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Clinical isolates of *Candida albicans*, *Candida tropicalis*, and *Candida krusei* have different susceptibilities to Co(II) and Cu(II) complexes of 1,10-phenanthroline

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Abstract The minimal inhibitory concentrations (MICs) of copper and cobalt based dimeric pyrophosphate complexes with capping 1,10-phenanthroline groups on clinical isolates of *C. albicans* (28 isolates), *C. krusei* (20 isolates) and *C. tropicalis* (20 isolates) are reported. *C. albicans* was inhibited by the cobalt complex better than by the copper complex, while *C. krusei* demonstrated the opposite results. *C. tropicalis* showed similar sensitivities to both metals in terms of calculated MIC_{50} values but was more sensitive to cobalt when MIC_{90} values were noted. Knockout strains of *C. albicans* that had the copper efflux protein P-type ATPase (CRP1), the copper binding metallothionein CUP1 or both CRP1/CUP1 removed clearly demonstrate that the origins of copper resistant in *C. albicans* lies primarily in the P-type ATPase, with the MT playing an important secondary role in the absence of the efflux protein. This study suggests that certain strains of *Candida* have evolved to protect against particular metal ions and that in the case of *C. albicans*, a primary invasive fungal species, cobalt may be a good starting-point for new therapeutic development.

Keywords *Candida* · Cobalt · Copper · MIC_{90} · MIC_{50} · Pyrophosphate

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Introduction

Fungal infections are on the rise and resulting diseases currently kill as many as those afflicted with tuberculosis or malaria (Brown et al. 2012). *Candida* species are typically the causative agent among these invasive fungal diseases (IFDs) (Lass-Flörl 2009), so much so that they are now the fourth most common nosocomial bloodstream infection in the United States (Wisplinghoff et al. 2004). Mortality rates in candidemic patients range from 46 to 75 %, with an estimated economic burden of $40,000 per hospital case (Lepak and Andes 2011). The increase in *Candida* incidence is usually attributed to antimicrobial agent administration, admittance to an intensive care unit, extensive surgeries, or chemotherapeutic and immunocompromising diseases, such as HIV (Bross et al. 1989; Hagerty et al. 2003; Karabinis et al. 1988; Kojic and Darouiche 2004; Richet et al. 1991; Samaranayake et al. 2002). *C. albicans* is the most common human disease causing yeast and the primary agent of fungemia (Calderone 2002). Infection from non-*C. albicans Candida* (NCAC) has also been on the rise (Kauffman et al. 2000; Manzano-Gayosso et al. 2008;

Ruan and Hsueh 2009). This increase in NCAC infection may result from better diagnostic tools used in differentiating *Candida* species and/or increased resistance of NCAC to antifungal therapies relative to *C. albicans* susceptibility (Gonzalez et al. 2008). The increase in emerging *C. albicans* and NCAC pathogens requires renewed efforts to develop effective therapeutics with activity against emerging NCAC as well as overcoming resistance observed against older drugs (Silva et al. 2012).

Among the NCAC, there has been increasing infection as a result of *C. tropicalis* and *C. krusei* (Brunke and Hube 2013; Kullberg and Oude Lashof 2002). As antifungal drugs have become more prevalent, a decrease in infection caused by fluconazole-susceptible *Candida* has been followed by a rise in azole-resistant *Candida* species such as *C. krusei* (Marr et al. 2000). Development of antifungal therapies is more difficult than antibacterials, because a compound needs to be effective against eukaryotic cells with limited host toxicity (Silva et al. 2012). This selectivity and oral bioavailability contributes to why the azoles were so beneficial as fungal therapeutics (Heeres et al. 2010).

Metallopharmaceuticals may become important fungicidal therapeutic agents. Metal complexes offer redox chemistry, protein interactions, metal influx/efflux effects, etc. as anti-fungal modalities (Graf and Lippard 2012; Guo and Sadler 1999; Ronconi and Sadler 2007; van Rijt and Sadler 2009). Cobalt complexes have been specifically highlighted as promising therapeutic candidates. In a recent review, Meade et al. highlighted cobalt complexes with varying modes of action, including acting on biomolecules through ligand exchange, modifying the activity of ligated drugs, and activation by bioreduction (Heffern et al. 2013). Copper has been known to be biologically damaging as high intracellular concentrations can lead to oxidized sulfhydryl-groups, destabilized iron-sulfur clusters, and Fenton and Haber–Weiss reactions producing reactive oxygen species (Dupont et al. 2011).

