Copper ions and coordination complexes as novel carbapenem adjuvants

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ABSTRACT

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

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

Cloning and expression of the *bla*$_{\text{NDM-1}}$ gene in MG1655. The *bla*$_{\text{NDM-1}}$ gene from MS6192 was amplified with primers 7414 (5'-tgataaggatccattcagctttcacccattgg) and 7415 (5'-tcgaaaaagcttgatggcagattgggggtga) and cloned between the *Bam*HI and *Hind*III sites of pSU2718. The resulting plasmid pSU2718::*bla*$_{\text{NDM-1}}$ was transformed into *E. coli* MG1655 by electroporation to generate the NDM-1-positive strain MS8485. MS8485 was cultured in the presence of chloramphenicol (30 µg/mL) and IPTG (0.1 mM) to maintain the plasmid and promote expression of NDM-1, respectively.

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

Overexpression and purification of recombinant NDM-1. To obtain the pure NDM-1 enzyme, the coding sequence for NDM-1 (residues 27-270) was amplified from strain MS6192 using primers 7456 (5'-TACTTCCAATCCAATGCGATGCCCGGTGAAATCC-3') and 7457 (5'-TTATCCACTTCCAATGTCAGCGCAGCTTGTCG-3') containing ligation-independent cloning (LIC) overhangs. The gene product was cloned into pAL vector encoding a N-terminal His_6-tag followed by a thioredoxin (TRX) domain and a TEV protease cleavage site. NDM-1 was expressed overnight at 37 ^{o}C in *E. coli* BL21 (DE3) host in the presence of 0.5 mM IPTG. Cells were lysed in 25 mM Tris-Cl buffer (pH 7.5, 150 mM NaCl, 20 μM $ZnCl_2$) by sonication and NDM-1 protein was purified on a Ni-NTA HisTrap column (GE Healthcare) using the same buffer and a gradient of 0-400 mM imidazole. The N-terminal His_6-tag was cleaved with TEV protease and re-purified by elution from a Ni-NTA HisTrap column.

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

RESULTS

Copper ions potentiate the antibacterial activity of carbapenems against NDM-positive *E. coli.* MS6192 is an NDM-positive, carbapenem-resistant isolate of *E. coli* that is also resistant to cephalosporins, fluoroquinolones and aminoglycosides (Table 1). To assess the effect of copper ions on this antibiotic resistance profile, we first employed a modified disc diffusion assay on solid media containing copper sulfate (0-2 mM). At these concentrations, copper alone did not inhibit the growth of MS6192 but it led to dose-dependent increases in the zones of clearance around carbapenem discs (ertapenem and meropenem) (Table 2). This potentiating effect of copper was also observed in 11 additional NDM-positive clinical isolates (Table S1 in Supplemental Material) but only strain MS6192 was selected for further study. The zones of clearance around other antibiotic discs, including other β-lactams, remained unchanged (Table 2), suggesting that the potentiating effect of copper was specific to carbapenems. Standard checkerboard assays further confirmed that addition of copper decreased the MIC of carbapenems against strain MS6192 in a dose-dependent manner (Figure 1A). The FIC indices were 0.17 ± 0.13 for the combinations of copper and ertapenem, and 0.11 ± 0.06 for copper and meropenem, suggesting that the interaction between copper and carbapenems was synergistic (FIC < 0.5) (Figure S1A).

Copper ions inhibit the activity of NDM-1 carbapenemase. Excess copper ions are known to inactivate a variety of metalloenzymes (19-22). Because the observed potentiating effect of copper was specific to carbapenems (Table 2), we hypothesised that this metal impacted the activity of NDM-1. To test this idea, strain MS6192 was cultured without and with copper sulfate (2 mM) to mid-exponential phase and total β-lactamase activities in cell-free extracts were measured using nitrocefin as the substrate. As predicted, growth in copper-rich medium

