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1 **Copper ions and coordination complexes as novel carbapenem adjuvants**

2

3 Karrera Y. Djoko<sup>1,2,3#</sup>, Maud E. S. Achard<sup>1,2#</sup>, Minh-Duy Phan<sup>1,2</sup>, Alvin W. Lo<sup>1,2</sup>, Manfredi  
4 Miraula<sup>1,2,4</sup>, Sasiprapa Prombhul<sup>1,2</sup>, Steven J. Hancock<sup>1,2</sup>, Kate M. Peters<sup>1,2</sup>, Hanna  
5 Sidjabat<sup>2,4</sup>, Patrick N. Harris<sup>2,4</sup>, Nataša Mitić<sup>5</sup>, Timothy R. Walsh<sup>6</sup>, Gregory J. Anderson<sup>1,7</sup>,  
6 William M. Shafer<sup>8</sup>, David L. Paterson<sup>2,4</sup>, Gerhard Schenk<sup>1,2</sup>, Alastair G. McEwan<sup>1,2</sup>, Mark  
7 A. Schembri<sup>1,2\*</sup>

8

9 <sup>#</sup>These authors contributed equally

10

11 <sup>1</sup>School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane,  
12 Queensland 4072, Australia.

13 <sup>2</sup>Australian Infectious Diseases Research Centre, The University of Queensland, Brisbane,  
14 Queensland 4072, Australia.

15 <sup>3</sup>Department of Biosciences, Durham University, Durham DH13LE, United Kingdom

16 <sup>4</sup>University of Queensland Centre for Clinical Research, The University of Queensland,  
17 Brisbane, Queensland 4029, Australia

18 <sup>5</sup>Department of Chemistry, Maynooth University, Maynooth, Co. Kildare, Ireland.

19 <sup>6</sup>School of Medicine, Cardiff University, Cardiff CF14 4XN, United Kingdom

20 <sup>7</sup>Iron Metabolism Laboratory, QIMR Berghofer Medical Research Institute, Herston,  
21 Queensland, Australia.

22 <sup>8</sup>Department of Microbiology and Immunology, and Emory Antibiotic Resistance Center,  
23 Emory University School of Medicine, Atlanta, Georgia, USA; Laboratories of Bacterial  
24 Pathogenesis, Veterans Affairs Medical Center, Decatur, Georgia, USA

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34 #Corresponding author:

35 Mailing address: School of Chemistry and Molecular Biosciences, Building 76, The

36 University of Queensland, Brisbane, Queensland 4072, Australia. Telephone: +6173365

37 3306; E-mail: [m.schembri@uq.edu.au](mailto:m.schembri@uq.edu.au)

38 **ABSTRACT**

39 Carbapenem-resistant *Enterobacteriaceae* are an urgent threat to global human health. These  
40 organisms produce  $\beta$ -lactamases with carbapenemase activity, such as the metallo- $\beta$ -  
41 lactamase NDM-1, which is notable due to its association with mobile genetic elements and  
42 the lack of a clinically useful inhibitor. Here we examined the ability of copper to inhibit the  
43 activity of NDM-1 and explored the potential of a copper coordination complex as a  
44 mechanism to efficiently deliver copper as an adjuvant in clinical therapeutics. An NDM-  
45 positive *Escherichia coli* isolate, MS6192, was cultured from the urine of a patient with  
46 urinary tract infection. MS6192 was resistant to antibiotics from multiple classes, including  
47 diverse  $\beta$ -lactams (penicillins, cephalosporins, and carbapenems), aminoglycosides and  
48 fluoroquinolones. However, in the presence of copper (range 0-2 mM), the susceptibility of  
49 MS6192 to the carbapenems ertapenem and meropenem increased significantly. In standard  
50 checkerboard assays, copper decreased the MIC of ertapenem and meropenem against  
51 MS6192 in a dose-dependent manner, suggesting a synergistic mode of action. To examine  
52 the inhibitory effect of copper in the absence of other  $\beta$ -lactamases, the *bla*<sub>NDM-1</sub> gene from  
53 MS6192 was cloned and expressed in a recombinant *E. coli* K-12 strain. Analysis of cell-free  
54 extracts prepared from this strain revealed copper directly inhibits NDM-1 activity, and this  
55 was further confirmed using purified recombinant NDM-1. Finally, delivery of copper at a  
56 low concentration of 10  $\mu$ M using the FDA-approved coordination complex copper-  
57 pyrrithione sensitised MS6192 to ertapenem and meropenem in a synergistic manner. Overall,  
58 this work demonstrates the potential use of copper-coordination complexes as novel  
59 carbapenemase adjuvants.

