

1 **Copper(II)-bis(thiosemicarbazonato) complexes as antibacterial agents: insights into their**  
2 **mode of action and potential as therapeutics**

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4 Karrera Y. Djoko<sup>1#</sup>, Maira M. Goytia<sup>2</sup>, Paul S. Donnelly<sup>3</sup>, Mark A. Schembri<sup>4</sup>, William M.  
5 Shafer<sup>5</sup>, and Alastair G. McEwan<sup>6#</sup>

6

7 **#Corresponding authors**

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9 <sup>1</sup>School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, QLD  
10 4072, Australia. Email: k.djoko@uq.edu.au

11 <sup>2</sup>Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta,  
12 GA 30322. Email: mgoytia@emory.edu

13 <sup>3</sup>School of Chemistry and Bio21 Institute of Molecular Science and Biotechnology, The  
14 University of Melbourne, Parkville, VIC 3010, Australia. Email: pauld@unimelb.edu.au

15 <sup>4</sup>School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, QLD  
16 4072, Australia. Email: m.schembri@uq.edu.au

17 <sup>5</sup>Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta,  
18 GA 30322. Laboratories of Bacterial Pathogenesis, Veterans Affairs Medical Center, Decatur,  
19 GA 30033. Emory Antibiotic Resistance Center, Emory University School of Medicine, Atlanta,  
20 GA 30322. Email: wshafer@emory.edu

21 <sup>6</sup>School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, QLD  
22 4072, Australia. Email: mcewan@uq.edu.au

23

24 **ABSTRACT**

25

26       There is increasing interest in the use of lipophilic copper (Cu)-containing complexes to  
27 combat bacterial infections. In this work, we showed that Cu complexes with  
28 *bis*(thiosemicarbazone) ligands [Cu(btsc)] exert antibacterial activity against a range of  
29 medically significant pathogens. Previous work using *Neisseria gonorrhoeae* showed that  
30 Cu(btsc) complexes may act as inhibitors of respiratory dehydrogenases of the electron transport  
31 chain. We now show that these complexes are also toxic against a range of bacterial pathogens  
32 some of which lack a respiratory chain. Respiration in *Escherichia coli* was slightly affected by  
33 Cu(btsc) complexes but our results indicate that, in this model bacterium, the complexes act  
34 primarily as agents that deliver toxic Cu ions efficiently to the cytoplasm. Although the  
35 chemistry of Cu(btsc) complexes may dictate their mechanism of action, their efficacy depends  
36 highly on bacterial physiology which is linked to the ability of the bacterium to tolerate Cu and  
37 additionally, the susceptibility of the respiratory chain to direct inhibition by Cu(btsc)  
38 complexes. The physiology of *N. gonorrhoeae*, including multidrug-resistant strains, makes it  
39 highly susceptible to damage by Cu ions and Cu(btsc) complexes, highlighting the potential of  
40 Cu(btsc) complexes as a potential treatment against this significant bacterial pathogen.

41

42 **Keywords:** copper, ionophores, antimicrobial, Cu(btsc), *Neisseria gonorrhoeae*

43

## 44 INTRODUCTION

45

46 Copper (Cu) is an essential trace micronutrient in bacteria but it is bacteriotoxic in  
47 excess. Nutrient Cu inserts into specific high-affinity sites in proteins, or it forms complexes with  
48 low molecular weight thiols such as glutathione. As a consequence, the concentration of “free”  
49 Cu ions in cells has been estimated to be vanishingly low (sub-femtomolar) (1). Any excess Cu  
50 beyond this normal binding capacity may be mislocated to nonspecific and low-affinity metal ion  
51 binding sites, thereby inactivating crucial enzymes and interfering with normal metabolism (2).  
52 As the mid-potential of the Cu(II)/Cu(I) redox couple is biologically accessible, these weakly  
53 bound Cu ions can also cause further toxicity by catalyzing gratuitous electron transfer and  
54 promoting redox stress (3). The widespread damage caused by excess Cu ions is termed Cu  
55 poisoning. Almost all bacterial species, including those that have no apparent use for Cu, possess  
56 homeostatic systems that protect them against an excess of this ion (4). These systems consist  
57 typically of a Cu ion efflux pump that operates under the control of a Cu-specific transcriptional  
58 regulator.

59 As a result of its chemistry, Cu is considered a promiscuous or broad-spectrum  
60 antimicrobial but there is concern regarding its universal toxicity. In the pre-antibiotic era,  
61 simple ionic Cu salts were used to treat a variety of infections (5), but these salts are now  
62 associated with significant toxicity (6). The difficulty in delivering bactericidal Cu at doses that  
63 are nontoxic to human tissues may explain the failure to translate Cu-based therapeutics into the  
64 modern medical setting. The emergence of bacteria with resistance to classical antibiotics,  
65 combined with the paucity of new compounds in the pipeline, has now renewed interest in the  
66 development of Cu as an antimicrobial (7-9). This research is motivated further by recent

67 findings that physiological Cu may be harnessed as a direct antimicrobial in innate immune cells  
68 (10, 11).

69 Ionic Cu salts have limited ability to cross membranes as their translocation relies on  
70 specific transport proteins, and so they have little potential to be developed as anti-infective  
71 drugs. Cu ions generally show restricted penetration into target tissues and bacteria, and thus  
72 exceptionally high concentrations are needed to achieve a bactericidal effect *in vitro* and *in vivo*.  
73 To assist in the delivery of Cu ions across lipid membranes, several lipophilic ligands or  
74 proligands have been developed (9, 12). These molecules are termed ‘Cu ionophores’ for their  
75 ability to act as carriers of Cu ions.

76 Of interest in this work are *bis*(thiosemicarbazone) ligands that bind Cu(II) as small,  
77 uncharged, lipophilic, and stable complexes (Cu(btsc), Figure 1). We and others have shown that  
78 Cu(btsc) complexes exert antimicrobial activity against several human pathogens, including  
79 Gram-negative bacteria such as *Neisseria gonorrhoeae* (13) and *Mycobacterium tuberculosis*  
80 (14), as well as the Gram-positive bacterium *Staphylococcus aureus* (15). However, the  
81 mechanism of antibacterial action of Cu(btsc) complexes has not been examined fully. Although  
82 it is assumed that they operate as Cu ionophores (14, 15), we have shown that Cu(btsc)  
83 complexes can also act as direct respiratory inhibitors in *N. gonorrhoeae* (13). Here we describe  
84 experiments using *Escherichia coli* that are aimed at determining whether Cu(btsc) complexes  
85 act primarily by inhibition of the respiratory chain or by delivery of bioavailable Cu ions. We  
86 also assess the susceptibility of a range of bacterial pathogens to these complexes, including  
87 multidrug-resistant strains of *N. gonorrhoeae*.

88

89 **MATERIALS AND METHODS**

90

91           **Cu stocks.** Stocks of aqueous Cu salts, supplied as CuCl<sub>2</sub> or Cu(NO<sub>3</sub>)<sub>2</sub>, were prepared in  
92 deionised water and their concentrations were standardised using bathocuproine disulfonate as  
93 described elsewhere (16). The two salts were used interchangeably in experiments. Stocks of  
94 Cu-pyrithione, Cu-neocuproine, and Cu-disulfiram complexes were prepared in DMSO by  
95 mixing standardised CuCl<sub>2</sub> with 2.5 molar equivalents of the respective free ligands. Stocks of  
96 Cu(atsm) and Cu(gtsm) were prepared in neat DMSO and standardised using their solution  
97 absorbance in DMSO (Cu(atsm):  $\lambda_{\max}$  457 nm,  $\epsilon$  7200 M<sup>-1</sup> cm<sup>-1</sup>; Cu(gtsm):  $\lambda_{\max}$  472 nm,  $\epsilon$  8700  
98 M<sup>-1</sup> cm<sup>-1</sup>). DMSO was used as a vehicle control in all experiments.

