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Disulfiram as a potent metallo-β-lactamase inhibitor with dual functional mechanisms

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We report a promising NDM-1 inhibitor, disulfiram, which could covalently bind to NDM-1 by forming an S-S bond with the Cys208 residue. Its copper-containing metabolite *in vivo*, $Cu(DTC)_2$ also inactivated NDM-1 through oxidizing the Zn(II) thiolate site of the enzyme, therefore exhibiting dual functional inhibitory potential against B1 and B2 subclasses MβLs.

Carbapenem-resistant Gram-negative pathogens have been gradually increasing in prevalence due to selection pressure caused by excessive consumption of carbapenem antibiotics (e.g., imipenem, meropenem, doripenem, and ertapenem), which poses a huge threat to public health worldwide.[1] Notably, carbapenem-resistant *Enterobacteriaceae* (CRE) in recent years are responsible for 9,000 annual nosocomial infections, kill almost half of in-patients who get bloodstream infections from these bacteria.[2] One of the main mechanisms of bacterial resistance to carbapenems is through the expression of plasmid-mediated carbapenem-hydrolyzing β-lactamases.[3] The carbapenemases are divided into four classes: A, B, C and D β-lactamases, the class A, C and D enzymes are serine β-lactamases (SβLs), including KPC, CMY and OXA-type, that contain a serine moiety in the active sites to covalently attack the C-N bond of β-lactam. Class B enzymes, also called metallo-β-lactamases (MβLs), are the most important type of carbapenemases, which use a nucleophilic water to cleave the β-lactam ring.[4] MβLs are further grouped into B1, B2 and B3 subclasses. The most clinically important MβLs include New Delhi metallo-β-lactamases (NDMs) and MβLs from Imipenem-resistant *Pseudomonas* (IMPs), conferring on bacteria the resistance to the last resort carbapenems and all other bicyclic β-lactams that are currently used.[5] Currently, few therapeutic options are available to treat infection from these so-called "superbugs". [6]

The current recommendation to treat carbapenem-resistant Gram-negative infections involves the use of antimicrobial combination therapy.[7] Several inhibitors in combination with antibiotics against the bacteria producing SβLs are being used clinically, such as clavulanic acid, sulbactam, tazobactam and relebactam.[8] For MβLs, several types of inhibitors have been reported, including metal-binding inhibitor (D-captopril, cyclic boronates[5]), metal-stripping inhibitor (aspergillomarasmine A[9]), covalent inhibitor (Ebselen[10]), allosteric inhibitor (arginine peptides, DNA nanoribbon[11]) and undefined inhibitor (dithiocarbamates[12]). However, to date, no available MβL inhibitor has been used clinically. Therefore, there is a pressing clinical demand for effective and safe drugs that can overcome the carbapenem-resistant Gram-negative pathogens mediated by MβLs.

In this study, we applied an isothermal titration calorimetry (ITC)-based approach to screen a panel of disulfide compounds (Fig. S1), and successfully identified a promising NDM-1 inhibitor, disulfiram (DSF), which has been used for treatment of alcohol dependence for over six decades, with well-established pharmacokinetics, safety and tolerance at the FDA-recommended dosage.[13] DSF inhibited NDM-1 activity by covalently binding to the Cys208 residue, in which one Zn(II) ion at the active site of enzyme was released. Importantly, $Cu(DTC)_2$, a copper-containing metabolite *in vivo* of DSF, exhibited broad-spectrum inactivation against the subclasses B1 and B2 MβLs with a nanomolar affinity, through a unique inhibition mechanism with oxidizing Zn(II) thiolate site of the enzymes (Fig. 1).

Using ITC-based approach as in our previous report,[14] the primary screen generated one promising hit, DSF, which significantly inhibited the hydrolysis of imipenem by NDM-1 (Fig. S2). Next, the inhibitor concentration causing 50% decrease of enzyme activity (IC_{50}) of DSF and its analogues were tested using imipenem as substrate.[15] The results indicated that DSF and all analogues exhibited potent inhibition against MβLs, with an IC_{50} value range of 0.12 ± 0.05-3.74 ± 1.24 μM for NDM-1, IMP-1, and ImiS, but no inhibition was observed on the class B3 MβL L1 (Fig. 2a and Table S1). In addition, DSF was shown to exhibit dose- and

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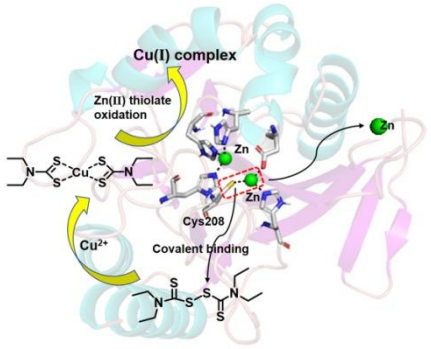

Fig. 1. Disulfiram inhibits NDM-1 with dual functional mechanisms.