In this work, we explored the susceptibility of *Candida* species to cobalt and copper using compounds that we had previously demonstrated had marked activity against Mycobacteria (Hoffman et al. 2013), namely $\{[Co(phen)_2]_2(\mu\text{-}P_2O_7)\}$ (**1**) and $\{[Cu(phen)]_2(\mu\text{-}P_2O_7)\}$ (**2**) (see Fig. 1) (Ikotun et al. 2008; 2009). **1** and **2** are composed of a cobalt or copper metal center bridged by the pyrophosphate (PPi) ligand with terminal 'capping' 1,10′-phenanthroline (phen) ligands. **1** and **2** were screened against at least 20 (and up to 28) clinical isolates of *C. albicans*, *C. tropicalis*, and *C. krusei*.

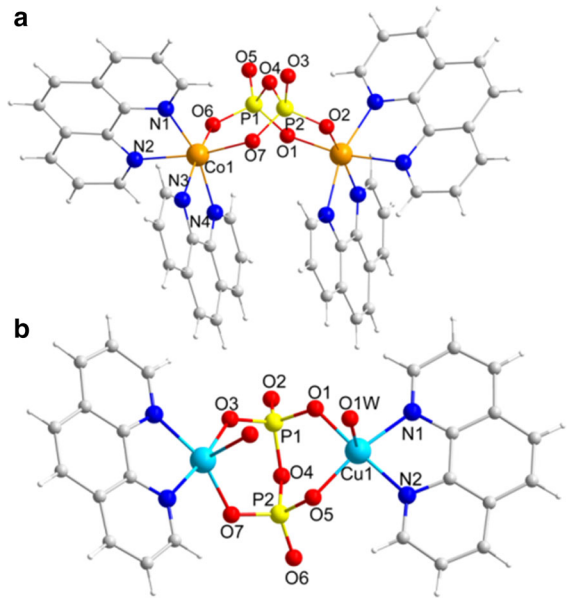

Fig. 1 Schematic of complexes **a** **1** and **b** **2** with atomic labeling scheme and solvent molecules omitted for clarity

Materials and methods

Strains and compounds

C. krusei ATCC 6258, *C. albicans* ATCC 90028, *C. tropicalis* ATCC 750, and *C. parapsilosis* ATCC 22019 purchased from the American Type Culture Collection (Manassas, VA) were used as quality control isolates. Clinical isolates of *C. tropicalis* and *C. krusei* were obtained from Barbara Body, LabCorp of America (Burlington, NC). Clinical isolates of *C. albicans* were obtained from LabCorp of America or the Clinical Microbiology Laboratory, SUNY Upstate Medical Center (Syracuse, NY). *C. albicans* KC knock-out and wild-type (WT) strains were kindly provided by Dr. Daniel Kornitzer of the Department of Molecular Microbiology, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa Israel and used for susceptibility testing. Strain number and Genotype are tabulated below.

Strain number	Genotype
KC643 (WT)	^ura3::imm434/^ura3::imm434 <CaURA3>
KC6 *CRP1*Δ	^ura3::imm434/^ura3::imm434 crp1^::hisG/crp1^::hisG-URA3-hisG
KC24 *CRP1*Δ/*CUP1*Δ	^ura3::imm434/^ura3::imm434 cup1^::hisG/cup1^::hisG crp1^::hisG/crp1^::hisG-URA3-hisG
KC2 (WT)	^ura3::imm434/^ura3::imm434
KC12 *CUP1*Δ	^ura3::imm434/^ura3::imm434 cup1^::hisG/cup1^::hisG

All metal pyrophosphate complexes were synthesized and characterized in accordance with the literature (Ikotun et al. 2009; Ikotun et al. 2008).

