A computational evaluation of targeted oxidation strategy (TOS) for potential inhibition of SARS-CoV-2 by disulfiram and analogues

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ABSTRACT

In the new millennium, the outbreak of new coronavirus has happened three times: SARS-CoV, MERS-CoV, and SARS-CoV-2. Unfortunately, we still have no pharmaceutical weapons against the diseases caused by these viruses. The pandemic of SARS-CoV-2 reminds us the urgency to search new drugs with totally different mechanism that may target the weaknesses specific to coronaviruses. Herein, we disclose a computational evaluation of targeted oxidation strategy (TOS) for potential inhibition of SARS-CoV-2 by disulfiram, a 70-year-old anti-alcoholism drug, and predict a multiple-target mechanism. A preliminary list of promising TOS drug candidates targeting the two thiol proteases of SARS-CoV-2 are proposed upon virtual screening of 32,143 disulfides.

1. Introduction

Aggressive RNA viruses are typically characterized by rapid reproductive activities in cytoplasm. Similar to normal cells, proliferations of contagious RNA coronaviruses, including SARS-CoV, MERS-CoV and SARS-CoV-2, heavily rely on the functions of proteins containing crucial thiol/zinc(II)-thiolate sites, such as thiol protease and RNA-dependent RNA polymerase (RdRP). Thus, thiol/thiolate oxidation to disulfide (TOD) caused by appropriate oxidants may transform the proteins from primitive active form to targeted inactive form (Fig. 1A), providing a potential new strategy to block the life cycle of RNA viruses.

However, such strategy is generally disabled by an intracellular reductive environment common in aerobic organisms, which is regulated by glutathione (GSH) in mM concentrations and high ratios of GSH to GSSG (glutathione disulfide, an oxidized form of GSH in cytoplasm [1,2]) (Fig. 1B). Oxidant quenching (OQ) by GSH can lower the accessibility of thiol/zinc(II)-thiolate sites of cytosolic proteins to oxidants to a relatively non-deleterious level. Additionally, disulfide reduction to

thiol/thiolate (DRT) by GSH in local cytosolic microenvironments with high GSH:GSSG ratios (*ca.* 30–100 [3]) can restore the primitive active form of proteins even if aberrant TOD occurs occasionally. We postulated that such combination of OQ and DRT may likely be an essential reductive-protection mechanism evolved by aerobic organisms to reconcile the conflicting vital needs of both oxygen and oxidatively-unstable intracellular functional sites as a response to the transition from an O_2-lean atmosphere to an O_2-rich one on ancient Earth. Consequently, how to overcome OQ/DRT on demand represents a key challenge for potential therapeutic applications of TOD, such as killing malignant cells and inhibiting cytosolic proliferation of RNA viruses *in vivo.*

Recently, we reported that targeted oxidation strategy (TOS I, Fig. 1C) can allow oxidants to avoid OQ and lead to intracellular TOD, unraveling a new general chemical mechanism for the universal anti-cancer activities of disulfiram and its metabolite Cu(DTC)$_2$. The coordination of DTC tunes the oxidation ability of Cu(II) abnormally pre-accumulated in tumors just to the right level between the oxidation

potentials of thiol and zinc(II) thiolate. Thus, the classical redox reactivity between Cu(II) and thiol is blocked while the ability to oxidize proteins containing zinc(II)-thiolate site to the corresponding disulfide form is preserved by Cu(DTC)$_2$, resulting in targeted oxidative damage of intracellular zinc-finger domains and other zinc-thiolate active sites [2]. In relatively non-reductive intracellular local microenvironments, such as endoplasmic reticulum where low GSH:GSSG ratios close to 1 have been observed [3], the oxidized disulfide form may be sufficiently stable to undermine the primitive protein activity and kill cancerous cells.