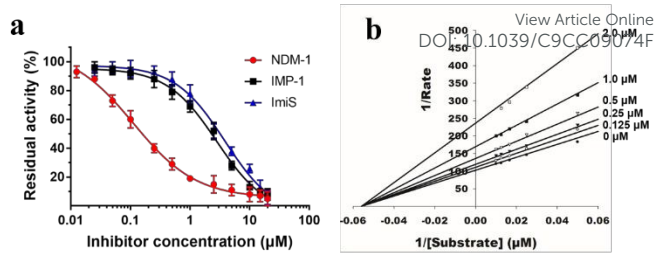

Fig. 2. (a) Inhibition curves of DSF against NDM-1, IMP-1 and ImiS. (b) Lineweaver–Burk plot of imipenem (0-100 μM) hydrolysis by NDM-1 (50 nM) in the absence and presence of DSF (0-2 μM) with 30 mM Tris at pH 7.0 at 298K.

time-dependent inhibition on NDM-1 (Fig. S5), implying that the binding of DSF to NDM-1 maybe covalent in nature.[10] We examined the change of inhibition kinetics of NDM-1 by varying DSF concentration. The addition of DSF resulted in a decrease of the apparent V_{max} from 9.8 to 4.2 μM/min. The Lineweaver–Burk plot indicated that it is either a typical irreversible or a non-competitive inhibition (Fig. 2b). Multiple studies reported that DSF spontaneously forms complexes with Cu(II) *in vivo* and in cell culture media, which enhances its anti-tumour activity via inhibition of aldehyde dehydrogenase.[16] Subsequently, we examined whether the Cu(II) is also capable of affecting inhibition of DSF on MβLs, the results showed that the $Cu(DTC)_2$ and its analogues had high inhibitory activities, with an IC_{50} range of 3.9 ± 1.6-77.5 ± 12.5 nM against NDM-1, IMP-1 and ImiS (Table S2), which indicated that Cu(II) is able to promote the inhibition of MβLs by DSF. As shown in Fig. S7, the gradual decrease of heat flow was clearly observed during titration of imipenem into NDM-1-DSF/NDM-1-$Cu(DTC)_2$ mixture with the gradually increase of inhibitor dose, verifying the inhibitory effect of DSF and $Cu(DTC)_2$ against NDM-1 thermodynamically. Also, it can be observed that $Cu(DTC)_2$ resulted in a gradually decrease in total heat release Q, but a slight heat decrease for DSF.[17]

We then investigated how the DSF and $Cu(DTC)_2$ exert their inhibitory activity. The addition of 2 eq Zn(II) to apo-NDM-1 treated by DSF led to only about 10% activity being restored. However, almost full activity of NDM-1 was recovered after addition of 2 eq Zn(II) to apo-NDM-1 and apo-NDM-1 treated with $Cu(DTC)_2$ (Fig. S6 and S8). These observations indicated that DSF may covalently bind to NDM-1 by forming an S-S bond with the Cys208 of the enzyme, but $Cu(DTC)_2$ did not directly target the Cys208.

NDM-1 is a carbapenem hydrolase with two Zn(II) in the active site. To confirm whether DSF and $Cu(DTC)_2$ target NDM-1 by removing Zn(II) from the active site of the enzyme, we used ICP-AES to measure the Zn(II) content of NDM-1 samples treated with DSF and $Cu(DTC)_2$.[18] The gradient addition of DSF to a sample of NDM-1 resulted in gradual decreases in the stoichiometry of Zn (II) in NDM-1, eventually 0.89 eq Zn(II) were released for DSF (Fig. S9). However, $Cu(DTC)_2$ was not enable to lead to the releases of Zn (II), implying that DSF and $Cu(DTC)_2$ exhibited different inhibition behavior.

