu influx due to these chelators.

2) Ligands that do not elicit antifungal activity in the presence of Cu are not altering the cellular delicate balance of metals.

   These studies show that ligands showing Cu-dependent antifungal activity are able to significantly disturb metal balance of fungal cells, but
further work needs to be done to determine how this hyper accumulation of Cu along with Zn and Fe causes fungal toxicity. It is intriguing to note that the hyper accumulation of intracellular Cu as a result of co-treatment of Cu with 8HQ, PyS, or ThM causes the accumulation of the other metals like Zn and Fe. This hyper accumulation of Zn and Fe does not occur when the cells are treated with ligand, 8HQ and PyS, alone suggesting that these ligands are unable to bring in Fe and Zn intracellularly. This inability of PyS and 8HQ alone to bring Fe and Zn from the media to the cell is probably due to their weaker affinity of Zn and Fe iron compared to Cu. On the other hand, ThM alone with no additional Cu increases not only Cu levels but also Zn and Fe suggesting that it can bring Cu, Zn, and Fe from the media to inside the cell. Therefore, ThM’s ability to bring Zn and Fe suggests that its affinity for these metals is higher compared to PyS and 8HQ as these ligands are unable to bring Zn and Fe from the media to the cell intracellular. To probe this idea, antifungal growth assays should be performed in Zn and Fe deplete media and see how these ligands along with Cu co-treatment are manipulating Cu, Zn, and Fe in such conditions.

3.4 Materials and Methods

The Thiele lab (Duke University) generously provided C. neoformans H99 strain wild type. This fungal strain was streaked onto solid Synthetic Somplete Media, SC Media (SC, MP Biomedicals) from frozen glycerol stocks at -80° C.
For experiments, one colony was inoculated and grown in SC Media overnight. An overnight culture of C. neoformans was diluted to OD_{600} 0.2 and incubated at 30 °C shaking at 200 rpm for 3 hr. 5 mL cultures treated with 10 µM CuSO_{4} and 10 µM ligand (PyS, PyO, Mal, ThM, Dfp, ThDfp, and 8HQ) as indicated in the figure legends, and each of these treatments were incubated for 1 hr at 30° C while shaking at 200 rpm. The fungal cell samples were transferred to pre-weighed acid washed Teflon tubes, washed twice in ddH_{2}O with 1 mM ethylenediamine tetraacetic acid (EDTA), once in phosphate buffer saline (PBS), and resuspended in 1 mL of PBS buffer. A 100 µL aliquot was removed and diluted in SC Media before plating on SC Media agar plates to determine CFUs and the remaining fungal cell solution was pelleted and dried. The dried cell lysate was digested in neat trace metal grade nitric acid (Fischer Scientific, Pittsburg, PA) overnight and heated for 30 min at 80° C. The acid solution was cooled and diluted with ultra pure laboratory grade water. Kim Hutchison (Department of Soil Science, North Carolina State University) performed metal content analysis on a Varian 820 ICP-MS. The following isotopes were utilized for data collection: $^{63}$Cu (monitored $^{65}$Cu), $^{56}$Fe, pooled $^{42,43,44}$Ca, and pooled $^{66,67,68}$Zn. The collected data were normalized to a non-analyte internal standard ($^{89}$Y) in order to correct for small differences due to instrument drift and sample versus standard matrices. A laboratory check standard of a different stock was prepared and used to verify the calibration standard solution. A continuing
laboratory standard was analyzed every 12 samples, and the calibration curve was repeated if the laboratory standard was off by 10% or more. All experiments were performed at least 4 times or more, and the mean and standard error of mean (S.E.M) are reported.
4. Thiohydroxamic Acids as a Potential New Class of Cu-based Antifungals

4.1 Introduction and Significance

Thiohydroxamic acids (N-hydroxythioamides) (Figure 30) are a class of compounds containing five different atoms (CH-sp², N, O, S). The close proximity of these four atoms gives them unique reactivity. These acids equilibrate between the thione and thiol form as shown in Figure 30. The specific bond system of sulfur-carbon-nitrogen-oxygen results in the higher acidity of thiohydroxyamide nitrogen compared to hydroxyamide nitrogen; hence the pKₐ of simple thiohydroxamic acids range from 4.5-5.6 compared to a pKₐ of the corresponding hydroxamic acids in the range of 8-9.²⁵ This reduced pKₐ of thiohydroxamic acid implies that the thiohydroxamic acids can bind metals at lower pH values than the corresponding hydroxamic acids. This property of the thiohydroxamic acids makes them excellent bidentate chelators.