Herein, we disclose a new targeted oxidation strategy (TOS II, Fig. 1D) based on special disulfide-type oxidants that may overcome both OD and DRT even in cytoplasm with high ratios of GSH:GSSG. The strategy essentially leverages specific intramolecular non-covalent interactions in proteins to stabilize the oxidized disulfide form and suppress DRT in GSH-rich microenvironments, potentially offering a new general pathway to inhibit cytosolic proliferation of RNA viruses. Quantum mechanical calculations suggest that it might be possible to develop such special disulfide-type oxidants selectively targeting thiol for a specific protein. Docking studies provide evidences to support a TOS II mechanism for the encouraging anti-SARS-CoV-2 activities recently observed for FDA-approved anti-alcoholism drug disulfiram [4], a special disulfide oxidant. A preliminary list of promising TOS II drug candidates targeting thiol proteases M^{pro} and PL^{pro} of SARS-CoV-2 are proposed.

2. Results and discussion

2.1. Chemical principles underlying TOS II and potential involvement of TOS II in inhibition of SARS-CoV-2

We first reasoned that there should be four distinctive types of disulfide oxidants with characteristic kinetic and thermodynamic behaviors in two competitive intracellular TOD/DRT reactions: 1) reduction of the disulfide oxidant by GSH to form a new small-molecule disulfide; and 2) reduction of the disulfide oxidant by protein thiol to form targeted protein disulfide (Fig. 2). Type-I disulfide oxidants correspond to the situation where reaction 1 is both kinetically and thermodynamically more favorable than reaction 2, while type-II disulfide oxidants show completely reverse kinetic and thermodynamic behaviors. For type-III disulfide oxidants, although reaction 1 is kinetically more favorable than reaction 2, the opposite is true thermodynamically. In contrast to type III, *vice versa* is true for type IV. In principle, types II and III should be more suitable for TOS II than types I and IV in cytosolic microenvironments with high ratios of GSH:GSSG. Furthermore, type III may be less desirable than type II due to kinetics.

