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Hydroxamate and thiosemicarbazone: Two highly promising scaffolds for the development of SARS-CoV-2 antivirals

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A B S T R A C T

The emerging COVID-19 pandemic generated by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has severely threatened human health. The main protease (Mpro) of SARS-CoV-2 is promising target for antiviral drugs, which plays a vital role for viral duplication. Development of the inhibitor against Mpro is an ideal strategy to combat COVID-19. In this work, twenty-three hydroxamates 1a-i and thiosemicarbazones 2a-n were identified by FBET screening to be the potent inhibitors of Mpro, which exhibited more than 94% (except 1c) and more than 69% inhibition, and an IC50 value in the range of 0.12–31.51 and 2.43–34.22 μM, respectively. 1a and 2b were found to be the most effective inhibitors in the hydroxamates and thiosemicarbazones, with an IC50 of 0.12 and 2.43 μM, respectively. Enzyme kinetics, jump dilution and thermal shift assays revealed that 2b is a competitive inhibitor of Mpro, while 1a is a time-dependent inhibitor; 2b reversibly but 1a irreversibly bound to the target; the binding of 2b increased but 1a decreased stability of the target, and DTT assays indicate that 1a is the promiscuous cysteine protease inhibitor. Cytotoxicity assays showed that 1a has low, but 2b has certain cytotoxicity on the mouse fibroblast cells (L929). Docking studies revealed that the benzylloxycarbonyl carbon of 1a formed thioester with Cys145, while the phenolic hydroxyl oxygen of 2b formed H-bonds with Cys145 and Asn142. This work provided two promising scaffolds for the development of Mpro inhibitors to combat COVID-19.

1. Introduction

The coronavirus disease (COVID-19) generated by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has spread widely and rapidly around the globe since it was found in December 2019 [1]. So far, millions of people have died because of the infection of COVID-19 [2]. Though some vaccines have been developed and widely vaccinated, the problems of this viral disease have not been essentially solved, especially now, the emergence of coronavirus variants such as delta and kappa [3–6]. Studies have found that the sera of individuals receiving a dose of Pfizer or AstraZeneca vaccine have limited inhibition of variant Delta [7]. Therefore, there is an urgent need to develop more effective antivirals.

SARS-CoV-2 is a positive-sense and single-stranded RNA virus belonging to the genus betacoronavirus and shares 96% sequence to a bat coronavirus [8]. The virus occupies roughly 26–32 kb in length and encodes some structural, non-structural and accessory proteins. The cleavage of pp1a and pp1ab polyproteins into a single non-structural protein is an essential process in virus replication, and the main protease (Mpro) plays a vital role in this process [9–11]. Also, there is no homology between Mpro and human protease [12]. Therefore, the Mpro is regarded as an important target for designing drugs to combat COVID-19 [13].

The Mpro, a nuleophilic cysteine protease, has three domains: I (resides 8–101), II (102–184), and III (201–306). The first two domains are responsible for the structure of protein and the last domain is in charge of the catalytic process [14]. In the active site, Cys145 and His41 form a catalytic binary. The thiol (–SH) group of cysteine is responsible for the hydrolysis and His41 provides the optimal pH conditions to activate the –SH group, thus it achieves a nuleophilic attack on the substrate [15].

So far, many types of Mpro inhibitors have been reported, such as peptides, non-peptides, drug molecules and natural products [13,16–18]. Recently, Wang and his colleagues discovered that
peptidomimetic molecules (boceprevir, GC-376, and calpain inhibitors II and XII) have an IC\textsubscript{50} value range from single-digit to sub-micromolar [19]. Masitinib, a specific kinase inhibitor, was identified to be effective on tested coronavirus variants (B.1.1.7 and B.1.351) in vitro [20]. More importantly, recently, Pfizer has released an inhibitor (PF-07321332), currently in Phase 3 clinical trials, is expected to be the first M\textsuperscript{pro} drug to treat SARS-CoV-2 [21,22]. Although it is early to determine potential inhibitors that would be very effective against the virus, they provide an encouraging start for further coronavirus therapeutics.