60

61 **INTRODUCTION**

62 Carbapenems (ertapenem, doripenem, imipenem, meropenem) are  $\beta$ -lactam antibiotics with  
63 broad-spectrum activity (1, 2). They are generally used as a last resort for treating infections  
64 caused by cephalosporin-resistant *Enterobacteriaceae*. Hence, it is alarming that resistance to  
65 carbapenems, primarily in Gram-negative bacteria, has now emerged and disseminated  
66 worldwide, leading to high rates of treatment failure and increased complications (3-6).  
67 Carbapenem-resistant *Enterobacteriaceae* (CRE), which include *Escherichia coli* and  
68 *Klebsiella pneumoniae*, are frequently associated with hospital-acquired lung, urinary tract,  
69 bloodstream, and device-related infections, with urinary tract infections (UTI), including  
70 catheter-associated UTI, being the most common infection acquired in the nosocomial setting  
71 (7). Combined with the asymptomatic carriage of CRE (8, 9) and the potential for  
72 transmission of resistance via mobile genetic elements (10, 11), it is not surprising that CRE  
73 are recognised as one of the most urgent threats to global human health today (12).

74

75 Mechanisms of carbapenem resistance in CRE frequently involve the expression of  
76 carbapenemases, which are broad-spectrum  $\beta$ -lactamases that hydrolyse carbapenems with  
77 high catalytic efficiency. These carbapenemases are diverse and include the Ambler class A  
78 (*e.g.* KPC) and class D (*e.g.* OXA-48) serine hydrolases, as well as class B metallo- $\beta$ -  
79 lactamases (MBLs, *e.g.* VIM, IMP, NDM) (13). One approach to combat carbapenem  
80 resistance would be to develop adjuvants that inhibit carbapenemases, thus restoring  
81 susceptibility to carbapenems and ultimately extending the use of these antibiotics. For the  
82 serine-dependent lactamases, this strategy is best exemplified by the use clavulanic acid and  
83 tazobactam in the clinic as  $\beta$ -lactam adjuvants that inhibit the activity of extended-spectrum  
84  $\beta$ -lactamases (ESBLs) and restore susceptibility of ESBL-positive strains to  $\beta$ -lactams (14).  
85 However, such inhibitors are ineffective against MBLs (15, 16). MBLs require up to two zinc

86 ions for their activity and thus are inhibited by reagents that disrupt zinc binding, either by  
87 complete chelation (*e.g.* EDTA) or partial coordination (*e.g.* thiol-containing compounds)  
88 (14, 15, 17). Despite their effectiveness *in vitro*, these inhibitors have not proven to be  
89 clinically useful (14, 15).

90

91 Here we present evidence for a possible approach to inactivate MBLs with copper ions.  
92 Using the New Delhi Metallo- $\beta$ -lactamase 1 (NDM-1) enzyme as our model, we show that  
93 copper ions inhibit the activity of this MBL *in vitro* and enhance the susceptibility of NDM-  
94 positive isolates of *E. coli* to carbapenems. Using pyrrhione, an FDA-approved antifungal  
95 agent that exerts an antimicrobial effect by acting as a copper delivery molecule (18), we also  
96 provide proof of concept that copper coordination complexes have the potential to be used as  
97 carbapenem adjuvants in the clinic.

