Spring 5-1-2014

Synthesis and Characterization of Co(II) and Cu(II) Pyrophosphate Coordination Complexes for Treatment of Pathogenic Agents.

Leann Jade Miles

Synthesis and Characterization of Co(II) and Cu(II) Pyrophosphate Coordination Complexes for Treatment of Pathogenic Agents.

A Distinction Project Submitted in Partial Fulfillment of the

Requirements of the Renée Crown University Honors Program at

Syracuse University

Leann Jade Miles

Candidate for B.S. Degree

and Renée Crown University Honors

May 2014

Honors Capstone Project in Biochemistry

Capstone Project Advisor: _____
Advisor Title & Name

Capstone Project Reader: _____
Reader Title & Name

Honors Director: _____
Stephen Kuusisto, Director

Date:

Abstract

Candidiasis, the yeast infection of the mouth, genitals, and blood can be fatal in individuals who are immunocompromised. In humans, Candidiasis is most commonly caused by the yeast, *Candida albicans* (*C. albicans*), but can also be caused by other *Candida* species .[1]

C. albicans demonstrates marked resistance towards Cu(II) but more sensitivity towards Co(II) compounds, especially in mutants that lack the Ca*CRP1* and Ca*CUP1* gene that encodes for the copper-transporting P-type ATPase and Metallothionein proteins, respectively.[2] Here, I will describe the effects of Co(II) and Cu(II) metal based complexes that incorporate a bridging pyrophosphate (PPi) moiety.

Currently, we are exploring potential mechanisms of copper resistance and cobalt sensitivity in *C. albicans*. We hope that in doing so, we can deepen our understanding of the nature of *C. albicans* sensitivity and shed more light on potential development of therapeutics.

The main focus of this report will focus on the relationship between Co(II) and Cu(II) during *in vitro* assays of *C. albicans* clinical isolates to measure growth and metal retention in *C. albicans*.

Executive Summary

Recent years have seen a rise in fungal infections due factors such as poor patient adherence to long term treatment and the increasing number of patients with severe immunosuppression. While life expectancy has increased by more than ten years, this has also led to an increase in the number of fungal infections and subsequent antimicrobial resistance. The rise in drug resistance is currently an issue of much concern in the public world health sector and emphasizes the crucial need for developments of new classes of antimicrobial therapies.

Of current fungal infections, candidiasis will be the focus of our discussion. Commonly caused by the opportunistic yeast, *Candida albicans*, candidiasis includes oropharyngeal/ esophageal, vulvovaginal, and invasive candidiasis which are infections of the mouth, genitals, and blood. *Candida* infections are commonly treated by azole-class drugs such as first line defense drugs, fluconazole and itronazole. However, the current trend in resistance to first- and second-line defense therapies has led our lab to explore the use of metal-based compounds as potential alternative antimicrobial therapeutics. We are particularly interested in the use of Co^{+2}- and Cu^{+2}- pyrophosphate (PPi) bridged complexes.

We hypothesize that specificity for and efficacy against *Candida albicans* can be achieved by using Co^{+2} and Cu^{+2} complexes featuring the PPi moiety. We also hypothesize that *C. albicans* will demonstrate higher sensitivity towards pyrophosphate-incorporated Co^{+2} than to Cu^{+2} complexes due to mechanisms associated with evolution of resistance.

The first aim of my project was to resynthesize and character Co^{+2} and Cu^{+2} complexes, $\{[Co(phen)_2]_2(\mu\text{-}P_2O_7)\}$ (**CoD**) and $\{[Cu(phen)]_2(\mu\text{-}P_2O_7)\}$ (**CuD**). My second aim was to work in collaboration with Dr. Michael Cynamon at the Veterans Affair Hospital (Syracuse, NY) to perform *in vitro* assays on *C. albicans* isolates and Saccharomyces cerevisiae. All *in vitro* assays

were done in the Cynamon group's Biosafety Level 3 (BSL 3) facility, thus allowing us to work with *Candida albicans,* a BSL 2 organism. My third aim was to perform *in vitro* assays of mutant *C. albicans* strains, KC643 WT, KC2 WT, KC6 *crp1*Δ, KC7 *crp1*Δ, KC24 *cup1*Δ/*crp1*Δ, and KC12 *cup1*Δ which were kindly donated to us by Dr. Daniel Kornitzer, Technion-Israel Institute of Technology, Israel. These mutant strains lack the gene required to synthesize metallothionein (Ca*CUP1* gene) and the gene responsible of the P-type ATPase transporter protein (Ca*CRP1* gene). My third goal is to perform Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) to measure metal retention in these organisms.

Once CoD and CuD were synthesized, *in vitro* assays consisted of microtiter assays that determined the minimum inhibitory concentrations (MIC) in µg/mL concentrations. Mueller-Hinton broth (MHB) was used for *S. cerevisiae*. *S. cerevisiae* contains two copper import transporters, Ctr1 and Ctr3, and was used as a quality control assay.

RPMI media was initially used during the *in vitro* assays because it was approved by the Clinical and Laboratory Standard Institute (CLSI). Strains tested in RPMI were isolate #1, KC643 WT, and KC24 *cup1*Δ/*crp1*Δ. All strains except for *S. cerevisiae* were also tested in YPD media with uridine (KC12 *cup1*Δ and KC2 WT) and without (KC643 WT, KC24 *cup1*Δ/*crp1*Δ, KC6 *crp1*Δ, and KC7 *crp1*Δ).