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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439 **Table 1.** Antibiotic resistance profile of *E. coli* strain MS6192.
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Antibiotic				MIC (µg/mL)
Class			Name	
β-lactams	Penicillins		Ampicillin	≥ 32
			Amoxicillin/Clavulanic acid	≥ 32
			Ticarcillin/Clavulanic acid	≥ 128
			Piperacillin/Tazobactam	≥ 128
	Cephalo-sporins	1st	Cefazolin	≥ 64
		2nd	Cefotixin	≥ 64
		3rd	Ceftazidime	≥ 64
		3rd	Ceftriaxone	≥ 64
		4th	Cefepime	≥ 64
	Carbapenems		Meropenem	≥ 16
Aminoglycosides			Amikacin	≥ 64
			Gentamicin	≥ 16
			Tobramycin	≥ 16
Fluoroquinolones			Norfloxacin	≥ 16
			Ciprofloxacin	≥ 4
Others			Nitrofurantoin	128
			Trimethoprim	≥ 16
			Trimethoprim/Sulfamethoxazole	≥ 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)[a]		
Class		Name	Amount (µg)	[copper sulfate] (mM)		
				0	1	2
β-lactams	Carba-penems	Ertapenem	10	8	11	26
		Meropenem	10	11	17	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

[a]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.

FIGURE LEGENDS

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

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

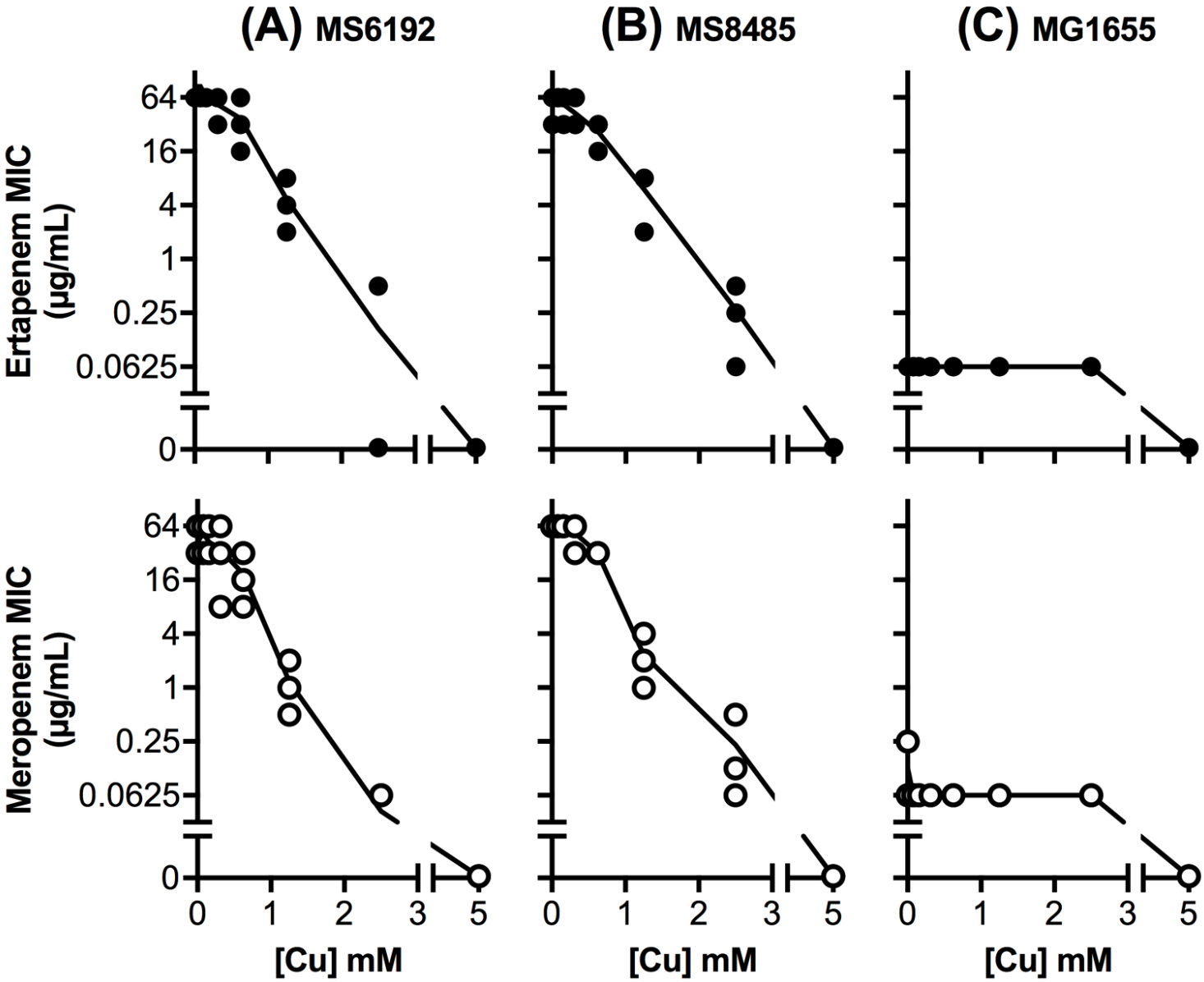
Figure 3. Effect of sub-inhibitory amounts of copper ions (0–2 mM) on the resistance of *E. coli* strains **(A)** MG1655 (black bars) and **(B)** MS8485 (white bars) to □-lactam antibiotics as determined by disk diffusion assays. Diameters of the zones of clearance were averaged from three independent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