Introduction of potential sites for non-covalent interactions at the targeted protein disulfide appears to be a natural approach to develop types II and III disulfide oxidants for TOS II. The encouraging cell-level anti-SARS-CoV-2 activity of disulfiram suggest the potential of using TOS II in inhibition of coronaviruses, where disulfide with hydrogen-bonding acceptors (N or S) two bonds away is a core structural unit. Such notion is supported by the observed inhibition of proteolytic activity of thiol protease M^{pro} of SARS-CoV-2 *in vitro* by disulfiram [4]. It should be noted that large excess amounts of a small cysteinyl fluogenic peptide with the sequence of MCA- AVLQSGFR-Lys(Dnp)-Lys-NH$_2$ was used as the substrate in the experiments, clearly showcasing the remarkable abilities of both disulfides to discriminate between M^{pro} thiols and the substrate thiol. Earlier *in vitro* experimental observations and docking analysis of the inhibition effect of disulfiram onto proteolytic activities of thiol proteases of the caspase family [5] and coronoviruses including MERS-CoV and SARS-CoV [6] further suggest that such inhibition might be relevant to oxidation of the catalytically active thiol of M^{pro} to a disulfide.
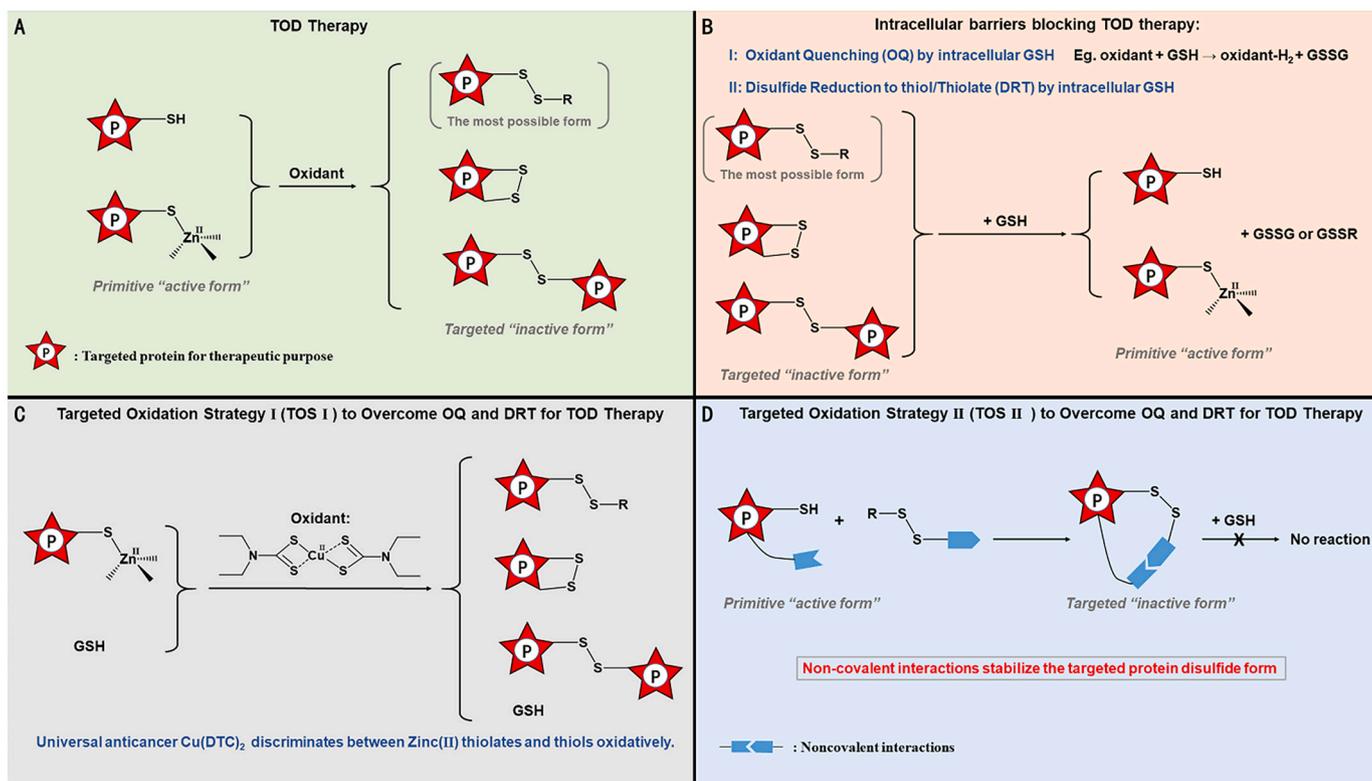
Fig. 1. Schematics illustrating the principle for target oxidation strategy (TOS): A, Thiol/thiolate oxidation to disulfide (TOD) caused by appropriate oxidants to transform proteins from primitive active form to targeted inactive form; B, Oxidant quenching (OD) and disulfide reduction to thiol/thiolate (DRT) blocking intracellular TOD; C, TOS I to overcome OD and DRT for TOD therapy; D, TOS II to overcome OD and DRT for TOD therapy.
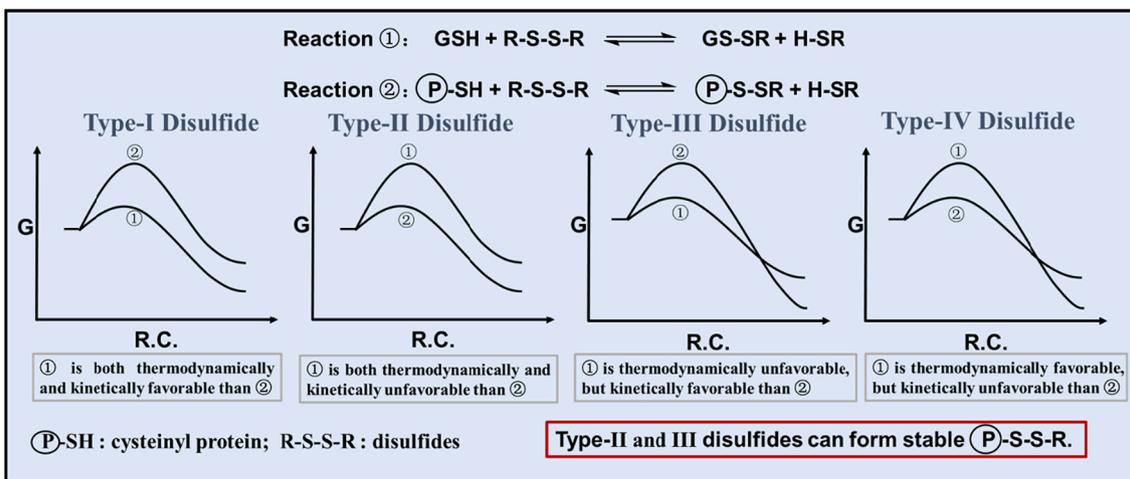
Fig. 2. Schematics illustrating the principle for special disulfide-type oxidants: Four distinctive types of disulfide oxidants.
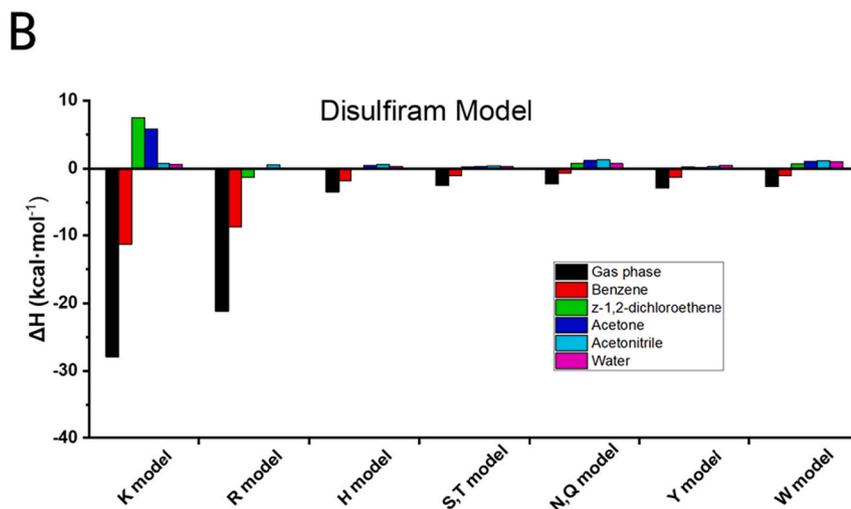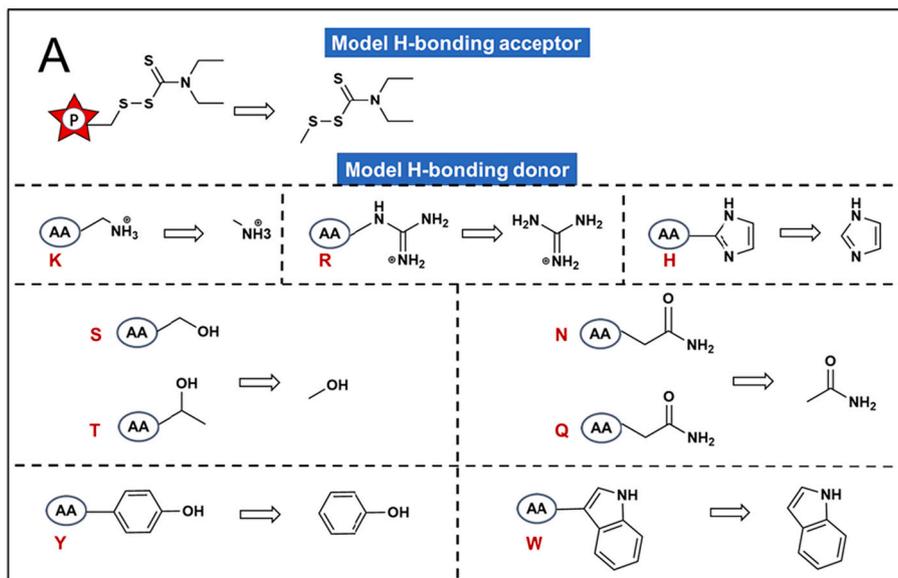
Fig. 3. A, Adapted model molecules for potential interactions arising from hydrogen-accepting disulfides covalently bonded to proteins and hydrogen-donating side chains of natural amino acids in proteins; B, Calculated hydrogen-bonding enthalpies with BSSE correction in various medium for model molecule of disulfiram.