Both New Delhi metallo-\beta-lactamase-1 (NDM-1) and M\textsuperscript{pro} are the cysteine proteases [23,24]. As the NDM-1 inhibitors, the Ebsulfur and Ebselen were reported to inhibit M\textsuperscript{pro} [25]. Recently, both hydroxamates and thiosemicarbazones were synthesized in our lab and characterized by NMR and mass spectrometry and evaluated to have inhibitory efficacy on NDM-1 [26,27]. In the same case, we expected these two classes of compounds to have inhibitory activity on M\textsuperscript{pro}, therefore evaluated them. In this work, we focus on screening the M\textsuperscript{pro} inhibitor by fluorescence resonance energy transfer (FRET) method [28,29]. The hydroxamates and thiosemicarbazones were found to be the potential scaffolds to target M\textsuperscript{pro}. Subsequently, the action mechanism of these molecules was characterized and analyzed by enzyme kinetics, jump dilution and thermal shift assays.

2. Results and discussion

2.1. Screening of M\textsuperscript{pro} inhibitor

The SARS-CoV-2 M\textsuperscript{pro} was expressed and purified by the method reported previously [29]. The M\textsuperscript{pro} gene was inserted into the vector PGEX-6P-1 and expressed in BL21 E. coli. The protein was purified by Ni-NTA and HiTrap Q FF columns, respectively. The SDS-PAGE of the purified protein is shown in Fig. 1a.

The enzyme activity was assayed by measuring \( K_m \) and \( V_{max} \) values as previously reported method [30]. The employed fluorescent substrate in this experiment was Mca- \( AVLQSGFRK(Dnp)K \). Various concentrations of the fluorescent substrate (1–100 \( \mu \)M) were premixed with M\textsuperscript{pro} sample (0.2 \( \mu \)M), respectively. The hydrolysis velocity of substrate was measured, and the data obtained were fitted to the Michaelis-Menten equation to give \( K_m \) and \( V_{max} \) values. The calculated \( K_m \) and \( V_{max} \) are 5.4 ± 4.13 \( \mu \)M and 0.68 ± 0.08 nM/s, respectively (Fig. 1b), and \( K_{cat} / K_m \) is 6296 M\textsuperscript{-1} s\textsuperscript{-1}, which is consistent with the data (6925 M\textsuperscript{-1} s\textsuperscript{-1}) previously reported [30].

The FRET experiments were performed to screen the potential M\textsuperscript{pro} inhibitors [28,29]. The hydroxamates and thiosemicarbazones were prepared in our lab, characterized by \( ^1 \)H and \( ^{13} \)C NMR, confirmed by HRMS, and reported to be the inhibitors of metallo-\beta-lactamases [26,27] (Fig. 2). To explore these molecules whether have potential inhibitory effects against M\textsuperscript{pro}. We firstly determined the percent inhibition of these compounds as previously reported method [31]. The hydroxamates 1a-i and thiosemicarbazones 2a-n were dissolved in a certain amount of DMSO, and then diluted with assay buffer (20 mM Tris, pH 6.5, 0.4 mM EDTA, 20% glycerol, 120 mM NaCl) [29]. It should be noted that the final concentration of DMSO was less than 0.5%, because the control experiments proved that DMSO at this concentration has no effect on enzyme activity. Percent inhibition of the tested compounds on M\textsuperscript{pro} is shown in Fig. 3. It is clearly to be observed that all hydroxamates (50 \( \mu \)M) exhibited more than 94% inhibition on M\textsuperscript{pro} (except 1c), and all thiosemicarbazones at same concentration showed more than 69% inhibition. Significantly, the thiosemicarbazones tested had better percent inhibition on M\textsuperscript{pro} than the thiosemicarbazone complexes with iron (III) recently reported (30.62% at 100 \( \mu \)M) [32].