99           **Strains and growth conditions.** *N. gonorrhoeae* strains (Table 3) were grown on GC  
100 agar (Oxoid) supplemented with 1 v/v % IsoVitaleX (Becton Dickinson). Liquid cultures were  
101 prepared in GC broth supplemented with IsoVitaleX and 0.042 w/v % sodium bicarbonate. *E.*  
102 *coli* strains MG1655 (laboratory strain K-12) and EC958 (fluoroquinolone-resistant strain  
103 ST131), and *Salmonella enterica* sv. Typhimurium SL1344 were propagated on LB agar or  
104 broth. *Haemophilus influenzae* RdKw20 was propagated on BHI medium (Oxoid) containing 10  
105  $\mu$ g/mL NAD<sup>+</sup> and 10  $\mu$ g/mL hemin. *Streptococcus pneumoniae* D39 was propagated on THY  
106 base (Oxoid) containing 10 U/mL catalase. *S. aureus* strain ATCC 22913 (methicillin-sensitive),  
107 and SR2852 (methicillin-resistant) strains were grown on Tryptic Soy medium (Becton  
108 Dickinson). *Lactobacillus acidophilus* NCFM was grown on L-MRS agar (Oxoid) under  
109 anaerobic growth conditions. All bacteria were grown for 12 – 16 h at 37 °C. *N. gonorrhoeae*, *S.*  
110 *pneumoniae*, and *S. aureus* were grown in the presence of 5 v/v % atmospheric CO<sub>2</sub>. Anaerobic  
111 growth was performed in an anaerobic jar containing an AnaeroGen sachet (Oxoid).

112           **Determination of MICs.** MICs were determined by agar dilution method. Briefly,  
113 bacterial lawns from an overnight agar culture were resuspended in the appropriate broth to  $10^7$  –  
114  $10^8$  CFU/mL and 5  $\mu$ L of each dilution was spotted onto new solid media containing various  
115 concentrations of the desired Cu source. The amount of DMSO was maintained at 0.5 v/v %. The  
116 MIC value was defined to be the minimum concentration at which no growth was visible after 24  
117 h.

118           **Assays of gonococcal killing kinetics.** *N. gonorrhoeae* lawns from an overnight agar  
119 culture were resuspended in broth to an OD<sub>600</sub> of 0.1 (ca.  $10^7$  CFU/mL). Cu complexes were  
120 added to the desired final concentration and the mixtures were incubated at 37 °C with gentle  
121 shaking. Survival was monitored for up to 7 h by conventional colony counting.

122           **Measurement of intracellular Cu levels in *E. coli*.** *E. coli* was resuspended in broth to  
123 an OD<sub>600</sub> of 0.1 (ca.  $10^8$  CFU/mL) and used to seed fresh solid media containing the desired Cu  
124 source. After an overnight growth, bacteria were harvested, washed once in PBS containing 10  
125 v/v % DMSO, once in PBS containing 1 mM EDTA, and finally once in PBS without any  
126 additive. The final pellet was dissolved in conc. nitric acid. Amounts of Cu and other transition  
127 metal ions were determined by Inductively-Coupled Plasma Optical Emission Spectrometry (ICP  
128 OES). Results were standardised to total biomass as represented by total protein content. The  
129 amount of protein was assayed using the Quanti BCA Kit (Sigma).

130           To evaluate the antibacterial activity of the Cu complexes under the same conditions, the  
131 initial inoculum was diluted serially up to  $10^{-7}$  and 5  $\mu$ L of each dilution was plated out on  
132 duplicate solid media containing the various Cu sources. Visible colonies were enumerated after  
133 24 h to determine the efficiency of plating.

134           **Assays of  $\beta$ -galactosidase activity in *E. coli*.** The promoter region of *copA* from *E. coli*  
135 MG1655 (150 bp upstream from the translation start codon ATG) was amplified using the primer  
136 pairs *PcopA-F* (5'-CATCAGGGATTTCAGATAAATGTCTAATCCTGA-3') and *PcopA-R*  
137 (5'-CTGATGAAGCTTAAAACACTCCTTTAAGACAG-3'). The PCR product was cloned  
138 between the *Bam*HI and *Hind*III sites of the low-copy vector pQF50 containing a promoterless  
139 *lacZ* gene (17). The resulting pQF50::*PcopA* plasmid was transformed into *E. coli* DH5 $\alpha$ .  
140 Colonies that were blue on agar plates containing ampicillin (100  $\mu$ g/mL), X-gal (40  $\mu$ g/mL),  
141 and CuCl<sub>2</sub> (500 – 1000  $\mu$ M) were selected and grown to the mid-exponential phase. Cultures  
142 were challenged with Cu complexes for 0 – 120 min without any shaking.  $\beta$ -galactosidase  
143 activities were measured using *ortho*-nitrophenyl- $\beta$ -galactoside following procedures for  
144 standard Miller assay. Raw values of  $\beta$ -galactosidase activity were normalised to OD<sub>600</sub>. Results  
145 were expressed as fold-induction over the uninduced control at the same time point.

146           **Assays of respiration in *E. coli*.** *E. coli* was cultured to the mid-exponential phase,  
147 harvested, resuspended in LB to 10% of the original volume, and kept on ice. Respiration was  
148 measured at 35 °C in 2 mL of broth containing 50  $\mu$ M of Cu salt, Cu(*gtsm*), or Cu(*atsm*).  
149 Consumption of O<sub>2</sub> was initiated by adding 10  $\mu$ L of the bacterial suspension. Respiration was  
150 monitored for 15 min using an S1/Mini Clark-type oxygen electrode (Hansatech Instruments) in  
151 conjunction with an Oxytherm control unit.

152           *E. coli* membrane vesicles were isolated as described previously for *N. gonorrhoeae* (13).  
153 Rates of NADH oxidation were measured spectrophotometrically at 340 nm following  
154 procedures described previously for submitochondrial particles from rat liver (18).

155           **Assays of using cervical epithelial cells.** Immortalised and adherent ME-180 cervical  
156 epithelial cells (ATCC® HTB33™) were cultured routinely according to manufacturer's

157 instructions in McCoy's 5a Modified Medium (ATCC 30-2007) containing fetal bovine serum  
158 (10 v/v %, ATCC 30-2020), and penicillin and streptomycin (100 IU/mL and 100 µg/mL,  
159 respectively, Corning-Cellgro 30-002-CI), Cells were seeded at  $1 \times 10^6$  cells/mL and allowed to  
160 adhere overnight. The resulting monolayers were transitioned into fresh medium containing  
161 Cu(gtsm), Cu(atsm), or Cu salt. The concentration of DMSO in all samples was 0.1 v/v %. After  
162 24 h, the supernatant was collected and checked for cells that may have detached during  
163 treatment. The remaining adherent cells were allowed to recover for 1 – 2 h and subsequently  
164 brought into suspension using trypsin/EDTA. Trypan Blue (0.2 w/v %) was added and cells were  
165 enumerated in a hemocytometer.

166

## 167 **RESULTS**

168

### 169 **Antibacterial activity of Cu(btsc) complexes towards select bacterial pathogens.**

170 Two prototypes of the Cu(btsc) family, Cu(gtsm) and Cu(atsm) (Figure 1), exerted dose-  
171 dependent antibacterial activity against *N. gonorrhoeae* strain 1291 (13). Both complexes were  
172 effective at concentrations where the unmetallated H<sub>2</sub>btsc ligands and the uncomplexed or ‘free’  
173 aqueous Cu<sup>2+</sup> ions were ineffective (Table 1) (13). The MICs were 0.1 µM (0.03 µg/mL) for  
174 Cu(gtsm) and 1 µM (0.3 µg/mL) for Cu(atsm) (Table 1). We also evaluated the anti-gonococcal  
175 activities of additional Cu ionophores, including Cu-disulfiram, Cu-neocuproine, and  
176 Cu-pyrithione. The MIC value for Cu-pyrithione (0.13 µM, ca. 0.04 µg/mL) was comparable to  
177 that for Cu(gtsm) (Table 1). For this study, we examined Cu(gtsm) in greater depth as the  
178 availability of the structural analogue Cu(atsm) with a lower efficacy allowed us to probe the  
179 mode of action in more detail.



180 The mode of action of Cu(gtsm) and Cu(at-sm) against *N. gonorrhoeae* was bactericidal.  
181 Complete killing of *N. gonorrhoeae* ( $\sim 10^7$  CFU/mL) by 1  $\mu$ M of Cu(gtsm) was achieved within  
182 1.5 h, while an equal dose of Cu(at-sm) required at least 5 h to elicit an equivalent effect (Figure  
183 2). By contrast, ionic Cu salts displayed no bactericidal activity within this time period (Figure  
184 2). The relative MICs and killing kinetics established that Cu(gtsm) and Cu(at-sm) were more  
185 toxic than Cu salts, and that Cu(gtsm) was more toxic than Cu(at-sm).