2.2. Equations quantum-mechanical studies of relevant non-covalent interactions

Using disulfiram as an example, we next performed quantum-mechanical calculations (Gaussian 09, Revision E.01) at the level of B3LYP/6-311+G(d,p) to gain fundamental insights into potential non-covalent interactions that may be involved in the formation of stable protein disulfides in general. Simplified model systems featured with relevant core functional groups were adapted to probe the interactions between the corresponding disulfide moiety covalently bonded to the proteins and various possible hydrogen-bonding donors in proteins, including the side chains of lysine, arginine, histidine, serine, threonine, asparagine, glutamine, tyrosine, and tryptophan (Fig. 3A). All optimized structures were confirmed to be real minima by frequency calculation. Notably, geometry optimization results indicate that thiocarbonyl S is preferred over the N as the hydrogen-bonding site in disulfiram. Solvent effects were further calculated by SMD method to estimate the impacts of other non-covalent interactions including both electrostatic and non-electrostatic items. Gas phase ($\varepsilon = 1$), benzene ($\varepsilon = 2.3$), Z-1, 2-dichloroethene ($\varepsilon = 9.2$), acetone ($\varepsilon = 20.5$), acetonitrile ($\varepsilon = 35.7$) and water ($\varepsilon = 78.4$) were chosen to mimic various protein local microenvironments ranging from low polarity to high polarity. In principle, the dielectric environment in proteins should be closer to the nonpolar solvents than the polar ones. But the polar solvents were still studied here to interrogate the impacts of other non-covalent interactions implicitly. The calculated hydrogen-bonding enthalpies corrected by basis set superposition error (BSSE) were summarized in Fig. 3B.

The calculated results indicate that disulfiram should be candidate type II/III disulfides. In general, hydrogen-bonding enthalpies decrease significantly as the polarity of the surrounding medium increases. Notably, the impact of solvation onto the magnitude of hydrogen bonding enthalpy can be even larger than the impact of different types of hydrogen donors. A non-polar environment is needed for the interaction between the disulfiram model molecule and the hydrogen donors to be energetically favorable. Thus, it's reasonable to anticipate that appropriate non-covalent interactions can endow protein disulfides with the ability to resist DRT even in cytosolic microenvironments with high ratios of GSH:GSSG (*ca.* 30–100).

Additionally, the relative magnitude of the hydrogen-bonding enthalpies for the hydrogen-bonding donor follows the order of ammonium > guanidinium >> imidazole ~ phenol > indole ~ alcohol ~ amide in nonpolar media. In sharp contrast, the difference in hydrogen-bonding enthalpies becomes much smaller in polar media. The large impact of hydrogen-bonding side chains of natural amino acids and polarity of the medium onto the hydrogen-bonding strength strongly suggests that it should be possible to develop type II/III disulfides selectively targeting thiol for a specific protein.

2.3. Potential TOS II targets of SARS-CoV-2 and docking studies of disulfides potentially targeting M^pro and PL^pro of SARS-CoV-2 via TOS II

Although experimental data have shown the oxidation of the catalytically active thiol of M^pro to disulfide as a potential mechanism for inhibition of SARS-CoV-2, it's likely that oxidation of other thiol/zinc-thiolate sites by disulfiram might also play important roles in the inhibitions. As listed in Table 1, there are a few important proteins of SARS-CoV-2 containing targets for disulfides, including two thiol proteases M^pro and PL^pro as well as several proteins clearly involved in viral replication, such as RdRP, helicase, nsp10 and 3′ to 5′ exonuclease. Notably, there are 12 cysteine residues in M^pro (Fig. 4A). Spike glycoprotein should also be a potential target since the abundant extracellular disulfides should be in corresponding thiol forms intracellularly. Considering the structural simplicity of disulfiram, the catalytic thiol of M^pro might just be one of the sites susceptible to TOD. But it should be emphasized that the disulfide bond in disulfiram can be reduced in the digestive processes and inner circulation, disabling its TOS II ability.

Table 1

Cysteine(s) in zinc fingers, active sites or disulfide bonds in proteins of SARS-CoV-2 that can be potentially oxidized by disulfides.