Antifungal susceptibility testing

All isolates were tested in accordance with the Clinical and Laboratory Standard Institute (CLSI) document M27-A3 (Institute 2008) using a 50 % turbidity endpoint at 48 h. Briefly, polystyrene 96-well round bottom plates (Corning Inc., Corning, NY) were prepared with 50 μL of modified RPMI 1640 media per well. 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 the fungal isolates, the frozen cultures were thawed and diluted to a final concentration of approximately 0.5×10^3–2.5×10^3 CFU/mL in media. The actual inoculums were measured by counting CFU on the respective agar after 1:10000 serial dilution in saline with 0.05 % Tween 80. The agar plates were incubated for 48 h at 37°. To each well, 50 μL of working stock was added. The microtiter plates were covered with SealPlate adhesive film (Excel Scientific, Wrightwood, CA) and incubated at 37 °C for 48 h. The MIC was defined as the lowest concentration of antifungal agent yielding at least 50 % or less of the growth. Each isolate was tested at least in triplicate and a standard antimicrobial was used as a positive control. RPMI 1640 (HyClone Laboratories, Logan, UT) was buffered to a pH of 7.0 with MOPS, 0.165 M. Fluconazole (Sigma, St. Louis, MO) and voriconazole (Sequoia Research, Pangbourne, UK) were purchased. The range of concentrations tested was 0.00625 –6.4 μg/mL for fluconazole against *C. albicans*, 0.0625–64 μg/mL for fluconazole against *C. tropicalis*, and was 0.0156–16 μg/mL for voriconazole against *C. krusei*. For each *Candida* species tested, one clinical isolate was used as an internal control in each set of experiments, and tested against the control drug (fluconazole or voriconazole), **1**, and **2**. Cell turbidity was determined using a Klett meter (Photoelectric Colorimeter; Manostat Corp., New York, NY). Mueller–Hinton (MH) agar (Becton–Dickinson and Company, Sparks, MD) was prepared as directed and supplemented with 2 % glucose (BBL Microbiology Systems, Cockeyesville, MD).

Knock-out antifungal susceptibility testing

KC643 WT, KC6 *CRP1*Δ, KC7 *CRP1*Δ, and KC24 *CRP1*Δ/*CUP1*Δ were cultured in yeast-peptone-dextrose (YPD) media without uridine. KC2 WT and KC12 *CUP1*Δ were cultured in YPD media supplemented with uridine (1 mg/mL).

Results

The MICs of *C. albicans* isolates were measured against **1** and **2** using fluconazole as a control drug. (Table 1). *C. albicans* isolate 1 was an. internal control. The range of inocula tested was 7.00×10^2–21×10^4 CFU/mL. The MICs for *C albicans* 1 (control isolate) 1 for fluconazole, **1**, and **2** were of 0.4, 15.63 and 62.5 μg/mL respectively.

The MICs of *C. tropicalis* isolates were assayed against **1** and **2**, with isolate 1 used as an internal control (Table 2). The range of inocula tested was 1.30×10^3–8.64×10^4 CFU/mL. The MICs for *C. tropicalis* 1 (control isolate) for fluconazole, **1**, and **2** were 2, 31.25, and 31.25 μg/mL respectively.

The MICs of *C. krusei* isolates were determined against **1** and **2** with voriconazole used as a control drug and *C. krusei* 1 as an internal control (Table 3). The range of inocula tested was 5.50×10^3–7.43×10^4 CFU/mL. Controls for *C. krusei* resulted in MICs for voriconazole ranging from 0.25–0.5 μg/mL,

Table 1 MICs (μg/mL) of fluconazole, **1** and **2** against *C. albicans*

Isolate	Fluconazole	**1**	**2**
1	0.4	15.63	62.5
2	0.4	31.25	31.25
3	0.4	31.25	15.63
4	0.4	31.25	15.63
5	0.8	15.63	15.63
6	0.8	7.81	62.5
7	0.8	15.63	15.63
8	0.4	15.63	31.25
9	0.4	31.25	31.25
10	0.4	15.63	62.5
11	0.4	15.63	31.25
12	0.8	31.25	125
13	0.4	31.25	125
14	0.8	15.63	125
15	0.4	15.63	62.5
16	0.4	62.5	62.5
17	0.1	3.91	31.25
18	0.4	31.25	62.5
19	0.4	31.25	125
20	0.4	31.25	125
21	0.4	15.63	62.5
22	0.2	31.25	15.63
23	0.8	7.82	62.5
24	0.8	31.25	250
25	0.2	31.25	62.5
26	0.2	15.63	31.25
27	0.8	15.63	62.5
28	0.4	15.63	62.5