To further probe the detail interaction between DSF and NDM-1, MALDI-TOF MS was employed to determine the binding mode of DSF to MβLs. As shown in Fig. 3a, the isolated NDM-1 showed a peak with a 24924 m/z, while addition of DSF analogue **3d** for 2 h appeared an clear peak at 25133 m/z, that is corresponding to NDM-1 with binding of one (dibenzyl)dithiocarbamate (272 Da), loss of one Zn(II) ion (65 Da) and two protons. This result suggested that DSF analogue could covalently bind to NDM-1 presumably through the formation of an S-S bond with the thiol of Cys208, and resulting in the release of one Zn(II) ion from the active site of the enzyme.

To corroborate the binding site of DSF and $Cu(DTC)_2$ on NDM-1, only one cysteine-to-alanine mutant (C208A mutant) was made via site-directed mutation. The residual activities of wild type and mutant enzyme in the presence of DSF are shown in Fig. 3b, it is observed that the displacement of Cys208 reduced ~75% activity of NDM-1 enzyme,[19] suggesting that Cys208 residue is essential to the full activity of NDM-1. Compared to wild-type NDM-1, the NDM-1-C208A mutant and B3 subclass MβL L1 (no cysteine at its active site) showed no significant change in inhibitory activity at a given inhibitor concentration (10 μM), which demonstrated that the Cys208 is responsible for inhibiting NDM-1 by DSF. In addition, 0.93 equivalent Cu(II) was found to finally bind to the wild-type NDM-1, but the binding capacity of Cu(II) to the NDM-1-C208A mutant was reduced significantly, with only ~0.1 equivalent Cu(II) bound to the mutant as testified by ICP-AES (Fig. S10). By thermal shift assay,[11] we found that supplementation of DSF and $Cu(DTC)_2$ to the purified NDM-1 sample led to the decreased shift in the Tm (protein-melting temperatures) of the enzyme by 2.6 and 3.4 ℃, making the enzyme instable (Fig. S11), while Tm of the NDM-1-C208A mutant remained almost unchanged upon treatment with DSF and $Cu(DTC)_2$ (Fig. S12). These results demonstrated that Cys208 is essential for DSF to inhibit NDM-1, and the Zn(II) thiolate (Zn(II)-binding cysteine) site of

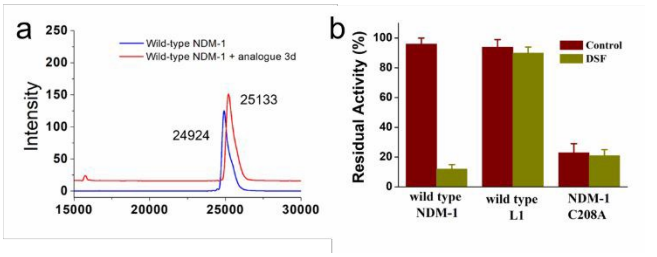

Fig. 3. (a) Analysis of interaction between NDM-1 and DSF analogue **3d** by MALDI-TOF-MS. 10 μM NDM-1 was incubated with 50 μM **3d** for 2h in 30 mM Tris-HCl (pH 7.5) at 298K. (b) Normalized residual activity of wild-type NDM-1, B3 subclass MβL L1, and NDM-1-C208A mutant in the absence and presence of DSF (10 μM).

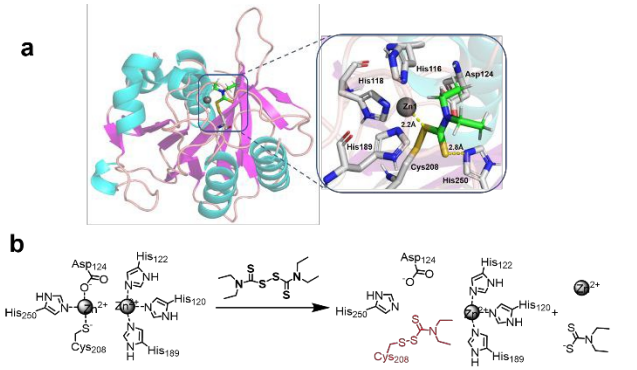

Fig. 4. The binding mode of DSF covalently targeting NDM-1 (a), and the proposed mechanism of NDM-1 inhibition by DSF (b).

the enzyme plays a pivotal role on $Cu(DTC)_2$ to inhibit NDM-1.

