Electronic Supplementary Material (ESI) for ChemComm.


# Ebselen as a potent covalent inhibitor for New Delhi Metallo-β-lactamase (NDM-1)

Jiachi Chiou[a,b,#], Shengbiao Wan[c,#], Pui-Kin So[a,b], Kin-Fai Chan[b], Dandan He[a,b], Edward Wai-chi Chan[a,b], Tak-hang Chan[b], Kwok-yin Wong[b], Jiang Tao[c,*], Sheng Chen [a,b,*]

## Materials and Methods

### Antibiotics, media and chemicals

Ampicillin and kanamycin were purchased from Tokyo Chemical Inc. (Tokyo, Japan). Meropenem and ebselen were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Luria broth (LB) and nitrocefin were purchased from BD (Franklin Lakes, NJ, USA). Mueller-Hinton broth (MHB) was purchased from Oxoid Co. (Hampshire, United Kingdom). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from IBI Inc. (Boca Raton, FL, USA).

### Bacterial strains and screening system

The $bla_{NDM-1}$ was constructed on the IPTG-inducible pET28b vector harboring a kanamycin resistant gene. *Escherichia coli* BL21(NDM-1) carrying the plasmid pET28b-$bla_{NDM-1}$, which encoded the full-length of NDM-1, was used in the *in vivo* screening of antibiotics or inhibitors; organisms harboring the plasmid pET28b-$bla_{H6\text{-}mNDM\text{-}1}$, which encoded $G^{36}$ to $R^{270}$ and carried an N-terminal $His_6$ tag were used in purification of NDM-1 as previously described.[1] The construct containing $bla_{NDM-1}$ was grown on a Mueller-Hinton agar (MHA) plate, incubated at 37ºC overnight and transferred to MHB medium supplemented with a serial concentration of β-

lactams alone, the test compound alone or a combination of both (ebselen:antibiotic of 1.4:1 to 14:1 molar ratio). The cultures were incubated at 37ºC overnight and the minimum inhibitory concentration (MIC) values were determined and interpreted according to the CLSI guidelines.[2]

**Kinetic assay of mNDM-1 and determination of inactivation constants of inhibitors**

Expression and purification of mNDM-1 (m refers to mature form) carrying no signal peptide and $His_6$ tag was performed as previously described. [1] The effects of inhibitors were monitored by observing the rate of hydrolysis of the reporter substrate, nitrocefin, by mNDM-1. Briefly, 1 nM of purified mNDM-1 was pre-incubated with various concentrations of ebselen at 25°C in 500 µl of assay buffer (50 mM phosphate buffer, pH 7.0 by mixing 61.5 ml of 50 mM $K_2HPO_4$ and 38.5 ml of 50 mM $KH_2PO_4$, 50 µM $ZnSO_4$) prior to the addition of ~7× Km of nitrocefin. The Km of nitrocefin is 1.74±0.47 µM in our assay condition. Bovine serum albumin (BSA) was added in the assay reaction with a final concentration of 100 µg/ml to maintain the stability of diluted mNDM-1. The initial velocity was determined by monitoring the wavelength change at 482 nm over the first 4 min in at least triplicate. The reversibility of inhibition by ebselen was performed as previously described [3]. Briefly, 100 nM of purified mNDM-1 was pre-incubated with 90 µM of ebselen at 25°C for 20 min, followed by diluting 100-fold with the addition of 35 µM of nitrocefin and monitored the absorbance change for 30 min. The residual activity was determined compared to the activity from the pre-incubation of enzyme following the same procedure except in the absence of ebselen. The effect of inactivation of NDM-1 as mediated by ebselen was determined by plotting the natural logarithm of the residual activity against incubation time. The $k_{obs}$, namely the observed rate constant, was determined by the negative slope for each line from the plot. Each $k_{obs}$ was plotted against the concentration of ebselen and

fitted nonlinear regression curve was constructed to determine the inactivation kinetic parameters $k_{inact}$ and $K_I$ according to the equation below. All the kinetic and inactivation parameters were determined using GraphPad Prism5 (San Diego, CA, US)