In RPMI media, isolate #1 and KC643 demonstrated higher sensitivity towards CoD than towards CuD. Meanwhile, mutant, *cup1*Δ/*crp1*Δ, exhibited greater sensitivity towards CuD than to CoD. This aligned with our expectations because the mutant lacked both genes responsible for sequestering or effluxing copper ions and Co^{+2} should therefore be more active. In YPD media, the same trend is observed again. MIC values of the remaining mutants, *cup1*Δ and *crp1*Δ, shows that the CuD complex has higher activity than the CoD. Interestingly, *crp1*Δ is more sensitive

towards CuD than was *cup1*Δ. This finding further suggests that the copper extrusion pump plays a large role than the copper binding metallothionein in copper resistance. The double gene knockout mutant, *cup1*Δ/*crp1*Δ, has the lowest MIC value of all the strains, suggesting that both types of resistant mechanism takes part in lowering intracellular copper toxicity.

It should also be noted that all isolates grown in YPD media showed higher growth than when grown in RPMI and CoD is significantly less active. A likely explanation is that the YPD media contain unspecified metal chelators that diminishes the cytotoxic affect of CoD and CuD. Varying the media emphasizes the importance of these the types of conditions in which these organisms thrive. *C. albicans* are found in the mammalian digestive tracts where copper concentrations are naturally high. While copper is an essential cofactor at low concentrations, it becomes toxic at higher concentrations. Thus, *C. albicans* must have adapted to its ecological niche to survive. In turn, *C. albicans* is not naturally exposed to toxic levels of cobalt metals in its environment. Therefore, it has not had to opportunity to evolve mechanisms of cobalt resistance.

To help explain the possible chelating effect of YPD media, future work includes performing assays in MHB where extracellular metal binding is not observed. Finally, ICP-AES assays will be performed to further understand the behavior of intracellular copper and cobalt metals.

Acknowlegement

I would like thank Professor Robert Doyle for welcoming me into his lab and for becoming my valued advisor and mentor. I am grateful for all of Professor Doyle's patience, support, and counsel throughout the three years that I have been a part of his lab. During my time as part of the Doyle group, I have had the pleasure of working with PhD candidate, friend and mentor: Amanda Hoffman. I would not be here today if not for Amanda's guidance, advice, and patience. In addition to Amanda's guidance, I will forever cherish the help and support from the rest of the Doyle have made my experience so memorable.

Of course, my project would not be complete if not for our collaboration with Dr. Michael Cynamon at the Veterans Affairs Hospital. I am grateful towards Dr. Cynamon collaborating for welcoming me into his lab. I have thoroughly enjoyed my time working with the Cynamon group and especially with Carolyn Shoen, to whom I owe much of my microbiology experience.

I would also like to thank the Coronat Scholars Program for granting me a financial means of attending Syracuse University.

Last, but not least, I would not be here today if not for the Renée Crown University Honors. Thank you for being a large of part of my SU experience. I would not be here today if not for your Crown Wise Award and supportive community.

Table of Contents

Introduction

1.0) Hypothesis and Specific Aims

We **hypothesize** that specificity for and efficacy against *C. albicans* can be achieved by using Co(II) and Cu(II) complexes featuring a pyrophosphate (PPi) moiety. We also hypothesize that *C. albicans* will demonstrate higher sensitivity towards pyrophosphate-incorporated Co(II) complexes as compared to the copper analogues through mechanisms associated with evolution of resistance.

My first aim was to synthesize and characterize the Co(II) and Cu(II) complexes that incorporated pyrophosphate. My second aim was to perform *in vitro* testing of these complexes against *Candida albicans*, *Candida krusei*, *Candida tropicalis*, and *Saccharomyces cerevisiae*. My final aim was to perform *in vitro* assays on single and double gene knockout strains of *C. albicans*: KC643 WT, KC2 WT, KC6 *crp1*Δ, KC7 *crp1*Δ, KC24 *cup1*Δ/*crp1*Δ, and KC12 *cup1*Δ.

1.1) Overview of Candidiasis[3]

Candidiasis, colloquially known as "yeast infection", is a type of fungal infection that occurs due to an imbalance of microorganisms at a particular location on the host and is most prevalent in the United States. Usually caused by the opportunistic yeast, *Candida albicans* of the *Candida* genus. This type of mycosis causes severe diseases in immunocompromised individuals which can be fatal. Oropharyngeal candidiasis, also known as 'thrush', is marked by the distinctive whitening of the tongue due to yeast overgrowth. Thrush is common in people undergoing immunosuppressive therapy for cancer, patients with HIV/AIDS, after antimicrobial therapy, or those receiving immune suppressive therapy after organ transplantation. *C. albicans* is also the pathogen involved in vaginal yeast infections.

Infections are diagnosed through either a microscopic examination or culturing a sample from the patient. During microscopic examinations, the affected area is swabbed or scraped and treated with potassium hydroxide (KOH). KOH dissolves the skin cells, leaving just *C. albicans* to be visualized and examined. In the culturing method, the infected area is swabbed, streaked onto a culture medium, incubated at 37 ˚C for several days, and the colonies are then examined. A *C. albicans* colony is round, smooth, and white.

C. albicans is present in healthy humans, especially in the oral and intestinal flora, and are in constant competition with other microorganisms for a particular location on the host. Until an environmental imbalance occurs, *C. albicans* lives with other organisms in a commensal relationship. An imbalance can be caused by stressors such as changes in pH or hormones, oftentimes allowing *C. albicans* to outcompete the other organisms and overpopulate, leading to disease.

1.4) *Candida albicans* and Treatment[4]

There are five main antifungal drug classes, each with its own targets. For example, fluorinated pyrimidine analogs (5-FC) target the synthesis of RNA and DNA by interfering with DNA replication. Polyene drugs, Nystatin and Amphotericin B (AMB), have heterocyclic moieties that allow them to insert into the lipid bilayers of *C. albican*'s, increasing the cell's permeability by binding to ergosterol, and forming pores. Evidence shows that polyenes cause oxidative damage and permit the efflux of cations, leading to cell death.