186 Cu(gtsm) and Cu(at-sm) were also effective against several additional and unrelated  
187 bacterial pathogens, including *S. aureus* (methicillin-sensitive and resistant strains), *S.*  
188 *pneumoniae*, and *H. influenzae*, but at MICs that were appreciably higher (Table 2). *E. coli*  
189 (fluoroquinolone-sensitive and resistant strains) and *S. Typhimurium* were the most resistant. In  
190 the case of *E. coli*, bacterial growth was observed even at Cu(gtsm) concentrations as high as 25  
191  $\mu$ M (7.5  $\mu$ g/mL), although there was a 1000-fold reduction in the plating efficiency when  
192 compared with the untreated control (Figure S1 in Supplemental Material). In comparison,  
193 exposure to 25  $\mu$ M of Cu(at-sm) had no effect (Figure S1). Due to limited solubility of Cu(btsc)  
194 complexes, higher concentrations were not tested.

195 Since *N. gonorrhoeae* and *E. coli* represented the most susceptible and most resistant test  
196 organisms, respectively, they were examined further; the former because it may be a promising  
197 target for Cu- and Cu(btsc)-based therapeutics and the latter because its resistance properties and  
198 its amenability to molecular analysis might help in determining the mode of Cu(btsc) action.

199

200 **Inhibition of respiration in *E. coli* by Cu(btsc) complexes and antibacterial activity under**  
201 **anaerobic growth conditions.**

202           Recently, we showed that Cu(gtsm) and, to a lesser extent, Cu(at-sm) suppressed aerobic  
203 respiration in *N. gonorrhoeae* (13) and mitochondria (18). Within the electron transport chain,  
204 NADH dehydrogenases (Nuo or Complex I (H<sup>+</sup>-translocating) and Nqr (Na<sup>+</sup>-translocating)) were  
205 identified as the primary targets of inhibition. Inhibition occurred at or near the site of  
206 ubiquinone reduction and it was independent of the release of ‘free’ Cu ions (18). Instead, an  
207 intact Cu(gtsm) or Cu(at-sm) molecule was determined to be the inhibitory species. Subsequently,  
208 we proposed that the action of Cu(btsc) complexes as respiratory inhibitors may be a major  
209 mechanism of their antibacterial activity (13). However, our present work has now shown that  
210 these complexes were also effective against bacteria that do not respire such as *S. pneumoniae*  
211 (Table 2).

212           Cu(gtsm) (50 μM) also suppressed respiration in *E. coli*, as indicated by a decrease in the  
213 total amount of O<sub>2</sub> consumed after 15 min, while equal concentrations of Cu salts or Cu(at-sm)  
214 had no effect (Figure 3A). *E. coli* possesses two respiratory NADH dehydrogenases, Nuo and  
215 Ndh-2 (single-subunit flavoenzyme, does not translocate H<sup>+</sup>) (19), which may be targets of  
216 Cu(gtsm) inhibition. However, Cu(gtsm) only weakly suppressed the rates of NADH oxidation  
217 in isolated membrane vesicles containing both Nuo and Ndh-2 (Figure 3B). The *I*<sub>50</sub> value was  
218 extrapolated to be >130 μM (Figure S2), well beyond the solubility limit for Cu(gtsm) indicating  
219 that the NADH dehydrogenases in *E. coli* were not major targets of respiratory inhibition by  
220 Cu(gtsm). This finding was not altogether surprising, as Nuo and Ndh-2 in *E. coli* were also less  
221 sensitive to inhibition by the classical Nuo or Complex I antagonist, rotenone. This difference  
222 has been ascribed to subtle structural differences at or near the sites of ubiquinone reduction (20).

223           More importantly, Cu(gtsm) was also inhibitory to *E. coli* under anaerobic growth  
224 conditions, as evidenced by a curve of plating efficiency that was essentially indistinguishable to

225 that obtained under aerobic growth conditions (Figure S1). Therefore, unlike the situation in *N.*  
226 *gonorrhoeae* (13), inhibition of aerobic respiration did not appear to contribute significantly to  
227 the antibacterial activity of Cu(gtsm) against *E. coli*.

228

### 229 **Boosting of intracellular Cu levels by Cu(btsc) complexes.**

230 The MICs for both Cu(gtsm) and Cu(atsm) were consistently lower than those for ionic  
231 Cu salts by >2 orders of magnitude (Table 2). Unlike charged Cu ions, neutral Cu(btsc)  
232 complexes are presumed to be readily membrane-permeable. Thus, treatment with Cu(btsc)  
233 complexes would be expected to drive a greater accumulation in bacterial Cu contents when  
234 compared with equal doses of Cu salts. However, previous analyses of total Cu by ICP OES  
235 detected no such effect in *N. gonorrhoeae* (13). As *N. gonorrhoeae* was killed by low nanomolar  
236 doses of Cu(btsc) complexes (Tables 1 and 2), we reasoned that any gain in intracellular Cu  
237 might remain below the detection limit of these measurements.

238 The analyses of Cu content were repeated here using *E. coli* because of its ability to  
239 tolerate micromolar concentrations of Cu(gtsm) and Cu(atsm) (Table 2). First, we established  
240 that treatment with 10 – 15  $\mu$ M of ionic Cu salts increased the total Cu content of *E. coli* by 2 –  
241 3-fold when compared with the untreated control (Figure 4A). These amounts of intracellular Cu  
242 were non-inhibitory and there was no decrease in plating efficiency (Figure 4B). Treatment with  
243 similar doses of Cu(atsm) induced a comparable rise in Cu levels (Figure 4A), again without any  
244 loss in plating efficiency (Figures 3B). By contrast, exposure to equal doses of Cu(gtsm) led to a  
245 greater accumulation of Cu that was 5 – 8-fold higher relative to the unchallenged control  
246 (Figure 4A). This modest boost in intracellular Cu levels was correlated with an antibacterial  
247 effect and there was a 5 – 10-fold reduction in plating efficiency (Figure 4B). There was no

248 change in the levels of other transition metal ions (Figure S3), confirming that the antibacterial  
249 effect of Cu(gtsm) was Cu-dependent.

250

### 251 **Dissociation of Cu from Cu(btsc) complexes as bioavailable ions.**

252 The relative ability of Cu(gtsm) and Cu(atsm) to promote intracellular accumulation of  
253 Cu matched their relative antibacterial potency (Table 2). In fact, for all bacterial pathogens that  
254 we tested, the MICs of Cu(gtsm) were invariably lower than those of Cu(atsm) (Table 2). This  
255 result was in line with the proposed action of Cu(btsc) complexes as Cu carriers. The Cu(II)  
256 center in Cu(btsc) is bound strongly and is not thought to be dissociated as Cu(II) ions. Instead,  
257 Cu is released as Cu(I) (see Figure 8 below). This occurs upon reduction of the Cu(II) center by  
258 biological reductants such as thiols (21). As a consequence of a higher Cu(II)/Cu(I) reduction  
259 mid-point potential for Cu(gtsm) (Figure 1), dissociation of Cu(I) ions from Cu(gtsm) is assured  
260 (21). By contrast, Cu(atsm) possesses a lower Cu(II)/Cu(I) mid-point potential, and thus  
261 dissociation of Cu(I) ions from Cu(atsm) is not thought to occur except in hypoxic cells (22, 23).

262 The final amounts of intracellular Cu delivered by Cu(gtsm) and Cu(atsm) (< 25 ng  
263 Cu/mg protein) were well below the maximum tolerable capacity of *E. coli*. Exposure to higher  
264 doses of Cu salts (1500  $\mu$ M) led to the accumulation of Cu to 200 ng Cu/mg protein (Figure S3),  
265 but the plating efficiency of bacteria remained unchanged (Figure S1). This apparent disconnect  
266 between total Cu content and antibacterial potency has been observed previously (13, 15). Here it  
267 must be noted that ICP OES measurements do not differentiate between Cu that is captured by  
268 the bacterium as bioavailable ions and Cu that remains coordinated as a Cu(btsc) complex.