Table 2 MICs (μg/mL) of fluconazole, **1** and **2** against *C. tropicalis*

Isolate	Fluconazole	**1**	**2**
1	2	31.25	31.25
2	0.25	3.91	31.25
3	0.5	7.81	31.25
4	0.5	15.63	31.25
5	0.5	7.81	15.63
6	0.5	7.81	15.63
7	1	7.81	15.63
8	0.5	62.5	31.25
9	1	31.25	31.25
10	0.5	31.25	31.25
11	1	62.5	15.63
12	8	62.5	15.63
13	1	31.25	31.25
14	4	62.5	31.25
15	0.5	15.63	31.25
16	0.25	62.5	15.63
17	0.25	15.63	31.25
18	0.25	31.25	31.25
19	32	31.25	15.63
20	0.125	62.5	31.25

1 consistently at 3.91 μg/mL, and **2** consistently at 3.91 μg/mL. The MICs for the control *C. krusei* isolate 1 for voriconazole, **1**, and **2** were 0.25, 3.91, and 3.91 μg/mL respectively.

To compare the most active drug across the various *Candida* species, Table 4 below outlines the MIC_{50} and MIC_{90} for the control drug, **1**, and **2**. This table illustrates that the cobalt complex **1** is four-fold more active against *C. albicans* than the copper complex **2**. The cobalt and copper complexes have comparable activity against *C. tropicalis*, with cobalt based **1** having slightly greater activity. However, against *C. krusei*, the copper complex **2** is more potent than the cobalt complex **1**. This led to the study of knock-out isolates in an effort to better understand the decreased activity of the copper based **2** against *C. albicans*.

To determine the effect of the pyrophosphate bridging ligand and the phen capping ligand, phen alone, as well as metal-phen salts, $[CoCl_2(phen)_2]$ (Rubin-Preminger et al. 2008) and $[CuCl(phen)_2]Cl$ (Lu et al. 2004), which maintain a single metal center and phen ligand but lack the PPi, were also tested against the three *Candida* species. Table 5 shows the MIC (μg/mL) results of a control drug, the MIC_{90} of **1** and **2**, the Co-phen salt $[CoCl_2(phen)_2]$, the Cu-phen salt $[CuCl(phen)_2]Cl$, and phen alone against *C. albicans*, *C. tropicalis*, and *C. krusei*. The range of inocula tested was 1.60×10^3–6.29×10^4 CFU/mL.

As the copper resistance-associated P-type ATPase (CRP1) extrusion pump and copper resistance-associated (CUP1) metallothionein (MT) play critical roles in resistance to copper, as evidenced by knock-out mutants being highly sensitive to copper (Hamer et al. 1985) knock-out mutant *C. albicans* isolates were obtained. These knock-out mutants consist of the single CRP1 knock-out KC6 *CRP1*Δ, the single CUP1 knock-out KC12 *CUP1*Δ, and a double knock-out, in

Table 3 MICs (μg/mL) of voriconazole, **1** and **2** against *C. krusei*

Isolate	Voriconazole	**1**	**2**
1	0.25	3.91	3.91
2	0.25	3.91	3.91
3	0.25	1.95	1.95
4	0.5	3.91	1.95
5	0.5	1.95	3.91
6	1	7.81	1.95
7	0.5	3.91	1.95
8	1	3.91	1.95
9	1	7.81	1.95
10	0.125	1.95	7.81
11	0.0625	7.81	1.95
12	4	62.5	1.95
13	0.125	7.81	1.95
14	0.125	7.81	3.91
15	0.25	3.91	3.91
16	0.5	3.91	3.91
17	1	3.91	3.91
18	0.5	3.91	7.81
19	0.5	1.95	3.91
20	0.5	3.91	3.91

which the KC12 *CUP1*Δ isolate was deleted for CRP1 as well, resulting in KC24 *CRP1*Δ/*CUP1*Δ. KC643 WT is the wild-type for the non-uridine dependent isolates and KC2 WT is the wild-type for the uridine dependent isolates. The testing of **2** against these strains aimed to determine if the deletion of these copper related genes had an effect on their sensitivity to **2**, compared to that of the WT, thus establishing a major reason for the greater sensitivity of *C. albicans* to cobalt over copper. While it was expected that **2**, being copper based, would be more potent in the knock-out strains, it was important to determine if administration of **2** resulted in increased sensitivity for the full pyrophosphate and phen ligated complex, since the initial studies using the knock-outs tested simple copper salt. It was also of interest to note whether the MT or efflux pump played any major role in resistance to the copper complex. Table 6 shows the results of this study and demonstrates that, compared to WT, the P-type ATPase (CRP1) knock-out isolate (minimally), and the double P-type ATPase (CRP1) and MT (CUP1) knock-out isolate (especially), are more sensitive to the complex, while the MT (CUP1) knock-out alone shows no difference in MIC.