Unlike polyenes, azoles are fungistatic meaning that growth is inhibited, and the organism is not killed. Fluconazole (Flu) is the azole first line of defense against *C. albicans* infections and interferes with sterol biosynthesis by inhibiting lanosterol demethylase, a key enzyme involved in ergosterol synthesis. Levels of ergosterol are depleted, which decreases

membrane integrity decreases and increases membrane permeability leading to fungal growth inhibition.

Unlike earlier azole drugs such as ketoconazole, the synthetic triazole fluconazole offers several advantages such as increased solubility, longer plasma half-life, and improved pharmacokinetic properties.

Need for New Drug Development to Combat Drug Resistance

2.0) Rise in Drug Resistance

The increase in drug-, multidrug-, and extensively drug resistant bacteria and yeast compromises the effectiveness of existing antibiotics and antifungal treatment. In recent years, there has been little effort in developing new classes of broth drugs. The resistance to current treatments emphasizes the crucial need for new drug development, unfortunately, there are relatively few new antibacterial compounds undergoing development, and few new classes of antibacterial and antifungal drug therapy has been introduced to the market in the past three decades.[5] The absence of new classes of drugs highlights the difficulties in discovering and clinically testing new therapeutic agents.[6]

Avenues of approach include biogenesis and examining genes thought to be directly involved in regulatory enzymes.[9] Researchers have also attempted to bypass bacteria such as *M. tuberculosis*'s ability to adapt by developing new analogues of established drugs and screening the compounds for activity using *in vitro* whole cell assays. As a result, private and public research groups focus on finding new targets for potential antibiotic compounds. However, many current treatments share similar or the same cellular targets, resulting in cross-resistance.[9]

2.1) Drug Resistance in Candidiasis [1,6,7]

Despite promising results from front line drugs, recent studies show that prolonged fluconazole treatment resulted in treatment failures and *C. albicans* eventually develops a higher resistance. It is possible that fluconazole is either not being retained (cellular uptake or efflux) or that the drug target (14α-sterol demethylase) has changed. It is also possible that the membrane composition has been altered and permeability towards fluconazole has decreased.

Recent studies found that at high fluconazole concentrations, the rate of accumulation plateaus, suggesting that the drug enters by facilitated diffusion. Additionally, there are two genes, *BEN*^{r} and the *CDR1* which encodes for proteins implicated in drug resistance and ATP-binding cassette-type transporter, respectively. Studies have also found that *C. albicans* isolates that are resistant to fluconazole also expressed high levels of *BEN*^{r}, while isolates that are resistant towards other azoles are possibly associated with elevated levels of *CDR1* expression. While increases in *CDR1* and *BEN*^{r} levels do not necessarily lead to increased levels of their products in *in vitro* systems, there is indirect evidence that Cdr1 and Ben^r multidrug transporters in *C. albicans* use azoles as substrates in drug efflux. It is also plausible that elevated levels of Ben^r and Cdr^1 proteins interfere with systems that are directly involved with multidrug resistance. If so, this regulatory function would follow recent evidence that suggest different efflux systems are working together to form drug resistance mechanisms.

2.2) Unusual Resistance of *Candida albicans* to Copper

Interestingly, *C. albicans* has a high level resistance to elevated concentrations of copper, especially when compared to *Saccharomyces cerevisiae*, and previous gene disruption studies indicate that the copper-transporting P-type ATPase, coded by Ca*CRP1*, is largely responsible for this unusual resistance. A second gene, Ca*CUP1* which codes for metallothionein, has also been implicated in copper resistance by functioning in residual copper binding. Under acidic, anaerobic conditions the function of Ca*CRP1* is crucial for survival, even in very low concentrations of copper.[1]

2.3) Metallotherapeutics: New Generation of Alternative Drugs[6]

Despite roadblocks in finding new treatments for various forms of infectious diseases, a growing area in drug discovery is bioinorganic chemistry. While metal-based therapeutics make up a small percentage of available drugs, utilizing metals in drug delivery offers numerous advantages over organic-based drugs. To begin, metals are cationic which allows them to attract towards the negatively charged yeast cell membranes while also maintaining the metal center(s)'s 3D geometry and ligand arrangements. Metals can also be active in their inert, reactive, and biotransformational forms and metal complexes can even be active in fragmented parts. Thus, metallopharmaceutical complexes offer varying redox states, extensive geometrical diversity, and a great sum effect of metal-ligand interactions.

2.4) Existing Applications of Metals in Medicinal Chemistry[8]

After the breakthrough discovery of cisplatin, metal medicinal chemistry has become a point of interest with a rise in effective compounds used in treatments against diseases such as cancer, diabetes, and rheumatoid arthritis (RA).

Discovered by accident, cisplatin was the first metal-based agent to be used effectively worldwide. A Pt(II)-based square planar compound, cisplatin can be administered alone or in combination with other drugs to treat various types of cancers such as ovarian, lung, and bladder. Cisplatin remains the 'gold-standard' despite the rise of second- and third-line Pt(II) analogues such as carboplatin and oxaliplatin.

In treating diabetes, vanadyl complexes uses V^{+4} and V^{+5} to mimic insulin behavior by oxidizing glucose in adipocytes and reducing blood glucose levels. Currently, bis(ethylmaltolato)oxovanadium(IV), "BEOV", is undergoing phase II clinical trials and is

orally administered. Results show that patients treated with BEOV have a 15% decrease in blood glucose levels relative to levels before the trials.

