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Short communication

Co(II) and Cu(II) pyrophosphate complexes have selectivity and potency against *Mycobacteria* including *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis (TB) causes up to 10 million incident cases worldwide per annum. Multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains are leading factors in the resurgence of TB cases and the need to produce new agents to combat such infection. Herein, we describe Co(II) and Cu(II) metal based complexes that feature the pyrophosphate ligand with notable selectivity and marked potency against *Mycobacterium tuberculosis*, including MDR strains. Such complexes are confirmed to be bacteriocidal and not affected by efflux inhibitors. Finally, while susceptibility to copper has recently been established for *M. tuberculosis*, the greater efficacy of cobalt observed herein is of considerable note and in line with the discovery of a copper metallothionein in *M. tuberculosis*.

1. Introduction

Mycobacterium tuberculosis (*M. tb*) causes approximately 8.9–9.9 million incident cases of tuberculosis (TB) worldwide per year. [1,2] *M. tb* has an infection rate of one per second, with one-third of the world's population infected as of 2010 [3]. The estimated annual death toll from TB is two million [4], most of which occur in underdeveloped nations. TB leads to increased healthcare and economic burdens [5], and is exacerbated in certain regions by the prevalence of co-infectious diseases such as HIV and malaria [1,6–9].

Poor patient adherence to the prolonged regimen currently required to treat TB has also resulted in the propagation of multidrug-resistant TB (MDR-TB; defined as resistance to isoniazid (INH) and rifampin [10,11]) and, more recently, extensively drug-resistant TB (XDR-TB; defined as resistance to ofloxacin or moxifloxacin and at least one of three injectable second-line drugs: amikacin, capreomycin or kanamycin) [12–16]. MDR- and XDR-TB pose a significant global threat, especially as resistance is outpacing development of new treatments [17]. There are approximately 500,000 cases of MDR-TB worldwide, yet fewer than 10% are treated with effective second-line therapeutics [16]. Even when patients have access to appropriate MDR-TB treatment, emerging strains of XDR-TB are expected to increase as these second-line therapies become more commonplace [18,19].

The MDR-TB and XDR-TB epidemics demand new and innovative solutions [17,20–25], yet no new effective TB drug has reached the market in decades [20,22,25]. Concomitant with the rise of MDR-TB strains is the advancement of medicinal inorganic chemistry. Recent reviews in the field have highlighted the advantages of metallopharmaceuticals as drug candidates. This is due to their accessible redox states, structural diversity, and the ability of the ligand to alter the reactivity of the metal as well as effects the metal ion can have on the ligands [26–29].

Herein, we combine the need for inventive TB treatment with metal-based drugs by utilizing coordination complexes of copper

Abbreviations: TB, tuberculosis; INH, isoniazid; MDR-TB, multidrug-resistant TB; XDR-TB, extensively drug-resistant TB; MOTT, *Mycobacteria* other than TB; MIC, minimum inhibitory concentration.

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and cobalt featuring the pyrophosphate (PPi) ligand. The complexes herein display significant specificity for, and efficacy against, *M. tb*, including clinical isolates associated with MDR-TB. The PPi ligand was chosen as a design tool to produce neutral mono or dinuclear complexes, with hydrophilic 'cores' and hydrophobic exteriors produced through aromatic 'capping' ligands. Such PPi complexes are typically water-soluble and capable of facile hydrolysis making such complexes potential pro-drugs, which, coupled with the redox properties of the metals complexed, is a strong approach to new metal based therapeutics [27]. Previous studies have shown some significant successes with cobalt and copper metal complexes as anti-tubular agents and this in part inspired our initial choice of these metals for PPi coordination complex development [30,31]. The metal-pyrophosphate coordination complexes described herein utilize Co(II) and Cu(II), and have previously been described by Doyle et al. These PPi containing complexes are {[Co(phen)$_2$]$_2$(μ-P_2O_7)} (**1**) and {[Cu(phen)]$_2$(μ-P_2O_7)} (**3**) (see Fig. 1; phen is 1,10′-phenanthroline) [32,33]. Both of these dimeric compounds, in addition to the monomeric analogs, [Co(phen)$_2$($H_2P_2O_7$)] (**2**) and [Cu(phen)(H_2O)($H_2P_2O_7$)] (**4**) (used for comparative purposes; see Fig. 1) [34], were previously shown to have significant antineoplastic activity. Such activity led us to consider them as antibacterial therapeutics [34].

