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

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3 **Running title:** Copper complexes are active against *Chlamydia*

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20 **ABSTRACT**

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

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34 **KEYWORDS**

35 *Chlamydia*, copper, copper ionophore, intracellular, respiration

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

49 *Chlamydia* infections are currently treated with 1 g of azithromycin. In some cases, a
50 seven day doxycycline regimen is used, although this is less preferred as non-compliance can
51 result in the induction of chlamydial persistence and treatment failure. The increasing
52 prevalence of *Neisseria gonorrhoeae* co-infections and a rise in resistance (or reduced
53 susceptibility) of gonococcus to azithromycin suggests that more effective combination
54 treatments that target both pathogens are needed. In this context, recent work has identified
55 that small and lipophilic copper complexes are highly effective against *N. gonorrhoeae*,
56 including multidrug-resistant strains (Djoko *et al.*, 2012, Djoko *et al.*, 2014, Djoko *et al.*,
57 2015).

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

64 lipophilic ligands or pro-ligands to facilitate delivery of copper ions across bacterial
65 membranes (termed as “copper ionophores”) (Speer *et al.*, 2013, Festa *et al.*, 2014, Haeili *et*
66 *al.*, 2014, Shah *et al.*, 2016). These copper ionophores are effective against a variety of
67 pathogens, including *Mycobacterium tuberculosis* and *Staphylococcus aureus* (Speer *et al.*,
68 2013, Haeili *et al.*, 2014, Shah *et al.*, 2016). Of interest to this work are copper ionophores
69 containing *bis*(thiosemicarbazonato) ligands (Cu(btsc)s) such as Cu^{II}-diacetyl- and Cu^{II}-
70 glyoxal-*bis*[*N*(4)-methylthiosemicarbazonato] (Cu(atsm) and Cu(gtsm), respectively) (Figure
71 1A), which showed activity *in vitro* against *N. gonorrhoeae* (Djoko *et al.*, 2014, Djoko *et al.*,
72 2015). It has previously been established that copper ions will inhibit *Chlamydia* if added
73 prior to cellular entry, and some reports indicate that women using copper intrauterine
74 devices as a contraceptive may have a lower frequency of contracting *Chlamydia* (Kleinman
75 *et al.*, 1989). To evaluate if copper ionophores could also be effective against *Chlamydia*, we
76 tested the *in vitro* effect of Cu(gtsm) and Cu(atsm) on *Chlamydia trachomatis*.

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

88 1B). Note that 10e3 is the limitation of detection for this assay as the number of IFU below
89 this threshold cannot be reliably quantified.

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

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

111 Next we assessed the impact on host cells, by pre-treating McCoy B host cells with
112 copper complexes 5 hrs prior to infection and then measured the viable infectious yield of
113 elementary bodies. We observed a loss of chlamydial infectious progeny at 5 μ M (Figure
114 1E), which is a slightly higher dose than the dose leading to loss of infectivity when
115 compounds were added to chlamydial cultures during the active growth phase. The host cell
116 live-dead assay (Figure 1D) indicated that in this cell model there was some toxicity that
117 likely contributed to the phenotypes. Additionally, some EBs were detected at the toxic host
118 cell concentration of 1.6 μ M suggesting that viable EBs are prevailing either in detached host
119 cell or in the media itself. This host cell effect differs from previous data on different cell
120 lines where minimal cell death was detected (Djoko *et al.*, 2015), indicating that further
121 understanding of host cell-specific impacts of copper ionophores is needed before
122 progressing to *in vivo* experiments.

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

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

146 These data indicate that these copper compounds are toxic to *Chlamydia*, although
147 here a host cell impact is also notable on McCoy B cells that contributed to this phenotype. It
148 was previously reported that other cells are not susceptible to these compounds (Djoko *et al.*,
149 2015), suggesting that the toxicity observed in this study may relate to host cell type. Overall,
150 whilst showing some promise much is needed to be done to unravel toxicity and metabolic
151 impacts of the copper ionophores on host cells before *in vivo* applications could be trailed.
152 Interestingly, these data could suggest that respiration is important for the RB phase of
153 chlamydial growth. One possible application of future derivatives of these copper complexes
154 could be as a component of a topical anti-microbial lubricant that could inactivate EBs before
155 they establish an infection from sexual transmission, and potentially in this application could
156 minimise any toxicity on the host cells.