2.2. Determination of IC\textsubscript{50}

The inhibitor concentrations causing 50% decrease of enzyme activity (IC\textsubscript{50}) of hydroxamates and thiosemicarbazones on M\textsuperscript{pro} were measured as previously reported method [33]. The concentration range of inhibitors was from 0 to 80 \( \mu \)M, and the substrate and protease concentrations were 20 and 0.2 \( \mu \)M, respectively. The measured IC\textsubscript{50} data are listed in Table. 1. The collected data show that all of these compounds exhibited potential inhibition against M\textsuperscript{pro}, with an IC\textsubscript{50} value in the range of 0.12–34.22 \( \mu \)M. The hydroxamates and thiosemicarbazones had an IC\textsubscript{50} value range of 0.12–31.51 and 2.43–34.22 \( \mu \)M, respectively, 1a (IC\textsubscript{50} = 0.12 \( \mu \)M) and 2b (IC\textsubscript{50} = 2.43 \( \mu \)M) were found to be the most effective inhibitors in the two classes of compounds, respectively. These assays revealed that both hydroxamates and thiosemicarbazones are attractive scaffolds for the development of M\textsuperscript{pro} inhibitors.

2.3. Inhibition mode assay

Given the best potency, the time-dependent inhibition of hydroxamate 1a on M\textsuperscript{pro} was assayed. As shown in Fig. 4a, the residual activity of M\textsuperscript{pro} decreased with the increase of premix time of protease with inhibitor (1.25 \( \mu \)M), and 1a exhibited about 90% inhibition after incubation for 100 min, indicating that 1a is a time-dependent inhibitor [34].

The reversibility of hydroxamate 1a and thiosemicarbazone 2b binding to M\textsuperscript{pro} was evaluated by jump dilution tests [29,35,36]. The M\textsuperscript{pro} sample was incubated with a high concentration of inhibitors

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Fig. 1. The SDS-PAGE of the purified SARS-CoV-2 M\textsuperscript{pro} (a). Lane 1: protein molecular weight marker, lane 2: purified M\textsuperscript{pro}, lane 3: M\textsuperscript{pro} before cleavage with HRV 3C-protease. Activity of M\textsuperscript{pro} was confirmed by quantification crack of the fluorescent decapeptide Mca–AVLQSGFRK(Dnp)K as substrate (b).
(equivalent to $50 \times IC_{50}$) 1a (6 $\mu$M) and 2b (122 $\mu$M) for 2 h, respectively, so that the inhibitor could fully occupy enzymatic active sites, the resulting mixtures were diluted 100-fold with the fluorescence substrate solution, and the enzymatic residual activity was determined by monitoring the fluorescence. It is clearly observed in Fig. 4b that in the presence of 1a, the enzyme activity did not recover after the dilution. However, the enzyme treated with 2b recovered about 60% activity after dilution for 4000 s. These results indicate that the thiosemicarbazone reversibly, but hydroxamate like the Ebselen and Ebsulfur, irreversibly inhibit $M_{\text{pro}}$.

To further study the inhibition mode of the hydroxamates and thiosemicarbazones on $M_{\text{pro}}$, 1a and 2b were chosen to determine the enzyme kinetic parameters [37–39]. The above assay reveals that 1a inhibit $M_{\text{pro}}$ in a time-dependent pattern, and the kinetic progression curves exhibited a biphasic character (Fig. 5a), suggesting the inactivation rate follows pseudo-first-order rate kinetics. These results imply that the hydroxamate may covalently bind to the target [38]. The $K_{\text{obs}}$ (observed rate constant) were fitted against inhibitor concentration by nonlinear regression to calculate $K_I$ (the concentration of inactivator at the half-maximum inactivation rate constant), $k_{\text{inact}}$, and $k_{\text{inact}}/K_I$ values, which are $1.18 \pm 0.43 \, \mu$M, $0.0075 \pm 0.0005 \, \text{s}^{-1}$, and $6.4 \times 10^3 \, \text{M}^{-1} \text{s}^{-1}$, respectively [37].

The inhibition mode of thiosemicarbazone 2b was identified by analyzing Lineweaver–Burk plots, and $K_I$ (inhibition constant) value was
determined by fitting initial velocity versus substrate concentrations at each inhibitor concentration using SigmaPlot 12.0. The concentrations of substrate and inhibitor were in the range of 2.5–20 and 0–10 µM, respectively. The enzyme (0.2 µM) was incubated with inhibitor for 2 h, and the reaction was monitored when substrate was added. The Lineeweaver–Burk plots of fluorescent substrate hydrolysis by Mpro in the absence and presence of 2b are shown in Fig. 5b, which indicate that 2b is a competitive inhibitor [39], and the calculated Ki value is 3.9 µM.