269 To examine the dissociation of Cu from Cu(btsc) complexes as bioavailable ions, we  
270 exploited the innate bacterial response to excess Cu. The system in *E. coli* is particularly well

271 characterized (Figure 5A) and amenable for analysis. In *E. coli*, increased intracellular Cu is  
272 sensed by the Cu(I)-specific transcriptional regulator CueR. In turn, CueR activates the  
273 expression of CopA, a membrane-bound P-type ATPase that exports Cu(I) out of the cytoplasm,  
274 and CueO, a periplasmic cuprous oxidase that oxidizes Cu(I) to the less toxic form Cu(II) (Figure  
275 5A) (24). In this work, we fused a plasmid-borne, promoterless *lacZ* transcriptional reporter gene  
276 with the promoter region of the *copA* gene (*PcopA-lacZ*, Figure 5A) and subsequently tested the  
277 ability of ionic Cu salts, Cu(gtsm) and Cu(atism) to induce  $\beta$ -galactosidase activity in *E. coli*.  
278 This opportunity was not available with *N. gonorrhoeae*, as the CueR regulon or any other  
279 recognizable Cu detoxification system is absent, with the sole exception of the efflux pump  
280 CopA (25). Furthermore, unlike the *copA* gene in *E. coli*, expression of gonococcal *copA* is  
281 controlled by an unidentified mechanism that does not appear to involve Cu (25).

282         Addition of Cu salts into the growth medium led to a dose-dependent increase in  
283  $\beta$ -galactosidase activity (Figure 5B). No induction was observed in the presence of other  
284 transition metal ions (Figure 5C), thus validating the Cu-specific response of the *PcopA-lacZ*  
285 fusion. More importantly, exposure to Cu(gtsm) also led to a robust induction of  $\beta$ -galactosidase  
286 activity (Figure 5B), consistent with the intracellular release of bioavailable Cu ions from  
287 Cu(gtsm), presumably as Cu(I). While the minimum dose of Cu salts required for induction was  
288  $> 1 \mu\text{M}$ , activation by Cu(gtsm) was observed at concentrations as low as  $0.1 \mu\text{M}$  (Figure 5B).  
289 Moreover, activation of *PcopA-lacZ* by Cu(gtsm) was rapid and the maximum response was  
290 achieved as early as 20 min post-exposure (Figure 5D). There was a detectable decrease in this  
291 response after 40 min, presumably due to the toxic effects of Cu(gtsm) or the dissociated Cu(I)  
292 ion. By comparison, induction of *PcopA-lacZ* by ionic Cu salts occurred gradually over a period

293 of at least 2 h (Figure 5D). These findings further confirmed that Cu(gtsm) is a more efficient  
294 source of intracellular bioavailable Cu(I) ions than uncomplexed Cu salts.

295 As mentioned earlier, the Cu center in Cu(atsm) is considered to display less dissociation  
296 intracellularly when compared to Cu(gtsm) (21-23). However, like Cu(gtsm), Cu(atsm) also  
297 activated the *PcopA* promoter as detected by an increase in  $\beta$ -galactosidase activity (Figure 5B).  
298 The maximum magnitudes of activation by Cu(atsm) and Cu(gtsm) were comparable (Figures  
299 4B and 4D). These results provided strong evidence that Cu was also released from Cu(atsm) as  
300 bioavailable Cu(I) ions. However, when compared with Cu(gtsm), there was a reproducible lag  
301 in the response to Cu(atsm) and a maximum was achieved only after 80 min of exposure (Figure  
302 5D). Taken together, this data is consistent with the view that Cu(gtsm) is a more efficient Cu  
303 delivery agent than Cu(atsm).

304

### 305 **Susceptibility of Cu tolerance mutants of *E. coli* to Cu(btsc) complexes and dissociation of** 306 **Cu ions in the cytoplasm.**

307 Activation of the *PcopA* promoter by Cu(gtsm) and Cu(atsm) implied that expression of  
308 *copA* and export of Cu(I) from the cytoplasm by CopA may mediate bacterial resistance to these  
309 Cu complexes. To test this proposal, we examined whether inactivation of *copA* in *E. coli*  
310 enhanced susceptibility to Cu(btsc) complexes. The effect of *copA* (and *cueO*) mutation on Cu  
311 tolerance in *E. coli* has been well characterised previously (26, 27). In this work, we confirmed  
312 that the *copA* mutant strain of *E. coli* was more sensitive to inhibition by Cu salts when  
313 compared with the isogenic parent strain (Figure 6A), consistent with the established role of  
314 CopA in tolerance to Cu ions. Importantly, the *copA* mutant was also sensitized to Cu(gtsm) and  
315 no bacterial growth was observed above 5  $\mu$ M (Figure 6B). This reduction in MIC when

316 compared with the wild type (MIC >25  $\mu$ M, Table 2) supports a role for Cu(I) ion efflux by  
317 CopA in the detoxification of Cu(gtsm).

318 A *cueO* mutant strain of *E. coli* that lacks the periplasmic cuprous oxidase (Figure 5A)  
319 also displayed a Cu salt-sensitive phenotype (Figure 6A). However, susceptibility of the *cueO*  
320 mutant to Cu(gtsm) was indistinguishable from that of the wild type (Figure 6B). This result  
321 indicated that, although oxidation of toxic Cu(I) to Cu(II) by CueO was required for tolerance to  
322 free Cu ions and salts, it was not essential for resistance to Cu(gtsm). While CopA protects  
323 against cytoplasmic Cu toxicity, CueO operates in the periplasm (Figure 5A). Thus, the apparent  
324 requirement for CopA but not CueO indicated that dissociation of bioavailable Cu ions from  
325 Cu(gtsm) occurred specifically in the cytoplasm and not in the periplasm.

326 In the case of Cu(atsm), there was no reduction in its MIC against the *copA* mutant  
327 (Figure 6C). However, there was a noticeable decrease in colony size (Figure 6C), which was  
328 consistent with a suppressed growth rate in liquid medium (Figure S4). These observations  
329 suggest that the CopA efflux pump may also confer tolerance to Cu(atsm), although the effect  
330 was subtle presumably because generation of bioavailable Cu ions from this complex was  
331 inefficient. As expected, Cu(atsm) had no observable effect on the *cueO* mutant (Figure 6C).

332 The toxic effects of salts of Cu and other metal ions are known to be affected severely by  
333 speciation or potential binding and buffering by components of the culture medium (28). Thus, it  
334 is often the case that the less complex the medium, the lower the MICs. The inhibitory effects of  
335 Cu(gtsm) and Cu(atsm) on the growth of the most sensitive mutant, *copA*, were similar when  
336 tested in LB or in M9 medium (Figure S4), suggesting that there was minimal release of free Cu  
337 ions from Cu(btsc) complexes in the extracellular medium.

338

339 **Efficacy of Cu(btsc) complexes against MDR strains of *N. gonorrhoeae*.**

340 The demonstrated action of Cu(gtsm) and Cu(at-sm) as carriers of Cu ions and the  
341 established mechanism of Cu ion poisoning by mis-metallation of enzymes and promotion of  
342 redox stress are distinct from the known modes of action of conventional antibiotics. Thus, we  
343 propose that these complexes may represent a promising new strategy for the treatment of  
344 antibiotic-resistant bacterial infections. Their low relative MICs against *N. gonorrhoeae* (Table  
345 2) indicated that Cu(gtsm) and Cu(at-sm) were highly potent against this bacterium. Therefore,  
346 we extended this work to test the potential for Cu(btsc) complexes to be used against multidrug-  
347 resistant (MDR) *N. gonorrhoeae*.

348 Cu(gtsm) showed robust activity against several antibiotic-resistant isolates of *N.*  
349 *gonorrhoeae* (Table 3), including the MDR strains F89 (29) and H041 (30), which are resistant  
350 to  $\beta$ -lactams (except carbapenems), fluoroquinolones, macrolides, tetracycline, chloramphenicol,  
351 trimethoprim-sulfamethoxazole, chloramphenicol, and nitrofurantoin. The MICs for these  
352 isolates were comparable to that for the drug-susceptible strain 1291 (Table 3), suggesting that  
353 the antibacterial activity of Cu(gtsm) was not diminished by enzymes and transporters that  
354 confer resistance to other antibiotics. In *N. gonorrhoeae*, these include the pilus secretin PilQ  
355 (31) and the MtrFCDE multidrug efflux pump system (32). To test this hypothesis, we  
356 determined the MICs of Cu(gtsm) for strains KH15 and DW120, which are isogenic mutants of  
357 the drug-susceptible strain FA19 that express higher basal levels of the MtrCDE pump (32).  
358 Although these strains showed increased resistance to multiple antibiotics and antimicrobial  
359 peptides when compared with the parent strain (32), they were no less susceptible to Cu(gtsm)  
360 (Table 3).