![Figure 30: Thiohydroxamic Acids](image)

Figure 30: Thiohydroxamic Acids
Naturally occurring thiohydroxamic acids are not very common; however, some naturally occurring metal complexes of thiohydroxamic acids were discovered in the early 1970's. The bis-Cu(II) complex (Fluopsin C) and tris-Fe(III) complex (Fluopsin F) of the ligand, N-methyl-N-thioformylhydroxylamine (thioformin) are naturally occurring metal chelates, which have been isolated from the culture supernatant fluids of *Pseudomonas* and *Streptomyces* species (Figure 31). Fluopsin F and C both have been shown to have antimicrobial activity against both gram-negative and gram-positive bacteria as well as some fungi species.\(^{26}\) Cu(II), Ni(II) and Pd(II) divalent complexes as well as the Fe(III), Co(III), and Cr(III) trivalent complexes with thioformin displayed antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*.\(^{26}\) However, the ligand thioformin itself showed no antifungal activity, hinting that the metal complex is essential for antimicrobial activity of these thiohydroxamic acids.\(^{26}\)

Inspired by these early studies and our lab’s identification of the Cu-dependent antifungal activity of the thiohydroxamic acid Pyrithione, we were encouraged to screen new thiohydroxamic acids against the opportunistic fungal pathogen, *C. neoformans*. In this chapter, several linear and cyclic thiohydroxamic acids are examined to test their effect on *C. neoformans* growth as determined in a growth assay (Figure 32). 3-hydroxy-4-methylthiazole-2(3H)-thione, TAT is a cyclic thiohydroxamic acid like pyrithione and similar to pyrithione, it forms divalent metal complexes with cobalt, zinc, and copper.\(^{27}\)
Based on these properties, we anticipate that it might show similar antifungal activity as Pyrithione. The Pierce lab graciously provided the linear thiohydroxamic acids, BL-09-94, BL-09-78, and BL-14-04, and BL-14-05 (Figure 32). It should be noted that thiohydroxamic acid, BL-09-78, has its N-hydroxide methylated and it is anticipated that this methylation will prevent metal binding; hence this hydroxamic acid was selected as control for non-metal binding thiohydroxamic acid. Further, BL-09-94, BL-14-04, and BL-14-05 all have variable R groups with BL-14-05 containing a heteroaryl R group; the variable nature of R groups in these thiohydroxamic acids will allow us also to elucidate the influence of the R group on antifungal activity. BL-09-94 and BL-14-04 both have a phenyl group in their R groups but each has a variable length of spacers separating the phenyl ring from the thiohydroxamic acid moiety; these were selected to probe the influence of flexibility of thiohydroxamic acid moiety and the attached R group on the compound’s antifungal activity. In order to further probe thiohydroxamic acids’ Cu facilitated antifungal activity, the growth assay of C. neoformans with various thiohydroxamic acids was performed with and without Cu(I) membrane impermeable chelator (BCS). Lastly, the ability of thiohydroxamic acids to provide bio-available Cu to C. neoformans lacking Cu(I) importers, Ctr1 and Ctr4, was examined. In a preliminary effort to understand their antifungal activity, the thiohydroxamic acids’ relative binding affinities for Cu(II) were measured using a calcein competition assay.
4.2 Results and Discussion