Accession	Protein name	Protein length	# of Cys	Cys in Zinc fingers (ZF), active sites, & disulfide bonds
YP_009724390.1	spike	1273	40	disulfide bonds (C15-C136, C131-C166, C291-C301, C336-C361, C379-C432, C391-C525, C480-C488, C538-C590, C617–649, C662-C671, C738-C760, C743-C749, C840-C851, C1032-C1043, C1082-C1126)
YP_009725299.1	nsp3	1945	51	Zinc finger (C934, C937, C969, C971); active site in the PL^pro domain (C856)
YP_009725301.1	M^pro	306	12	active site (C145)
YP_009725306.1	nsp10	139	13	Zinc fingers (C74, C77, H83, C90; C117, C120, C128, C130)
YP_009725307.1	RdRP	932	29	Zinc fingers (H295, C301, C306, C310; C487, H642, C645, C646)
YP_009725308.1	helicase	601	26	Zinc fingers (C5, C8, C26, C29;C16, C19, H33, H39; C50, C55, C72, H75)
YP_009725309.1	3′ to 5′ exonuclease	527	23	Zinc fingers (C207, C210, C226, H229;H257, C261, H264, C279; C452, C477, C484, H487)
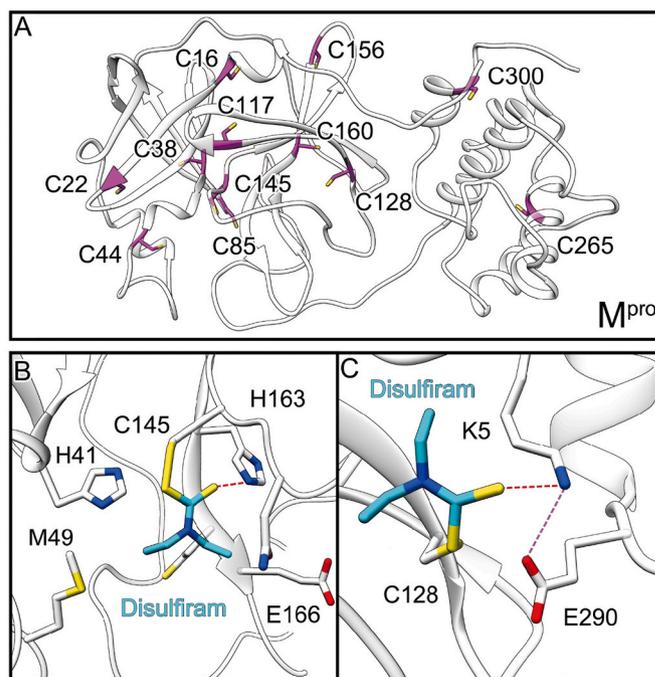
Fig. 4. A, there are 12 cysteines in M^pro. B, covalent docking poses of disulfiram in the active site of M^pro. C, covalent docking poses of disulfiram at Cys128 of M^pro.

Alternatively, the zinc-thiolate sites may be selectively oxidized *via* TOS I after coordination the reduced product of disulfiram (DTC) with Cu(II) *in vivo*, potentially resulting in inhibition of the virus proliferation. Experimental evaluation of such possibility is recommended at least at cell level.

To further investigate the mechanism of experimentally identified anti-SARS-CoV-2 drug disulfiram *via* inhibition of M[pro], we performed covalent docking of disulfiram to M[pro] (PDB ID: 6LU7 [4]). First we focused on Cys145 – the catalytic cysteine of this thiol protease, and later we explored the possibility of other cysteines. In order to perform an ensemble docking, 200 ns molecular dynamics simulation was performed on the crystal structure of M[pro] (apo form) through Amber18, using ff14SB force field. Four M[pro] conformations were obtained from trajectory clustering (100–200 ns), based on conformations of residues in the ligand binding pocket. The four protein conformations and the two ligands were then prepared by Protein Preparation Wizard (Schrödinger, LLC) and LigPrep (Schrödinger, LLC), respectively. Covalent docking was performed by Glide (Schrödinger, LLC), disulfide bond is requested to form between ligand and the cysteine in active site of M[pro] (Cys145). Representative docking poses were shown in Fig. 4B. Beyond the covalent disulfide bond, disulfiram adduct may also form a hydrogen bond with His163 in the binding pocket of M[pro]. Next we explored the possibility of other cysteines in M[pro] that can also be oxidized by disulfide drugs and may lead a negative allosteric modulation to the protease function of M[pro]. Indeed there are a few sites that can accommodate the drugs quite well. A representative pose of disulfiram binding to Cys128 was shown in Fig. 4C. A strong interaction with Lys5 is observed, and such interaction is further stabilized by Glu290, directly or indirectly.