361 Like Cu(gtsm), Cu(atasm) was also effective against MDR isolates and MtrFCDE-  
362 overexpressing strains of *N. gonorrhoeae* (Table 3). However, there were > 2-fold increases in  
363 MICs when compared to the antibiotic-susceptible strains (Table 3). This loss of efficacy  
364 indicated that Cu(atasm) may be a substrate for the MtrCDE efflux pump. Consistent with this  
365 proposal, inactivation of the *mtrD* gene (strain KH14) (33) led to a modest but reproducible  
366 decrease in the MIC of Cu(atasm) when compared to the isogenic parent strain (FA19) (Table 3).  
367 The loss of *mtrD* did not have any effect on the MIC of Cu(gtsm) (Table 3).

368

369 **Viability of host epithelial cells in the presence of antimicrobial doses of Cu(btsc)**  
370 **complexes.**

371 To ascertain the potential of Cu and Cu(btsc) complexes as a clinically useful anti-  
372 gonococcal agent, we examined whether Cu(gtsm) and Cu(atasm) exert an effect on the viability  
373 of cervical epithelial cells (ME-180 epithelial cells) *in vitro*. As shown in Figure 7, incubation of  
374 ME-180 monolayers with up to 0.5  $\mu$ M of Cu(gtsm) and 5  $\mu$ M of Cu(atasm) (ca. 5X the MIC  
375 against *N. gonorrhoeae*, see Table 3) for 24 h did not result in loss of cell viability. There was no  
376 loss in cell numbers (Figure 7) and more than 95% of these cells retained the ability to exclude  
377 Trypan Blue (Figure 7). Under our experimental conditions, the ME-180 cell line did not  
378 withstand treatment with DMSO beyond 0.1 v/v %. Thus, higher concentrations of the Cu  
379 complexes were not tested because of poor solubility in the culture media without DMSO.  
380 Nevertheless, previous work with human prostate epithelial cells were had used up to 100  $\mu$ M  
381 without any observable loss of viability (34), indicating a potential therapeutic index of 1000 for  
382 *N. gonorrhoeae* These results indicate that at antimicrobial doses, the test compounds exert  
383 minimal toxicity towards host cells.

384

## 385 **DISCUSSION**

386

### 387 **Insight into the mode of antibacterial action of Cu(btsc) complexes.**

388         *Dynamics of “Cu-boosting” by Cu(btsc) complexes.* The use of lipophilic ligands to  
389 deliver metal ions into cells is an established concept, particularly in the detection and  
390 intervention of cancers (35). In terms of antimicrobial applications, this concept has been  
391 demonstrated by the use of zinc pyrithione (Zn-PYT) as an antifungal in soaps and shampoos.  
392 The mode of action of Zn-PYT depends on transchelation with free Cu ions, presumably from  
393 the extracellular environment, and subsequent delivery of these Cu ions into the target organism  
394 (36). Coordination complexes that act as carriers for Cu ions are now being increasingly  
395 investigated as a novel approach to combat bacterial infections (9, 13-15).

396         The bis(thiosemicarbazone) family of Cu carriers displays anticancer activities (37).  
397 There is also interest in their potential as neurotherapeutics and as imaging agents for hypoxia.  
398 Our group and others have now demonstrated that Cu(btsc) complexes are also promising  
399 antimicrobials (13-15). However, while studies with mammalian systems have established the  
400 action of these complexes as agents that alter the bioavailability of Cu, studies with bacteria have  
401 not been equally conclusive, as the final Cu content of treated bacteria did not always correlate  
402 with survival or viability (13, 15). These studies have focused on the total amounts of Cu at the  
403 ‘end point’ (eg. the ICP OES measurements in Figure 4A) but the present work suggested that  
404 the kinetics of Cu influx and potential efflux by detoxification systems must also be considered  
405 (Figure 8). This model is likely to be universal to all lipophilic Cu carriers and not limited to  
406 those containing btsc ligands.

407 Our results using *E. coli* showed that both Cu(gtsm) and Cu(at-sm) enter the bacterial  
408 cytoplasm more rapidly than do ionic Cu salts. These complexes are membrane-permeable,  
409 probably *via* passive diffusion as an uptake system has not been identified (38). As these  
410 complexes are uncharged, they would equilibrate rapidly across bacterial membranes and would  
411 not accumulate as intact molecules to a high intracellular concentration. However, reduction of  
412 the Cu(II) center and subsequent dissociation as bioavailable Cu(I) ion would generate a  
413 powerful mass-action effect (Figure 8). This thermodynamically-driven influx of Cu ions may  
414 overwhelm basal Cu tolerance and cause Cu poisoning. By comparison, the more restricted entry  
415 of ionic Cu salts may allow activation of dedicated Cu detoxification mechanisms, which would  
416 enable the bacterial cell to amass and, more importantly, survive higher final amounts of total  
417 Cu.

418 The above model extends to the observed difference between the antibacterial activities  
419 of Cu(gtsm) and Cu(at-sm). While studies with mammalian systems suggest little intracellular  
420 dissociation of bioavailable Cu ions from Cu(at-sm) (21-23), our work with *E. coli* indicated that  
421 it does occur, although it is less efficient than the equivalent process from Cu(gtsm). We cannot  
422 discount possible variations in the rates of membrane penetration by the two complexes as a  
423 result of subtle differences in lipophilicity (39). Nevertheless, it is more likely that, as a  
424 consequence of a lower Cu(II)/Cu(I) mid-point potential for Cu(at-sm) (Figure 1), the rate of  
425 reduction (and, subsequently, dissociation) of Cu from Cu(at-sm) is also lower. The bacterial cell  
426 thus has more time to respond and detoxify the excess Cu, resulting in a lower antibacterial  
427 potency of Cu(at-sm).

428 Our results with MtrCDE-overexpressing strains of *N. gonorrhoeae* (Table 3) also  
429 indicate that potential efflux of Cu(gtsm) and Cu(at-sm) as intact molecules out of the cytoplasm

430 must not be overlooked. Cu(gtsm) rapidly and efficiently dissociates within the cytoplasm and  
431 thus this complex may evade active export by promiscuous efflux transporters. By comparison,  
432 Cu(atsm) may linger as an intact molecule and thus be exported prior to dissociation and  
433 subsequent release of bioavailable Cu(I) ions (Figure 8). This removal of Cu(atsm) from the  
434 cytoplasm, either by MtrCDE or other efflux systems, would further reduce its antibacterial  
435 efficacy. Although we have not tested this idea directly, the AcrAB-TolC multi-drug efflux  
436 pump system (ref) it is likely that in this transporter will contribute to the observed resistance of  
437 this bacterium to Cu(atsm) (40).

438 ***Correlation between bacterial physiology and susceptibility to Cu(btsc) complexes.*** The  
439 antibacterial activity of Cu(btsc) complexes has now been tested against several important  
440 human pathogens, including *S. pneumoniae*, *H. influenzae*, uropathogenic *E. coli*, *Salmonella*  
441 (Table 2), as well as *M. tuberculosis* (14) and *S. aureus* (15). Not all of these showed equal  
442 promise as targets for Cu(btsc) therapeutics, but all showed less susceptibility when compared  
443 with *N. gonorrhoeae*. The explanation for these differences may relate to bacterial physiology.

444 Compared with most other bacterial pathogens, *N. gonorrhoeae* possesses a Cu  
445 detoxification system that is unusually underdeveloped. It consists of a single Cu efflux pump,  
446 CopA, and no additional cytoplasmic or periplasmic accessories (25). Importantly, Cu does not  
447 induce the expression of the *copA* gene. Thus, while gonococcal CopA may participate in general  
448 maintenance of Cu levels during regular metabolism (25, 41), it may be unable to confer  
449 resistance to severe Cu stress. This absence of an inducible resistance system coincides with the  
450 availability of targets of poisoning by Cu or Cu(btsc) complexes (Figure 8). These targets  
451 include iron-sulfur (Fe-S) cluster-containing enzymes such as coproporphyrinogen(III) oxidase in  
452 the pathway for heme biosynthesis (41). In addition, *N. gonorrhoeae* depends on two NADH

453 dehydrogenases that are both susceptible to inhibition by Cu(btsc) complexes (13). As a  
454 consequence, *N. gonorrhoeae* displays hypersensitivity to inhibition by Cu salts and Cu(btsc)  
455 complexes, particularly Cu(gtsm) (Tables 1 – 3).

