Copper(II)-*bis*(thiosemicarbazonato) complexes as anti-chlamydial agents

Running title: Copper complexes are active against *Chlamydia*

James W. Marsh[1], Karrera Y. Djoko[2], Alastair G. McEwan[2], and Wilhelmina M. Huston[3*]

[1]The ithree institute, University of Technology Sydney, 15 Broadway, Ultimo, NSW 2007, Australia

[2]School of Chemistry and Molecular Biosciences and Australian Centre for Infectious Disease Research, The University of Queensland, St Lucia, QLD, 4072, Australia

[3]School of Life Sciences, University of Technology Sydney, 15 Broadway, Ultimo NSW 2007, Australia.

*Corresponding author: wilhelmina.huston@uts.edu.au; School of Life Sciences, Faculty of Science, City Campus, University of Technology Sydney, PO BOX 123, Broadway, NSW 2007, Australia.

ABSTRACT

Lipophilic copper (Cu)-containing complexes have shown promising antibacterial activity against a range of bacterial pathogens. To examine the susceptibility of the intracellular human pathogen *Chlamydia trachomatis* to copper complexes containing *bis*(thiosemicarbazone) ligands [Cu(btsc)], we tested the *in vitro* effect of Cu^{II}-diacetyl- and Cu^{II}-glyoxal-*bis*[*N*(4)-methylthiosemicarbazonato] (Cu(atsm) and Cu(gtsm), respectively) on *C. trachomatis*. Cu(atsm) and to a greater extent, Cu(gtsm), prevented the formation of infectious chlamydial progeny. Impacts on host cell viability and respiration were also observed in addition to the *Chlamydia* impacts. This work suggests that copper-based complexes may represent a new lead approach for future development of new therapeutics against chlamydial infections, although host cell impacts need to be fully explored.

KEYWORDS

Chlamydia, copper, copper ionophore, intracellular, respiration

Chlamydia trachomatis is the most common sexually transmitted bacterial infection worldwide. As an obligate intracellular bacterial pathogen, *Chlamydia* has a unique developmental cycle that consists of an extracellular, non-replicative, infectious form (elementary body) and an intracellular, replicative form (reticulate body). *Chlamydia* relies heavily on the host for nutrition and energy and ATP/ADP transporters have been identified. Yet, metabolic data has demonstrated that *Chlamydia* is capable of generating energy through substrate level phosphorylation and oxidative phosphorylation with a respiratory chain terminating in a cytochrome *bd* oxidase (reviewed in (Omsland *et al.*, 2014)).

Chlamydia infections are currently treated with 1 g of azithromycin. In some cases, a seven day doxycycline regimen is used, although this is less preferred as non-compliance can result in the induction of chlamydial persistence and treatment failure. The increasing prevalence of *Neisseria gonorrhoeae* co-infections and a rise in resistance (or reduced susceptibility) of gonococcus to azithromycin suggests that more effective combination treatments that target both pathogens are needed. In this context, recent work has identified that small and lipophilic copper complexes are highly effective against *N. gonorrhoeae*, including multidrug-resistant strains (Djoko *et al.*, 2012, Djoko *et al.*, 2014, Djoko *et al.*, 2015).

Copper is essential for bacterial metabolism but it is bacteriotoxic in excess. The antimicrobial properties of excess copper ions have been documented for centuries. Copper primarily poisons bacteria by displacing other metal ions in metalloproteins and inactivating key bacterial metabolic pathways (Macomber & Imlay, 2009). In the pre-antibiotic age, ionic copper salts were used to control bacterial infections but due to poor membrane permeability, there was a high dose requirement. Recently, we and others have explored the use of small,

lipophilic ligands or pro-ligands to facilitate delivery of copper ions across bacterial membranes (termed as "copper ionophores") (Speer *et al.*, 2013, Festa *et al.*, 2014, Haeili *et al.*, 2014, Shah *et al.*, 2016). These copper ionophores are effective against a variety of pathogens, including *Mycobacterium tuberculosis* and *Staphylococcus aureus* (Speer *et al.*, 2013, Haeili *et al.*, 2014, Shah *et al.*, 2016). Of interest to this work are copper ionophores containing *bis*(thiosemicarbazonato) ligands (Cu(btsc)s) such as Cu^{II}-diacetyl- and Cu^{II}-glyoxal-*bis*[*N*(4)-methylthiosemicarbazonato] (Cu(atsm) and Cu(gtsm), respectively) (Figure 1A), which showed activity *in vitro* against *N. gonorrhoeae* (Djoko *et al.*, 2014, Djoko *et al.*, 2015). It has previously been established that copper ions will inhibit *Chlamydia* if added prior to cellular entry, and some reports indicate that women using copper intrauterine devices as a contraceptive may have a lower frequency of contracting *Chlamydia* (Kleinman *et al.*, 1989). To evaluate if copper ionophores could also be effective against *Chlamydia*, we tested the *in vitro* effect of Cu(gtsm) and Cu(atsm) on *Chlamydia trachomatis*.

