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Heparin interacts with the main protease of SARS-CoV-2 and inhibits its activity

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HIGHLIGHTS
- Heparin is a sulfated polysaccharide used in clinic as an intravenous anticoagulant.
- Mpro of SARS-CoV-2 is a key enzyme for the viral replication and transcription.
- Heparin strongly binds with the main protease of SARS-CoV-2.
- Heparin effectively inhibits activity of SARS-CoV-2 main protease.
- Heparin might have a potential role in inhibiting SARS-CoV-2 infection.

ABSTRACT
The ability of SARS-CoV-2 to replicate in host cells is dependent on its main protease (Mpro, also called 3Cpro) that cut the viral precursor polyproteins and is a major target for antiviral drug design. Here, we showed that heparin interacts with the Mpro of SARS-CoV-2 and inhibits its activity. Protein fluorescence quenching showed that heparin strongly binds to the Mpro protein with dissociation constants KD of 16.66 and 31.60 μM at 25 and 35 °C, respectively. From thermodynamic parameters of the interaction, there are hydrophobic and hydrogen bond interactions between them. Fluorescence resonance energy transfer (FRET) assay demonstrated that heparin inhibits the proteolytic activity of Mpro with an inhibition constant Ki of 6.9 nM and a half maximal inhibitory concentrations (IC50) of 7.8 ± 2.6 nM. Furthermore, molecular docking analysis revealed that the recognition and binding groups of heparin within the active site of SARS-CoV-2 Mpro provide important new information for the characteristics of heparin.
1. Introduction

Coronavirus infects humans and other animals and causes a variety of highly prevalent and severe diseases, including severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) [1]. A novel coronavirus, called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the pathogen of the COVID-19 pandemic of 2019–2021 [2,3].

SARS-CoV-2 is an RNA virus from the Betacoronavirus genus [2,3]. The genome of this virus is about 88% homologous to the bat coronavirus, but only 79% to SARS-CoV and 50% to MERS-CoV [4]. SARS-CoV-2 has a typical coronavirus gene sequence, and the genome contains about 30,000 nucleotides. About two thirds of the genome is occupied by two open reading frames ORF1a and ORF1b, which encode the non-structural proteins, while the remainder of the region next to the 3′ end encodes structural proteins [4]. ORF1b is translated into two polyproteins PP1a and PP1ab by host ribosomes, which are processed by the virus’ main protease (Mpro, also called 3Clpro) and a papain-like protease (PLpro). PLpro cleaves the first three sites at the N-terminus and Mpro cuts at the remaining 11 sites at the C-terminus, which form 15 non-structural proteins (NSP) [3,4]. The Mpro of SARS-CoV-2 contains three domains (domains I to III) and has a 6-stranded β-barrel chymotrypsin-like fold with a homology to the monomeric picornavirus 3C protease, which forms a homodimer and whose active site contains a cysteine-histidine catalytic dyad [5,6]. The sequence recognized by Mpro contains Leu-Gln-(Ser/Ala/Gly) with a cleavage site occurring after the Gln residue [5,6]. Because Mpro of SARS-CoV-2 is necessary for the viral replication and transcription and no human homologues [3], it is one of the most characteristic and ideal antiviral targets in the virus [7,8].

Heparin is a sulfated polysaccharide widely used in clinic as an intravenous anti-coagulant [9,10], which is mainly composed of a tri-sulfated disaccharide repeating unit, 2-O-sulfato-N-sulfo-D-glucosamine (Fig. 1) [10-12]. In addition to tri-sulfated disaccharides, heparin also contains disaccharides with a lower degree of sulfation, resulting in a heterogeneous structure, named heparin sulfate (HS) (Fig. 1) [10,12]. HS forms the precursor polysaccharide which is transformed by a series of enzymes into the main repeating unit of heparin [10]. The structural distinction between heparin and HS is subtle; Over 70% of heparin consists of the disaccharide shown in the top panel of Fig. 1, but HS contains much less of the main repeating unit of heparin, which has a higher proportion of the many intermediate structures resulting from incomplete action of the postpolymerization enzymes [10,12]. Heparin together with HS forms a glycosaminoglycan family, which have a same biosynthesis pathway [11]. COVID-19 is usually accompanied by coagulation dysfunction and disseminated intravascular coagulation (DIC). For these reasons, heparin is used as a therapeutic drug for the treatment of COVID-19 [13]. Heparin can be directly targeted to the surface of the airway lumen by atomization, reducing both the infection of the surface of the airway lumen and the thrombosis in the air sac [14]. These data suggested that the current use of systemic unfractionated heparin (UFH) in the treatment of patients with COVID-19 in an ICU setting may provide useful antiviral benefits. In addition, various commercially and clinically available UFH have an antiviral effect against live wild-type SARS-CoV-2 through inhibiting the binding of Spike RBD to human ACE2 [15]. However, the interactions between heparin and Mpro of SARS-CoV-2 and the effects of heparin on the Mpro activity remain unknown.