To quantitatively compare these sites, the non-covalent-interacting disulfide core was taken out of the docking structures (Supplementary Fig. 1) for quantum-mechanical calculations at the level of B3LYP/6-311+G(d,p). It should be noted that these calculations might underestimate the strength of the non-covalent interactions because the surrounding amino acid residues might be actually flexible to adjust to better positions to interact with the disulfides than the docking structures. The calculated results show that the energetic benefits gained from the non-covalent interactions follow the order of C145/disulfiram < C128/disulfiram (Supplementary Table 1). Again, non-polar media provide much stronger driving forces for the non-covalent interactions than polar media. These results further support our hypothesis that non-covalent interactions contribute to the binding of disulfiram and the catalytically active C145 of M[pro] should not be the sole targeting site for TOS II. A mixture of both competitive and non-competitive inhibitions is

expected for the kinetics of *in vitro* inhibitions of the proteolytic activities of M[pro] of SARS-CoV-2 by disulfiram. Further experimental verifications are suggested.

In order to identify more disulfide drug candidates, we performed large scale ensemble docking for both thiol proteases M[pro] and PL[pro]. Structure model of PL[pro] was obtained from the C-I-TASSER website [7,8]. Four conformations for each thiol protease obtained from MD sampling were used for docking. We built a large disulfide ligand library from filtering Enamine REAL set [9], one of the largest enumerated databases of synthetically feasible molecules which comply with "rule of 5" and Veber criteria. The focused ligand library has 32,143 disulfides, and they were prepared by LigPrep for docking use.

Non-covalent docking was performed as the first step to pick ligands which could form good noncovalent interactions with the active site before covalent binding. This step aims to obtain ligands that are more likely to access the binding pocket, reducing the false positive rate of covalent docking due to high energy barrier for disulfide ligand to access the binding pocket. Then top 10% ranked ligands were used for further covalent docking. Our results showing that top ranked ligands tend to form polar interactions with His41 and Gln189 in M[pro] (Fig. 5A), as well as His1017 and Trp851 in PL[pro] (Fig. 5B). Finally, we proposed a list of molecules which may act as inhibitor of M[pro] or PL[pro] based on TOS II through virtual screening (Supplementary Table 2 and Supplementary Table 3). Notably, many of the predicted top-ranked potential inhibitors for M[pro] contain amide or thiourea, while many of those for PL[pro] have aromatic ring (Supplementary Fig. S2). The binding pocket of our MD sampling structures are similar to the pockets in the later reported crystal structures with the catalytic C145 site of M[pro] modified covalently by various molecules (Supplementary Fig. S3), showing that such covalent modification doesn't alter the pocket significantly.

3. Conclusions

In summary, we have described the fundamental principles to overcome the intracellular reductive-protection mechanism for potential therapeutic purposes *via* TOS II. The encouraging cell-level anti-SARS-CoV-2 activity of disulfiram suggests the potential of using TOS II in inhibition of coronaviruses. In particular, disulfiram is a simple and cheap drug that has been approved by FDA for anti-alcoholism purpose
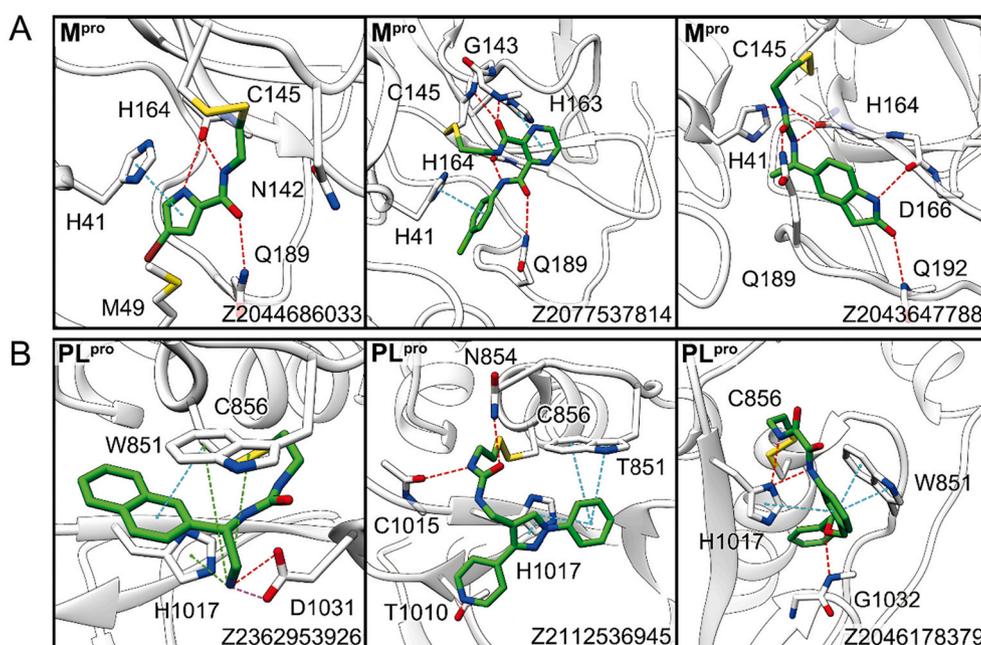
Fig. 5. Selected top ranked hits for M[pro] (A) and PL[pro](B) from a two-step virtual screening: first non-covalent docking and then covalent docking.