98

## 99 MATERIALS AND METHODS

100 **Bacterial strains, reagents, and culture conditions.** *E. coli* MS6192 is an NDM-1-positive  
101 strain isolated from the urine of a patient with UTI. *E. coli* MG1655 is a K-12 strain and is  
102 susceptible to all antibiotics. All strains were propagated from frozen glycerol stocks on  
103 Luria-Bertani (LB) agar at 37 °C. Liquid cultures were prepared in LB broth and grown at 37  
104 °C with shaking at 200 rpm. Antibiotic discs (Sensi-discs) were purchased from BD  
105 Biosciences (Australia). Copper(II) sulfate (C8027), zinc(II) pyrithione (H6377), ertapenem  
106 sodium (SML1238), and meropenem trihydrate (M2574) were purchased from Sigma  
107 (Australia). Stocks of reagents were prepared in deionised water except zinc pyrithione,  
108 which was dissolved in DMSO. Copper(II) pyrithione was prepared by adding equimolar  
109 amounts of copper sulfate to a solution of zinc(II) pyrithione.

110

111 **Cloning and expression of the *bla*<sub>NDM-1</sub> gene in MG1655.** The *bla*<sub>NDM-1</sub> gene from MS6192  
112 was amplified with primers 7414 (5'-tgataaggatccattcagcttcacccattgg) and 7415 (5'-  
113 tcgaaaaagcttgatggcagattgggggtga) and cloned between the *Bam*HI and *Hind*III sites of  
114 pSU2718. The resulting plasmid pSU2718::*bla*<sub>NDM-1</sub> was transformed into *E. coli* MG1655  
115 by electroporation to generate the NDM-1-positive strain MS8485. MS8485 was cultured in  
116 the presence of chloramphenicol (30 µg/mL) and IPTG (0.1 mM) to maintain the plasmid and  
117 promote expression of NDM-1, respectively.

118

119 **Antibiotic susceptibility assays.** The antimicrobial susceptibility profile of MS6192 was  
120 determined using the Vitek 2 automated AST-N426 card (bioMérieux). The E-test was used  
121 to determine MICs for meropenem, imipenem and ertapenem. Disc diffusion assays were  
122 performed by seeding LB agar containing copper sulfate (0-2 mM) or copper pyrithione (0-20  
123 µM) with bacterial suspensions at an OD<sub>600</sub> of 0.18 (~1.5 x 10<sup>8</sup> CFU/mL, equivalent to 0.5  
124 McFarland standard). Zones of clearance around antibiotic discs were measured after  
125 incubation at 37 °C for 24 h. Checkerboard assays were also performed in LB. Bacterial  
126 suspensions were prepared to an OD<sub>600</sub> of 0.001 (ca. 5 x 10<sup>5</sup> CFU/mL) and exactly 100 µL  
127 was dispensed into each well of a U-bottomed 96-well microtiter plate. To each well were  
128 also added 50 µL of LB broth containing ertapenem or meropenem (0-64 µg/mL) and 50 µL  
129 of LB broth containing copper sulfate (0-5 mM) or copper pyrithione (0-20 µM). Turbidity in  
130 each well was measured using a microtiter plate reader after incubation at 37 °C for 24 h. The  
131 minimum inhibitory concentration (MIC) was defined as the lowest concentration of agent  
132 that completely inhibited bacterial growth. The fractional inhibitory concentration (FIC) for  
133 each agent was defined as its MIC in combination divided by its MIC alone. The FIC index  
134 was the sum of the FIC values for both agents.