COVID-19 pandemic is becoming one of the largest in the history of global public health crisis. There are currently no targeted therapies for the disease, and effective treatment options remain very limited. Finding an effective drug to prevent or treat infections is a top priority for health care providers, government officials and the pharmaceutical industries. As a viral protease Mpro of the SARS-CoV-2, is a most prominent target for antiviral drug screening [5,6,8]. In the present work, we showed that heparin binds with the Mpro of SARS-CoV-2 and inhibits the activity of the protease. Our findings suggested that heparin might have a potential role in inhibiting SARS-CoV-2 infections through inhibiting the viral replication.

2. Material and methods

2.1. Chemicals and reagents

Heparin was purchased from Sigma-Aldrich (Shanghai, China), and GC376 from BioChemPartner (Shanghai, China). The fluorescence substrate MCA-AVLQSGFR-Lys(DNP)K-NH2 was synthesized by Nanjing Peptide Bio-tech Ltd. (Nanjing, China). All other chemicals and reagents used were of HPLC or analytical grade.

2.2. Protein expression and purification

To express the Mpro of SARS-CoV-2, the gene encoding the protein (GenBank, accession number MN908947.3) was synthesized and cloned into pGEX6P-1 vector between BamHI and EcoRI sites to create a fused protein with glutathione S-transferase (GST) and a preScission protease cleavage site for the removal of the GST moiety. Escherichia coli strain BL21(DE3) transformed with the plasmid was cultured in LB medium containing 100 µg/ml ampicillin at 30 °C to an absorbance of 1.0 at 600 nm and then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18 °C for 20 h. The cells were harvested by centrifugation at 8000×g for 10 min, and washed with wash buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl) and then pelleted again for protein purification.

Fig. 1. Structures of the repeating disaccharide motifs in heparin and heparin sulphate (HS).
The harvested cells resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM β-mercaptoethanol, 1 mM EDTA, pH 8.0, 200 mg/l lysozyme, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 3 μg/ml leupeptin, 3 μg/ml pepstatin A) were lysed by sonication and the cell debris was discarded after centrifugation at 15,000×g for 30 min. The supernatant was filtered by a filter of 0.22 μm and loaded onto a Glutathione-Sepharose 4B affinity column equilibrated with cleavage buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, pH 8.0, and 5 mM β-mercaptoethanol). The resin bound with the fused protein was washed with cleavage buffer and digested using preScission protease at 4 °C for 16 h. The digested Mpro was eluted and concentrated using Amicon Ultra-4 (0.15 M NaCl, 1 mM EDTA, pH 8.0, and 5 mM β-mercaptoethanol). The resin bound with the fused protein was washed with cleavage buffer and digested using preScission protease at 4 °C for 16 h. The digested Mpro was concentrated to 10 mg/ml and stored in liquid nitrogen for use. The concentrations of the protein solution were determined by the BCA method using BSA as a standard [16].

2.3. Fluorescence quenching

To assess the interaction of heparin with Mpro, fluorescence quenching was carried out at 25 and 35 °C with an RF-5301 PC spectrofluorometer (SHIMADZU, Japan), as previously described with some modifications [17, 18]. In brief, heparin of 66.7 μM was titrated to a cuvette containing 2 ml of 1.5 μM of Mpro in 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT. Mixing and allowing the mixture to equilibrate for 5 min at the selected temperatures of 25 and 35 °C. The bulk of the added heparin solution was less than 5% of the total bulk. The fluorescence of the fluorescence emission were scanned from 300 to 500 nm with a slit width of 15 nm, excited by a wavelength of 290 nm with a slit width of 15 nm. The corresponding spectra of the buffers in the absence of the protein were subtracted from the corresponding spectra of the samples.