4.2.1 Screening Thiohydroxamic Acids for Their Antifungal Activity

*C. neoformans* needs to maintain a delicate balance of Cu and Fe for both its virulence and survival; this fungal requirement is particularly alluring as we can screen metal binding compounds that can manipulate the delicate balance of these metals within the fungi thereby causing fungal death. As mentioned earlier, thiohydroxamic acids are particularly attractive compounds for manipulating metals because of their ability to bind metals. Here, we assayed linear and cyclic thiohydroxamic acids for their ability to show antifungal activity
dependent upon Cu. The Pierce lab (North Carolina State University) graciously provided the linear thiohydroxamic compounds. All compounds in Figure 12 were assayed for their effect on *C. neoformans* growth by measuring optical density of liquid cultures incubated at 30 °C for 48 hr in the presence and absence of 100 µM CuSO₄ along with 0-100 µM thiohydroxamic acid. The minimum inhibition concentration (MIC) is defined as the lowest concentration at which no detectable growth was seen compared to the positive control. Without supplemental Cu, the MICs of the linear thiohydroxamic acids were BL-14-05, BL-14-04, BL-09-78, BL-09-94 are 12.5 µM, 7.5 µM, 50 µM, 12.5 µM, respectively (Figure 33). In the presence of 100 µM CuSO₄, the MICs of the linear thiohydroxamic acid were unchanged (Figure 33). This lack of change in MIC in the presence of CuSO₄ suggests that these linear thiohydroxamic acids might not be showing Cu-dependent antifungal activity. It should be noted that among the thiohydroxamic acids, the methylated N-hydroxide in thiohydroxamic acid (BL-09-78) shows the lowest antifungal activity suggesting that the apparent lack of metal binding might be influencing antifungal activity.

Interestingly, MIC of the cyclic thiohydroxamic acid, TAT, in the absence of CuSO₄ is 1.5 µM while the presence of 100 µM CuSO₄ changes the MIC to 0.78 µM (Figure 34). Among the thiohydroxamic acids tested, it appears that linear thiohydroxamic acids show lesser antifungal activity whereas cylic
thiohydroxamic acid show greater higher antifungal activity (both PyS and TAT have MICs of 0.78 µM in the presence of 100 µM CuSO₄).

Figure 33: Linear Thiohydroxamic Acids’ Effect on the Growth of *C. neoformans*

Growth, as monitored by optical density after 48 h, of wild type *C. neoformans* H99 strain grown in SC medium at 30°C with BL-14-05, BL-09-94, BL-14-04, and BL-09-78 and co-incubated with or without 100 µM CuSO₄.
Figure 34: TAT’s Affect on Growth of Wild Type C. neoformans

Growth, as monitored by optical density after 48 h, of wild type C. neoformans H99 strain grown in SC medium at 30°C with TAT and co-incubated with or without 100 µM CuSO₄.

To further probe the Cu-dependence of the antifungal activity of the linear thiohydroxamic acids, the C. neoformans’ growth assays were conducted in the presence and absence of 500 µM BCS with varying concentrations of the thiohydroxamic acids. BCS, as mentioned earlier, is a cell-impermeable Cu(I) chelator, so addition of high concentration of BCS will sequester Cu from the media thereby creating Cu-limiting growth conditions for the fungi. As reported previously by the Thiele lab, C. neoformans grows well in both low Cu and high Cu conditions.⁹b In the presence of BCS, chelators requiring Cu for their antifungal activity would have higher MICs while chelators not requiring Cu for

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their antifungal behavior will have their MICs unchanged. For all the linear thiohydroxamic acids tested in this screen, their MICs remained unchanged upon the addition of 500 µM BCS as compared to no additional BCS (Figure 35). TAT’s antifungal activity in the presence and absence of BCS was not probed as the initial growth assay in the presence and absence of CuSO₄ had indicated a Cu-dependence in its antifungal behavior.
Figure 35: Effect of Thiohydroxamic Acids on Growth of *C. neoformans* in the Presence and Absence of BCS

Growth, as monitored by optical density after 48 h, of wild type *C. neoformans* H99 strain grown in SC medium at 30°C with BL-14-05, BL-09-94, BL-14-04, and BL-09-78 and co-incubated with or without 500 µM CuSO₄.
4.2.2 Ctr1ΔCtr4Δ Mutant and Wild Type Growth Assays