Aside from vanadium and platinum compounds, gold has two oxidation states that are found in biological conditions; Au^{+} and Au^{+3}, and forms drugs, myochrysine, solganol, sanochrysine, and auranofin that are used to treat rheumatoid arthritis. Of these compounds, only myochrysine, solganol, and sanochryine are soluble and are thus injected into patients while auranofin is administered orally. Gold functions by targeting proteins and small molecules with thiols or thioether functional groups and by saturating throughout the body, accumulating most at sites of inflammation.

2.5) Efficacy of Inorganic Pyrophosphate Complexes[9]

We wanted to synthesize neutral dinuclear divalent compounds by using pyrophosphate as the hydrophilic core. In addition to pyrophosphate, the metal centers would be chelated to aromatic 'capping' ligands to create a hydrophobic component. Pyrophosphates also serve as excellent 'bridging' ligands due to their level of protonation on the ligand species.

Pyrophosphate is a diphosphatetra-anion ($[O_3P\text{-}O\text{-}PO_3]^{4-}$) and plays an important role in bioenergetics processes. Found ubiquitously in nature the –P-O-P— moiety is a major form of energy bond that circulates throughout living cells. Compounds with pyrophosphate have been studied extensively, especially for their roles in coordination chemistry with possible applications as treatment against infectious diseases. However, only 33 metallo-pyrophosphate complexes have been properly characterized and reported to date, and of these compounds, only 18 complexes incorporate pyrophosphate as a bridging construct.

Despite the modest numbers in pyrophosphate complexes, PPi coordination chemistry has garnered much attention, specifically for their roles as potential drugs/prodrugs. More recently, research from Doyle *et al.* and Bose *et al.* have contributed to interest revolving around these pyrophosphate complexes as potential antitumor agents.[11,12] Work in these two labs show that pyrophosphate systems involving Pt(I)/Pt(IV) or dimeric Co(II)/Ni(II)/Cu(II) demonstrated extraordinary toxicity in drug-resistant cancer cell lines. The concentration of Pt-based pyrophosphate compounds of the Bose group at which 50% of cisplatin/carboplatin-resistant A2780/C30 cancer cell line was inhibited (IC_{50} value) was approximated half that of the required cisplatin concentration. These complexes showed little DNA binding, suggesting that pyrophosphate complexes to be potential alternative anticancer agents.[10]

Research performed in our lab utilizes and examines the added cytotoxicity of 1,10-phenanthroline (phen) in dimeric compounds, $\{[Co(phen)_2]_2(\mu\text{-}P_2O_7)\}$ (**CoD**) and $\{[Cu(phen)]_2(\mu\text{-}P_2O_7)\}$ (**CuD**). These compounds appear to have unusually high toxicity against Adriamycin-resistant A2780/AD cell line within the time dependent nano- and pico-molar toxicity range. Meanwhile, cisplatin control has an inhibitory concentration of 11μM. The overall toxicity is the sum of DNA interactions via binding or intercalation, oxidative stress, and topoisomerase I inhibition. Internationalization of these complexes remains relatively unclear, but a possibility is hydrolysis of the pyrophosphate upon entering and activation.[12]

The extraordinary degree of antineoplastic activity of CoD and CuD has led the Doyle group to consider them as possible antibacterial therapeutics.

Materials and Methods

3.0) Materials

Candida albicans ATCC 90028 and *Saccharomyces cerevisiae* were purchased from the American Type Culture Collection (Manassas, VA) and were used as quality control strains. Additional isolates were obtained from the Clinical Microbiology Laboratory, SUNY Upstate Medical Center in Syracuse, NY *Candida albicans* #1 and *C. albicans* strains: KC643 WT, KC2 WT, KC6 *crp1*Δ, KC7 *crp1*Δ, KC24 *cup1*Δ/*crp1*Δ, and KC12 *cup1*Δ were kindly donated to us by Dr. Daniel Kornitzer, Technion-Israel Institute of Technology, Israel.

Susceptibility tests were performed on the isolates according to the Clinical and Laboratory Standard Institute (CLSI) document M27-A3 (Institute 2008). RPMI 1640 from Hyclone Laboratories in Logan, UT was buffered at pH=7 with MOPS, 0.165 M. The control drug, fluconazole (Sigma, St. Louis, MO) was made at 25.6 µg/mL in dimethyl sulfoxide. Finally, cell turbidity was determined by a Klett colorimeter.

3.1) Synthesis {[Co(phen)$_2$]$_2$(µ-P_2O_7)} (CoD)

$CoSO_4$ hexahydrate (0.5622g, 2 mmol) was dissolved in 15 mL of deionized H_2O resulting in a pale pink solution. Solid 1,10-phenanthroline (0.7208 g, 4 mmol) was next added to the solution and allowed to fully dissolve before adding 5mL of aqueous tetrasodium pyrophosphate (0.1659 g, 1 mmol) for a 2:4:1 ratio. The mixture was allowed to stir until a solid pale pink solution with pink precipitate formed. The supernatant and precipitate were separated via centrifugation and the supernatant was filtered for crystallization while the precipitate was dried. Crystallization was conducted through slow evaporation in 25 mL glass beakers, resulting in red-pink crystals after *ca.* 3-4 days. Crystals were also obtained through liquid-liquid

diffusion. This was achieved by dissolving dried precipitate in water and minimal methanol. Crystals formed in about 2-3 days. $C_{54}H_{56}Co_2N_8O_{13}P_2$ (1204.87): calculated. C 53.9, H 4.70, N 9.30; found C 53.8, H 4.69, N 9.31. FTIR (KBr): = 1625 (w), 1516 (s), 1497 (s), 1424 (s), 1342 (s), 1180 (sh), 1103 (br), 1021 (s), 906 (m), 849 (s), 725 (s), 566 (w) cm^{-1}.

