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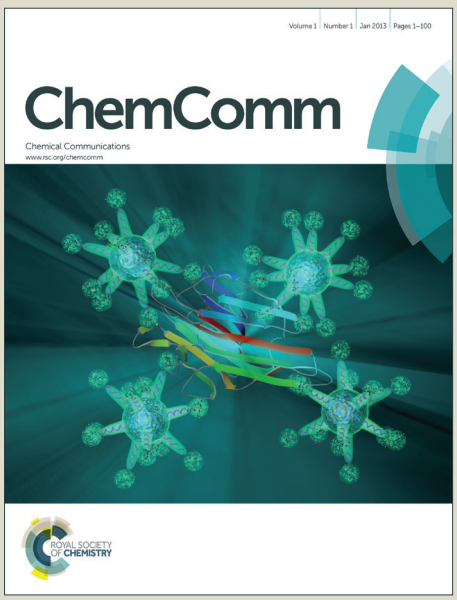

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Ebselen as a potent covalent inhibitor for New Delhi Metallo-β-lactamase (NDM-1)

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

We report the discovery of a promising NDM-1 inhibitor, ebselen, through a cell-based screening approach. Enzymatic kinetic study and ESI-MS analysis suggested that ebselen could bind to NDM-1 by forming a S–Se bond with residue Cys^{221} at the active site, thereby exhibiting a new inhibition mechanism with broad spectrum inhibitory potential.

β-Lactams have been a cornerstone in treatment of infections caused by both Gram-positive and Gram-negative bacterial pathogens due to their high efficacy and low toxicity to humans, among which carbapenems are considered agents of the last resort, especially in cases where extended-spectrum β-lactamase producing organisms are involved.[1] In the past two decades, usage of carbapenems such as imipenem and meropenem has been substantially increased due to the emergence of multidrug-resistant organisms.[2] However, increased carbapenem consumption in turn initiated a vicious cycle that the carbapenem-resistant Gram-negative pathogens, which often cause untreatable hospital infections,[3] further gained selection advantage. Recently the Center for Disease Control and Prevention declared that the age of antibiotics has come to an end since human have run out of treatment options for these so-called "superbugs".[4]

Metallo-β-lactamases (MBLs) represent the most important type of carbapenemases, the determinants of which are known to spread among different Gram negative bacterial species.[5] The phenomenon of worldwide transmission of organisms producing NDM-1 further highlighted the clinical significance of this type of carbapenemases in mediating bacterial antimicrobial resistance.[6] MBLs are further grouped into three subclasses, B1, B2 and B3.[7] Subclass B1 MBLs, including the most clinically important MBLs such as NDM-1, VIM-1 and IMP-1, contain two zinc ions interacting with various amino acids in the active site.[8] Subclass B2 MBLs are mono-zinc carbapenemases that possess the ability to hydrolyse carbapenems exclusively, whereas subclass B3 is mostly intrinsically encoded on the chromosome.[5,7] Therefore subclass B1 MBLs are the most clinically significant MBLs and are major targets for development of inhibitors of therapeutic values. To date, several inhibitors against the serine β-Lactamases are being used clinically, such as clavulanic acid, sulbactam and tazobactam, yet effective inhibitors for MBLs are currently not available.[9]

Albeit attempts to search for effective compounds for NDM-1 inhibition, limited achievement has been made. Traditional strategies to synthesize new antibiotics by developing structural analogues appear to be impeded by the flexible and relatively extensive active site of MBLs.[9] In addition, while β-lactam analogues have been adopted as inhibitors for MBLs, their performance varied between different subclasses of MBLs; worse still, structural variations are known to occur even within the same subclass.[10] Dozens of other compounds including single strand DNAs, peptides, natural products and chemically synthesized inhibitors have also been reported, yet there is no single inhibitor that exhibits promising potential for clinical application.[9,10b,11] Among all the reported MBL inhibitors, D- and L-Captopril (Fig. 1) and their analogues may be the most promising candidates.[12] L-Captopril, an inhibitor of angiotensin-converting enzyme, is a FDA-approved drug used to treat hypertension clinically. The half-maximal inhibition concentration (IC_{50}) values for inhibition of NDM-1 upon imipenem hydrolysis by D- and L-captopril are 7.9 and 202.0 μM, respectively.[12a] However, they are not effective on carbapenemase-carrying bacteria probably due to the low permeability of bacterial cell membrane.

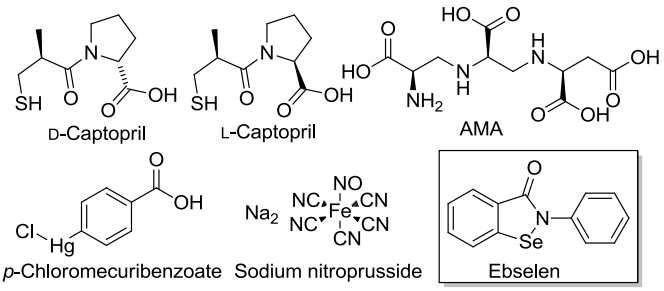

Fig. 1 Chemical structures of ebselen and other NDM-1 inhibitors reported in the literature.