Fluorescence quenching data in the presence of the heparin were fitted to the Stern-Volmer equation and the static quenching equation. The Stern-Volmer equation [19] is

\[ F_0/F = 1 + K_q[Q] = 1 + K_{SV}[Q] \]  

(1)

and the static quenching equation is

\[ 1/(F_0 - F) = 1/F_0 + 1/(K_q[Q]) = 1/F_0 + K_0/(F_0[Q]) \]  

(2)

where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of heparin, respectively; \( K_q \) is the quenching rate constant of the biomolecule; \( \tau_0 \) is the average lifetime of the molecule without quencher; \( [Q] \) is the free concentration of heparin; \( K_{SV} \) is the dynamic quenching constant; \( K_A \) is the formation constant; and \( K_0 \) is the dissociation constant.

In order to determine the interaction characteristics between heparin and Mpro, such as hydrogen bond, van der Waals force, and electrostatic and hydrophobic interaction, the following equations were used:

\[ \ln K_A = -\Delta H/R + \Delta S/R \]  

(3)

\[ \ln (K_2/K_1) = (1/T_1 - 1/T_2) \Delta H/R \]  

(4)

\[ \Delta G = \Delta H - T\Delta S = -RT\ln K \]  

(5)

where, \( \Delta H \), \( \Delta G \), and \( \Delta S \) are enthalpy, free energy, and entropy change, respectively.

2.4. Mpro activity assay

Proteolytic activity of Mpro was determined by a fluorescence resonance energy transfer (FRET) assay using a peptide substrate MCA-AVLQ|SGFR-Lys(DNP)K-NH₂ in which MCA is a fluorescent donor and DNP is a fluorescent receptor or known as a quencher [5]. The two groups are closely enough to produce a FRET, and the fluorescence from MCA is very small and almost not detected, when the substrate not to be digested by the Mpro. When the substrate is digested by the protease, the separation of the two groups induces an increase in MCA fluorescence and the more substrate is digested, the stronger the MCA fluorescence is. The maximum excitation wavelength of MCA was 325 nm and the maximum emission wavelength was 393 nm.

The activity of Mpro was measured at 37 °C with a cuvette added a reaction mixture of 2 ml using an RF-5301 PC spectrofluorometer (SHIMADZU, Japan). The fluorescence spectra of the reaction mixture (final concentrations: 1.5 μM of Mpro, 20 μM of substrate) were recorded every 5 min in a reaction buffer (10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl, 1 mM EDTA and 1 mM DTT). The excitation wavelength was set at 325 nm with a slit width of 15 nm, and the fluorescence emission spectra were scanned from 300 to 500 nm with a slit width of 15 nm. The fluorescence spectra at the beginning of the reaction as a background were subtracted from these after reaction. The slope of the curve of fluorescence intensity at 393 nm with time quantitatively reflects the activity of the enzyme.

For assay of heparin against Mpro activity, a heparin solution (66.7 μM) was added into the Mpro solution in the reaction buffer, mixing and allowing the mixture to equilibrate for 5 min at 37 °C, and then initiated by adding the substrate solution (1 mM). The concentrations of Mpro and substrate were 1.5 μM and 20 μM, respectively, with various concentrations of heparin. As a positive control, the known Mpro inhibitor GC-376 was used. The slope ratio in the presence and absence of an inhibitor provides the percentage of inhibition against the enzyme.

2.5. Kinetic analysis of substrate

To determine the kinetic parameters of the substrate, a variety of concentrations of the substrate (0.2–80 μM) and a fixed concentration of Mpro (1.5 μM) were used. Different concentrations of the substrate were hydrolyzed at 37 °C for 30 min and then measured by an RF-5301 PC spectrofluorometer (SHIMADZU, Japan) at an excitation wavelength of 325 nm and emission of 393 nm, using a cuvette added 2 ml reaction mixture. The experiment was repeated three times. Kinetic parameters were determined using the Michaelis–Menten equation and GraphPad Prism software.