3.2) Synthesis of $[Cu(phen)]_2(\mu\text{-}P_2O_7)$ (CuD)

CuD was synthesized in a similar procedure as the synthesis of CoD. First, CuCl (0.2689 g, 2 mmol) was dissolved in in 15 mL of deionized water to create a faint blue solution that was free of precipitates. Next, solid 1,10-phenanthroline (0.3604 g, 2 mmol) was added to the first solution. Finally, 5 mL of aqueous tetrasodium pyrophosphate (0.1659 g, 1 mmol) was added to the solution for a final volume of 20 mL and yielded blue solution with no precipitate. The solution was centrifuged at 4000 rpm for 10 minutes and the supernatant filtered out and crystallized *via* ambient conditions for approximately 3 days. FTIR (KBr): = 1625 (w), 1516 (s), 1497 (s), 1424 (s), 1342 (s), 1180 (sh), 1103 (br), 1021 (s), 906 (m), 849 (s), 725 (s), 566 (w) cm^{-1}.

3.3) Preparation of Antifungal Agents

A dry stock solution of fluconazole was prepared in DMSO for a final concentration of 25.6 µg/mL The CoD drug solution was prepared by dissolving 10 mg of CoD in 10mL of MeOH for a 1000 µg /1mL concentration. Additional CoD drug solutions were prepared at a final concentration of 2000 µg /1mL and 5000 µg /1mL in 10% MeOH. Two CuD drug solutions were prepared as well. The first was performed by adding10 mL of water to 10 mg of CoD (1000 µg/mL) and the solution was placed in an oil bath at 80 ˚C overnight or until fully dissolved. The

second CoD solution was also dissolved in an oil bath, but this time the concentration was 20 mg of CoD in 10 mL of water (2000 µg/mL). All of the solutions were filter-sterilized before using in the *in vitro* assays. A methanol control was also used.

3.4) Determining MICs and CFU of Antifungal Agents

Saccharomyces cerevisiae was cultured and tested in Mueller Hinton Broth (MHB). *Candida albicans* isolates (ATCC 90028, isolate #1, KC643 WT, KC2 WT, KC6 *crp1*Δ, KC7 *crp1*Δ, KC24 *cup1*Δ/*crp1*Δ, and KC12 *cup1*Δ) were cultured in 10 mL of media and incubated overnight. RPMI-MOPS was initially used for *C. albicans* ATCC 90028, isolate #1, KC643 WT, and KC24 *cup1*Δ/*crp1*Δ. YPD(-) uridine media was used for KC643 WT, KC6 *crp1*Δ, and KC7 *crp1*Δ. The *C. albicans* KC643 is the wildtype strain for the KC6 *crp1*Δ and KC7 *crp1*Δ gene knockout mutants. KC2 is the wildtype strain of the *cup1*Δ mutant, KC12. The KC24 *cup1*Δ */crp1*Δ mutant is missing both Ca*CUP1* and Ca*CRP1* genes.

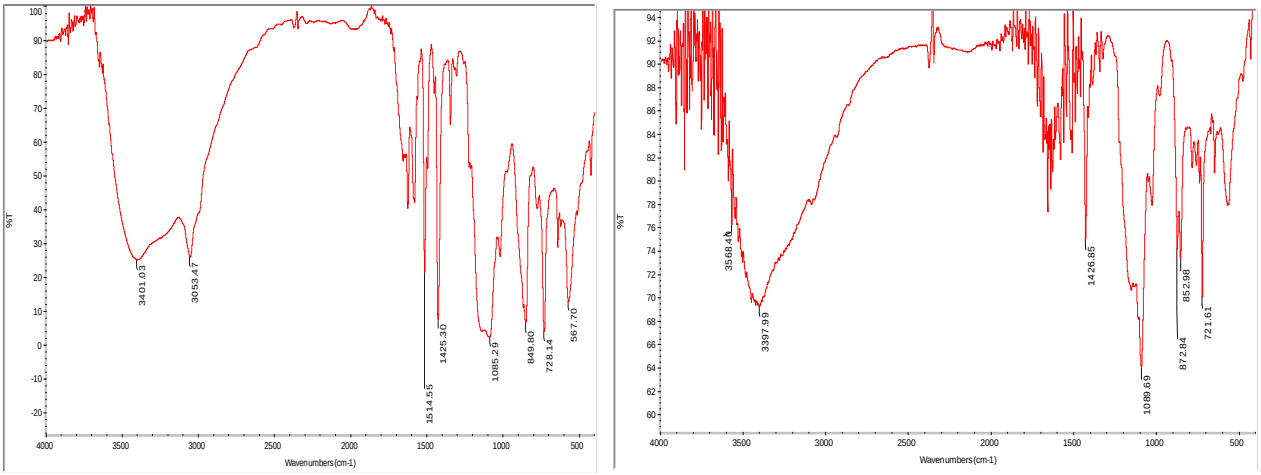
The drugs were prepared at four times the maximum concentration tested and 50 µL of each drug stock solution was added respectively to the first well and serially diluted two-fold, until the last well in which no drug was added to serve as a positive growth control. To prepare fungal isolates, overnight cultures were diluted to a final concentration of approximately 5×10^5 CFU/mL in the respective broth. The actual inoculums were measured by plating and counting CFU on the respective agar after 1:10000 serial dilution in saline with 0.05% Tween 80. The agar plates were incubated for 2-4 days at 37 °C. The microtiter plates were covered with SealPlate adhesive film (Excel Scientific, Wrightwood, CA) and incubated at 37 °C for the same time period as the respective agar plates. The MIC was defined as the lowest concentration of

antifungal agent yielding no visible turbidity. Each isolate was tested in at least one triplicate and a known antifungal was used as a positive control.