Cu(btsc) complexes were added to *Chlamydia trachomatis* serovar D/UW-3/Cx cultures (in McCoy B cells) at the mid-replicative phase (20 h PI). Cu(atsm) and Cu(gtsm) were provided as powders by Dr Paul Donnelly from The University of Melbourne (Gringras *et al.*, 1962, Paterson & Donnelly, 2011). Cultures were propagated using standard conditions at a multiplicity of infection of 1 (Huston *et al.*, 2008). The compounds were left in the cultures until the conclusion of the developmental cycle and cultures were harvested and re-infected onto fresh McCoy B monolayers to enumerate infectious progeny (previously described protocols (Huston *et al.*, 2007, Huston *et al.*, 2008, Lawrence *et al.*, 2016)). The compounds were highly effective with a loss of infectious progeny detected at the low micromolar range for both compounds (Figure 1B). The Cu(gtsm) had a greater impact on chlamydial infectious progeny production (1.6 µM), compared to Cu(atsm) (3.2 µM, Figure

1B). Note that 10e3 is the limitation of detection for this assay as the number of IFU below this threshold cannot be reliably quantified.

The elementary body (EBs) does not undergo cellular division, but does have metabolic activity (Omsland *et al.*, 2012). To evaluate whether the Cu(btsc) complexes are also effective against the elementary body, we incubated elementary bodies with each compound in Sucrose Phosphate Glutamate (SPG) media for 30 mins, washed, and immediately added to a McCoy B cell monolayer to commence a chlamydial infection. The infectious progeny formed from this infection were then enumerated. The treatment of elementary bodies was effective, although almost 50x higher dose (50 µM) (compared to treatment of the intracellular Reticulate body (RB) phase; Figure 1C). As was the case during the intracellular growth phase, Cu(gtsm) was more effective against the *Chlamydia* elementary bodies compared to Cu(atsm), consistent with earlier observations in *N. gonorrhoeae*, *Mycobacteria tuberculosis*, and *Staphylococcus aureus* (Speer *et al.*, 2013, Haeili *et al.*, 2014, Djoko *et al.*, 2015). The ionophores showed similar effects against the RB phase of a distinct strain of *Chlamydia* (*C. trachomatis* L2, data not shown).

It is not yet certain if the *Chlamydia* reticulate body is completely or partially reliant on host cell ATP (Tipples & McClarty, 1993, Omsland *et al.*, 2014). It has been previously demonstrated that, in addition to the release of bioavailable copper ions into the cytoplasm, Cu(btsc) complexes partition to the membranes of *N. gonorrhoeae* where they inhibit the activity of Nuo and Nqr, two NADH dehydrogenases of the gonococcal electron transport chain (Djoko *et al.*, 2015). Since *Chlamydia* possesses an Nqr as its sole NADH dehydrogenase, it is tempting to suggest that this is potential target for Cu(btsc) complexes in this bacterium.

Next we assessed the impact on host cells, by pre-treating McCoy B host cells with copper complexes 5 hrs prior to infection and then measured the viable infectious yield of elementary bodies. We observed a loss of chlamydial infectious progeny at 5 µM (Figure 1E), which is a slightly higher dose than the dose leading to loss of infectivity when compounds were added to chlamydial cultures during the active growth phase. The host cell live-dead assay (Figure 1D) indicated that in this cell model there was some toxicity that likely contributed to the phenotypes. Additionally, some EBs were detected at the toxic host cell concentration of 1.6 µM suggesting that viable EBs are prevailing either in detached host cell or in the media itself. This host cell effect differs from previous data on different cell lines where minimal cell death was detected (Djoko *et al.*, 2015), indicating that further understanding of host cell-specific impacts of copper ionophores is needed before progressing to *in vivo* experiments.