The efficacies of **1**–**4** against a range of bacteria including known human pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *M. tb* and *Mycobacteria* other than TB (MOTT) were assayed and unexpected, but highly significant, selectivity and efficacy against specific *Mycobacteria* was observed. The organisms studied were *M. bovis* bacillus Calmette-Guérin (BCG) Tice, *M. kansasii* ATCC 35775, *M. abscessus* MC 6005, *Mycobacterium smegmatis* ATCC 700084, *E. coli* ATCC 25922, and *S. aureus* ATCC 29213. To quantify these effects, the minimum inhibitory concentrations (MICs) of **1**–**4** were determined for a range of *Mycobacteria* (including a MDR-TB strain), as well as Gram-positive and Gram-negative bacteria, as listed in Table S1 and shown in Fig. 2.

1–**4** were least effective against *S. aureus*, with MIC values in the range 525 μM (312.50 μg/mL; **2**) and 44.79 μM (31.25 μg/mL; **3**). Additionally, **1**-**4** had limited efficacy against *E. coli* with MICs ranging from 143.06 μM (62.51 μg/mL; **4**) to 32.8 μM (19.52 μg/mL; **2**). The complexes were also only moderately effective against the fast-growing mycobacteria, *M. smegmatis*, and *M. kansasii*.

Four different strains were used to elucidate the potency of **1**-**4** against *M. tb* in liquid media. Strains were tested at one of two facilities in the US and South Africa using two methods to confirm reliability (see Supplementary materials). The drug susceptible strains of *M. tb* Erdman ATCC 35801, *M. tb* H37Rv [35] and *M. tb* HN878 [36] were used to determine if **1**–**4** inhibited *M. tb* growth. Additionally, the drug-resistant MDR *M. tb* ATCC 49967, which is moderately resistant to INH, ethionamide, capreomycin, clarithromycin, and resistant to pyrazinamide, cycloserine, and rifampin [37] was studied.

M. tb H37Rv had MIC values range from 2.05 μM (1.22 μg/mL) for **2**–71.53 μM (31.25 μg/mL) for **4**. The MICs against *M. tb* Erdman ranged from 35.8 μM (15.6 μg/mL) for **4**–4.82 μM (4.88 μg/mL) for **1**. *M. tb* HN878 had similar MIC values for **1** to *M. tb* Erdman, namely 2.70 μM (2.73 μg/mL). Consistent with its close genetic relatedness to *M. tb*, *BCG* exhibited comparable values at 18.3 μM (8 μg/mL) and 1.98 μM (2 μg/mL) for **4** and **1**, respectively. Of significance, the *M. tb* MDR strain had low observed MIC values with 2.8 μM (1.95 μg/mL) for **3** and 2.41 μM (2.44 μg/mL) for **1**.

Simple cobalt ($CoCl_2 \cdot 6H_2O$) and copper ($CuCl_2 \cdot 2H_2O$) salts were administered to *Mycobacterium smegmatis* to determine the toxic effect of metal salt as compared to the complexes. Whereas the MIC for **1** in *M. smegmatis* is 19.08 μM, that of $CoCl_2$ is 2.63 mM. The MIC for **3** is 13.63 μM in *M. smegmatis*, but that of $CuCl_2$ is 3.67 mM. The MIC values of the respective metal salts are significantly higher than those of the corresponding metal containing complexes, suggesting that the full complex is important in inhibiting bacterial growth, rather than simply the metal alone. As a class of control compounds, [$CoCl_2$(phen)$_2$] [38] and [CuCl(phen)$_2$]Cl [39] were synthesized and tested against a sampling of the bacteria, including *M. smegmatis*, *Escherichia coli*, *M. tb* Erdman, and MDR *M. tb*, to understand the activity of hydrolytic products or fragments of **1**–**4**. In *M. smegmatis*, [$CoCl_2$(phen)$_2$] performed less effectively in terms of μM, with an activity of 19.21 μM (9.77 μg/mL). This was also true for [CuCl(phen)$_2$]Cl in *M. smegmatis* with an activity of 17.92 μM (9.44 μg/mL.