Results and Discussion

4.0) Synthesis and Characterization of Compounds CoD and CuD

The CoD compound was synthesized from an aqueous suspension of heptahydrate cobalt (II) sulfate, phen, and tetrasodium pyrophosphate in a 2:4:1 stoichiometric ratio, resulting in a pale pink solution with pink precipitate. Slow crystallization yielded pink-red crystalline blocks after allowing to supernatant to evaporate at room temperature. The crystals were separated from the remaining mother liquor, dried, and characterized by infrared spectroscopy (IR), elemental analysis, and ultraviolet-visible spectroscopy. Meanwhile, the Cu(II) analog was synthesized in a similar manner, but this time with a 2:2:1 stoichiometric ratio. The IR spectrum showed characteristic [P—O—P] stretches at approximately 1103 and 840 cm^{-1} with shoulders at approximately 1164 and 1021 cm^{-1}. The presence of a phenanthroline ring was indicated by bands near 1517 and 1426 cm^{-1}. Water molecules that are shown by the broad band at approximately 3400 cm^{-1} (figure 1).



(a) **(b)**

Figure 1. (a) Infrared spectrum of CoD (b) Infrared spectrum of CuD.

4.1) Crystal Structures of CoD and CuD

Single crystal X-ray diffraction studies of compounds CoD and CuD (see figure 2) show neutral species with binuclear cobalt(II) and copper(II) centers (respectively) and each metal center is chelated to 1,10-phenanthroline rings: two ligated to each cobalt(II) and one ligated to copper(II) ions. Additionally, each metal center is bridged by the hydrophilic pyrophosphate moiety which forms a bis-bidentate structure and forms a six member-chelate ring to each cobalt(II) and copper(II) centers. The overall geometries of CoD and CuD are a distorted octahedral and a distorted square pyramidal with water molecules filling in the axial positions for the copper analogue.

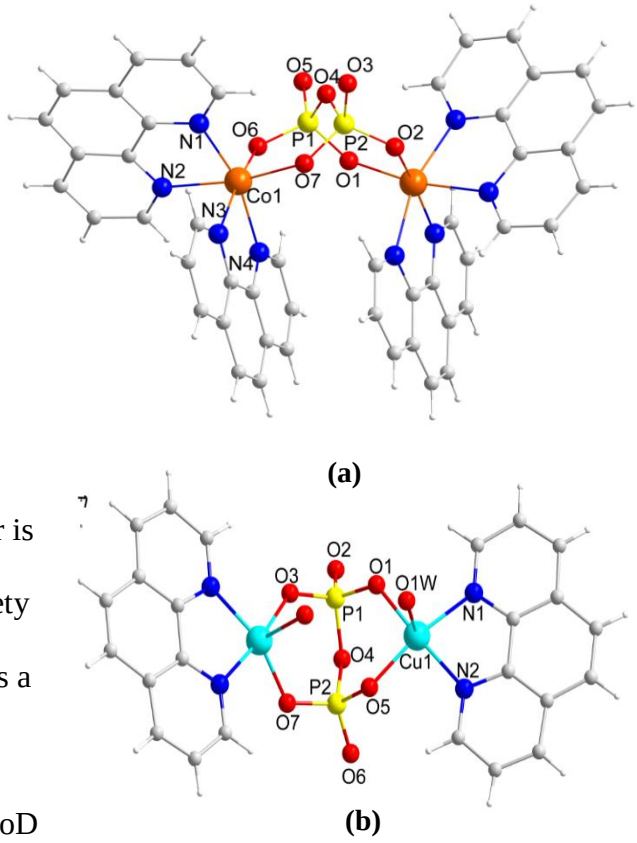


Figure 2. (a) CoD complex (b) CuD complex

The resulting structures have pseudo-layers that contain water molecules in between these layers. This supramolecular effect arises from a combination of intermolecular π- π stacking interaction between the phen ligands.

4.2) *In vitro* Cell Cytotoxicity

When *S. cerevisiae* demonstrated relative resistance towards both the copper dimer and cobalt dimer complexes, but with little variations in MIC between the two (Table 1). According

to the results, *S. cerevisiae* appears to be slightly more susceptible to copper than to the cobalt analogue. This was expected because *S. cerevisiae* possess two copper import proteins, Ctr1 and Ctr3.

When *C. albicans* isolate #1 was tested in RPMI media, CoD had MIC values between 0.977 and 1.9531 µg/mL while the copper dimer's MIC was between 31.25 and 62.5 µg/mL (Table 1). Based on the relative MIC values, *C. albicans* isolate #1 is 32-64 times more sensitive to CoD than to CuD. When the KC643 WT (wildtype for the KC6 and KC7 *crp1*Δ mutants) was tested in the same conditions, CoD demonstrated 32 to 128 times more activity than CuD with an MIC range of 0.244 to 0.488 µg/mL for the CoD and 15.625 to 32.25 µg/mL for the CuD. The maximum fluconazole control (maximally at 6.4 µg/mL) was found to be at 0.8 µg/mL for both wildtype strains

C. albicans isolate #1 and KC643 WT were also tested in minimal YPD (lacking uridine) media using the same assay procedures (Table 2). The results show that the yeast organisms demonstrated higher tolerance towards CuD (MIC at 125-250 µg/mL) than towards CoD (MIC at 62.5 µg/mL). The MIC for the fluconazole control was 0.8 µg/mL. Interestingly, these strains showed significantly enhanced growth in the minimal YPD media than in RPMI (Table 2 and 3).