Promisingly, an elegant study has recently identified a natural product, aspergillomarasmine A (AMA), which is produced by a fungus (Fig. 1).[13] AMA has been shown to effectively inhibit the activities of the carbapenemases NDM-1 and VIM-2, and restore the activity of meropenem against *Enterobacteriaceae*, *Acinetobacter* spp. and *Pseudomonas* spp. possessing either of these two carbapenemases. Its mechanism of action was further confirmed by inductively coupled mass spectrometry that showed a loss of two zinc ions, indicating that AMA was able to extract zinc ions from the enzyme. Importantly, AMA also displayed the potential to restore carbapenem activity against NDM-1-borne *Klebsiella pneumoniae* in mouse model, suggesting that it can be developed into a therapeutic agent in the future.[13] Our long term goal is to search for clinically applicable MBL-inhibitory agents that interacted with the cysteine residue at the active site of these enzymes with the potential of broad spectrum inhibition to all B1 and B2 MBLs. The common structural

feature for B1 and B2 MBLs, in which interaction between zinc ion and residue Cys^{221} plays a role in coordination of the active site, represents a good target for broad-spectrum inhibitor design. Successful cases came from the prior study that identified two thiol-modifying, NDM-1 metal-containing compounds, namely *p*-chloromercuribenzoate and sodium nitroprusside (Fig. 1).[14] In the present study, we successfully identified a potent inhibitor, namely Ebselen (Fig. 1), through a cell-based screening. We also obtained evidence which suggests that ebselen can act as a potent inhibitor for NDM-1 by covalently binding to the cysteine residue at the active site, thereby exhibiting a new inhibition mechanism with highly promising, broad spectrum inhibitory potential.

To obtain a clean background of the *E. coli* strain for *in vivo* screening, *Escherichia coli* BL21 (NDM-1) carrying an isopropyl β-D-1-thiogalactopyranoside-inducible plasmid pET28b-bla_{NDM-1}, which encoded the full-length of NDM-1, was generated in our laboratory.[15] We screened this strain in the presence of a sub-lethal concentration of meropenem or ampicillin in combination with 50 different compounds including 11 ebselen analogues, 17 thiol-containing compounds and 22 general compounds which were commercially available or synthesized in the Ocean University of China. The screen generated one promising hit, ebselen, which reduces the minimum inhibition concentrations (MICs) of ampicillin and meropenem by 16-fold and 128-fold respectively (Sup Table 1). The MICs of ampicillin and meropenem in combination with ebselen at ratio of 1.3:1 and 1.4:1 (ebselen:antibiotic) reduced from 1465 μM and 333 μM to 92 μM and 2.6 μM respectively. Increasing the ratio of ebselen to meropenem from 1.4:1 to 14:1 further reduced the MIC of meropenem from 2.6 μM to 0.65 μM. Ebselen alone does not have any cytotoxic effect (MIC ≥7469 μM). These data clearly demonstrated that ebselen can restore the activity of meropenem on NDM-1 positive *E. coli*.

To confirm whether ebselen can neutralise NDM-1 activity *in vitro*, purified enzyme and the colorimetric β-lactamase substrate nitrocefin were employed. The reversibility of inhibition by ebselen was first tested by measuring the recovering enzymatic activity after rapid dilution as described previously[16]. Approximate 11% residual activity was obtained after 100-fold dilution of a 20min pre-incubation mixture (100-fold of enzyme of normal assay condition and 10-fold IC_{50} of ebselen for the amount of the enzyme) compared to non-ebselen control in the same assay condition, implying that ebselen employed an irreversible or slow reversible binding with NDM-1 (Sup. Fig. 1). To confirm whether ebselen can neutralise NDM-1 activity *in vitro*, purified enzyme and the colorimetric β-lactamase substrate nitrocefin were employed. Ebselen was found to inhibit the activity of NDM-1 in a time-dependent and concentration-dependent manner (Fig. 2A). Plots of the natural logarithm of the residual activity against incubation time were linear, suggesting the observed rate of inactivation follows *pseudo*-first order kinetics. This also implies that interaction of ebselen with the enzyme may be covalent in nature. The k_{obs} (observed rate constant) was determined by the negative slope for each of the line from the plot. Each k_{obs} was plotted against the concentration of ebselen and fitted nonlinear regression curve plotted to determine the inactivation kinetic parameters revealed a K_I of 0.38 ± 0.03 μM, k_{inact} of 0.034 ± 0.002 min^{-1} and k_{inact}/K_I of 1.496 $mM^{-1}s^{-1}$ within 95% confidence intervals (Fig. 2B).