Table 4 MIC_{50} and MIC_{90} (μg/mL) of *C. albicans*, *C. tropicalis*, and *C. krusei*

Organism	Control		**1**		**2**	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
C. albicans[a]	0.4	0.8	15.63	31.25	62.5	125
C. tropicalis[a]	0.5	4	31.25	62.5	31.25	31.25
C. krusei[b]	0.5	1	3.91	7.81	3.91	3.91

[a] Indicates control used is fluconazole

[b] Indicates control used is voriconazole

Table 5 MIC (μg/mL) and MIC_{90} (μg/mL, for **1** and **2**) of *C. albicans*, *C. tropicalis*, and *C. krusei*

Organism	Control	**1**	$[CoCl_2(phen)_2]$	**2**	$[CuCl(phen)_2]Cl$	phen
C. albicans[a]	0.4	31.25	7.81	125	1.95	0.488
C. tropicalis[a]	0.25	62.5	15.63	31.25	7.81	0.488
C. krusei[b]	0.25	7.81	3.91	3.91	1.95	0.488

[a] Indicates control used is fluconazole

[b] Indicates control used is voriconazole

Table 6 MIC (μg/mL) of *C. albicans* wild-type (WT) and knock-out (Δ) strains

	Fluconazole (μg/mL)	2 (μg/mL)	Inoculum (10^3)
Isolate (−)			
KC643 WT	0.8	125	1.85
KC6 *CRP1*Δ	0.8	62.5	1.55
*CRP1*Δ/*CUP1*Δ	0.8	15.63	2.40
Isolate (+)			
KC2 WT	0.8	125	1.85
KC12 *CUP1*Δ	0.8	125	2.50

KC643 is the wild-type for non-uridine dependence and KC12 is the wild-type for uridine dependence.The inoculum is presented in colony forming units/milliliter (CFU/mL)

− Indicates YPD media without uridine

+ Indicates YPD media with uridine

Discussion

Upon examining the MIC_{90} data for *C. albicans*, the activity of the cobalt based **1** is approximately four-fold greater than the copper based analogue **2**. The MIC_{50} and MIC_{90} for **1** and the MIC_{50} and MIC_{90} for **2** differ by one dilution from one another, suggesting a similar level of activity for the clinical isolates tested. When examining **1** in *C. tropicalis*, the MIC_{50} is lower than the MIC_{90} by one dilution. For **2**, the MIC_{50} and MIC_{90} is the same, showing a smaller range in the activity of **2**. While it appears that the cobalt based **1** has comparable activity in *C. albicans* as it does in *C. tropicalis*, with the MIC_{50} and MIC_{90} both showing a single dilution difference, the copper containing **2** appears to have greater activity in *C. tropicalis* than it does in *C. albicans*. The MIC_{90} of **2** is about four-fold lower in *C. tropicalis* than in *C. albicans*.

An intriguing shift in activity is observed when comparing **1** and **2** in *C. krusei* (see Table 3). The activity of **1** and **2** are both greater in *C. krusei* when compared to *C. albicans* and *C. tropicalis*. Additionally, the activity of **2** in *C. krusei* is the first instance in which the copper complex (**2**) is actually more active than the cobalt complex (**1**) so much so in fact that **2** is comparable to the voriconazole control. Evolutionarily, *Candida* species have developed proteins to traffic metals. *C. albicans* contains multiple copper related proteins including copper resistance-associated P-type ATPase (Weissman et al. 2000), copper transport protein 1 (Marvin et al. 2003), copper resistance-associated MT (Weissman et al. 2000), and copper-binding MT (Riggle and Kumamoto 2000). Homologous proteins were likewise found in *C. tropicalis*, with a 78 % amino acid homology to the copper resistance-associated P-type ATPase (Butler et al. 2009), a 60 % amino acid homology to copper transport protein 1 (Butler et al. 2009), and a 80 % amino acid homology to the copper resistance-associated MT (Butler et al. 2009). However, a BLAST search performed on the copper related proteins did not reveal homologs in *C. krusei*. This lack of homologs of copper associated proteins could perhaps explain why the increased sensitivity to both metals, and particularly copper, was pronounced in *C. krusei*.