456 In *E. coli*, major targets of Cu poisoning are available, such as the Fe-S cluster enzymes  
457 fumarase in the TCA cycle and isopropylmalate dehydratase in the pathway for branched-chain  
458 amino acid synthesis (42). However, this bacterium also possesses sophisticated and robust,  
459 inducible defenses against Cu toxicity (24). In addition, *E. coli* uses a versatile respiratory  
460 electron transport system and fermentative systems that are less sensitive to Cu(btsc) complexes.  
461 These arguments correlate well with our finding that *E. coli* is more resistant to inhibition by  
462 Cu(btsc) complexes, even Cu(gtsm) (Table 2). Similarly, the Cu detoxification system in *S.*  
463 *pneumoniae* is relatively well-developed compared with the system in *N. gonorrhoeae*. However,  
464 *S. pneumoniae* has a relatively low dependence on Fe-S cluster enzymes and it does not contain a  
465 respiratory chain. This may explain the limited sensitivity of this bacterium to Cu(atms) and  
466 Cu(gtsm) (Table 2). For these Cu-tolerant bacteria, the antimicrobial efficacy of Cu delivery  
467 agents might be enhanced if used in conjunction with a CopA antagonist to trap excess Cu ions.

468

#### 469 **Cu delivery agents as a novel concept for the topical treatment of *N. gonorrhoeae*.**

470 Gonorrhea is the second most prevalent sexually transmissible infection worldwide and  
471 management of this disease represents significant challenge to public health. There is no vaccine  
472 and thus antibiotic treatment remains the only method to control the spread of infection.  
473 However, MDR strains have developed resistance to virtually all first-line antibiotics (43). Our  
474 work suggests that delivery of bioavailable Cu ions may represent a new approach to combat  
475 gonococcal infections. The application of copper in the cervix and vagina is an established

476 concept and intrauterine devices containing elemental copper is one of the most common and  
477 most effective non-hormonal contraceptives worldwide (44). Here we showed that Cu salts and  
478 Cu(btsc) complexes did not affect the viability of cervical epithelial cells *in vitro* at  
479 concentrations that were inhibitory to the gonococcus (Figure 7). Moreover, Cu salts and  
480 Cu(btsc) complexes were ineffective against lactic acid bacteria, as exemplified by *Lactobacillus*  
481 *acidophilus* (see Table 2), suggesting that Cu delivery agents can be used to target gonococci  
482 without significantly affecting the commensal flora. Crucially, unlike other bacterial pathogens  
483 that have been identified as potential targets for treatments by Cu delivery agents, including *M.*  
484 *tuberculosis* (14), *S. aureus* (15), and *Cryptococcus neoformans* (9), *N. gonorrhoeae* is primarily  
485 an extracellular mucosal pathogen that colonises surfaces of the genitourinary epithelium.  
486 Gonococcal infections are thus amenable to topical drug formulations and would bypass many of  
487 the challenges of a systemic route for the delivery of Cu.

488

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504 strains.

505

## 506 **CORRESPONDENCE**

507 Address all correspondence to KYD (k.djoko@uq.edu.au) or AGM  
508 (mcewan@uq.edu.au). The authors declare no competing financial interest.

509

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652

653

654 **TABLES**

655

656 **Table 1.** Antibacterial activity of several Cu complexes against *N. gonorrhoeae*.

657

<b>Complex<sup>a</sup></b>	<b>MIC (<math>\mu</math>M)</b>
Cu(gtsm)	0.10
Cu-pyrithione	0.13
Cu(atsm)	0.80
Cu-neocuproine	0.80
Cu-disulfiram	15 – 20
Cu salt	250

658

659 <sup>a</sup>The complexes were prepared and their concentrations standardised as described in Materials  
660 and Methods.

661

662 **Table 2.** Susceptibility of select bacterial pathogens to Cu(atSm) and Cu(gtSm).

663

<b>Organism<sup>a</sup></b>	<b>MIC (<math>\mu</math>M)</b>	
	<b>Cu(atSm)</b>	<b>Cu(gtSm)</b>
<i>N. gonorrhoeae</i>	0.8	0.1
<i>H. influenzae</i>	10	1
<i>S. aureus</i>	>10	1.5
<i>S. pneumoniae</i>	>10	2
<i>L. acidophilus</i>	>25	5
<i>S. Typhimurium</i>	>25	>25
<i>E. coli</i>	>25	>25

664

665 <sup>a</sup>Strain information is available in Materials and Methods.

666

667 **Table 3.** Susceptibility of multidrug-resistant strains of *N. gonorrhoeae* to Cu salt, Cu(atsm) and  
 668 Cu(gtsm). Data presented were averaged from three independent experiments. Standard  
 669 deviations from the mean are shown in brackets.

670

671

	Strain <sup>a</sup>	Ref	MIC (μM)		
			Cu salt	Cu(atsm)	Cu(gtsm)
antibiotic-sensitive	1291	(45)	250 (0)	0.8 (0.1)	0.1 (0.0)
	FA1090	(46)	250 (0)	0.9 (0.1)	0.1 (0.0)
	F62	(47)	250 (0)	0.7 (0.1)	0.1 (0.0)
antibiotic-resistant	MS11	(48)	250 (0)	2.3 (0.2)	0.1 (0.0)
	FA6140	(49)	250 (0)	3.0 (0.3)	0.1 (0.2)
	F89	(29)	250 (0)	1.7 (0.3)	0.1 (0.0)
	H041	(30)	250 (0)	1.9 (0.2)	0.1 (0.0)
	FA19	(50)	250 (0)	0.9 (0.1)	0.1 (0.0)
	DW120	(32)	250 (0)	2.2 (0.2)	0.1 (0.0)
	KH15	(32)	250 (0)	2.7 (0.3)	0.1 (0.0)
	KH14	(33)	250 (0)	0.7 (0.1)	0.1 (0.0)

672

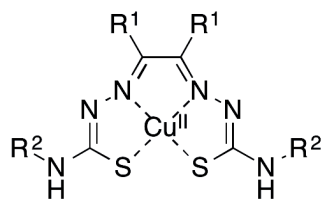
673 <sup>a</sup>Antibiotic resistance profiles of select strains are shown in Table S1.

674



675 **FIGURES**

676



**Cu(gtsm):**  $R^1 = \text{H}; R^2 = \text{CH}_3$   
 $E_{1/2} = -440 \text{ mV}$

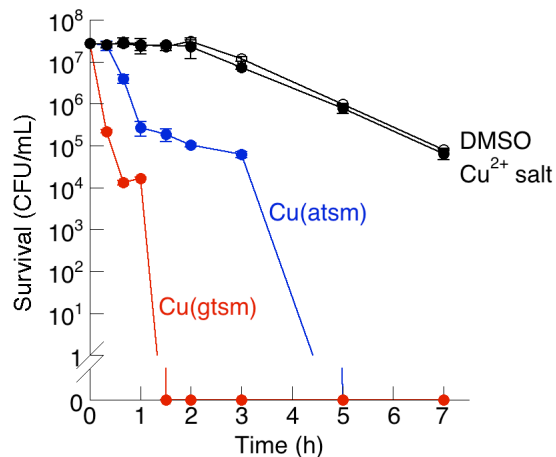
**Cu(at-sm):**  $R^1 = R^2 = \text{CH}_3$   
 $E_{1/2} = -600 \text{ mV}$

677

678 **Figure 1.** Structure of Cu(btsc) complexes. Mid-point reduction potentials are vs Ag/AgCl.<sup>21</sup>

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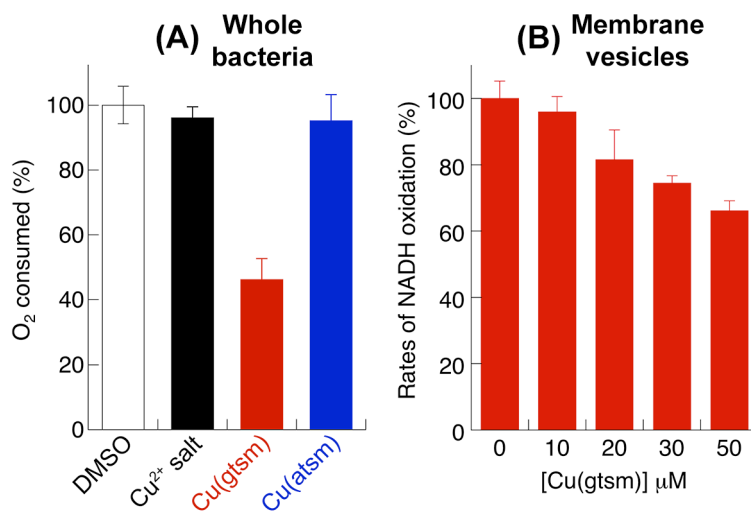
683 **Figure 2.** Time-dependent bactericidal effects of Cu(btsc) complexes (1  $\mu$ M each) against *N.*

684 *gonorrhoeae* 1291. Number of surviving CFUs was plotted against time post-challenge. The

685 effects of Cu salt (1  $\mu$ M) and DMSO control were also shown. Each data point was averaged

686 from three independent replicates. Error bars represent  $\pm$  standard deviation from the mean.