157

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164

165 **CONTRIBUTORS**

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

169

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224

FIGURE

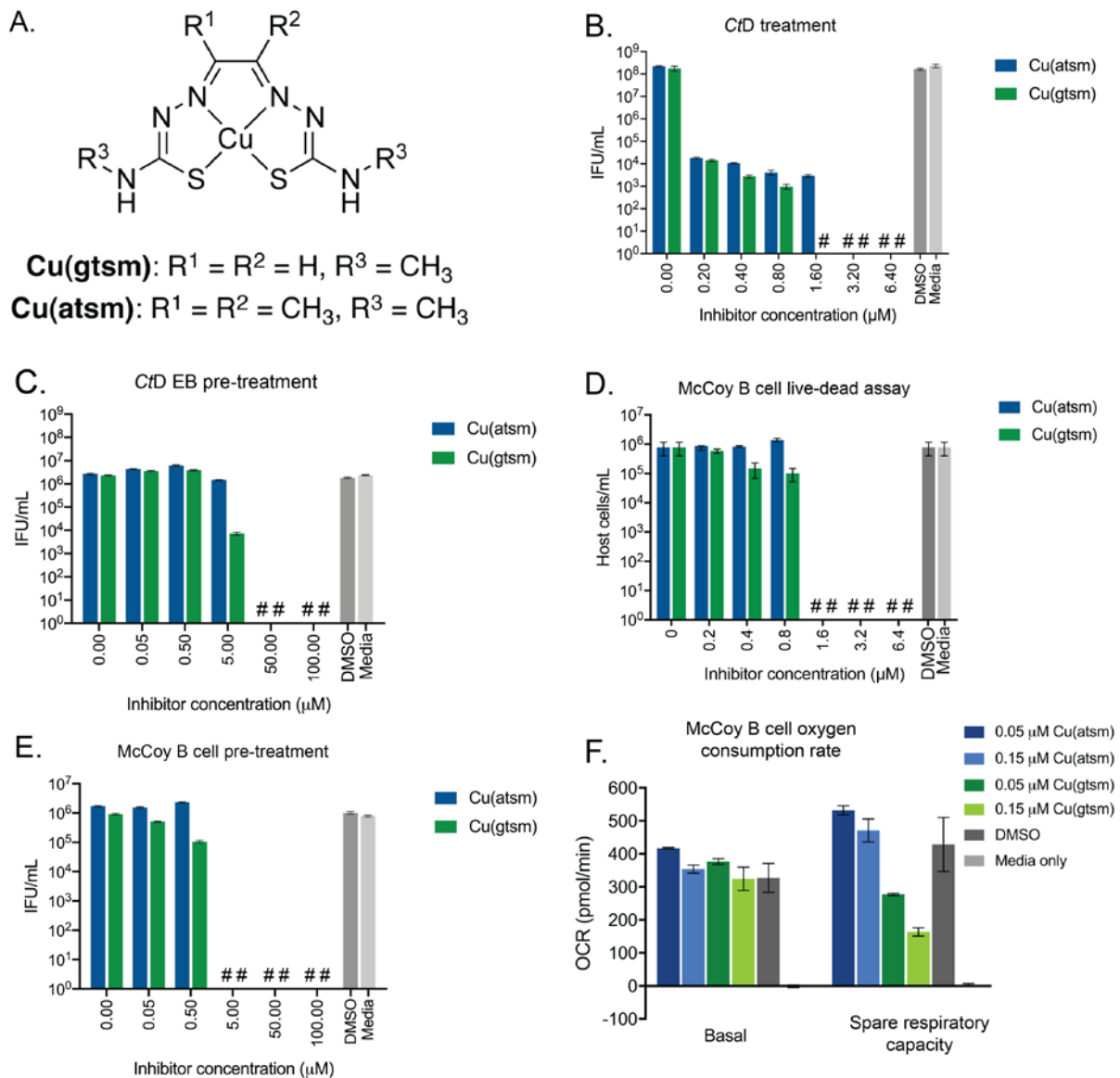


Figure 1 **A.** Structure of Cu(gtsm) and Cu(atsm) molecules. **B.** Inclusion forming units (IFU) produced after *C. trachomatis* D/UW/3/CX (*CtD*) cultures were treated with Cu(gtsm) or Cu(atsm) at the mid-replicative phase (20 h PI). Infectious progeny were measured at completion of the developmental 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(atasm). **E.** Inclusion forming units after McCoy B host cells were pre-treated with Cu(gtsm) or Cu(atasm) 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. Error bars depict the standard error of the mean. # indicates no growth detected. **F.** Impact of Cu(gtsm) and Cu(atasm) 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 ($2 \mu\text{M}$; targets ATP synthase), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone ($2 \mu\text{M}$; targets inner mitochondrial membrane), and rotenone/antimycin A ($0.5 \mu\text{M}$; 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).