135

136 **Overexpression and purification of recombinant NDM-1.** To obtain the pure NDM-1  
137 enzyme, the coding sequence for NDM-1 (residues 27-270) was amplified from strain  
138 MS6192 using primers 7456 (5'-TACTTCCAATCCAATGCGATGCCCGGTGAAATCC-  
139 3') and 7457 (5'-TTATCCACTTCCAATGTCAGCGCAGCTTGTCG-3') containing  
140 ligation-independent cloning (LIC) overhangs. The gene product was cloned into pAL vector  
141 encoding a N-terminal His<sub>6</sub>-tag followed by a thioredoxin (TRX) domain and a TEV protease  
142 cleavage site. NDM-1 was expressed overnight at 37 °C in *E. coli* BL21 (DE3) host in the  
143 presence of 0.5 mM IPTG. Cells were lysed in 25 mM Tris-Cl buffer (pH 7.5, 150 mM NaCl,  
144 20 μM ZnCl<sub>2</sub>) by sonication and NDM-1 protein was purified on a Ni-NTA HisTrap column  
145 (GE Healthcare) using the same buffer and a gradient of 0-400 mM imidazole. The N-  
146 terminal His<sub>6</sub>-tag was cleaved with TEV protease and re-purified by elution from a Ni-NTA  
147 HisTrap column.

148  
149 **Assays of NDM-1 activity.** To prepare cell-free extracts, bacteria (50 mL) were cultured  
150 without or with copper sulfate (2 mM) to the mid-exponential phase (OD<sub>600</sub> ~ 0.4-0.5),  
151 centrifuged (5000 g, 10 min), washed with PBS, resuspended in 500 μL of HEPES buffer (50  
152 mM, pH 7.4), lysed by sonication (5 × 10 s bursts at 10 W each), and clarified by  
153 centrifugation (20,000 g, 5 min). β-lactamase activity in these cell-free extracts was measured  
154 by following the hydrolysis of nitrocefin (0-250 μM) in HEPES buffer (50 mM, pH 7.4) at 35  
155 °C. Copper sulfate (0-100 μM) was added into the nitrocefin solution immediately before  
156 addition of the cell-free extracts to initiate hydrolysis. Absorbance values at 485 nm ( $\epsilon$ , 17.5  
157 mM<sup>-1</sup> cm<sup>-1</sup>) were monitored continuously for 2 min in a spectrophotometer. Initial rates (up to  
158 30 s) were normalised to total protein concentration as determined by BCA assay. Data were  
159 fitted to the Michaelis-Menten equation that incorporates terms describing either  
160 noncompetitive or competitive inhibition using the software package Prism 7 (GraphPad).



161

162 **RESULTS**163 **Copper ions potentiate the antibacterial activity of carbapenems against NDM-positive**164 *E. coli*. MS6192 is an NDM-positive, carbapenem-resistant isolate of *E. coli* that is also

165 resistant to cephalosporins, fluoroquinolones and aminoglycosides (Table 1). To assess the

166 effect of copper ions on this antibiotic resistance profile, we first employed a modified disc

167 diffusion assay on solid media containing copper sulfate (0-2 mM). At these concentrations,

168 copper alone did not inhibit the growth of MS6192 but it led to dose-dependent increases in

169 the zones of clearance around carbapenem discs (ertapenem and meropenem) (Table 2). This

170 potentiating effect of copper was also observed in 11 additional NDM-positive clinical

171 isolates (Table S1 in Supplemental Material) but only strain MS6192 was selected for further

172 study. The zones of clearance around other antibiotic discs, including other  $\beta$ -lactams,

173 remained unchanged (Table 2), suggesting that the potentiating effect of copper was specific

174 to carbapenems. Standard checkerboard assays further confirmed that addition of copper

175 decreased the MIC of carbapenems against strain MS6192 in a dose-dependent manner

176 (Figure 1A). The FIC indices were  $0.17 \pm 0.13$  for the combinations of copper and177 ertapenem, and  $0.11 \pm 0.06$  for copper and meropenem, suggesting that the interaction

178 between copper and carbapenems was synergistic (FIC &lt; 0.5) (Figure S1A).