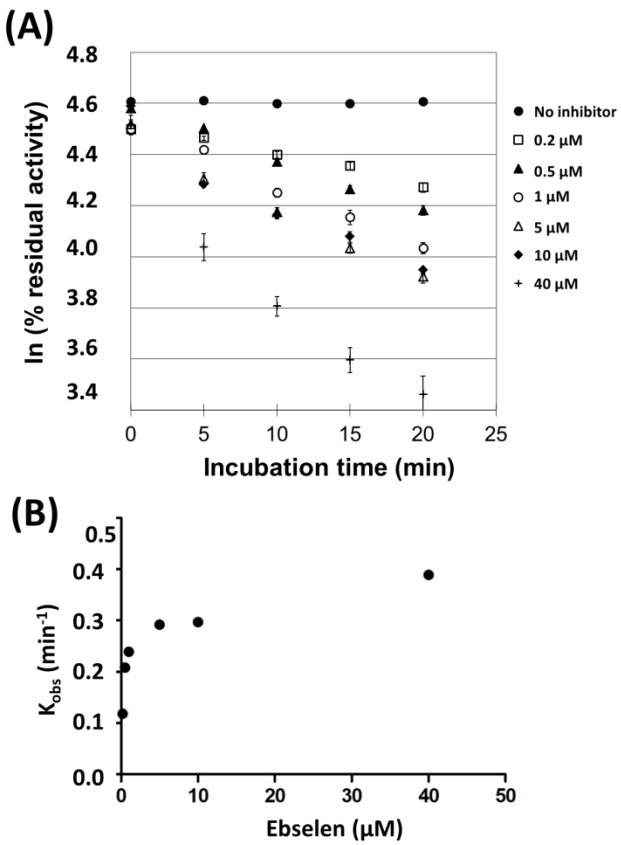

Fig. 2 (A) Time- and concentration-dependent inhibition of NDM-1 by ebselen; (B) The hyperbolic plot of k_{obs} of ebselen against ebselen concentrations

To further understand the details of interaction between ebselen and NDM-1, ESI-MS analysis was performed to determine the mode of binding of ebselen to NDM-1. Compared to NDM-1 under denaturing condition (25866 Da, Fig. 3C), the molecular weight (MW) of native NDM-1 (25991 Da) is in agreement with attachment of two zinc ions and loss of four or more protons, which is consistent with previous studies (Fig. 3A).[17] Addition of ebselen in NDM-1 for 20 minutes resulted in a complete mass shift of the protein complex to a clear peak with MW of about 26202 Da, which was the sum of MW of NDM-1 (25866 Da), one zinc ion (65 Da), ebselen (274 Da) and loss of three protons (Fig. 3B). The data suggested that ebselen not only exhibited strong binding activity to NDM-1, but also competed out one zinc ion from the active site of NDM-1. This could be due to the binding of ebselen to the thiol group of Cys^{221}, which disrupted the coordination of Zn with Asp^{120}, Cys^{221} and His^{263} during the process. To further confirm whether ebselen can bind to Cys^{221} in the active site of NDM-1, ESI-MS was used to check the mode of binding of ebselen to NDM-1 in a denaturing condition. Our data showed that under denaturing condition, NDM-1 exhibited a MW of 25866 Da, suggesting that zinc ions could not bind to the enzyme under this condition. The addition of ebselen in NDM-1 in a denaturing condition showed a clear shift of the complex to a peak with MW of 26140 Da, which is the exactly sum of MW of NDM-1 without zinc ion (25866 Da) and ebselen (274 Da) (Fig. 3D). These data suggested that ebselen could bind to NDM-1 in a covalent manner, which was most likely formed by covalent interaction of the selenium moiety of ebselen with the thiol group of Cys^{221} in the active site of NDM-1, since only one cysteine molecule was present in the entire NDM-1 protein. These results are consistent

with those of the enzyme kinetic study which suggests covalent interaction of ebselen with NDM-1.

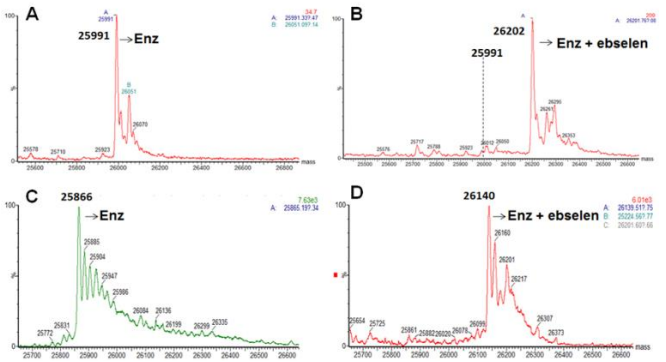

Fig. 3 Analysis of interaction between NDM-1 and ebselen by ESI-MS: (A) mass spectrum of native NDM-1 (Enz); (B) mass spectrum of native NDM-1 (Enz) after incubation with ebselen; (C) mass spectrum of denatured NDM-1 (Enz); (D) mass spectrum of denatured NDM-1 (Enz) after incubation with ebselen.