*C. neoformans* uses two functionally redundant Cu transporters, called Ctr1 and Ctr4, which have high affinity for Cu(I) to import Cu inside the cell. We were interested to know if the thiohydroxamic acids are able to transfer Cu to these Cu importers or if they can gain entry into the cell as the metal complex independent of these importers. In order to probe if these ligands require Cu importers for their antifungal activity, a growth assay was performed using a mutant of *C. neoformans* lacking these transporters. Yeast protein ethanol glycerol containing media, YPEG media, is used in these growth experiments as this media contains ethanol and glycerol as the sole-carbon source for respiration. These carbon sources force *C. neoformans* to become dependent on Cu’s bioavailability, as Cu is needed for cytochrome-c oxidase dependent respiration. The wild-type *C. neoformans* grows in the YPEG media as they do not lack Cu importers; however, the mutant strains are unable to grow in the YPEG media as they lack Cu(I) transporters.

The linear thiohydroxamic acids were not able to restore the growth of the mutant strain (Figure 36), suggesting that either these ligands are unable to deliver Cu to the cell, or they are unable to make Cu bioavailable to cytochrome-c oxidase. These results further suggest that these thiohydroxamic acids do not require Cu to exhibit their antifungal behavior. On the other hand, the cyclic thiohydroxamic acid, TAT, was able to recover the growth of the mutant
strains to almost the same level as the wild-type strain (Figure 37). This experiment indicates that TAT is able to deliver Cu present in YPEG to the cells without using the Ctr1/Ctr4 import mechanisms. Further TAT is not only able to bypass the Cu-import mechanisms, but it is also able to make Cu bio-available for cytochrome-c oxidase dependent respiration. This ability of TAT to make Cu bio-available suggests that it can exchange Cu with the intracellular Cu binding proteins.
Figure 36: Linear Thiohydroxamic Acids are Unable to Provide Bio-available Cu to C. neoformans

Comparison of the growth of WT C. neoformans to the ctr1Δctr4Δ strain as a function of [BL-09-94], [BL-14-04], [BL-14-05], and [BL-09-78] in YPEG medium without supplemental Cu. Due to the loss of cell-surface Cu transporters to import Cu, ctr1Δctr4Δ cells are unable to grow in these conditions. None of these thiohydroxamic acid are able to overcome this growth defect.
Figure 37: TAT is Able to Provide Bioavailable Cu to *C. neoformans*

Comparison of the growth of WT *C. neoformans* to the *ctr1Δctr4Δ* strain as a function of [TAT] in YPEG media without supplemental Cu. Due to the loss of cell-surface Cu transporters to import Cu, *ctr1Δctr4Δ* cells are unable to grow in these conditions. TAT is able to overcome this growth defect suggesting that these ligands are able to transport trace Cu from the media to the fungal cell.

4.2.3 Calcein Florescence Recovery Experiments

In order to further understand the antifungal activity of the linear and cyclic thiohydroxamic acids and their possible link to Cu, we wanted to probe the relative binding affinity of these thiohydroxamic acids. A competition assay using the fluorophore calcien was designed where calcien serves as a both a competitor and an indirect indicator of Cu binding to the ligands (Figure 38). Calcein’s fluorescence was quenched by the addition of 1.1 equivalents of
CuSO₄; next increasing aliquots of ligands of interest were equilibrated with the Cu and calcein solution. Calcein’s fluorescence will be recovered if the ligands are able to transfer Cu from the calcien. In addition to the linear and cyclic thiohydroxamic acids, PyS, ThM, and ThDfp were also examined by this assay for comparison as they also contain O,S binding motive similar to the thiohydroxamic acids.