2.4. Thermal shift assay

Thermal shift analysis, a powerful technique, is used to screen molecules that impact protein stability via monitoring a shift in the melting temperature (Tm) of the protein [40]. In general, the binding of a small molecule stabilizes protein, leading to an increased Tm value. However, a decreased Tm value results in destabilization of the protein [41]. To investigate the interaction of Mpro with inhibitors, the Mpro (20 µM) was premixed with hydroxamate 1a (20 µM) and thiosemicarbazones 2b (20 µM) for 2 h, respectively, and then the mixtures were treated with the SYPROR orange dye. The reaction of the protein and inhibitor was

Table 1
The inhibitory activities (IC50, µM) of hydroxamates and thiosemicarbazones on Mpro.

| Compd. | IC50 (µM) | Compd. | IC50 (µM) | Compd. | IC50 (µM) | Compd. | IC50 (µM) |
|--------|----------|--------|----------|--------|----------|--------|----------|
| 1a     | 0.12     | 1g     | 18.78    | 2d     | 17.61    | 2j     | 3.25     |
| 1b     | 4.3      | 1h     | 11.7     | 2e     | 8.45     | 2k     | 28.81    |
| 1c     | 31.51    | 1i     | 3.6      | 2f     | 32.94    | 2l     | 9.11     |
| 1d     | 0.15     | 2a     | 3.61     | 2g     | 32.33    | 2m     | 33.40    |
| 1e     | 0.42     | 2b     | 2.43     | 2h     | 19.10    | 2n     | 20.74    |
| 1f     | 1.46     | 2c     | 4.37     | 2l     | 34.22    |        |          |

Fig. 3. Percent inhibition of hydroxamates 1a-i and thiosemicarbazones 2a-n (50 µM) against Mpro. 0.5% DMSO was used as negative control and ebselen was used as positive control.

Fig. 4. Time-dependent inhibition curve of hydroxamate 1a (1.25 µM) on Mpro (a). Progress curves of Mpro activity change in the presence of hydroxamate 1a and thiosemicarbazone 2b (b). 0.5% DMSO was used for the blank control.
heated from 25 to 80 °C in 0.8 °C increment. As shown in Fig. 6a, the $T_m$ of Mpro was 54.49 °C. While in the presence of 1a, the determined $T_m$ value of protein decreased to 50.81 °C, indicating that binding of hydroxamates to protein leads to destabilization, like the Mpro inhibitors ebselen and disulfiram previously reported [42]. In contrast, in the presence of 2b, the $T_m$ of protein increased from 54.49 to 56.11 °C, suggesting that the tightly binding of thiosemicarbazones to Mpro increases the stability of the protein.

Moreover, we performed a dose-dependent determination of $T_m$ as the reported method [33]. The Mpro (20 µM) was mixed with various concentrations of 1a and 2b (10–100 µM) for 2 h, respectively. As shown in Fig. 6c, the melting temperature shifts ($\Delta T_m$) of Mpro increased with the increase of inhibitor concentration (10–100 µM), implying that the stabilization of Mpro to thermal denaturation is concentration-dependent.

2.5. Dithiothreitol (DTT) assay

To verify the action site of hydroxamates 1a to Mpro, DTT experiments were performed as previously reported method [42]. The Mpro (0.2 µM) was premixed with 1a (1.25 µM) in the assay buffer (see above) supplemented with and without DTT (4 mM) for 2 h, respectively. The fluorescent substrate (20 µM) was added to the mixture solution and then the initial reaction rate was determined. As shown in Fig. 7a, 1a had a potential inhibitory effect on the protein in the absence of DTT. Nevertheless, 4a did not show a significant inhibition on the protein in the presence of DTT.

Meanwhile, we also carried out a dose-dependent inhibition experiments of Mpro. As shown in Fig. 7b, the residual activity of protein decreased with the increase of 1a concentration (0–10 µM) in the absence of DTT. In contrast, in the presence of DTT, the enzymatic activity was not effectively inhibited. Also the residual activity of enzyme

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**Fig. 5.** The hyperbolic plots of $K_{obs}$ against concentrations of hydroxamate 1a (a). The Lineweaver-Burk plots of Mpro catalyzed hydrolysis of thiosemicarbazone 2b. The concentrations of inhibitors were 0 (●), 2.5 (○), 5(▼), 10 (▽) µM (b).