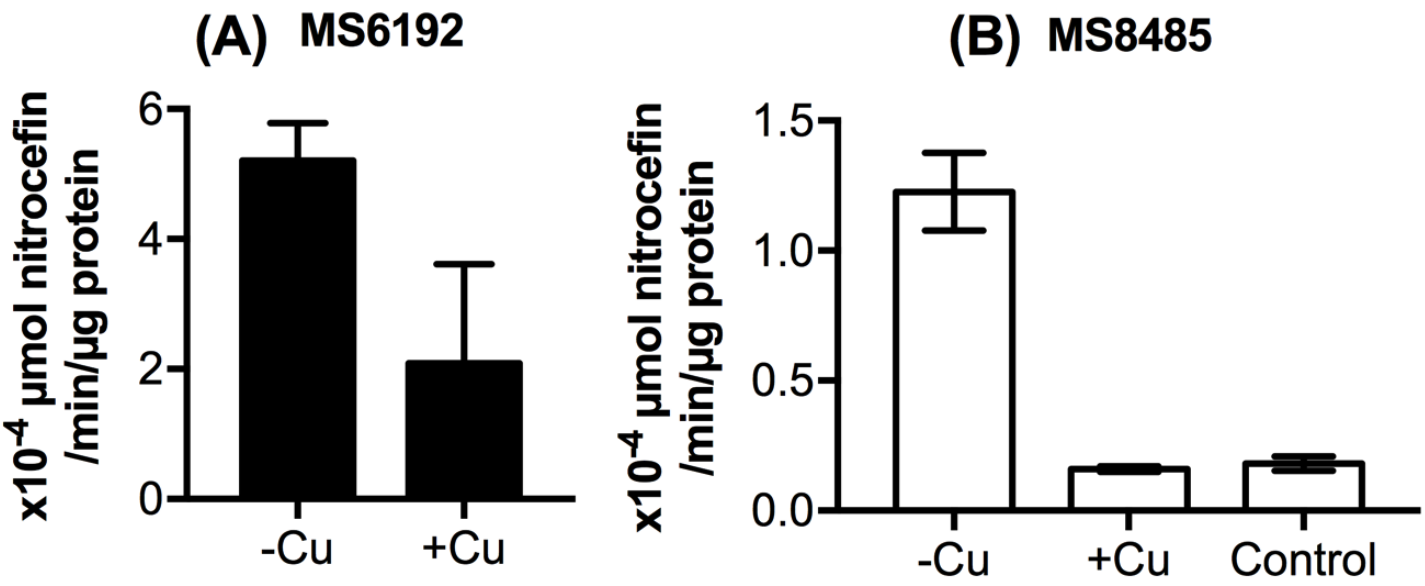
Figure 4. Direct inhibitory effects of copper ions on NDM-1 activity. Enzyme activity in **(A)** cell-free extracts of MS8485 or **(B)** purified NDM-1 from MS6192 was measured in the presence of copper sulfate using nitrocefin as substrate. The concentrations of copper sulfate