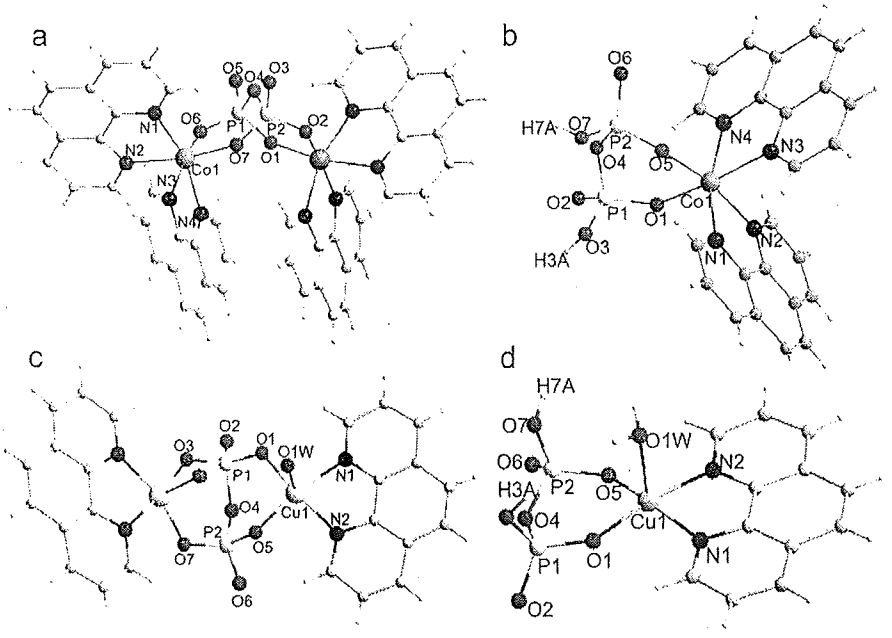

Fig. 1. Schematic of complexes (a) **1**, (b) **2**, (c) **3**, and (d) **4** with atomic labeling scheme and solvent molecules omitted for clarity.

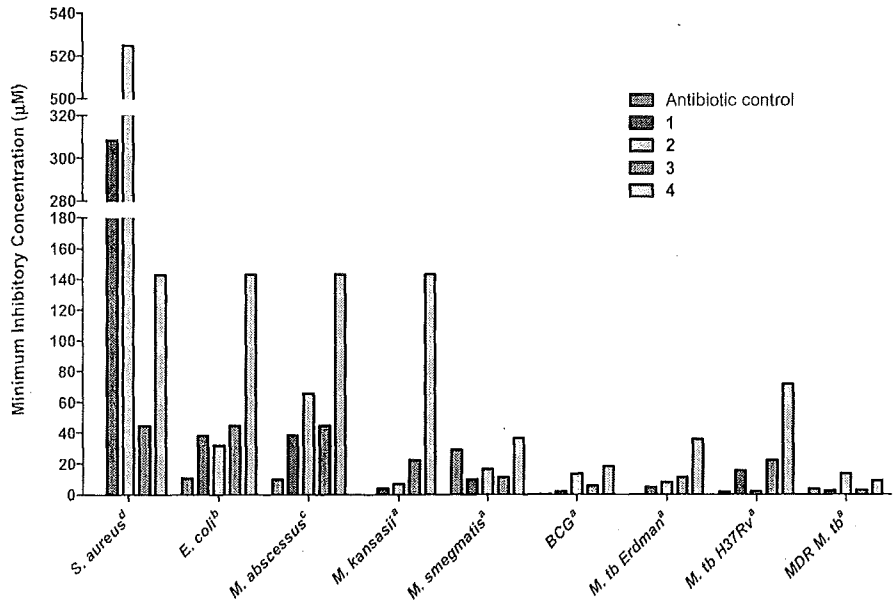

Fig. 2. Bar graph of MICs (μM) of 1, 2, 3, and 4 in screened bacteria. [a]Indicates control used was INH; [b]indicates control used was doxycycline; [c]indicates control used was moxifloxacin; [d]indicates control used was rifampin. Note the control for *M. tb* Erdman is not visible with an MIC value of 0.11 μM. Only **1** was tested against *M. tb* HN878 for comparative purposes. Table S1 of μM and μg/mL MIC values are provided in the Supplementary materials.

Controls were less active in *E. coli*, with $[CoCl_2(phen)_2]$ having an MIC of 76.85 μM (39.07 μg/mL) and $[CuCl(phen)_2]Cl$ showing activity of 35.85 μM (18.89 μg/mL). Interestingly, when applied to *M. tb*, the efficacy of **1** was greater than that of the component controls. $[CoCl_2(phen)_2]$ MIC in *M. tb* Erdman was 12.5 μM (6.35 μg/mL) and the $[CuCl(phen)_2]Cl$ MIC was 25 μM (13.17 μg/mL), whereas in *M. tb* Erdman **1** was 4.82 μM (4.88 μg/mL). In MDR *M. tb*, $[CoCl_2(phen)_2]$ showed no activity difference from drug-susceptible *M. tb*, maintaining the same MIC, 12.5 μM (6.35 μg/mL). Akin to the greater activity of Cu based **3** in drug-resistant *M. tb*, $[CuCl(phen)_2]Cl$ did have an increase in activity from *M. tb* Erdman to MDR *M. tb*, with an MIC of 12.5 μM (6.59 μg/mL) in the MDR line. This activity was still not greater than **3**, however, which had an MIC of 2.8 μM (1.95 μg/mL) in the MDR line. Phen alone was also administered to *M. tb* Erdman and MDR *M. tb*, with an observed MIC of 12.5 μM, again considerably less effective than **1**.