Calcein fluorescence recovery experiment results are shown in Figure 38. The assay results show that BL-09-78 was unable to restore calcein’s florescence signal even after addition of 32 equivalents of the ligand relative to Cu concentration. BL-14-05 and BL-09-94 were able to recover calcein florescence to approximately 20% when 32 equivalents of ligand are added relative to Cu. On the other hand, 32 equivalents of ThDfp and ThM relative to Cu were able to bring about almost 100% recovery in calcein florescence signal whereas PyS and BL-14-04 are able to bring about approximately 80% recovery under similar ligand concentrations. TAT is able to bring about 50% florescence recovery when there are 32 equivalents of ligand relative to Cu.
Figure 38: Relative Cu(II) Binding Strength of Select O,S Ligands

Competition assay using calcein as chromophore and Cu(II) competitor. Calcein’s florescence quenches in presence of 1.1 equivalent of Cu; increasing equivalents of ligands are titrated into the Cu and Calcein solution and if ligands can remove Cu from calcein, florescence of calcien is recovered. But, if ligands are unable to exchange Cu from calcein, no recovery of calcien florescence signal is observed. Conditions: 2 µM calcein; 2.2 µM CuSO$_4$ with ligand concentration from 0.25 µM to 64 µM in PBS, pH 7.4

From this assay, it can be inferred that the tightest Cu(II) binders under these conditions are ThDfp and ThM, whereas the weakest are BL-14-05 and BL-09-94. It is interesting to note that even though linear thiohydroxamic acid BL-14-04 is a tighter Cu(II) binder than the cyclic thiohydroxamic TAT, BL-14-04 does not show Cu-dependence in its antifungal activity, whereas TAT does. This
discrepancy in Cu binding ability and Cu-dependent antifungal activity of TAT and BL-14-04 is not unexpected. We have seen similar discrepancies earlier with PyS, ThM, and ThDfp that despite similar Cu(II) binding affinities only ThM and PyS exhibit Cu-dependent antifungal activity whereas ThDfp does not show any antifugal behavior. This discrepancy hints to other factors besides relative Cu(II) binding strength that might influence antifungal activity.

In case of cylic and linear thiohydroxamic acids, antifungal activity of these ligands might be influenced by their relative susceptibility to hydrolysis over the course of the growth assay; further work needs to be done to explore how stable these thiohydroxamic acids are in aqueous solution, especially at pH 4.5 (pH of SC Media). Among other factors influencing the Cu-dependent antifungal activity, lipophilicity of the linear and cylic thiohydroxamic acids and their bis-Cu(II) complexes should be explored. Preliminary work with the bis-Cu(II) complex of TAT (data not shown here) shows that it has poor solubility in aqueous solutions. This poor solubility hints towards the lipophilic nature of this Cu complex further validating our earlier studies that lipophilic bis-Cu(II) complexes facilitate Cu delivery into the fungal cells thereby eliciting antifungal activity.

BL-09-78’s inability to compete for Cu from calcein is not surprising considering that one of its metal binding groups is methylated. It should be noted that among the other thiohydroxamic acids it appears that flexibility of the R
groups (R is bonded to carbon bearing the sulphur) influences relative Cu binding affinity as BL-14-04 has the highest relative affinity, which has the most flexible R group (R is group is propylbenzene compared to methylbenzene of BL-09-94).

4.3 Summary

Here we have screened a new group of thiohydroxamic acids against C. neoformans to explore if these ligands showed any Cu-dependent antifungal activity. Results from this small screen indicate that cyclic thiohydroxamic acids exhibit higher antifungal activity than the linear thiohydroxamic acids. This difference might point towards their variable susceptibility to hydrolysis suggesting that cyclic thiohydroxamic acids are more stable and hence showing higher antifungal activity. Further, it was observed that only the cyclic thiohydroxamic acids, TAT and pyrithione, exhibited Cu-dependent antifungal activity, whereas the linear thiohydroxamic acids showed antifungal activity but did not exhibit any Cu-dependence again eluding to differences in their predisposition to hydrolysis. Among the thiohydroxamic acids, the relative Cu binding affinity followed this trend (highest to lowest): PyS > BL-14-04 > TAT > BL-09-94 > BL-14-05 > BL-09-78. These results are particularly interesting since BL-14-04 shows higher Cu binding than TAT, but it did not show Cu-dependence in it’s antifungal behavior, so it will be intriguing to see how these thiohydroxamic acids effect intracellular metal levels.
4.4 Materials and Methods