**Fig. 6.** The melting temperature ($T_m$) of Mpro in the absence and presence of 1a and 2b (a). Fluorescence based thermal shift assays of 2b interaction with Mpro as indicated by $dF/dT$ (b). Dose-dependent melting temperature shift (c).
was not associated with inhibitor concentration. The inhibition of 1a on the enzyme was abolished, probably because the binding site of 1a on enzyme was disrupted by DTT. The above experimental results implied that the inhibition of hydroxamate 1a on Mpro was realized by cysteine modification and 1a is also the promiscuous cysteine protease inhibitor [29].

2.6. Molecular modeling

To predict the binding affinity and pose of both hydroxamates and thiosemicarbazones to Mpro, 1a and 2b were docked into the active sites of the crystal structure of Mpro (PDB ID: 6LU7) [43]. The minimized binding free energy of 1a and 2b were calculated to be −4.53 and −6.64 kcal/mol, respectively. Docking studies revealed that the carbonyl and amine group of 1a first approach Cys145 through H-bond, and 1a also interacted with His41 residue, increasing the affinity of this substructure to the protein. Subsequently, the action mechanism might be as previously reported [15,19,44–46]. The SH group of Cys145 was deprotonated by His41, initiated a nucleophilic attack on benzylxycarbonyl carbon to form thioester (Fig. 8a), and control experiments proved that N-hydroxy-3,3-diphenylpropanamide had no inhibitory effect on Mpro, which also proved that the interaction site of the hydroxamate and protease is the benzylxycarbonyl instead of amide carbonyl.

For the complex Mpro/2b (Fig. 8b), the phenolic hydroxyl oxygen formed H-bond with Cys145 (2.6 Å) and Asn142 (3.0 Å), also two nitrogen atoms of thiourea interacted with Glu166 (2.3 Å, 2.6 Å) through H-bond, tightly anchoring the 2b complex in the active site of Mpro [47].

2.7. Cytotoxicity assay

The potential toxicity of compounds is a vital criterion to evaluate their clinical medical applications. The cytotoxicity of the hydroxamate 1a and thiosemicarbazone 2b (1–400 μM) were assayed by using mouse fibroblast (L929) cells [48,49]. As shown in Fig. 9, the cell viability was over 98% in the presence of 25 μM 1a, but only 80% of cells tested maintained viability in the presence of 2b at same concentration, indicating that the hydroxamate has low cytotoxicity, and the thiosemicarbazone has certain cytotoxicity on L929 cells.

Given that both hydroxamates and thiosemicarbazones were reported to have anticancer efficacy [50–53], we performed toxicity assay and fluorescence microscopy images of the human breast cancer cells (MCF-7) treated with these compounds (see supporting information) [54,55]. As shown in Fig. S1, more than 98% of cells maintained viability in the presence of 1a and 2b (25 μM). However, the cell viability was over 85% for 100 μM inhibitors (Fig.S2) and less than 30% for 800 μM inhibitors, indicating that 1a and 2b have low cytotoxicity at a low concentration.
3. Materials and methods

3.1. Enzymatic activity assay

The enzyme activity was evaluated according to previously described method [30]. The fluorescence substrate Mca–AVLQSGFR-K (Dnp)K was prepared into samples with different concentration (1–100 μM). The MPro (0.2 μM) was added to the assay buffer containing substrate, and then the fluorescence change of substrate was monitored on Microplate Reader (Var isoskan flash, emission, 405 nm / excitation, 320 nm) for 4 min. The initial reaction rate of enzyme hydrolyzing substrate was calculated by linear regression (Graphpad Prism 5) and the Michaelis-Menten equation was used to plot against the substrate concentration.

3.2. Enzymatic inhibition assay

To obtain the percent inhibition, the hydroxamates 1a-i and thiosemicarbazones 2a-n were first dissolved in DMSO and then diluted with assay buffer. The protease (0.2 μM) was mixed with inhibitors (50 μM) at 37 °C for 2 h. Finally, the substrate (20 μM) was added to the mixture solutions, and then the hydrolysis of fluorescence substrate was monitored on Microplate Reader for 1 min. The enzyme was treated with 0.5% DMSO as the negative control and ebselen was used as the positive control [31].