2.6. Molecular modeling

The crystal structure of the SARS-CoV-2 Mpro used was taken from the RCSB Protein Data Bank (http://www.rcsb.org) (PDB ID:7bro). A tetrasaccharide fragment derived from heparin (DP4) was docked to the Mpro of the SARS-CoV-2. After removing the water molecules in the crystal structure and adding the H atoms, the docking studies were performed using Autodock Vina software [20]. The exhaustiveness number is set to 20. By seeing the position of active site region, the center of the grid box was chosen to be at X: 10.643, Y: -13.867, Z: 20.125 with a suitable grid box volume where the ligand can easily be fitted and which covers the entire active site pocket. And its size has been set to 30×25×25 Angstroms to cover the active site of the protease. The nature of interactions of heparin with the protease was evaluated and visualized by Edu PyMOL software (version 2.4.2).
2.7. Statistical analysis

All statistics were performed using GraphPad Prism 8 software. Data were represented as mean ± SD. Comparison of the mean between groups was performed by t test. P values < 0.05 were considered significant.

3. Results

3.1. Heparin interacts with SARS-CoV-2 Mpro

The Mpro protein was expressed with a GST-tag and purified with Glutathi-one-Sepharose 4B affinity column and gel filtration column to high purity with a molecular weight about 35 kDa, which is consistent with 34.436 kDa of the theoretical molecular weight (Fig. 2A). To investigate whether there is an interaction between heparin and the Mpro of SARS-CoV-2, the fluorescence quenching of the Mpro protein was employed. As observed in Fig. 2B, C and D, the fluorescence intensity of Mpro was gradually decreased with an increase in the concentrations of heparin, demonstrating that the tryptophan fluorescence of the Mpro protein was quenched by heparin and an interaction between the heparin and Mpro occurred.

Fluorescence quenching includes dynamic and static modes, in which the static mode the fluorophore and quencher form a complex [17,19]. As shown in Fig. 2E, the Stern-Volmer curves were linear, and the slope decreased with increasing temperature. The Stern-Volmer quenching constant KSV is equal to Kq ear, and the slope decreased with increasing temperature. The static quenching plots for the fluorescence quenching of the Mpro protein was employed. As observed in Fig. 2B, C and D, the fluorescence intensity of Mpro was gradually decreased with an increase in the concentrations of heparin, demonstrating that the tryptophan fluorescence of the Mpro protein was quenched by heparin and an interaction between the heparin and Mpro occurred.

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There are mainly interacting forces of four kinds: hydrophobic interactions, hydrogen bonding, van der Waals forces, and electrostatic interactions, between biological macromolecules. The signs and magnitudes of the thermodynamic parameters (ΔH and ΔS) associated with various individual kinds of interaction that may occur during molecule binding have been characterized [23]: ΔH > 0 and ΔS > 0, hydrophobic interaction; ΔH < 0 and ΔS > 0, van der Waals and hydrogen-bonding interactions; ΔH < 0 and ΔS < 0, electrostatic forces. In order to determine the interaction characteristics of heparin with Mpro, the enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) change, were calculated according to the Eqs. (3), (4) and (5), as our previous described [17,18]. From the values of ΔH, ΔS and ΔG, the interactions between heparin and Mpro are mainly hydrogen bond and van der Waals force.
Table 1
Parameters of fluorescence quenching for the interactions of heparin with Mpro protein.

| Temp (°C) | NaCl (M) | Ksv (1 mol⁻¹) | Kq (1 mol⁻¹ s⁻¹) | Ke (μM) |
|----------|----------|---------------|------------------|--------|
| 25       | 0.15 M  | 9.38 x 10⁴    | 9.38 x 10⁻⁴      | 16.66  |
| 35       | 0.15 M  | 8.03 x 10⁴    | 8.03 x 10⁻²      | 31.60  |
| 25       | 1.5 M   | 14.74 x 10⁴   | 14.74 x 10⁻¹²    | 12.69  |

*The fluorescence quenching of Mpro was carried out at 25 and 35 °C in 10 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM DTT, in the presence of 0.15° or 1.5° M NaCl, at a concentration of 1.5 μM Mpro.