687



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689

690 **Figure 3.** Inhibitory effects of Cu(btsc) complexes on aerobic respiration by *E. coli*. **(A)**

691 Amounts of O<sub>2</sub> consumed by whole bacteria over 15 min in the presence of various Cu sources

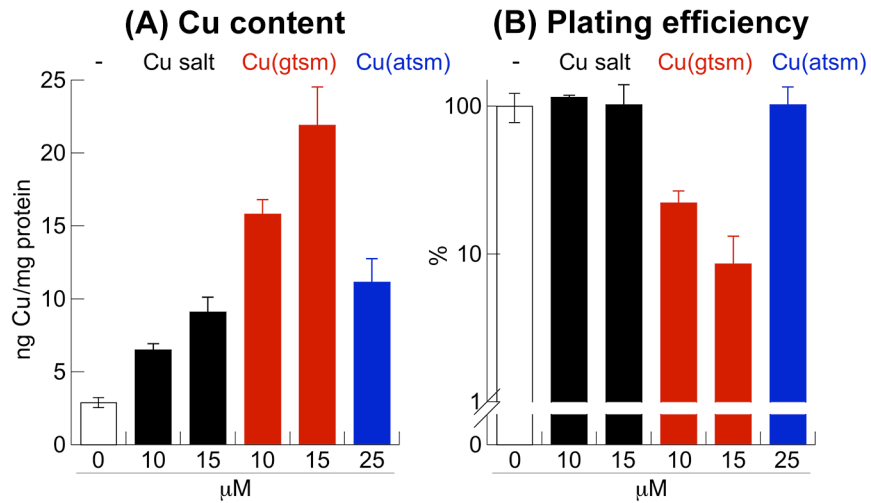
692 (50 μM). **(B)** Rates of NADH oxidation by isolated membrane vesicles in the presence of

693 Cu(gtsm) (0 – 50 μM). **(A and B)** Results were shown as a percentage of the unchallenged

694 control. Each data point was averaged from three independent replicates. Error bars represent ±

695 standard deviation from the mean.

696

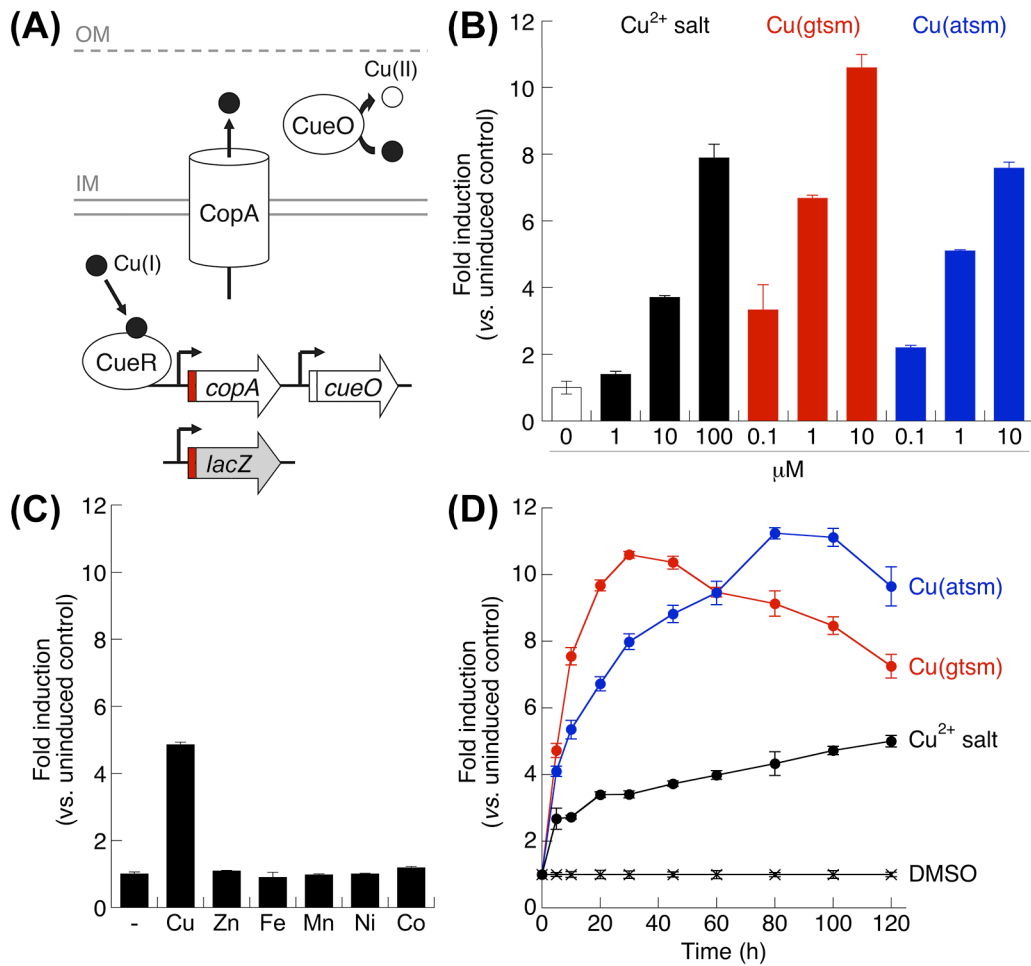


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699 **Figure 4.** Effects of Cu(btsc) complexes on **(A)** total intracellular Cu content and **(B)** plating  
 700 efficiency of *E. coli*, presented as a percentage of vs. the unchallenged control. **(A and B)**  
 701 Treatment time was 24 h. Each data point was averaged from three independent replicates. Error  
 702 bars represent  $\pm$  standard deviation from the mean.

703



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706 **Figure 5.** Release of Cu ions from Cu(btsc) complexes. **(A)** Cu detoxification system in *E. coli*.

707 To determine the presence of bioavailable Cu(I) ions in the cytoplasm, the promoter region of  
 708 *copA* (in red) was fused to a promoterless *lacZ* gene (in grey). IM, inner membrane; OM, outer

709 membrane. **(B)** Response of *PcopA-lacZ* fusion to divalent transition metal ions (supplied as

710 chloride salt, 100  $\mu\text{M}$  each) at t = 2 h post-exposure. **(C)** Dose-dependent induction of the *copA*