3.3. Inhibition mode assays

The inhibition mode of hydroxamate 1a on MPro was evaluated and the kinetic parameters were determined [37,38]. In detail, the substrate (20 μM) was added into assay buffer supplement with 1a at different concentrations (1–30 μM), respectively. When the enzyme sample was added and then the hydrolysis of fluorescence substrate was monitored on Microplate Reader for 5 min. The Kobs was obtained by fitting the enzyme inhibition progress curves to the equation (1).

\[ R_t - R_0 = \frac{V_0}{K_{obs}} (1 - e^{-k_{obs} t}) \]  
\[ (1) \]

Where \( R_t \) is the fluorescence value at time \( t \), \( R_0 \) is the initial fluorescence value at time 0, \( V_0 \) is the initial reaction rate, \( K_{obs} \) values was obtained and then fitted into Equation (2) to acquire \( k_{inact} \) and \( k_f \) values.

\[ K_{obs} = \frac{k_{inact}[I]}{K_f + [I]} \]  
\[ (2) \]

Where [I] is inhibitor concentration, \( k_{inact} \) is the rate constant of inactivation.

3.4. Thermal shift assay

Thermal shift assay was performed according to the previously described [33,40]. The dyes used in this experiment was SYPRO Orange (10 X final concentration), MPro (20 μM) was premixed with hydroxamate 1a and thiosemicarbazone 2b in buffer (60 mM, 200 mM NaCl, pH 7.5) for 2 h, respectively. When the SYPRO Orange was added in 96-well plate and then the fluorescence was monitored on an iCycler (Bio-Rad, emission, 570 nm / excitation, 300 nm) from 25 to 80 °C in steps of 0.8 °C. The protein melting temperature (Tm) was obtained by using the Boltzmann model (Protein Thermal Shift Software v1.3) to analyze the mid log of the transition state of protein from the nature to the denatured. The enzyme sample in wells was treated with 0.5% DMSO as blank controls, and both ligands only control and no protein control were used as the negative control to exclude the contamination in wells and ligand-dye interactions interference.

3.5. Cytotoxicity assay

The cytotoxicity of hydroxamate 1a and thiosemicarbazone 2b were tested with L929 cells. The cells were cultured into 96-well plates (1.0 × 10^3 cells/well) containing culture medium for 2 days. Subsequently, the cells were premixed with inhibitors (1–400 μM) for another 24 h, respectively. The cell supernatant was removed and added MTT solution (10 μL/well) for 4 h, and then added DMSO (100 μL/well) for 20 min. The OD values (optical density) were measured on Microplate Reader [48].

4. Conclusions

The main protease (MPro) that the SARS-CoV-2 viral replication employed was expressed and purified by Ni-NTA and HiTrap Q FF columns, and \( K_m \) and \( V_{max} \) were determined to be 5.4 ± 4.13 μM and 0.68 ± 0.08 nM/s, respectively. Twenty-three hydroxamates 1a-i and thiosemicarbazones 2a-n were identified by FRET screening to be the potent inhibitors of MPro, which exhibited more than 95% (except 1e) and more than 60% inhibition, and an IC50 value in the range of 0.12–31.51 and 2.43–34.22 μM, respectively, the hydroxamate 1a (IC50 = 0.12 μM) and thiosemicarbazone 2b (IC50 = 2.43 μM) were found to be the most effective inhibitors. The enzyme kinetics, jump dilution and thermal shift assays showed that 2b is a competitive inhibitor, while 1a is a time-dependent inhibitor; 2b reversibly but 1a irreversibly bound to the target; the binding of 2b increases but 1a decreases the stability of the
protein, and DTT assays indicate that 1a is the promiscuous cysteine protease inhibitor. Cytotoxicity assays showed that 1a has low cytotoxicity and 2b has certain cytotoxicity on the mouse fibroblast cells (1929). Docking studies revealed potential binding modes of the two most potent inhibitors to MPRO, in which the benzylxycarbonyl carbon of 1a might be formed a thiester bond with Cys145, while the phenolic hydroxyl oxygen of 2b formed H-bonds with Cys145 and Asn142.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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