179

180 **Copper ions inhibit the activity of NDM-1 carbapenemase.** Excess copper ions are known

181 to inactivate a variety of metalloenzymes (19-22). Because the observed potentiating effect of

182 copper was specific to carbapenems (Table 2), we hypothesised that this metal impacted the

183 activity of NDM-1. To test this idea, strain MS6192 was cultured without and with copper

184 sulfate (2 mM) to mid-exponential phase and total  $\beta$ -lactamase activities in cell-free extracts

185 were measured using nitrocefin as the substrate. As predicted, growth in copper-rich medium

186 led to a decrease in lactamase activity in MS6192 (Figure 2A). However, only a partial  
187 reduction was achieved (ca. 50%, Figure 2A), likely because MS6192 possesses multiple  
188  $\beta$ -lactamase enzymes (NDM-1, CTX-M-15, OXA-1), some of which are not MBLs and thus  
189 may be insensitive to copper.

190

191 To simplify this analysis, we cloned the *bla*<sub>NDM-1</sub> gene of MS6192 under an IPTG-inducible  
192 promoter and transformed the resulting plasmid (pSU2718::*bla*<sub>NDM-1</sub>) into the K-12 strain  
193 MG1655. The resulting NDM-positive recombinant strain MS8485 was resistant to penicillin,  
194 cephalosporins and carbapenems (Figure 3), as expected from the broad-spectrum activity of  
195 NDM-1. Disc diffusion and checkerboard assays confirmed that addition of copper to the  
196 culture medium restored the susceptibility of MS8485 to all  $\beta$ -lactams to levels that were  
197 comparable to MG1655 (Figure 1 and Figure 3). The mode of action was again synergistic  
198 with FIC indices of  $0.11 \pm 0.03$  for ertapenem and  $0.11 \pm 0.04$  for meropenem (Figure S1B).  
199 These results were consistent with a loss of NDM-1 activity in the presence of copper.  
200 Indeed, extracts of copper-treated MS8485 did not display appreciable NDM-1 activity when  
201 tested using nitrocefin as the substrate (Figure 2B).

202

203 As a control, we measured the production of NDM-1 in MS8485 by immunoblot analysis.  
204 Expression of the *bla*<sub>NDM-1</sub> gene in strain MS8485 is induced by IPTG but to our surprise,  
205 copper treatment led to a reduction in the amount of NDM-1 enzyme (Figure S2). This may  
206 account, at least partially, for the loss of  $\beta$ -lactamase activity in copper-treated cells (Figure  
207 2B). Similar observations were made using strain MS6192 (Figure S2 and Figure 2A), but the  
208 *bla*<sub>NDM-1</sub> gene in this strain is expressed from its native promoter. It is possible that copper  
209 exerts an effect at the step of enzyme folding, maturation, or secretion.

210

211 Although we observed the production (albeit reduced) of NDM-1 in copper-containing  
212 MS8485 cultures, we did not detect appreciable NDM-1 activity above background level  
213 (Figure 2B). Thus, we tested the possibility that copper also directly inhibited the activity of  
214 NDM-1 by measuring the kinetic properties of this MBL in cell-free extracts of MS8485  
215 prepared following culture in copper-free medium. Addition of copper (0-80  $\mu\text{M}$ ) to the  
216 reaction buffer led to a dose-dependent decrease in NDM-1 activity (Figure 4A). The data  
217 were best fitted to a noncompetitive model of inhibition ( $R^2 = 0.97$ ) with an apparent  
218 inhibition constant ( $K_i$ ) of  $47 \pm 5 \mu\text{M}$  in these cell-free extracts (Figure 4A and Figure S3A).  
219 A noncompetitive mode of inhibition was confirmed by repeating these measurements using  
220 purified recombinant NDM-1 (Figure 4B and Figure S3B). A lower  $K_i$  of  $3.7 \pm 0.3 \mu\text{M}$  ( $R^2 =$   
221  $0.99$ ) was obtained, confirming that copper strongly inhibits NDM-1 activity. A  
222 noncompetitive mode of inhibition by Cu(II) with similar magnitude was recently reported  
223 for the MBL AIM-1 (23).