4.4.1 Strains, Media and Compounds

The Thiele lab of Duke University generously provided two strains of C. neoformans H99 strain: wild type and a double copper transporter knockout strain, ctr1Δctr4Δ (geneotype ctr1::NAT;ctr4::NEO;NAT represents native strain and NEO represents strain resistant to neomycin). These fungal strains were streaked onto solid SC Media (SC, MP Biomedicals) from frozen glycerol stocks at -80°C. For experiments, one colony was inoculated and grown in SC Media overnight at 30°C at 200 rpm; these overnight cultures were diluted, washed, or aliquoted according to appropriate protocol for each experiment described below.

The Pierce Group, Department of Chemistry, North Carolina State University, generously provided the following thiohydroxamic acids as solids BL-14-04, BL-14-05, BL-09-78, and BL-09-94; 10 mM stock solutions of these hydroxamic acids were prepared in DMSO and used as needed in the experiment. Marian Helsel generously provided 100 mM stock solutions of ThM and ThDfp in DMSO. Phosphate buffer saline, PBS (PBS, Lonza) was used as received.
4.4.2 Growth Curve Assay for Wild Type C. neoformans in SC Media

In order to determine the susceptibility of various test compounds, the broth micro-dilution method described in the Clinical and Laboratory Standards Institute (CLSI).²³

A single colony from C. neoformans grown on solid SC Media was inoculated in liquid SC Media and the liquid SC Media culture was grown overnight at 30 °C at 200 rpm. The overnight cell suspensions was diluted to 0.002 OD₆₀₀ using fresh SC Media and then subsequently aliquots of this 0.002 OD₆₀₀ mixture were pipetted into 96-wells to give a final OD₆₀₀ value of 0.001. Test compounds from stocks solutions in DMSO were added to the 96-wells to give a final concentration ranging from 0-100 µM with less than 1% DMSO. For each growth assay experiment, a compound-free positive growth control was included along with cell-free negative control. In experiments involving Cu(II) ions, a fresh stock solution of Cu(II) was prepared in de-ionized water with CuSO₄, and Cu(II) stock solution was added to the appropriate wells in the 96-well plate to achieve the appropriate final concentration of Cu(II) as indicated in the figures. Similarly in experiments involving BCS, a fresh BCS stock solution was prepared using de-ionized water and BCS was added to the appropriate wells in the 96 well plate to get the concentration desired as indicated in the figure legends.
96-well plates containing fungal cells treated with and without compounds and/or Cu(II) were incubated at 30 °C; OD$_{600}$ values were recorded every 4-6 hr for 48 hr. The OD$_{600}$ values at 48 hr are plotted under various conditions with and without compound along with and without Cu(II). The minimal inhibition concentration was defined as the concentration at which no growth was detected compared to the positive control. For a single experiment, each condition was performed in triplicate and at least two separate experiments were carried out (biological replicates). For a single experiment, the three replicates for each given condition were averaged and the error was calculated as standard deviation. The last time points of each of the biological replicates were compared to see if they have similar outcomes.

4.4.3 Ctr1ΔCtr4Δ Mutant and Wild Type Growth Assays

Overnight cultures of ctr1Δctr4Δ and wild type C. neoformans were washed thrice in PBS, pH 7.4 and after washing suspended again in YPEG media (BD, Franklin Lakes, NJ). Cells suspensions were then diluted to OD$_{600}$ 0.001 using fresh YPEG; wild-type cells were plated on the top-half of the 96-well plates while the bottom half of the 96-well plates were plated with ctr1Δctr4Δ mutant. Test compounds from stocks solutions in DMSO were added to the 96-wells to give a final concentration ranging from 0-100 µM with less than 1% DMSO. For each growth assay experiment, a compound-free positive growth control was included along with cell-free negative control. For the wild-type strain
the compound-free positive control normal growth was observed; on the other hand for the ctr1Δctr4Δ mutant compound-free positive control, no growth in YPEG was observed. Plates were incubated at 30 °C and OD₆₀₀ readings were be taken at 24 and 48 hr. The percentage growth plots were obtained by plotting OD₆₀₀ readings for each condition normalized against wild type compound free positive control. For a single experiment, each condition was performed in triplicate and at least two separate experiments were carried out (biological replicates). For a single experiment, the three replicates for each given condition were averaged and the error was calculated as standard deviation. The last time points of each of the biological replicates were compared to see if they have similar outcomes.