(Table 2), which is in accordance with the result from in the presence of higher salt concentration.

3.2. Heparin inhibits Mpro enzymatic activity

To determine the Mpro activity, a commercially available FRET based substrate was used [5]. To calculate Kcat, a standard curve was produced and the fluorescence intensities were converted into the amount of cleaved substrate according to the standard curve (Fig. 3A). And then Ksv and Vmax values were measured to characterize the enzyme activity of SARS-CoV-2 Mpro. The initial velocity of the enzyme was measured under a fixed concentration of Mpro (1.5 μM) and various concentrations of FRET substrate (0–80 μM), and the curve against substrate concentration was plotted. Fitting the curve with Michaelis Menten equation, the best-fit values of Ksv and Vmax were 70.75 ± 3.6 μM and 1.386 ± 0.9 FI/s (FI, fluorescence intensity), respectively (Fig. 3A), and the calculated kcat/Km was 13062.9 s⁻¹ M⁻¹, which is similar to the previously reported value by Yang et al [5].

On the basis of establishing the experimental conditions for detecting protease activity, the inhibitory effects of different concentrations of heparin on the protease activity were investigated. Heparin was pre-incubated with Mpro in the reaction buffer to ensure the full interaction between them before the addition of the FRET substrate. As shown in Fig. 3B, in the absence of the inhibitor, the fluorescence of the substrate was gradually increased with time, indicating an increase in the amount of cleaved substrate with time. However, in the presence of the inhibitor, we can clearly observe that the fluorescence of the substrate remarkably attenuated, suggesting that heparin has a prominent intervention effect on the activity of Mpro (Fig. 3C). When the concentration of heparin is high enough, the fluorescence of the substrate was almost completely suppressed (Fig. 3D). In the presence of a variety of concentrations of heparin, the fluorescence intensities at a wavelength of 393 nm excited by a wavelength of 325 nm proceeding over time up to 50 and 20 min were shown in Fig. 4A and B, respectively. When the substrate hydrolysis reaction exceeded 20 min, significant substrate consumption was observed. Therefore, the reaction progress curve in the first 20 min is selected for fitting (Fig. 4B). The slope ratio in the presence and absence of the inhibitor reflects the inhibition rate of the inhibitor to the enzyme. The curve of the enzyme activities under different heparin concentrations was plotted, and fitted to use the Michaelis Menten equation (Eq. 4C and D). From the Morrison equation the inhibition constant Ki and the half maximal inhibitory concentrations (IC50) for heparin were 6.9 nM and 7.8 ± 2.6 nM, respectively, indicating that heparin nobly inhibits Mpro activity. As a positive control, the inhibitor of Mpro GC-376 was used. As shown in Fig. 4E, when addition of GC-376 to Mpro solution containing the FRET substrate, the fluorescence intensities of the substrate were decreased, and the reduction levels depended on the concentrations of GC-376. The IC50 of GC-376 against Mpro activity was 0.32 ± 0.05 μM, as shown in Fig. 4F, demonstrating that GC-376 inhibits the activity of Mpro protein.

3.3. Molecular modeling reveals a binding site of heparin

To obtain the structural basis about the interactions of heparin with Mpro, molecular docking was employed using the Autodock Vina software [20]. The SARS-CoV-2 Mpro protein consists of three domains, in which domains I and II have an antiparallel β-barrel structure, and domain III contains five α helices arranged into a large antiparallel globular cluster [57]. Mpro has a Cys–His catalytic dyad, and the substrate-binding site is located in a cleft between domain I and domain II. Docking studies using DP4 demonstrated preferred interactions with the substrate-binding pocket (Fig. 5). Evaluation of heparin-protein contacts and energy contributions using the Autodock Vina and Edu PyMOL suggested strong hydrophobic interactions with the residues Thr24, Cys44, Thr45, Phe140, Leu141, and hydrogen bonds with Thr25, Thr26, His41, Ser46, Asn142, Gly143, Glu166, Cys189, Cys145, His164 (Fig. 5). By our calculation, the free binding energy of heparin with SARS-CoV-2 Mpro is 7.40 kcal/mol. Recently studies using protein crystallography showed that Mpro inhibitors such as bocprevir bind to Thr26, Ty54, His41, Asn142, Gly143, Cys145 and Glu166 in the substrate binding site of the protease through hydrogen bonds [25,26]. Glu166 residue is a key amino acid involved in the dimerization of Mpro and creation of substrate binding pocket. Cys145 and His41 residue forms a catalytic dyad on the active site of the protein essential for its catalytic function. Similar results were obtained from molecular docking with different molecules against the Mpro protein [27].