A time-kill experiment was also performed using **1** (the most effective compound) to measure whether **1** inhibits growth or is bactericidal. *M. smegmatis* was incubated with **1** at 3×, 5×, and 15× the calculated MIC (19.32 μg/mL; see Fig. 3). Moxifloxacin at 15× MIC [40] was also incubated with *M. smegmatis* as internal control, producing a bactericidal killing curve in accordance with prior

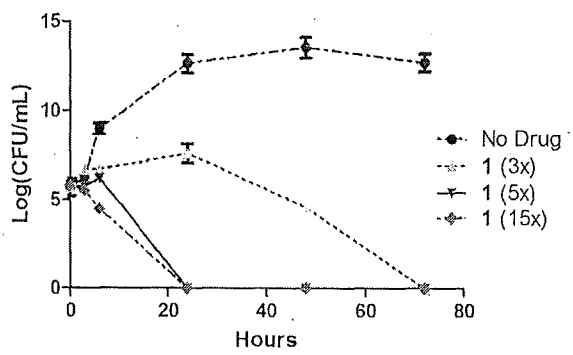

Fig. 3. Time-kill curve of *M. smegmatis* with **1**.

literature [40]. It is apparent that **1** inhibits further growth of *M. smegmatis* over the first 24 h at 3× MIC. Within 48 h, **1** at 3× produced a bactericidal effect, causing a log (CFU/mL) reduction of ~1.1. Rapid bactericidal activity was observed with **1** at 5× and 15× within 24 h 15× **1** is bactericidal yielding a log (CFU/mL) reduction of ~1.25 at 6 h, and below the level of detection at 24 h.

The lung cancer cell line A549 was treated with **1**–**4** with a one and 6 h incubation period. After incubation with the drug for 1 h, the dimeric copper based **3** was the most toxic of the four complexes, having an IC_{50} value of 13.4 ± 4.7 μM, followed by the monomeric copper based **4**, exhibiting an IC_{50} of 130.2 ± 56.0 μM. The cobalt containing complexes **1** and **2** had IC_{50} values an order of magnitude higher, being 342.9 ± 87.5 μM and 479.3 ± 50.9 μM respectively. At 6 h of incubation with the complexes, **3** was once again most lethal with an IC_{50} value (μM) of 0.0115 ± 0.0105 whereas **1** had a much higher IC_{50} value (μM) of 53.1 ± 9.9 indicating the cobalt complexes, while most effective against *M. tb*, were significantly less toxic to human lung cells. This observation suggests the exploration of cobalt over copper in anti-TB metallopharmaceutical development.

In both the mammalian cytotoxicity work and this bacterial work, the most competitive copper-containing compound is the dimeric complex **3**, which has comparable MICs to the dimeric cobalt complex **1**. A recent report highlighting copper resistance as essential for *M. tb* to maintain virulence may explain why **3** produces such effective MIC values in *M. tb* [41]. The outer membrane channel protein Rv1698 of *M. tb* (MctB; mycobacterial Cu^{2+} transport protein B) is reported to efflux Cu^{2+} from the mycobacterial cell envelope. This Cu^{2+} efflux is deemed necessary for mycobacterial survival [41]. Therefore, administration of a drug delivering a copper load, such as that of **3**, can theoretically overload the efflux capacity of MctB, rendering the bacteria susceptible to copper toxicity. Additionally, it has been reported that *M. tb* is susceptible to Cu metal, but that a monodrug-resistant *M. tb* strain is more susceptible to Cu than an MDR-TB strain [42]. Herein, we observed that the MDR-TB strain was still highly susceptible to the Cu-complexes.

To ascertain the effect of efflux on the activity of **1**–**4**, we determined the MIC of each compound in *Mycobacterium smegmatis* in combination with the efflux pump inhibitor, verapamil at 125 μg/mL (0.5 MIC) [43]. Verapamil is a non-competitive inhibitor of P-glycoprotein [44]. Amikacin was used as a control in these experiments since combined treatment with verapamil is known to result in a 0.5 fold reduction in the MIC of this compound [43]. While the MIC of amikacin was reduced as expected, those of **1**–**4** were unaffected by the addition of verapamil when compared to a side-by-side control containing no verapamil in the media (see Table S3), confirming no significant efflux of **1**–**4** occurs in *M. smegmatis* under the conditions trialled.