224

225 **Susceptibility of NDM-positive *E. coli* to carbapenems is enhanced using a copper**  
226 **coordination complex.** Ionic copper salts are lipid-insoluble and so are poorly membrane-  
227 permeable. As a consequence, high doses are often required to achieve an antibacterial effect  
228 *in vitro* (e.g. > 2 mM copper sulfate in our assays), hampering the development of copper-  
229 based antibiotics in clinical medicine. We and others have used small (<500 Da) and  
230 lipophilic compounds with high binding affinities to copper to act as membrane-permeable  
231 carriers of copper ions (24-26). These copper coordination complexes are under investigation  
232 as clinical therapeutics (27-29) and some are potent antibacterial agents, at least *in vitro* (24-  
233 26). One such carrier, pyrithione (Figure 5A), has been marketed for decades as an antifungal  
234 agent in healthcare and consumer products. Pyrithione is usually supplied in a zinc-

235 coordinated form but its action relies on trans-metallation by trace exogenous copper ions and  
236 subsequent delivery of antimicrobial copper (18).

237

238 To determine if pyrithione can deliver copper ions to CRE and inhibit NDM-1  
239 carbapenemase activity, we repeated our checkerboard assays in the presence of copper-  
240 loaded pyrithione (0-20  $\mu$ M). As anticipated, the copper-pyrithione complex increased the  
241 susceptibility of MS6192 and MS8485 to ertapenem and meropenem (Figure 5B and Figure  
242 5C). The FIC indices were  $0.18 \pm 0.07$  (ertapenem) and  $0.13 \pm 0.05$  (meropenem) for  
243 MS6192, and  $0.28 \pm 0.11$  (ertapenem) and  $0.17 \pm 0.08$  (meropenem) for MS8485 (Figure S4),  
244 again suggesting that the mode of interaction was synergistic. Copper was required for this  
245 synergy, as zinc-loaded pyrithione had little effect on carbapenem resistance (Figure 5B and  
246 Figure 5C). It must be noted that, in contrast to copper ions (Figure 1), copper-pyrithione did  
247 not decrease the MIC values of carbapenems against MS8485 to MG1655 levels (Figure 5).  
248 At 20  $\mu$ M, copper-pyrithione completely suppressed growth of *E. coli* even in the absence of  
249 carbapenems (Figure 5B and Figure 5C). Therefore, the potentiating effect of copper-  
250 pyrithione is a combination of its direct antibacterial action and the inhibition of  
251 carbapenemase activity.

252

## 253 **DISCUSSION**

254 The antibacterial properties of copper have been recognized for millenia and in the pre-  
255 antibiotic age, simple ionic salts and complexes of copper were used to control bacterial  
256 infections (30). It is now established that excess copper ions poison bacterial cells by  
257 inactivating key metalloenzymes, particularly those containing solvent-accessible iron (19)  
258 and zinc (20). This is a consequence of the high relative affinity of copper to these metal-  
259 binding sites, which leads to metal exchange and displacement of the cognate but weaker

260 binding metals (21). Here we showed that copper ions (as Cu(II)) can directly inactivate the  
261 metalloenzyme NDM-1. The precise mechanism remains to be elucidated but we propose that  
262 copper may disrupt binding of one or both of the zinc ions in the active site of NDM-1, in  
263 agreement with a recent study with the MBL AIM-1, which demonstrated that two Cu(II)  
264 ions bind to the enzyme in close vicinity (23). Alternatively, copper ions may bind to an  
265 allosteric site outside the zinc-containing pocket. Both scenarios (summarised in Figure 6)  
266 would be consistent with the observed noncompetitive mode of enzyme inhibition but  
267 detailed structural and biochemical studies of the purified enzyme will be required to describe  
268 the molecular basis of this inhibition.

269

270 Our immunoblot results indicated that copper may also affect the synthesis, maturation  
271 (enzyme folding and zinc site assembly), or stability of NDM-1. This MBL is anchored to the  
272 outer membrane and secreted in outer membrane vesicles (31). However, NDM-1 is folded  
273 and metallated in the periplasm (32). These processes are universal to all MBLs, including  
274 the VIM, IMP and AIM carbapenemases, and they can also be disrupted by excess copper  
275 (23, 33, 34). Indeed, a recent study has suggested that NDM-1 enzymes lacking the zinc  
276 centres are degraded in the cell (31).