4. Discussion

Coagulation disorder is a major problem in late stage of COVID-19 disease [28]. Some reports showed that systematic heparin treatment can reduce mortality in hospitalized patients with COVID-19, which is considered to be a consequence of the known anticoagulant effect [29,30]. The Mpro of SARS-CoV-2, as a key enzyme of coronaviruses, plays an essential role in mediating viral replication and transcription, making it an attractive drug target for SARS-CoV-2 [5,6,8]. In this report, we found that heparin binds to the SARS-CoV-2 Mpro and inhibits its proteolytic activity in vitro. Our findings suggested that heparin might inhibit SARS-CoV-2 replication and transcription through inhibiting activity of the SARS-CoV-2 Mpro protein.

Heparin was discovered in 1916 and is still in widespread clinical use as an intravenous anticoagulant [9], which has an average of four negative charges for each disaccharide unit, can interact with a wide range of proteins, with interactions that exhibit a range of specificities [31] and induce several associated biological

Table 2
Thermodynamic parameters of the interaction between heparin and Mpro determined by fluorescence quenching.

| Temp (°C) | NaCl (M) | ΔH (kJ mol⁻¹) | ΔS (J K⁻¹ mol⁻¹) | ΔG (kJ mol⁻¹) | ΔG (kcal mol⁻¹) |
|----------|----------|---------------|------------------|---------------|-----------------|
| 0.15 M   | NaCl⁰   | -48.8         | -72.4            | -27.3         | -6.52           |
| 1.5 M    | NaCl⁰   | ND            | ND               | -27.9         | -6.67           |

* Determined in the presence of 0.15° and 1.5° M NaCl, respectively. ND, not determined.
activities. These involve plasma or tissue proteins such as heparin cofactor II (HCII), tissue factor plasminogen inhibitor (TFPI), lipoprotein lipase, growth factors and heparinase [32]. As a therapeutic agent, heparin inhibits thrombosis by accelerating the binding of the protease inhibitor, antithrombin III, to thrombin and to other proteases involved in coagulation. Because of its anticoagulant activity, heparin carries a risk of excessive bleeding complications [33].
Some studies have reported the protective effect of heparin on patients with COVID-19 based on its anticoagulant and anti-inflammatory activities [28-30]. Heparin interacts with chemokines and DAMPs released during infection, thereby inhibiting the pro-inflammatory activities of these proteins [34]. Clinical data from patients with COVID-19 with coagulopathy have attributed the beneficial effects of LMWH (low-molecular-weight heparin) to its anti-inflammatory effects [29,30]. The inhibitory effects of...
UFH and LMWH on antiviral activity against live SARS-CoV-2 have been studied using a plaque inhibition assay with Vero E6 cells, which showed that heparin interacts with the spike protein of SARS-CoV-2 to prevent viral binding to host cellular receptor angiotensin-converting enzyme 2 (ACE2) [15]. Moreover, study showed that the cellular heparin promotes the combination of SARS-CoV-2 spike protein with ACE2 through enhancing the open of the conformation of its receptor-binding domain (RBD) and the spike protein binding to ACE2 depends on both heparan sulfate and ACE2 [34]. In a recent clinical trial, the prophylactic anticoagulation is similar to therapeutic anti-coagulation in the patients with COVID-19 [35], suggesting that in the actual use of heparin for COVID-19 treatment, it may increase the bleeding of the treated patients is also a problem that needs to be considered in the future.