When comparing the phen ligand alone to the metal-phen complexes or **1** and **2**, phen has the lowest MIC value. However, as seen in Table 5, the consistent activity of phen across all three *Candida* species suggests that the metal center plays a vital role in the differing susceptibilities of the complexes to the various *Candida* species, and it is likely that the metal center is a key factor in the resulting specificity of a particular metal to a particular species. The metal-phen salts also have impressive activity, perhaps reflecting the potency seen in phen alone. Interestingly, both the Co-phen and the Cu-phen salt have greatest activity against *C. albicans*, and *C. krusei*, and are less potent against *C. tropicalis*. The Cu-phen salt is equally active against *C. albicans* and *C. krusei*, suggesting that the PPi plays a role in the specificity of **2** toward *C. krusei*. The Cu-phen salt and **2** have comparable MICs for *C. krusei*. It is important to note that the MIC90 values used in Table 5 are the results

of **1** and **2** used against multiple clinical isolates, whereas only controls were tested with these other compounds.

It has previously been reported that knock-out isolates lacking the copper related proteins including copper resistance-associated P-type ATPase and copper resistance-associated MT are more sensitive to copper salts.(Weissman et al. 2000) The *CUP1*Δ MT knock-out showed slightly increased susceptibility to copper, with a reduction in the MIC from 20 to 10 mM, the *CRP1*Δ knock-out had a dramatic reduction in MIC to 0.5 mM. The heterozygotes showed no growth on copper at all (Weissman et al. 2000). In testing **2**, comparing the MIC of the knock-out strains against those of the WT, the results show the same increased susceptibility to that of the copper salt (Weissman et al. 2000). The *CUP1*Δ isolate lacking the copper MT showed no change as a result of the gene deletion, with the MIC in KC2 WT being the same as the KC12 *CUP1*Δ knock-out. The *CRP1*Δ isolate had a moderate increase in sensitivity, with the average MIC value about half that of the KC643 WT. Also in line with the copper salt sensitivities, the double *CRP1*Δ/*CUP1*Δ knock-out proved to be the most susceptible, with the MIC decreasing about eight-fold that of the KC643 WT. The fluconazole MIC did not change among these strains.

These findings support the concept that there is a similar mechanism at play for detoxifying copper in fungal species exposured to $CuSO_4$ and to **2**. It also supports the idea that the P-type ATPAse is the primary mode of defense against copper, with the MT presumably up-regulated in its absence. With both the CRP1 and CUP1 absent, there is a marked increase in copper sensitivity greater than the sum of either KO individually, suggesting both work in concert to protect *Candida* against copper.

C. albicans also has been found to have a cobalt uptake protein, COT1 (Butler et al. 2009), with *C. tropicalis* having a homologous protein, with 62 % amino acid homology, known as zinc/cadmium resistance protein (Butler et al. 2009). The trafficking of cobalt in these species may help explain why *C. albicans* and *C. tropicalis* are more susceptible to **1** over **2**. However, delivering a load of cobalt or copper containing drugs could potentially overload the resistance or transport proteins, and render the organism susceptible. The evolution of these metal pathway proteins within *Candida* highlight their vulnerability to metals.

These first generation metal centered complexes provide intriguing results against *Candida*. The work provides an interesting result of cobalt and copper complexes having activity against *Candida* species, but more specifically, that the activity of these two metals is not consistent across various *Candida* species, with the cobalt centered **1** having greater activity in *C. tropicalis* and *C. albicans*, and the copper centered **2** having greater activity in *C. krusei*. While copper is essential in trace amounts, high copper concentrations can quickly become toxic, as copper can catalyze the production of reactive oxygen species (Strain and Culotta 1996). As a result, *C. albicans* has evolved proteins, including CRP1 and CUP1, to mitigate these toxic copper related effects. It is feasible to consider cobalt-containing complexes as lead compounds to combat *C. albicans* infection, as over 28 clinical isolates have demonstrated preferential sensitivity to cobalt as compared to copper. This increased sensitivity to cobalt may be due to *C. albicans* having copper detoxifying proteins, a hypothesis supported by results from analysis of copper MT and efflux protein knock-out *C. albicans* strains. Understanding the mechanism of (or basis for) this difference of particular metal activity in various *Candida* species is of great interest and is currently being explored further. Complex stability is also of interest since hydrolysis will presumably play an important role in uptake/function of the PPi bridged species (Prodrugs). Ongoing work will focus on the mechanism(s) of action of **1** and **2**, in addition to monomeric analogues of these complexes and translating the work to in vivo models.

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