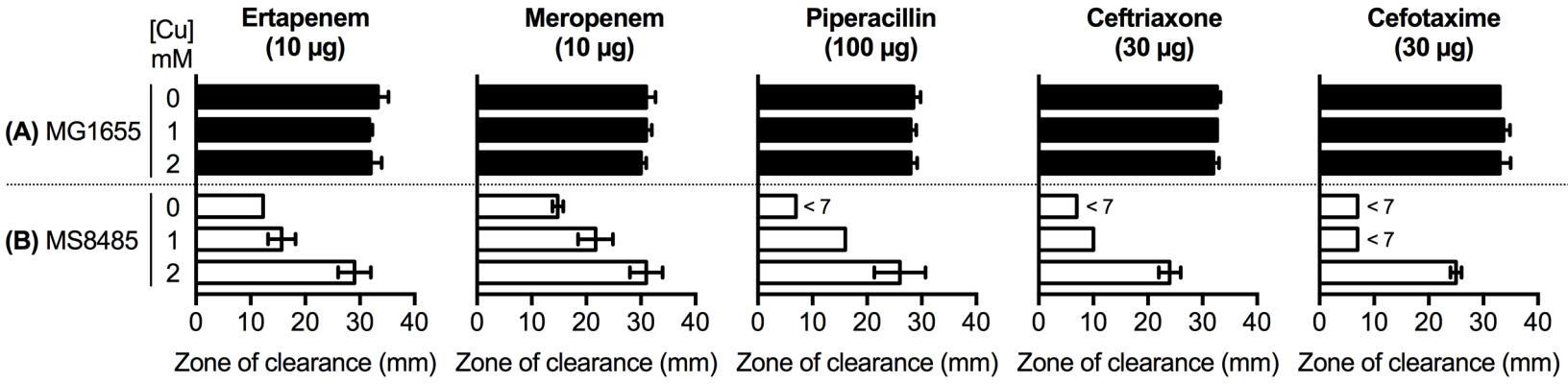
in micromolar were indicated to the right of each curve. Each data point was averaged from three independent replicates. Error bars represent ± SEM. Data were fitted to noncompetitive (solid lines) and competitive models of enzyme inhibition (Figure S3).