To explore the potential binding mode of DSF to NDM-1, we performed molecular docking studies.[20] The docking studies revealed that one sulfur atom of DSF coordinated with two Zn(II) ions (2.2 and 2.3 Å, respectively), one sulfur atom of disulfide bond also bound to the Zn(II) ion (2.9 Å), so as to anchor in the active site of NDM-1 (Fig. S13), which provided a basis for the covalent binding of thiol of Cys208 by DSF. Subsequently, Zn(II) coordinated with Cys208 was removed and (diethyl)dithiocarbamate (DTC) was covalently docked to the active site of NDM-1. In the covalent model where a bond between Cys208 and DSF was imposed, a conformation where the coordination of one sulfur atom of DTC with Zn(II) (2.2 Å) and the interaction of another sulfur atom with iminazole of His250 (2.8 Å), as illustrated in Fig. 4a. This conformation is consistent with the mechanism that DSF was nucleophilicly attacked by the thiol of Cys208 and formed an S-S bond with the enzyme. Based on these analyses, an inhibition mechanism of NDM-1 by DSF is proposed in Fig. 4b. The two sulfur atoms of DSF firstly coordinate to both Zn(II) at the active site of NDM-1, resulting in DSF being subjected to nucleophilic attack by the nearby thiol of Cys208, and then forming an S–S bond with Cys208 of the enzyme and releasing one Zn(II) ion from the active site.

Considering the structural complexity of Zn(II) thiolate site of MβLs and the lack of characteristic spectroscopic features to monitor chemical changes of the Zn(II) sites, to better understand the reactivity between $Cu(DTC)_2$ and Zn(II) thiolate in the active site of NDM-1, we synthetized a Zn(II) complex (SZn) as model which furnish a hybrid N/O/S coordination sphere in mimicking Zn(II)-binding His/Asp/Cys active site of NDM-1 (Scheme. S1). The black precipitate formed immediately upon mixing $Cu(DTC)_2$ with 2 eq SZn complex in acetonitrile (Fig. S14).

Furthermore, X-ray photoelectron spectroscopy (XPS) was employed to characterize the structure of the precipitate. As showed in Fig. 5a and S15, there are two strong peaks emerged at 932.3 and 952.3 eV, corresponding to Cu $2p_{3/2}$ and Cu $2p_{1/2}$, respectively, which are characteristic peaks of Cu. The satellite peak around 942 eV (Fig. S15) is attributed to the characteristic peak of Cu (II) in XPS spectrum of $Cu(DTC)_2$.[21] However, the lack of a satellite peak around 942 eV indicates the absence of Cu(II) in the precipitate (Fig. 5a). It is worth pointing that, compared to the auger spectra of $Cu(DTC)_2$ at 917.7 eV, the peak at 916.2 eV is attributable to the auger spectra of the precipitate (Fig. 5b), indicating the existence of Cu(I) in the precipitate,[22] thereby revealing that the reaction of $Cu(DTC)_2$ and SZn complex generated Cu(I) complex. These characterizations suggested a unique inhibition mechanism, which is that $Cu(DTC)_2$ is able to oxidize Zn(II) thiolate in the active site of the enzyme, therefore exhibiting broad-spectrum inhibitory potential against the B1 and B2 subclass MβLs, which is distinct from inhibitory mechanism on NDM-1 previously reported. This mechanism will provide a novel strategy for the future development of clinically used MβLs inhibitor. Given the lack of characterization method for Cu(I) in aqueous solution due to its disproportionation reaction, how the Zn(II) thiolate site of MβLs to exert oxidation by $Cu(DTC)_2$ in buffer solution, which remains an important open question that deserves extensive future investigations.

In view of the existence of many intracellular thiols such as glutathione and cysteinyl thiols of proteins, to test whether intracellular thiol is able to affect the inhibitory activity of $Cu(DTC)_2$ against NDM-1, the competitive experiment was performed. The β-Mercaptoethanol (β-ME) reduced $CuCl_2$ almost completely within 10 min under the same conditions (Fig. S16a), but only about 5% $Cu(DTC)_2$ was reduced by the β-ME even for 3 h (Fig. S16b). In the enzymatic inhibition assay, both thiols, dithiothreitol (DTT) and β-ME, had no effect on the inhibitory activity of $Cu(DTC)_2$ against NDM-1, but $CuCl_2$ is susceptible to thiols (Fig. S17). These results indicated that the reaction capacity of the thiols with $Cu(DTC)_2$ is much less than that of the Zn(II) thiolate, and showcased that DTC exhibits a unique redox-tuning ability that makes Cu(II) to avoid the reduction by intracellular thiols, and retains its ability to oxidize Zn(II) thiolate site of the enzyme. This is because that, compared to the hard Cl donors, DTC has two soft sulfur donors coordinated with one lone-pair-donating nitrogen. The coordination bonds in $Cu(DTC)_2$ are more likely to be covalent and its copper center is less electron-deficient than $CuCl_2$, which possibly render the oxidization of $Cu(DTC)_2$ less than that of $CuCl_2$.