The KC24 *cup1*Δ/ *crp1*Δ double gene knock-out mutant was tested in both RPMI and minimal YPD media. In RPMI media, the *cup1*Δ/ *crp1*Δ mutant showed higher sensitivity towards CuD than towards the CoD analogue (0.488 µg/mL and 3.125 µg/mL, respectively). This was expected because the *cup1*Δ/ *crp1*Δ mutant lacked the ca*CRP1* and ca*CUP1* genes that encode for the copper resistant mechanisms, metallothionein and P-type ATPase transporter protein. This trend of decreased copper sensitivity is also seen when the *cup1*Δ/ *crp1*Δ mutant was assayed in minimal YPD media. As was the case with wildtype KC643 and isolate #1, there

was significantly more growth in YPD media than in RPMI with the fluconazole showing an MIC value at 0.8 µg/mL instead of the 0.1 µg/mL in RPMI.

Organism	Isolate	Fluconazole (µg/mL)	CoD (µg/mL)	CuD (µg/mL)	Inoculum (10^3)
S. cerevisiae		0.4	3.906	1.953	4.95
S. cerevisiae		0.2	3.906	1.953	5.8
S. cerevisiae		0.8	3.906	1.953	5.1

Table 1. MIC values of *S. cerevisiae* isolates in MHB

Organism	Isolate	Fluconazole (µg/ mL)	CoD (µg/ mL)	CuD (µg/ mL)	Inoculum (10^3)
C. albicans	1	0.1	0.977	31.25	4.8
C. albicans	1	0.1	1.953	62.5	7.95
C. albicans	1	0.1	0.977	62.5	3
C. albicans	1	0.1	0.977	31.25	0.3
C. albicans	1	0.025	0.977	62.5	0.35
C. albicans	KC643 WT	0.05	1.953	15.625	0.1
C. albicans	KC643 WT	0.05	0.244	31.25	0.15
C. albicans	*cup1*Δ/ *crp1*Δ	0.1	3.125	0.488	0.5
C. albicans	*cup1*Δ/ *crp1*Δ	0.1	0.244	0.977	0.15
C. albicans	ATCC 90028	0.2	4.883	15.625	2.2

Table 2. MIC values of *C. albicans* isolates in RMPI-MOPS

Organism	Isolate (-)	Fluconazole (µg/ mL)	CoD (µg/ mL)	CuD (µg/ mL)	Inoculum (10^3)
C. albicans	1	0.8/04	62.5	250	3.65
C. albicans	KC643 WT	0.8	62.5	62.5	0.9
C. albicans	KC643 WT	0.8	62.5	250	0.3
C. albicans	KC643 WT	0.8	62.5	125	3.6
C. albicans	KC643 WT	0.8	62.5	125	1.85
C. albicans	KC643 WT	0.8	(+)	31.25	2.6
C. albicans	KC643 WT	0.8	(+)	125	2.25
C. albicans	KC6 *crp1*Δ	0.8	(+)	62.5	21
C. albicans	KC7 *crp1*Δ	0.8	(+)	62.5	1.55
C. albicans	*cup1*Δ/ *crp1*Δ	0.8	31.25	15.625	2.4
C. albicans	*cup1*Δ/ *crp1*Δ	0.8	125	15.625	2.5
Organism	**Isolate (+)**	**Fluconazole (µg/ mL)**	**CoD (µg/ mL)**	**CuD (µg/ mL)**	**Inoculum (10^3)**
C. albicans	KC12 WT	0.8	(+)	31.25	TBD
C. albicans	KC2 *cup1*Δ	0.8	(+)	31.25	TBD

Table 3. MIC values of *C. albicans* isolates in YPD (+)/(-) Uridine as indicated.

Conclusion

Resistance to fluconazole by *Candida albicans* is a major issue for patients undergoing long term treatment. *C. albicans*, as an opportunistic organism, provides several methods of mutagenesis and offers great promise in deepening our understanding this yeast's resistance. The mutant strains that we used had homozygous *URA3* disruptions. The *URA3* disruption was created by genetically transforming and selectively complimenting a *gal1* mutation. The subsequent recombination with a *CAT* sequence led *GAL1* and one *CAT* copy to be excised.[13]

The mutant strains we used contained had the deletion and complementation of this *URA3* disruption, making the organisms dependent on outside sources of uridine, and were used as quality control constructs. The wildtype KC2 (wildtype for mutants lacking the Ca*CUP1*, "*cup1*Δ") lack the *URA3* gene while the wildtype KC643 (wildtype for mutants lacking the Ca*CRP1* gene, "*crp1* Δ") had the *URA3* disrupted and recomplemented to ensure that the *URA3* disruption itself did not have any effect on cell growth.[13]

Aside from fluconazole, studies by Kornitzer *et al*. have found that *Candida albicans* demonstrated increased resistance against copper(II) metal. The Ca*CUP1* gene sequence is identical to the protein sequence of *C. albicans* copper binding metallothionein and thus works by chelating intracellular copper ions. The Ca*CRP1* gene has all the signature domains of copper transporting ATPases, functions catalytically, and is therefore thought to play a more active role in copper resistance. Of the two main copper mediators, Ca*CRP1* acts by effluxing copper while the Ca*CUP1* functions stoichiometrically.[1]

MIC values obtained for the two wildtype strains and the *Candida albicans* isolate #1 shows that the CoD complex was consistently 32 to 62 times lower than the MIC values required of the CuD. The KC643 WT also demonstrated marked sensitivity towards CoD with MIC 32 to

132 times lower than the MIC values of CuD. Additionally, the double knockout mutants ("*cup1*Δ */crp1*Δ") showed greater sensitivity towards the copper analogue than towards the cobalt complex. This further suggested that CaCRP1 and/or Ca*CUP1* were responsible for the higher resistance in the WT strains. However, according to table 3, CuD was more active in *cup1*Δ */crp1*Δ than in *crp1*Δ and *cup1*Δ alone. This suggests that metallothionein and the copper extrusion pump are working together to reduce intracellular copper concentrations.