A number of compounds, including, boceprevir, an FDA-approved HCV drug; GC-376, an experimental veterinary drug; MG-132, calpain inhibitors II and XII, three calpain/cathepsin inhibitors, have been shown to bind to the MPro of SARS-CoV-2 and inhibit its activity with \( IC_{50} 4.13 \pm 0.61 \mu M, 30 \pm 8 \text{ nM, } 3.90 \pm 1.01 \mu M, 0.97 \pm 0.27 \mu M, \) and 0.45 \pm 0.06 \mu M, respectively, in which GC-376 exhibits a strongly suppressing activity against MPro of SARS-CoV-2 [25]. In our case, \( IC_{50} \) of heparin against MPro activity is 7.8 \pm 2.6 \text{ nM, suggesting that heparin also displays a strongly inhibitory effect on the MPro activity of SARS-CoV-2.} \) The GC-376 has a sulfite base group that involves in a hydrogen bond with Cys145 residue in the active site of MPro [25]. Recent study showed that sulfated polysaccharides such as fucoidan from brown algae and iota-carrageenan from red algae have an obviously antiviral activity against SARS-CoV-2 [36]. Heparin, as one of the sulfated polysaccharides, is higher sulfated in each disaccharide unit than HS, fucoidan, iota-carrageenan and chondroitin sulfate [28,36]. So, the existence of multiple sulphuric acid groups in heparin may be the reason for its binding to MPro and the better inhibitory effect against MPro activity.

In our previous works, we have developed the method of protein fluorescence quenching to investigate the interactions between proteins and other molecules [17]. In the present work, we have determined the dissociation constant (Kd) and interaction characteristics between heparin and MPro protein, using the method. Our results showed that the dissociation constant between heparin and MPro protein is 16.66 \mu M at 25 °C, indicating that heparin strongly binds to MPro protein [17,18]. Thermodynamic parameters calculated from the data of the fluorescence quenching reveals that heparin interacts with MPro protein through hydrogen bond and hydrophobic interactions, but not electrostatic interaction, although having negative charges in heparin due to sulfation. The free binding energy and interaction features obtained from molecular docking are consistent with the results from fluorescence quenching, which provides a structural basis for the interaction between heparin and MPro protein. Nevertheless, to crystallize the heparin-MPro complex and resolve its crystal structure are necessary to elucidate the mechanism that heparin inhibits MPro activity, and the work is developing in our lab.

Heparin belongs to the glycosaminoglycan family and consists of long repeating disaccharide units with variable sulfate groups [11]. The structure and size of the molecules are important to their biological properties. Recently, many different origins and kinds of heparin including UFH and LMWH from pig and cattle have been studied for antiviral activity against live SARS-CoV-2 [15]. All the kinds of UFH have potent antiviral activities against SARS-CoV-2, with \( IC_{50} \) values ranging between 25 and 41 \mu g/ml, whereas the \( IC_{50} \) values for the kinds of LMWH range from 3.4 to 7.8 mg/ml, less inhibitory effects by -- 150-fold, demonstrating that UFH has a significantly strong anti-SARS-CoV-2 activity compared to LMWH. Tree et al. [15] attributed this antiviral activity of UFH to that UFH directly inhibits the binding of spike protein to the human ACE2 protein receptor. In our present work, we showed that UFH binds to MPro protein of SARS-CoV-2 and inhibits its activity in vitro. The processing of the SARS-CoV-2 polyproteins depends on MPro to a great extent [5]. SARS-CoV-2 MPro has higher proteolytic efficiency and can accelerate the life cycle of the virus. Therefore, the anti-SARS-CoV-2 activity of UFH might be contributed by the inhibitory effects of UFH against SARS-CoV-2 activity.

CRediT authorship contribution statement

Jinwen Li: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft. Yantao Zhang: Methodology, Software, Formal analysis, Investigation. Huimin Pang: Conceptualization, Methodology, Investigation, Visualization, Supervision, Project administration, Funding acquisition. Shu Jie Li: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Acknowledgements

This work was partly supported by the Science and Technology Program of Jiangsu Province (LYL-SZ201915) and the Natural Science Foundation of Shandong Province (ZR2020MC056).

Author contributions

JWL, HMP and SJL conceived and designed the study. JWL, YTZ and HMP performed the experiments. JWL wrote the paper. SJL and HMP reviewed and edited the manuscript. All authors were involved in data analysis, read and approved the manuscript.

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