In an effort to generate *M. tb* resistant mutants to these compounds two strategies were adopted. To elucidate the potential target(s) of **1** in *M. tb* a random transposon (Tn) library of wild-type *M. tb* was plated on solid medium containing 50 μg/ml of **1** (10× MIC) at a density of 20,000 CFU/plate. No viable colonies were obtained on any of the plates. Therefore, in the second approach, we attempted to isolate spontaneous mutants by plating wild-type *M. tb* H37Rv(JO) on increasing concentrations of **1**. Although no viable colonies were obtained at the higher concentrations (5× and 10× MIC), eight discrete colonies were observed after selection at the lower concentration of 2× MIC. Five of these were picked and re-grown in standard liquid medium without **1** before testing in the liquid MIC assay to determine the level of resistance. The MICs of the putative spontaneous resistant mutants were identical to the parental strain, *M. tb* H37Rv(JO), suggesting non-heritable phenotypic resistance. The failure to isolate resistant mutant by either approach –Tn insertion library or spontaneous resistance - implies that **1** interferes with multiple pathways in *M. tb*.

High intracellular copper concentrations can result in destabilized iron-sulfur clusters, Fenton and Haber–Weiss reactions producing reactive oxygen species, and oxidized sulfhydryl-groups, all resulting in cellular damage [45]. Additionally, the Cu(II) and Co(II) center of a complex that utilized INH as a ligand was believed to enhance the capacity of the complex to cross the mycobacterial cell wall and produce a longer acting anti-TB agent [46–48]. Initial work focusing on $Cu(phen)_2^{2+}$ and $Cu(phen)^{2+}$ complexes demonstrated increased antimicrobial activity upon Cu(II) coordination, indicating that the ligand-metal complex influence one another, and are greater than the sum of their parts [49]. This is supported by the work of Devereux et al. in which Cu(II)phen complexes coordinated to varying ligands, such as octanedioate, phthalate, terephthalate, and salicylic acid, display notable cytotoxicity in multiple cancer cell lines, determining DNA interactions and ROS generations as likely causes for activity [50–53]. These copper complexes were more effective than the phen ligand alone, reinforcing that the full coordination complex is the source of potency [50–53].

A recent study by Prodius et al. reports hetero-trinuclear Fe(III) furoates featuring Mn(II), Co(II), or Ni(II), in which only the Co(II) complexes displayed anti-tuberculosis activity [54]. Such selective sensitivity to cobalt as we reported herein has not been reported for *M. tb* and is an intriguing observation. One possible explanation for the increased Co sensitivity compared to Cu in *M. tb* is the report on the presence of a Cu-binding metallothionein (MT), designated MymT [35]. MymT is the first MT reported in a Gram positive organism [35]. MymT was observed to bind 4–6 coppers (and to a less significant degree to bind cobalt) and as such could more greatly reduce oxidative damage from copper exposure. A putative role for MymT in reducing the effectiveness of **3** in *M. tb* is suggested then. (See also a recent review of resistance mechanisms to copper in *M. tb*) [55].

There have also been indications that *M. tb* CorA protein, a member of the 2TM-GxN family of integral membrane proteins that are responsible for metal ion transport of mostly Mg^{2+}, transports Co^{2+} at the same binding site. [56] This transport protein could potentially encourage high intracellular Co^{2+} concentrations.

Evidence suggests that removal of intracellular copper is necessary for virulence in *M. tb*, and the bacterium utilizes P-type ATPases as a means to export damaging levels of metals such as copper [57]. Indeed, metal ion transporters represent 24% of all transporters in the *M. tb* genome, considerably higher when compared to 11% for *Escherichia coli*, 16% for *Pseudomonas aeruginosa*, or 18% for *Mycoplasma pneumoniae*. [58] Incorporated in this high percentage of metal transporters are 12 P-type ATPases, more than any other sequenced prokaryotic genome [58]. In addition, known influx and efflux proteins for Co^{2+} and Cu^{2+} have been characterized or postulated through amino acid homology including NiCoT, MgtE, MIT and CDF [58]. The abundance of *M. tb* metal transporters, coupled with the results described herein, underscores why approaching the treatment of *M. tb* infection with metallopharmaceuticals may prove highly fruitful, not just in efficacy but also in selectivity.

Ongoing work in the lab is focusing on mechanistic studies of **1**–**4**, evaluating analogs of **1**–**4** to probe structure/activity relationships, exploring the hypothesis that the compounds target multiple pathways, and translating the work into in vivo studies.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.10.044.

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