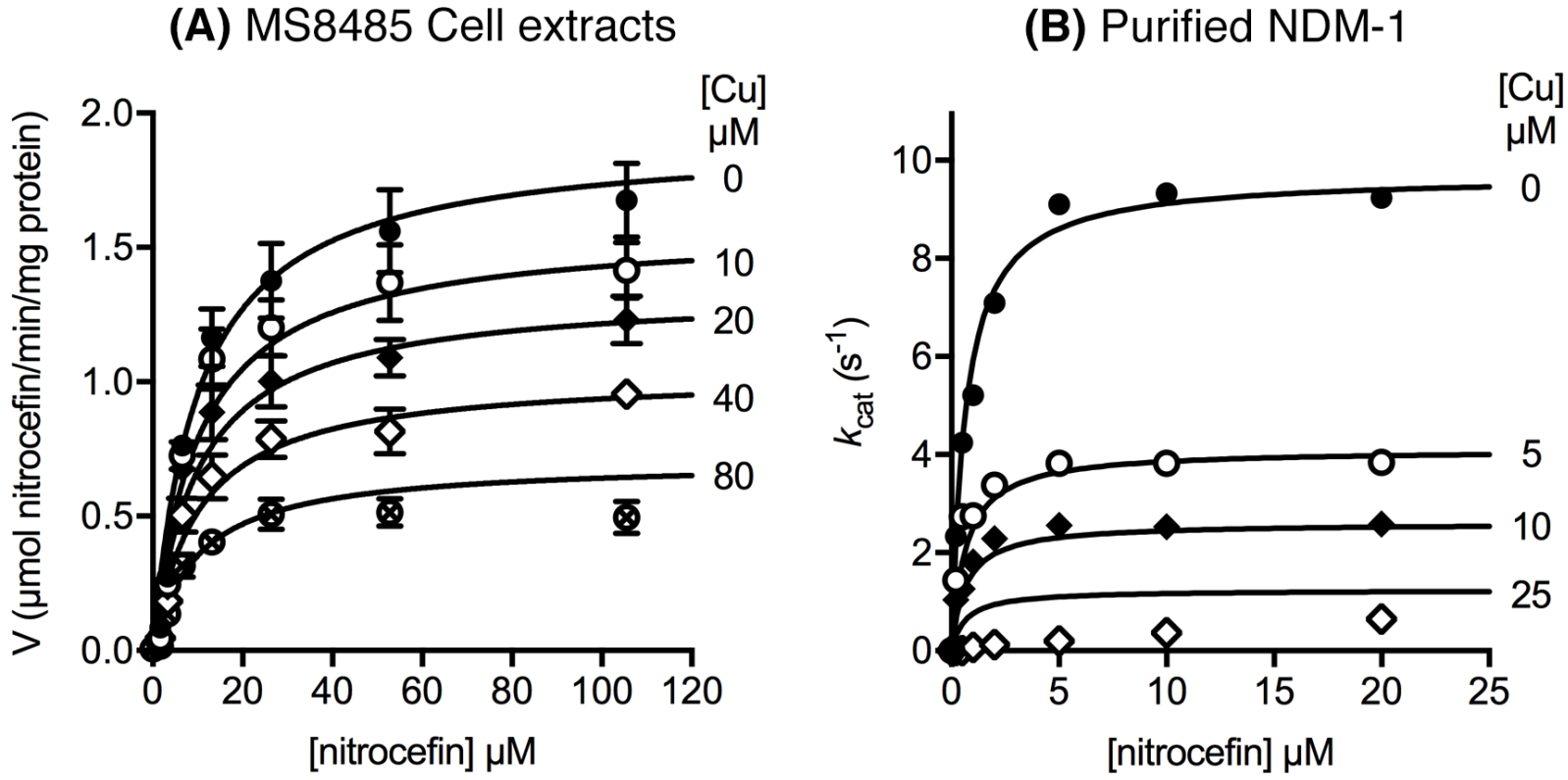
Figure 5. Structure of copper-pyrithione complex **(A)** and the effects of sub-inhibitory amounts of copper-pyrithione (0–20 μM, solid lines) and zinc-pyrithione (0–20 μM, dotted lines) on the MIC values of ertapenem **(B)** and meropenem **(C)** against *E. coli* strains MS6192, MS8485, and MG1655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