When *in vitro* assays were performed in YPD, overall increased growth of all the strains was observed, especially in *Candia albicans* isolate #1 and the two wildtype strains. In these three strains, higher MIC values were observed for CuD than for CoD, as expected, because all three strains should still contain their copper resistance proteins. When comparing the growth rates of the *cup1*Δ and *crp1*Δ mutants, it was found that the strains missing the Ca*CRP1* gene was consistently more susceptible to the Cu(II) complex than to the CoD analogue. Additionally, CuD was more active than CoD was in the *cup1*Δ */crp1*Δ mutant. This suggests that both genes function together for a greater total sum of copper resistance.

Another interesting observation is the lowered activity of CoD when *in vitro* assays were performed in YPD media. The maximum CoD dimer solutions that were used contained both 25% and 2.5% methanol concentrations (drug vehicle control). Slight growth inhibition was observed in 25% methanol, but the MIC values were comparable with those of the methanol control that was also tested (data not shown) and no growth inhibition was seen in 2.5% methanol. This observation is different from that seen when *in vitro* assays were performed in RPMI media in which the CoD demonstrated substantially lower MIC values than its methanol control. Along with decreased susceptibility towards CoD, the *C. albicans* strains also

demonstrated lower MIC values for CuD, indicating a lower sensitivity to both CoD and CuD in YPD media.

A possible explanation for the lowered susceptibility towards CuD and CoD in YPD media could be that YPD contains metal chelators that in turn allows *Candida albicans* to grow. This chelator effect is not observed in RPMI media or in MHB.

The MIC values observed for *S. cerevisiae* showed that the yeast is slightly more sensitive to CuD than to CoD. Additionally, *S. cerevisiae* is more susceptible to CuD than was *C. albicans* in RPMI assays. This observation aligned with our expectations because *S. cerevisiae* possess two copper import transporters, Ctr1 and Ctr3. It is also possible that the actual substrate for these transporters is Cu(I), suggesting that and Cu(II) is therefore reduced in the process.

Candida albicans contains a copper detoxification mechanism through an extensive copper extrusion pump. However, at high copper concentrations, copper catalyzes reactive oxygen species and is especially toxic in anaerobic conditions (such as in the digestive tract). Therefore, in eukaryotes, intracellular copper concentration must be maintained through controlling copper influx and the synthesis of copper chelators such as metallothionein.

It is likely that *C. albicans's* ecological niche has led it to develop a dedicated resistant mechanism. *Candida albicans* thrives in the digestive tract which contains the highest concentration of copper. It was also found that copper becomes even more toxic in anaerobic conditions, which is the case in the digestive tract. As a result, copper extruding pumps may have developed through evolution to let *Candida albicans* survive in its environment. Acting alone, the metallothionein would be unable to handle the amount of entering copper, leading to the shutdown of the system. Therefore, *Candida albicans* likely adapted by developing a second mechanism to pump out a continuous influx of copper: the P-type ATPases extrusion pump.[1]

The observations of *C. albicans* growth in RPMI suggest that *C. albicans* does not have similar resistance mechanisms for cobalt as it does for copper. *In vitro* assays of the Kornitzer strains were discontinued in RPMI because minimal growth was seen all the microtiter plate wells. However, observations of *C. albicans* in YPD media follows our expectations that the mutant strains are more susceptible to copper due to the absence of a copper extrusion pump and/or the copper binding protein, metallothionein.

Routes of investigation for the near future would be completing *in vitro* assays of all strains as well as performing *in vitro* assays in MHB to determine if YPD contain metal binders. Additionally, inductively coupled plasma atomic emission spectroscopy (ICP-AES) will be performed to determine the metal retention time in all seven isolates of *C. albicans.*

References

1. Available at: http://www.cdc.gov/fungal/diseases/candidiasis/. Accessed May 5, 2014

2. Weissman Z, Berdicevsky I, Cavari BZ, Kornitzer D. The high copper tolerance of Candida albicans is mediated by a P-type ATPase. *Proc Natl Acad Sci*., **2000**, *97,* 3520-3525.

3. Williams D, Lewis M., Pathogenesis and treatment of oral candidosis. *J Oral Microbiol*. **2011**, *3*, 5771 – DOI.

4. Cannon RD, Lamping E, Holmes AR, et al., Efflux-mediated antifungal drug resistance. *Clin Microbiol Rev*. **2009**, *22,* 291-321.

5. Duncan K., Barry C.E., 3rd Prospects for new antitubercular drugs, *Curr. Opin. Microbiol.*, **2004**, *7,* 460–465.

6. Graf, N., Lippard, S.J., Redox activation of metal-based prodrugs as a strategy for drug delivery. *Adv. Drug Delivery Rev*. **2012**, *64*, 993-1004.

7. Cannon RD, Lamping E, Holmes AR, et al., Efflux-mediated antifungal drug resistance. *Clin Microbiol Rev*. **2009**, *22,* 291-321.

8. Metals in Medicine?

9. Ikotun, O.F.; Armatus, N.G.; Julve, M.;Kruger, P.E.; Lloret, F.; Nieuwenhuyzen, M.; Doyle, R.P., Coordiation complexes incorporating pyrophosphate: structural overview and exploration of their diverse magnetic, catalytic, and biological properties. *Inorg. Chem*, **2007**, *46*, 6668-6674.

10. Doyle et al

11. Bose et al

12. Hoffman, A.E., DeStefano, M., Shoen, C., Gopinath, K., Warner, D.F., Cynamon, M., Doyle, R.P. Co(II) and Cu(II) pyrophosphate complexes have selectivity and potency against *Mycobacteria* including multidrug-resistant *M. tuberculosis*. *Eur. J. Med. Chem.* **2013**. *70*, 589-93.

13. Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in Candida albicans. *Genetics*. **1993**, *134*, 717-28.