277

278 In our experiments, copper was supplied in the growth medium as Cu(II) ions. Inactivation of  
279 NDM-1 via the various potential routes as described above would rely on diffusion of these  
280 Cu(II) ions into the periplasm. However, the concentration of copper required to restore  
281 susceptibility of MS6192 to carbapenems (~2 mM) in our study was high and unlikely to be  
282 tolerated physiologically. We were able to reduce this amount by two orders of magnitude to  
283 10  $\mu$ M by coordinating the copper ion to pyrithione, an FDA-approved antifungal agent that  
284 acts as a membrane-permeable carrier of copper. Pyrithione and its zinc-coordinated form are

285 currently approved for topical administration. While most formulations in consumer goods  
286 and healthcare products contain up to 2% of this compound, it is unknown whether the  
287 copper form is equally tolerated. However, a variety of other copper carriers are currently  
288 being investigated for their therapeutic potential (24-26). The results presented here provide  
289 an early proof-of-concept that a ligand or carrier-mediated delivery of copper ions to the  
290 bacterial cell, in this case to target NDM-1 carbapenemase activity, is possible. Our data also  
291 add to an emerging role of metal ions in enhancing the action of antibiotics. For example,  
292 silver can potentiate vancomycin activity by disrupting multiple bacterial cellular processes,  
293 including disulfide bond formation, metabolism, and iron homeostasis (35).

294

295 The ability of copper to inhibit NDM-1 carbapenemase activity also provides an opportunity  
296 to develop therapeutics that work in concert with the host innate immune system. Although  
297 the availability and location of copper in the human body are tightly regulated, copper is  
298 mobilized in response to inflammation, leading to increased copper concentrations at the site  
299 of infection. For instance, mobilization of copper occurs in infected macrophages (36).  
300 Infection by a variety of pathogens also results in increased copper levels in the serum, liver,  
301 and spleen of animals (37, 38). In the case of *E. coli*, particularly uropathogenic strains, it has  
302 been shown that copper levels are elevated (to ~ 0.3  $\mu\text{M}$ ) in the urine of patients with UTI  
303 compared to healthy controls (39, 40). Thus, delivery of membrane-permeable copper carriers  
304 such as pyrithione into the urinary tract may allow us to exploit this host-derived copper and  
305 enhance its action against CRE.

306

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439 **Table 1.** Antibiotic resistance profile of *E. coli* strain MS6192.

440

Antibiotic			MIC ( $\mu\text{g/mL}$ )	
Class		Name		
$\beta$ - lactams	Penicillins		Ampicillin	$\geq 32$
			Amoxicillin/Clavulanic acid	$\geq 32$
			Ticarcillin/Clavulanic acid	$\geq 128$
			Piperacillin/Tazobactam	$\geq 128$
	Cephalo- sporins	1st	Cefazolin	$\geq 64$
		2nd	Cefotixin	$\geq 64$
		3rd	Ceftazidime	$\geq 64$
		3rd	Ceftriaxone	$\geq 64$
		4th	Cefepime	$\geq 64$
	Carbapenems		Meropenem	$\geq 16$
Aminoglycosides		Amikacin	$\geq 64$	
		Gentamicin	$\geq 16$	
		Tobramycin	$\geq 16$	
Fluoroquinolones		Norfloxacin	$\geq 16$	
		Ciprofloxacin	$\geq 4$	
Others		Nitrofurantoin	128	
		Trimethoprim	$\geq 16$	
		Trimethoprim/Sulfamethoxazole	$\geq 32$	

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**Table 2.** Effect of copper ions on the resistance of *E. coli* MS6192 to antibiotics as determined by disc diffusion assays.