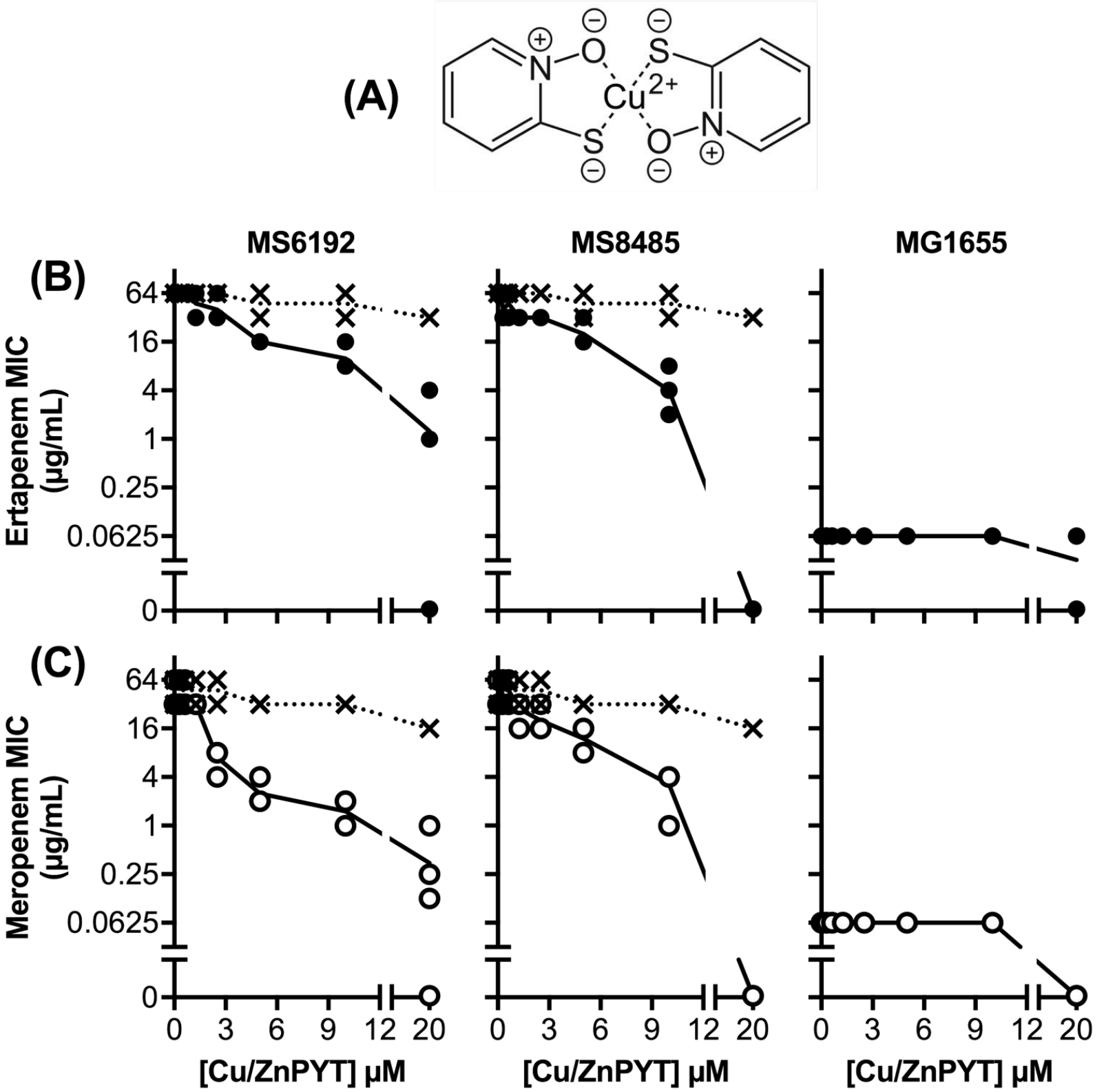
Figure 6. General schematic of the effect of copper ions on NDM-1 activity. Our data indicates that copper can directly inhibit the carbapenemase activity of NDM-1, and that it may also may also affect NDM-1 synthesis, maturation, or stability. Cu(II), and Zn(II) ions are depicted by black circles and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