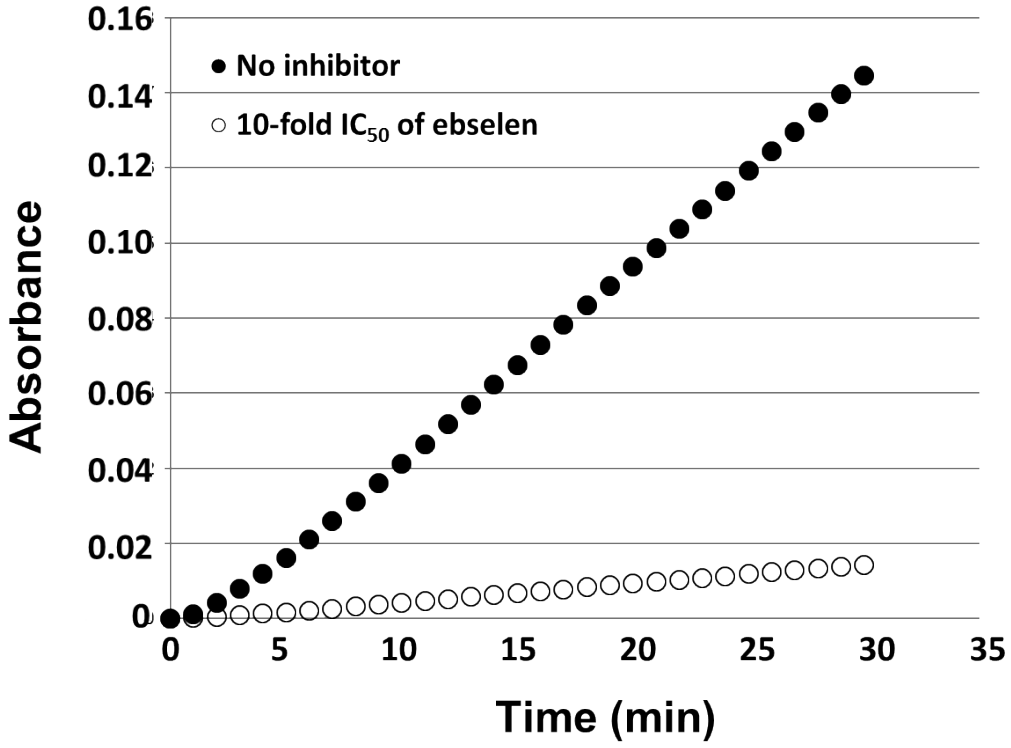
$$k_{obs} = \frac{k_{inact} \times [I]}{K_I + [I]}$$

**ESI-MS**

Electrospray ionization mass spectrometry (ESI-MS) experiments were performed with a Waters-Micromass Q-TOF2 quadrupole time-of-flight mass spectrometer. For detection of NDM-1/ebselen complex, 10 μM of mNDM-1 enzyme in 20 mM ammonium acetate was incubated with equal molar of ebselen in the same buffer system for 30 minutes. For analysis under non-denaturing conditions, the mNMD-1 enzyme or the mNDM-1/ebselen reaction mixture in ammonium acetate buffer were directly loaded into a nanospray emitter (Econo10, New Objectives, Woburn, USA), which was subsequently mounted onto the nano-ESI source for analysis. For analysis under denaturing conditions, the NMD-1 enzyme or the NDM-1/ebselen reaction mixture were first mixed with equal volume of acetonitrile with 0.5% formic acid (v/v). Subsequently, the sample was infused into the standard ESI source at a flow rate of 5 μL/min for analysis. During data acquisition, the mass spectrometer was operated in positive ion mode in the m/z range of 600 – 4,000 for detection of multiply charged ions. The raw multiply charged mass spectra obtained were deconvoluted with the Transform program (MassLynx 4.1, Waters).