We then investigated the ability of DSF and $Cu(DTC)_2$ to restore the antimicrobial activity of imipenem against *E. coli*-MβLs and a panel of clinical CRE.[23] Checkerboard MIC studies confirmed the expected synergy between imipenem and DSF only in NDM-1-expressing *E. coli* and not in *E. coli*-BL21 (Fig. S18). All DSF analogues exhibited their potent synergy with imipenem against NDM-1-, IMP-1-, ImiS-expressing *E. coli* and clinical CRE producing-NDM-1, *K. pneumoniae* and *P. aeruginosa*, with a 2-32-fold

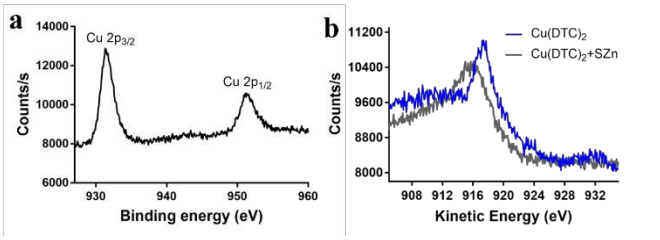

Fig. 5. High-resolution Cu XPS spectrum of the precipitate (a) and auger spectrum of $Cu(DTC)_2$ and the precipitate (b).

decrease in MIC of imipenem (Table S3 and S4). Also, $Cu(DTC)_2$ and its analogues exhibited potential in restoring imipenem efficacy, with a 8-64-fold reduction in MICs against *E. coli*-MβLs (Table. S6). Such excellent synergistic antibacterial efficacy is also verified by gradually elevated cellular uptake of Cu(II) and time kill curves (Fig. S20 and S21). The MIC of imipenem remained unchanged in the presence of DSF and $Cu(DTC)_2$ at a dose of 16 μg/mL against *E. coli*-NDM-1-C208A and *E. coli*-L1 (Table S7), also implying that the Cys208 is essential for inhibition of NDM-1 by DSF and $Cu(DTC)_2$. Importantly, $Cu(DTC)_2$ is able to circumvent the reduction by the intracellular thiols and retain its ability to oxidize Zn(II) thiolate site of NDM-1 to specifically kill carbapenem-resistant bacteria (Fig. S22a and Table S8).

Just as it was observed in the case of inhibitor-resistant TEM-1 and KPC-2,[24, 25] owing to the rapid evolution of variant MβLs, most organic molecule-based inhibitors may easily encounter resistance. The combination of $Cu(DTC)_2$ with imipenem significantly suppressed the development of high-level resistance in NDM-1 producers, compared to that of original strain treated by imipenem alone (Fig. S22b), which may be due to the reduced protein levels of NDM-1 in the combination therapy.

In conclusion, our results validated that the disulfiram is a promising candidate for the development of NDM-1 inhibitor, which covalently bind to NDM-1 by forming an S-S bond with the Cys208 residue at the active site. $Cu(DTC)_2$, a copper-containing metabolite *in vivo* of DSF, also inactivated NDM-1 through a unique mechanism with oxidizing Zn(II) thiolate in the active site of enzyme, thereby exhibiting dual functional inhibitory mechanisms against both B1 and B2 subclasses MβLs. Also, DSF and $Cu(DTC)_2$ effectively restored imipenem efficacy against clinical isolates *E. coli, K. pneumoniae* and *P. aeruginosa* producing-NDM-1. Importantly, $Cu(DTC)_2$ could avoid the reduction by the intracellular thiols to specifically kill CRE and slow down the development of higher-level resistance in bacteria producing NDM-1. Although the disulfiram and $Cu(DTC)_2$ are already used in clinic or in clinical phase II, their toxicity is a concern. Therefore, future strategy should focus on enhancing their specificity to carbapenem-resistant Gram-negative pathogens mediated by MβLs and reducing their toxicity.

Conflicts of interest

There are no conflicts to declare.

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