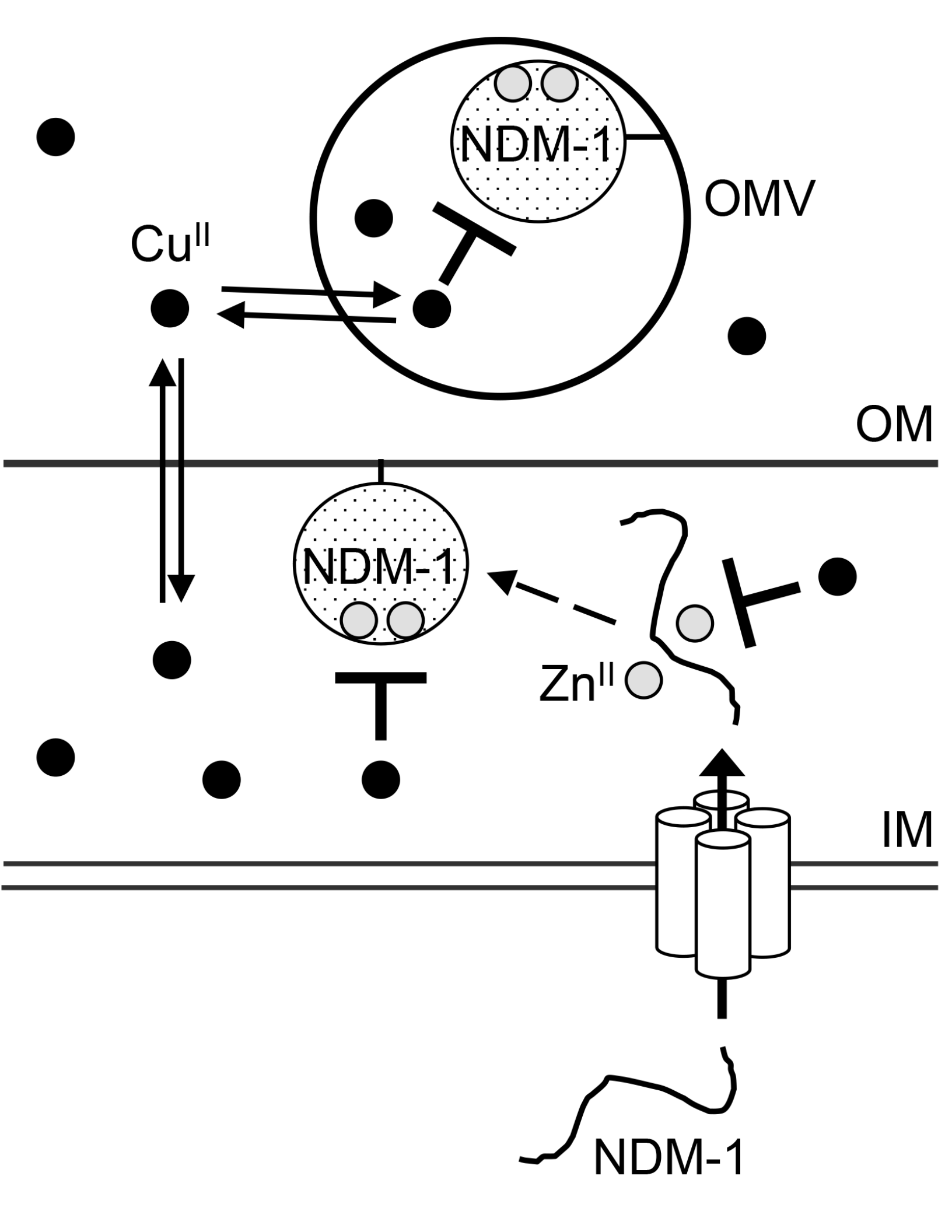

(A) MS6192
(B) MS8485
(C) MG1655
Ertapenem MIC (µg/mL)
Meropenem MIC (µg/mL)
64
16
4
1
0.25
0.0625
0
0
1
2
3
5
[Cu] mM


(A) MS6192
x10-4 µmol nitrocefin /min/µg protein
6
4
2
0
-Cu
+Cu
(B) MS8485
x10-4 µmol nitrocefin /min/µg protein
1.5
1.0
0.5
0.0
-Cu
+Cu
Control


[Cu] mM
Ertapenem (10 µg)
Meropenem (10 µg)
Piperacillin (100 µg)
Ceftriaxone (30 µg)
Cefotaxime (30 µg)
(A) MG1655
(B) MS8485
0
1
2
< 7
0 10 20 30 40
Zone of clearance (mm)


(A) MS8485 Cell extracts
V (µmol nitrocefin/min/mg protein)
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
[nitrocefin] µM
[Cu] µM
0
10
20
40
80
(B) Purified NDM-1
k_{cat} (s^{-1})
10
8
6
4
2
0
0
5
10
15
20
25
[nitrocefin] µM
[Cu] µM
0
5
10
25


(A)
Cu2+
(B)
MS6192
MS8485
MG1655
Ertapenem MIC (µg/mL)
64
16
4
1
0.25
0.0625
0
(C)
Meropenem MIC (µg/mL)
0 3 6 9 12 20
[Cu/ZnPYT] µM


NDM-1
OMV
Cu^{II}
OM
NDM-1
Zn^{II}
IM
NDM-1