To further confirm whether covalent binding of ebselen to Cys^{221} of NDM-1 occurred, a competition assay with different thiol compounds was employed. Our data showed that in the presence of thiol compounds such as dithiothreitol (DTT), β-mercaptoethanol or L-cysteine, NDM-1 hydrolysed substrate in the same manner as NDM-1 alone, suggesting that these thiol compounds did not affect the activity of NDM-1 (Sup. Fig. 1). The addition of these thiol compounds with ebselen could rescue NDM-1 hydrolysis of the substrate nitrocefin, with a strength approaching the same level as that without ebselen inhibitor (Sup. Fig. 1). These data suggested that these thiol compounds could compete with the thiol group of Cys^{221} for ebselen binding, attenuating its inhibitory effect on nitrocefin hydrolysis. This observation also implies that ebselen binds to the thiol group of Cys^{221} to inactivate the enzyme activity. Moreover, adding excessive zinc (100 μM $ZnSO_4$) to a mixture of ebselen and NDM-1 could not restore the activity of NDM-1 (Data not shown), indicating that the binding of ebselen to Cys^{221} of NDM-1 is covalent in nature.

Based on the above data, the mechanism of NDM-1 inhibition by ebselen was proposed in Fig. 4. Both zinc ions in the active site of enzyme can act as Lewis acid and may coordinate to the oxygen atom of ebselen, resulting in ebselen being subjected to nucleophilic attack. It is likely that the nearby nucleophilic thiol group of Cys^{221}, which is an essential residue for coordination with zinc ion, reacts with ebselen to form a covalent S─Se bond with the enzyme and results in the loss of one zinc ion. The resulting chemically modified Cys^{221} may completely destroy the conformation of the active site of NDM-1, thereby explaining its effects on inhibition of MBLs.

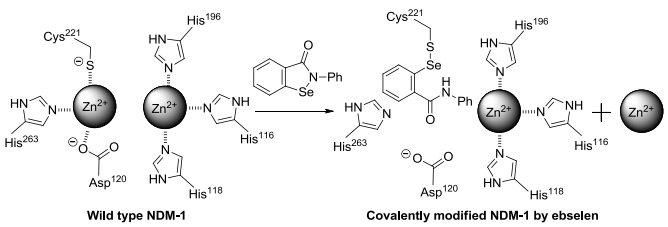

Fig. 4 Proposed mechanism of NDM-1 inhibition by ebselen.

Ebselen is a drug in human clinical trials for treatment of cerebral ischemia and stroke. It has very low toxicity (LD_{50} in rat >6180 mg per os) and is effective and well tolerated in animal models.[18] In cells, ebselen reduces hydrogen peroxide by mimicking the activity of the selenoenzyme glutathione peroxidase.[19] Therefore it is a very promising drug candidate for further development. Although several irreversible inhibitors have been reported for NDM-1 by metal ion sequestration (IC_{50} of AMA = 4 μm for NDM-1),[13] thiol modification on Cys^{221} and covalent modification on Lys^{211} (k_{inact}/K_I of 0.17 $M^{-1}s^{-1}$), conclusions were either inconclusive or exhibited poor inhibitory effects with low k_{inact}/K_I values.[20] Most importantly, ebselen could restore the activity of carbapenem when tested against *E. coli* expressing NDM-1, with higher efficiency than AMA (Table 1).[13]

In conclusion, our data showed that ebselen is a promising candidate for future NDM-1 inhibitor development. Its ability to target the Cys residue in MBLs may enable it to be developed into a broad spectrum inhibitor to subclasses B1 and B2 MBLs since these two subclasses of MBLs contain a cysteine residue to coordinate the functions of the zinc ion at the active site. However, its antioxidant activity might abrogate its activity to other enzymes such as MBLs when used *in vivo*. In addition, the selenium moiety is a concern for toxicity, limiting its potential for drug development. Future strategy should focus on improving its specificity to MBLs and reducing its toxicity to other cysteine-containing enzymes in human.

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Notes and references

[a] Shenzhen Key Laboratory for Food Biological Safety Control, Food Safety and Technology Research Centre, The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen, P.R. China.
[b] State Key Laboratory of Chirosciences, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong.
[c] Key Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao, China.

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# Jiachi Chiou and Shengbiao Wan contributed equally to this work.

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