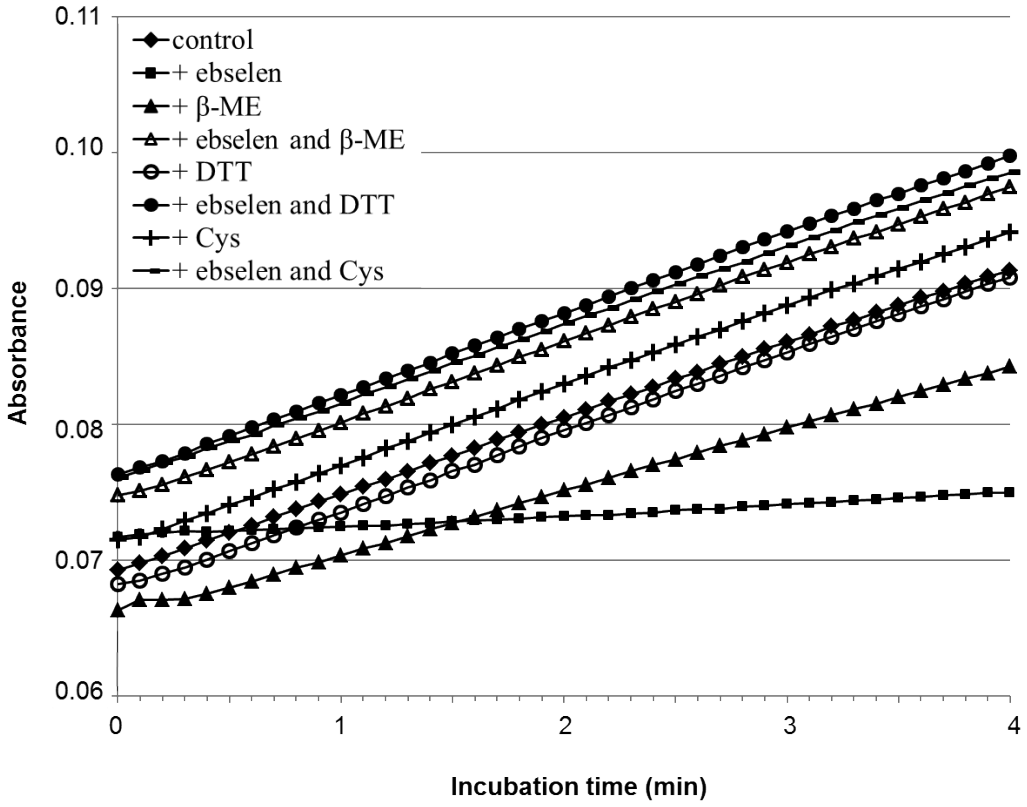
**Supplemental Table 1** MICs (μM) of β-lactams in *E. coli* expressing NDM-1 carbapenemase and its parent strain.

| Treatments (molar ratio) | *E. coli* strains | |
|---|---|---|
| | BL21 | BL21 (NDM-1) |
| Ebselen alone | ≥7469 | ≥7469 |
| Ampicillin alone | 2.86 | 1465 |
| Meropenem alone | <0.33 | 333 |
| Ebselen + ampicillin (1.3:1) | 2.86 | 92 (16-fold) |
| Ebselen + meropenem (1.4:1) | <0.33 | 2.6 (128-fold) |
| Ebselen + meropenem (2.8:1) | <0.33 | 2.6 (128-fold) |
| Ebselen + meropenem (5.6:1) | <0.33 | 1.3 (256-fold) |
| Ebselen + meropenem (8.4:1) | <0.33 | 1.3 (256-fold) |
| Ebselen + meropenem (11.2:1) | <0.33 | 0.65 (512-fold) |
| Ebselen + meropenem (14:1) | <0.33 | 0.65 (512-fold) |




**Supplemental Fig. 1. Residual activity after rapid dilution of enzyme-inhibitor complex.** One hundred nanomoles of NDM-1 was pre-incubated with 10-fold $IC_{50}$ of ebselen for 20 min, followed by the addition of nitrocefin and monitored the absorbance change for 30 min. The

absorbance changes from the co-incubation of NDM-1 and NCF (control, ●) and NDM-1 + NCF + ebselen, ○) were shown. Slope of the line represents the hydrolytic activity of NDM-1.




**Supplemental Fig. 2. Effect of dithiothreitol (DTT), β-mercaptoethanol (β-ME) and cysteine (Cys) on the inhibition of NDM-1-catalyzed nitrocefin hydrolysis by ebselen.** The absorbance changes due to nitrocefin (NCF) hydrolysis by NDM-1 were monitored for 4 min. The absorbance changes from the co-incubation of NDM-1 and NCF (control, ♦), NDM-1 + NCF + ebselen (■), NDM-1 + NCF + β-ME (▲), NDM-1 + NCF + ebselen + β-ME (Δ), NDM-1 + NCF + DTT (○), NDM-1 + NCF + ebselen + DTT (●), NDM-1 + NCF + Cys (┼), and NDM-1 + NCF + ebselen + Cys (─) were shown. Slope of the line represents the hydrolytic activity of NDM-1.

**References:**


1. Chiou, J., T.Y. Leung, and S. Chen, *Molecular mechanisms of substrate recognition and specificity of New Delhi metallo-beta-lactamase.* Antimicrob Agents Chemother, 2014. **58**(9): p. 5372-8.
2. Tsui, K., et al., *Laboratory identification, risk factors, and clinical outcomes of patients with bacteremia due to Escherichia coli and Klebsiella pneumoniae Producing Extended-Spectrum and AmpC type beta-Lactamases.* Journal of Microbiology Immunology and Infection, 2012. **45**(3): p. 193-199.
3. Copeland, R.A., *Evaluation of enzyme inhibitors in drug discovery: A guide for medichinal chemist and pharmacologist.* Wiley intersciences, 2005. **ISBN 0-471-68696-4**.