Antibiotics			Zone of clearance (Diameter, mm) <sup>a</sup>			
Class	Name	Amount ( $\mu$ g)	[copper sulfate] (mM)			
			0	1	2	
$\beta$ - lactams	Carba- penems	Ertapenem Meropenem	10 10	8 11	11 17	26 32
	Penicillins	Ampicillin	10	< 7	< 7	< 7
	Cephalo- sporins	Ceftriaxone	30	< 7	< 7	< 7
		Cefotaxime	30	< 7	< 7	< 7
Monobactams	Aztreonam	30	< 7	< 7	< 7	
Aminoglycosides	Gentamicin	10	< 7	< 7	< 7	
	Tobramycin	10	< 7	< 7	< 7	
Fluoroquinolones	Ciprofloxacin	5	< 7	< 7	< 7	

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<sup>a</sup>Values are representative of three independent experiments. The diameter of the disc was 7 mm and a value of < 7 mm indicated that no zone of clearance around was observed around the disc.

455 **FIGURE LEGENDS**

456

457 **Figure 1.** Effects of sub-inhibitory amounts of copper ions (0 – 2.5 mM) on the MIC values  
458 of ertapenem (top panels) and meropenem (bottom panels) against *E. coli* strains (A)  
459 MS6192, (B) MS8485, and (C) MG1655 as determined by standard checkerboard assays.  
460 Data shown were from three independent replicates. An MIC value of 0 µg/mL indicated that  
461 growth was inhibited in the absence of the carbapenem.

462

463 **Figure 2.** Effects of copper ions on  $\beta$ -lactamase activity in *E. coli* strains (A) MS6192 and  
464 (B) MS8485. Bacteria were cultured without (-Cu) and with sub-inhibitory amounts of  
465 copper (2 mM, +Cu) to the mid-exponential phase and lysed by sonication. As a negative  
466 control, MG1655 was also cultured without any copper (Control). Lactamase activities were  
467 measured in cell-free extracts and averaged from three independent replicates. Error bars  
468 represent  $\pm$  SD.

469

470 **Figure 3.** Effect of sub-inhibitory amounts of copper ions (0–2 mM) on the resistance of *E.*  
471 *coli* strains (A) MG1655 (black bars) and (B) MS8485 (white bars) to  $\beta$ -lactam antibiotics as  
472 determined by disk diffusion assays. Diameters of the zones of clearance were averaged from  
473 three independent replicates. Error bars represent  $\pm$  SD. The diameter of the disk was 7 mm  
474 and a value of < 7 mm indicated that no zone of clearance around was observed around the  
475 disc.

476

477 **Figure 4.** Direct inhibitory effects of copper ions on NDM-1 activity. Enzyme activity in (A)  
478 cell-free extracts of MS8485 or (B) purified NDM-1 from MS6192 was measured in the  
479 presence of copper sulfate using nitrocefin as substrate. The concentrations of copper sulfate

480 in micromolar were indicated to the right of each curve. Each data point was averaged from  
481 three independent replicates. Error bars represent  $\pm$  SEM. Data were fitted to noncompetitive  
482 (solid lines) and competitive models of enzyme inhibition (Figure S3).

483

484 **Figure 5.** Structure of copper-pyrithione complex (A) and the effects of sub-inhibitory  
485 amounts of copper-pyrithione (0–20  $\mu$ M, solid lines) and zinc-pyrithione (0–20  $\mu$ M, dotted  
486 lines) on the MIC values of ertapenem (B) and meropenem (C) against *E. coli* strains  
487 MS6192, MS8485, and MG1655 as determined by standard checkerboard assays. Data shown  
488 were from three independent replicates. An MIC value of 0  $\mu$ g/mL indicates that growth was  
489 inhibited in the absence of added carbapenem.

490

491 **Figure 6.** General schematic of the effect of copper ions on NDM-1 activity. Our data  
492 indicates that copper can directly inhibit the carbapenemase activity of NDM-1, and that it  
493 may also may also affect NDM-1 synthesis, maturation, or stability. Cu(II), and Zn(II) ions  
494 are depicted by black circles and light grey circles, respectively. IM, inner membrane. OM,  
495 outer membrane. OMV, outer membrane vesicle.

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