4.4.4 Calcein Florescence Recovery Experiments

The calcein florescence recovery experiments were performed in 96-well plate format using a florescence plate reader (Perkin-Elmer Victor 3V); each experiment was performed at least twice. For these experiments, 10 mM stock solution of calcein in DMSO, 10 mM CuSO₄ stock solution and 10 mM stock solution of chelators in DMSO was used. In these experiments, each row of the 96-well plate corresponds to a chelator being tested for its ability to remove Cu from calcein and in turn recovering calcein florescence. In the assay, 100 µL of 4µM calcein solution in PBS, pH 7.4 was added to wells A1 through G1 followed by an addition of 100 µL PBS, pH 7.4 to all these wells; these wells serve as the
positive control of total unquenched calcein florescence. Next, using a multichannel pipette, 100 µL solution containing 4 µM calcein and 4.4 µM CuSO₄ in PBS, pH 7.4 (prepared using the 10 mM calcein and 10 mM CuSO₄ stock solution) was added to wells A11 through G2. Next 100 µL of 132 µM (prepared using 10 mM stock solution) compound of interest was added to well A11 and serial dilutions were performed down the entire row until well A3, when the 100 µL was discarded. Similar serial dilutions were performed with the rest of the compounds in rows B-G. Next, 100 µL of PBS, pH 7.4 was added to wells A11-G2. The wells A3 through G3 serve as the negative control because they have no chelator present, so calcein’s florescence is quenched to the maximum extent. The plates were then shaken for 30 seconds in the plate reader and then scanned using excitation/emission wavelength of 495/515 nm. An initial florescence reading was taken and the plates were covered in foil and incubated at room temperature for 1 hr followed by another florescence measurement.
Appendix A: List of compounds tested in further biological screen against wild type *C. neoformans*, H99

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum Inhibitory concentration (MIC)</th>
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<tr>
<td></td>
<td>0 mM CuSO₄</td>
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<tr>
<td>5-nitroquinolin-8-ol (NX)</td>
<td>3.12 µM</td>
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<tr>
<td>1-benzyl-3-(pyridine-2-yl)thiourea (BPTU)</td>
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</tr>
<tr>
<td>1-H-imidazole-2-thiol (2MI)</td>
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<tr>
<td>2-mercaptobenzothioazole (2MBT)</td>
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<tr>
<td>2-mercaptoimidazole (2MBI)</td>
<td>&gt;100 µM</td>
</tr>
<tr>
<td>2-(1H-tetrazol-5-yl)-phenol (2TPh)</td>
<td>&gt; 100 µM</td>
</tr>
</tbody>
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*at 1 mM CuSO₄ and 100 µM (the highest concentration of compound tested), the *C. neoformans* start to grow again.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimum Inhibitory concentration (MIC)</th>
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<tr>
<td></td>
<td>0 mM CuSO$_4$</td>
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<tr>
<td>2-(4-methoxy-6-methyl pyrimidin-2-yl)phenol (2MMPP)</td>
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</tr>
<tr>
<td>2-(1H-tetrazol-5-yl)pyridine (2TPy)</td>
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<tr>
<td>Ethyl Maltol (EMal)</td>
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<th>Compound</th>
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<tr>
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References


24. Caragounis, A.; Du, T.; Filiz, G.; Laughton, Katrina M.; Volitakis, I.; Sharples, Robyn A.; Cherny, Robert A.; Masters, Colin L.; Drew,