for *ca.* 70 years. As a typical oxidant, disulfiram may be reduced under intracellular reductive environment. A later study showed that some small-molecule inhibitors including disulfiram lost their inhibitory effect onto some proteins such as SARS-CoV-2 M^{pro} in the presence of 1,4-dithiothreitol (DTT) or GSH. [12] However, a recent statistical analysis suggests that patients with alcohol use disorder (AUD) under disulfiram treatment have a reduced COVID-19 infection rate and less symptoms compatible with COVID-19 than those without the treatment. [13] Clearly, the effect and the acting mechanism of disulfiram require further studies. Given the urgent needs of therapeutic methods to treat SARS-CoV-2 in the ongoing international outbreak, it's of great significance to further explore its potential anti-SARS-CoV-2 activity at clinical levels. However, further animal evaluations and clinical trials to develop appropriate methods to prevent the gradual degradation of disulfiram in digestive processes/inner circulation and allow its transport to infected tissues is recommended if its potential anti-SARS-CoV-2 activity *via* TOS II would be leveraged to a large extent.

In addition to potential interference of cytosolic proliferations of coronaviruses, TOS II might also be envisioned to trigger other significant physiological consequences such as inhibiting protein ubiquitination *via* oxidation of the active cysteinyl thiols of ubiquitin-activating enzyme E1 and ubiquitin-conjugating enzyme E2 to form stable disulfides that can resist the reductive environments in cells. Considering the ubiquity of thiol/zinc-thiolate sites in proteins and their important biological roles, we hope that more research attention can be devoted to the development of TOS II drugs for various therapeutic applications.

Author contributions

LX performed the quantum-mechanical calculation. JT performed the docking analysis. YW performed the searching of disulfide, thiol and zinc thiolate sites in proteins of SARS-CoV-2. SZ directed the analysis of protein thiol/zinc thiolate and the docking study. BLL directed the quantum-mechanical study. BLL conceptualized TOS and proposed TOS as a potential anti-SARS-CoV-2 mechanism. SZ proposed thiol proteases of coronaviruses as potential TOS targets. BLL and SZ co-wrote the manuscript. LX and JT also contributed to the preparation of the manuscript. BLL thank Prof. Lei Liu from Department of Chemistry of Tsinghua University for helpful discussions regarding the potential roles of p97 and ROS.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

target mechanism proposed in the original manuscript [11]. A phase-II clinical trial is also currently ongoing to evaluate disulfiram as a drug candidate for COVID-19 treatment (clinicaltrials.gov; identifier: NCT04485130). Finally, a recent statistical analysis suggests that patients with alcohol use disorder (AUD) under disulfiram treatment have a reduced COVID-19 infection rate and less symptoms compatible with COVID-19 than those without the treatment [13]. We wanted to note that disulfiram and PX-12 were both studied in the original ChemRxiv manuscript. In the current manuscript, the PX-12 part is deleted while the disulfiram part remains virtually the same as the original manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bpc.2021.106610.

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