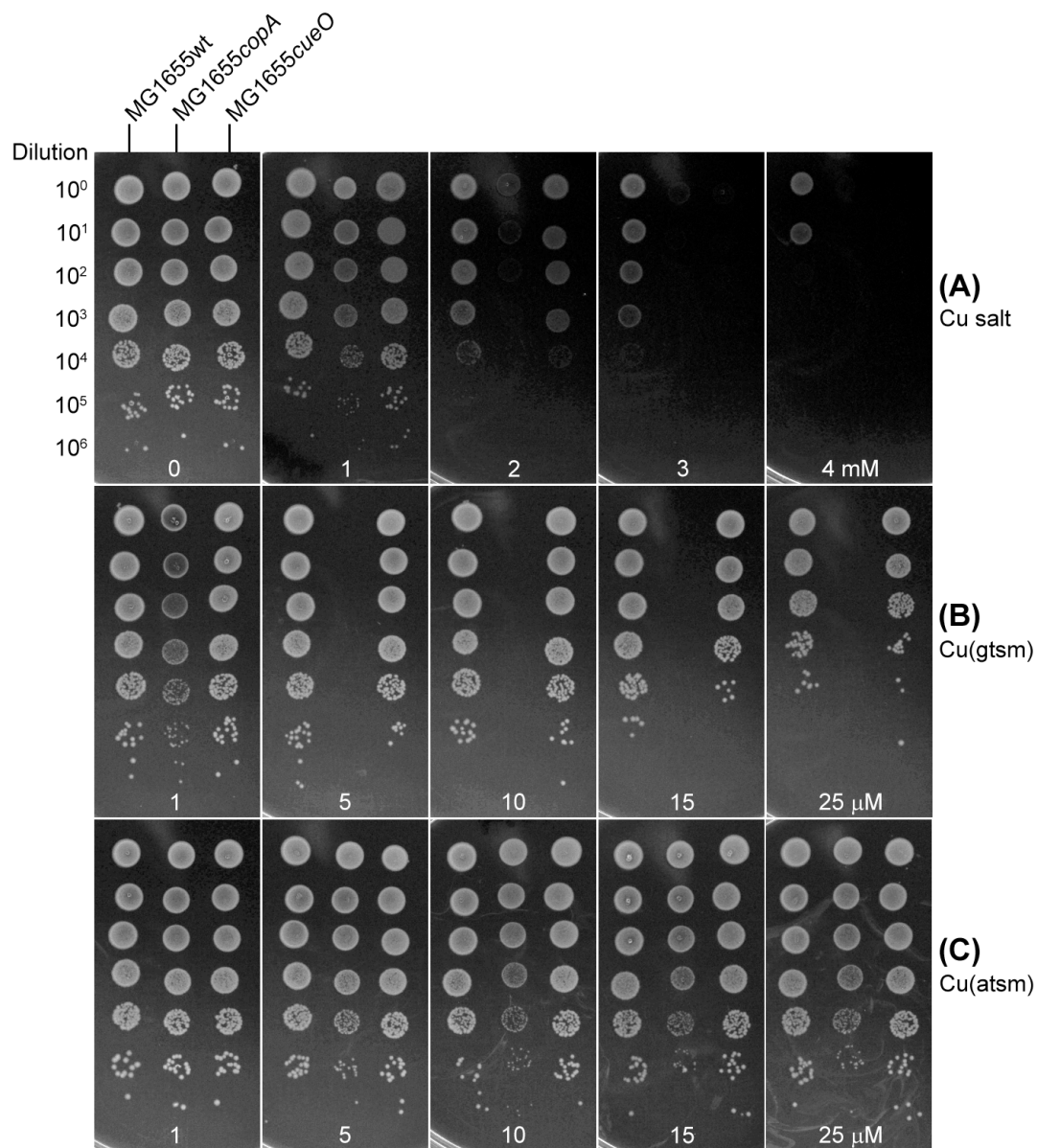
711 promoter.  $\beta$ -galactosidase activity was assayed at t = 2 h post-exposure. **(D)** Time-dependent

712 induction of the *copA* promoter in response to Cu salt (100  $\mu\text{M}$ ),  $\text{Cu}(\text{gtsm})$  (10  $\mu\text{M}$ ) or  $\text{Cu}(\text{atism})$

713 (25  $\mu\text{M}$ ). **(B – D)** Each data point was averaged from three replicates. Error bars represent  $\pm$

714 standard deviation from the mean. The results shown were representative of at least three  
715 independent experiments.

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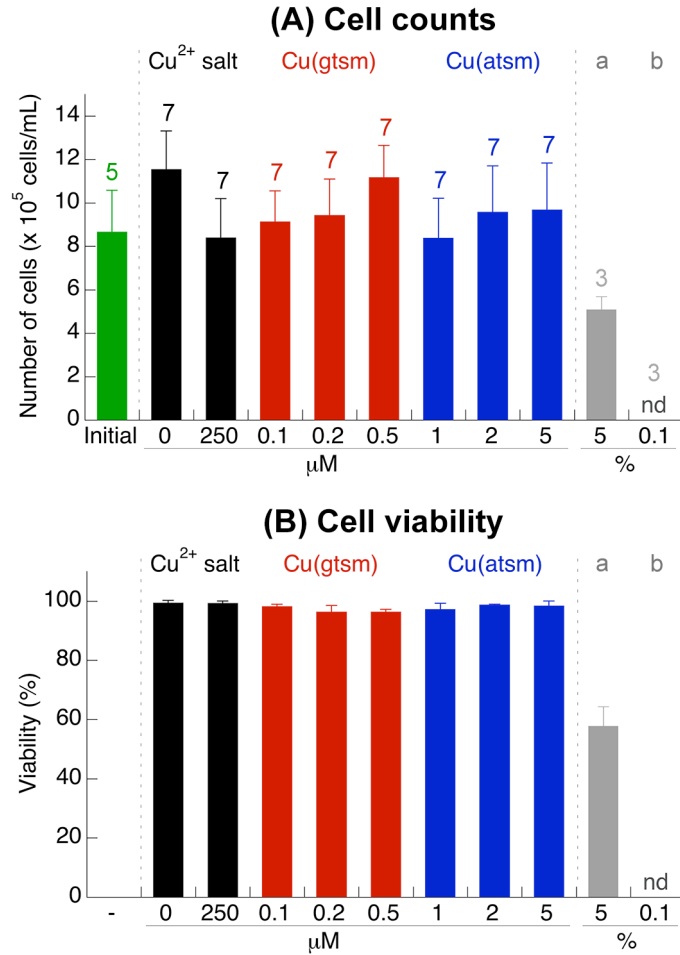
719 **Figure 6.** Susceptibility of *E. coli* mutant strains to (A) Cu salt, (B) Cu(gtsm), and (C) Cu(at sm)

720 as determined by efficiency of plating on solid medium. Serial dilutions of bacteria were shown

721 on the left. Concentrations of the various Cu sources were indicated at the bottom. Treatment

722 time was 24 h. The results shown were representative of at least three independent experiments.

723



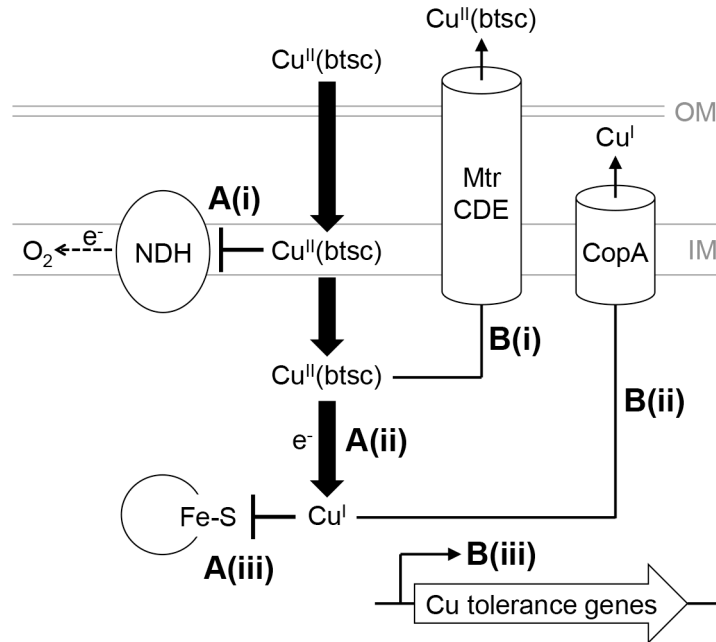
724

725

726 **Figure 7.** Effects of Cu(btsc) complexes on **(A)** total cell counts and **(B)** cell viability of ME-180  
 727 cervical epithelial cells. The treatment time was 24 h. The viability of cells in panel **B** was  
 728 determined using the same corresponding set of cells used for counting in panel **A**. The number  
 729 of replicates is shown above each column. Error bars represent  $\pm$  standard deviation from the  
 730 mean. There was no statistically significant difference in the cell counts of viability from the  
 731 different treatments. The results shown were representative of at least three independent  
 732 experiments. DMSO (5 v/v %, columns a) and Triton X-100 (0.1 v/v %, columns b) were also  
 733 included as positive controls of cell death.

734





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737 **Figure 8.** Schematic representation for the antibacterial action of Cu(btsc) complexes. **A.**

738 Mechanisms of action: **(i)** Inhibition of membrane-bound NADH dehydrogenases of the electron

739 transport chain by the intact Cu(btsc) complex, **(ii)** reduction of the  $\text{Cu}^{\text{II}}$  centre and dissociation

740 of  $\text{Cu}^{\text{I}}$  as bioavailable ions, and **(iii)** poisoning of enzymes by  $\text{Cu}^{\text{I}}$  ions. **B.** Mechanisms of

741 tolerance: **(i)** Efflux of the intact Cu(btsc) complex by the MtrCDE efflux pump or other

742 promiscuous drug transporters, **(ii)** efflux of bioavailable  $\text{Cu}^{\text{I}}$  ions, and **(iii)** activation of other

743 dedicated Cu ion tolerance genes.