The Cu(btsc) complexes, particularly Cu(gtsm), are known to inhibit of Complex I in isolated rat liver mitochondria (Djoko *et al.*, 2014) but it has not been determined whether Cu(gtsm) also inhibits mitochondrial function in intact cells and tissues. Therefore, we measured cellular respiration to assess whether host cell mitochondrial impacts could explain the loss of infectious progeny. We measured the impact of Cu(gtsm) and Cu(atsm) on host cell mitochondrial respiration using the Seahorse XF Cell Mito Stress Test modulators kit (Agilent Technologies). McCoy B cells were seeded 96-well plates with 20,000 cells per well and Cu(atsm) and Cu(gtsm) copper complexes were added at 50 and 150 nM for 30 mins prior to commencing the assay. While Cu(atsm) did not affect the spare respiratory capacity and ATP production of the host, Cu(gtsm) resulted in a reduction in spare respiratory capacity at both 50 nM and 150 nM (Figure 1F). Lower doses were tested than those where complete lethality was observed in Figure 1B as we wished to tease out the role of respiratory impacts in the doses where loss of progeny was observed.

In summary, Cu(btsc) were effective against both the intracellular replicative form of the *Chlamydia* and the extracellular form. The reduced effect seen following the pre-treatment of EBs (extracellular form) may be attributable to the reduced metabolic activity of this development form and possible reduced access due to the structural density of the EB outer membrane. However, given that the copper complexes were also demonstrated to impact on respiratory capacity of the host cell, it is hard to differentiate the role of host cell impact from anti-chlamydial impact for loss of chlamydial infectious progeny. It is important to note, however, that the pre-treatment of host cells is not the same as treatment during an active infection as the chlamydial burden is likely to alter the respiration rate of the host and reduce the amount of complex accessing the mitochondria.

These data indicate that these copper compounds are toxic to *Chlamydia*, although here a host cell impact is also notable on McCoy B cells that contributed to this phenotype. It was previously reported that other cells are not susceptible to these compounds (Djoko *et al.*, 2015), suggesting that the toxicity observed in this study may relate to host cell type. Overall, whilst showing some promise much is needed to be done to unravel toxicity and metabolic impacts of the copper ionophores on host cells before *in vivo* applications could be trailed. Interestingly, these data could suggest that respiration is important for the RB phase of chlamydial growth. One possible application of future derivatives of these copper complexes could be as a component of a topical anti-microbial lubricant that could inactivate EBs before they establish an infection from sexual transmission, and potentially in this application could minimise any toxicity on the host cells.

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CONTRIBUTORS

JM and KYD conducted and analysed the experiments and interpreted the data. AGM and WMH conceived and designed the study and contributed to interpretation of the results. All authors contributed to the writing of the manuscript and have approved the manuscript.

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FIGURE

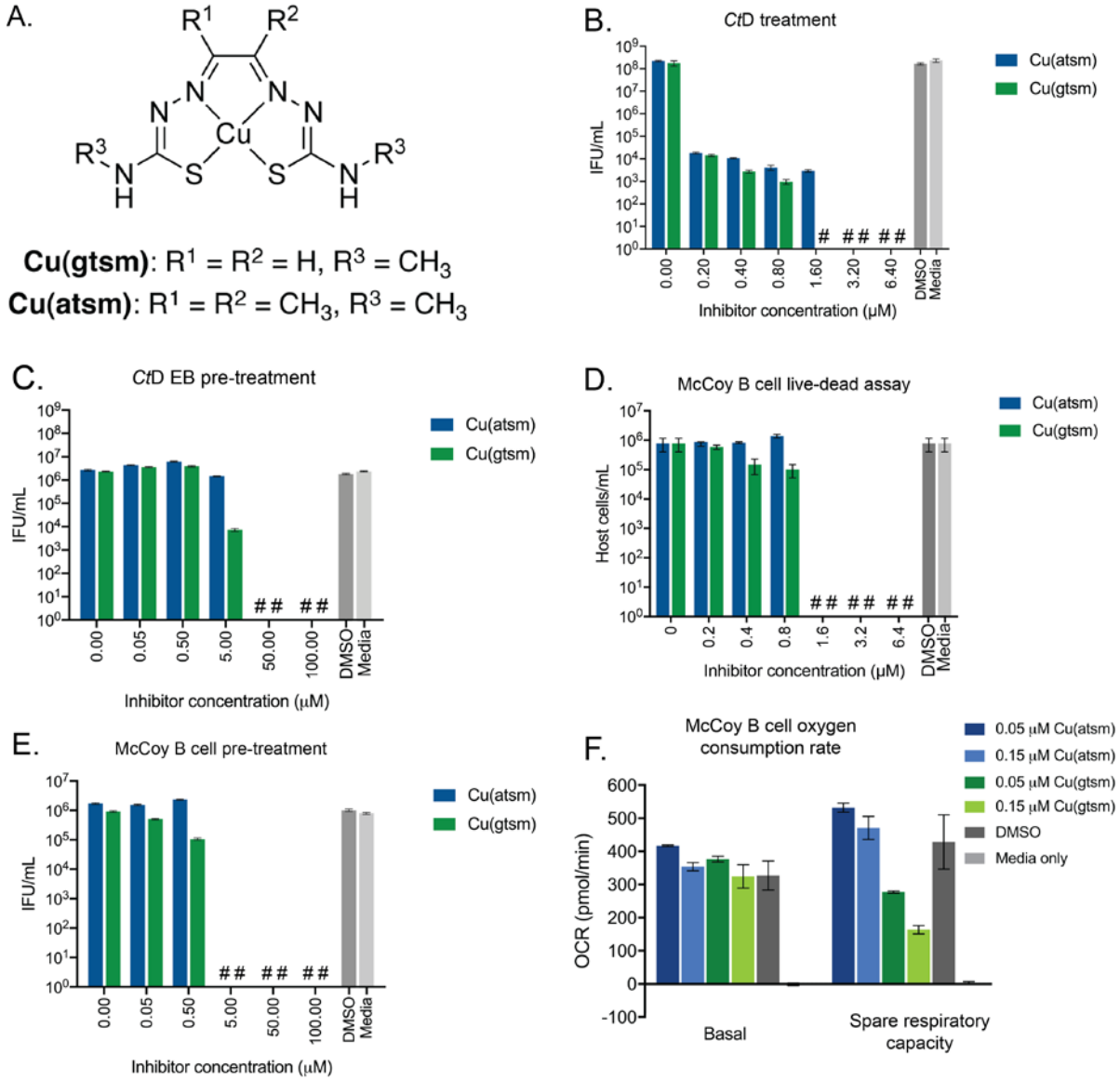

Figure 1 A. Structure of Cu(gtsm) and Cu(atsm) molecules. **B.** Inclusion forming units (IFU) produced after *C. trachomatis* D/UW/3/CX (*Ct*D) cultures were treated with Cu(gtsm) or Cu(atsm) at the mid-replicative phase (20 h PI). Infectious progeny were measured at completion of the developmental cycle (44 h PI). **C.** Inclusion forming units after *C. trachomatis* D/UW/3/CX elementary bodies were treated with Cu(gtsm) or Cu(atsm) for 30 min prior to infection of host cells. Infectious progeny were determined from cultures

harvested at 44 h PI. **D.** Live host cell counts after 24 h exposure to Cu(gtsm) or Cu(atsm). **E.** Inclusion forming units after McCoy B host cells were pre-treated with Cu(gtsm) or Cu(atsm) prior to the chlamydial infection (for 300 min). **A-E.** Results are representatives of experiments repeated in independent triplicate with n = 27 in each bar. Errors bars depict the standard error of the mean. # indicates no growth detected. **F.** Impact of Cu(gtsm) and Cu(atsm) on the basal (energetic demand of the cell under baseline conditions) and spare respiratory capacity (capacity of the cell to respond to energetic demands) of the host cells when treated in the absence of chlamydial infection (oxygen consumption rate) on the *y* axis. The rate of oxygen consumption was measured following the sequential addition of oligomycin (2 µM; targets ATP synthase), carbonyl cyanide-*4* (trifluoromethoxy)phenylhydrazone (2 µM; targets inner mitochondrial membrane), and rotenone/antimycin A (0.5 µM; targets complex I and III respectively. Concentrations were as per the manufacturer's recommendation. The key to the right indicates the colour corresponding to each compound on the graphs. Graphical presentation of the data and statistical analysis was conducted using Graphpad Prism (v7).