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From quantum-derived principles underlying cysteine reactivity to combating the COVID-19 pandemic

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Abstract

The COVID-19 pandemic poses a challenge in coming up with quick and effective means to counter its cause, the SARS-CoV-2. Here, we show how the key factors governing cysteine reactivity in proteins derived from combined quantum mechanical/continuum calculations led to a novel multi-targeting strategy against SARS-CoV-2, in contrast to developing potent drugs/vaccines against a single viral target such as the spike protein. Specifically, they led to the discovery of reactive cysteines in evolutionary conserved Zn^{2+}-sites in several SARS-CoV-2 proteins that are crucial for viral polypeptide proteolysis as well as viral RNA synthesis, proofreading, and modification. These conserved, reactive cysteines, both free and Zn^{2+}-bound, can be targeted using the same Zn-ejector drug (disulfiram/ebselen), which enables the use of broad-spectrum anti-virals that would otherwise be removed by the virus's proofreading mechanism. Our strategy of targeting multiple, conserved viral proteins that operate at different stages of the virus life cycle using a Zn-ejector drug combined with other broad-spectrum anti-viral drug(s) could enhance the barrier to drug resistance and antiviral effects, as compared to each drug alone. Since these functionally important nonstructural proteins containing reactive cysteines are highly conserved among coronaviruses, our proposed strategy has the potential to tackle future coronaviruses.

This article is categorized under:

Structure and Mechanism > Reaction Mechanisms and Catalysis
Structure and Mechanism > Computational Biochemistry and Biophysics
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KEYWORDS

COVID-19, cysteine reactivity, labile Zn-sites, SARS-CoV-2, structural Zn

1 | INTRODUCTION

The cell in all the complexity of its constituents and physiological processes can be thought of as a microcosm populated by various biological entities including organelles, proteins, lipids, and nucleic acids where the information flows as chemical signals.[1] Experimental methods generate a plethora of quantitative data on DNA/RNA/protein sequences,

Ting Chen and Karen Sargsyan contributed equally to this study.

structures, and dynamics, revealing intricate patterns of the genome and protein universe.[2] The vast and sheer complexity of such data is quite overwhelming. Computational approaches can complement experimental data on complex biological processes by revealing their key underlying physical principles and elucidating recognition/reaction mechanisms. For example, experimental methods are limited in describing short-lived transition states/intermediates in enzyme-catalyzed reactions, whereas QM/MM methods can reveal how enzymes stabilize transition states/intermediates and help inhibitor design as well as test hypotheses/mechanisms and identify new reaction pathways.[3–7] As another example, computational approaches have elucidated the physical origins of noncovalent aromatic (π) interactions,[8] and the relationship between the sequences, structural properties, and functions of intrinsically disordered proteins.[9]

At the atomic level, covalent, ionic, and metallic bonds as well as hydrogen-bonding and van der Waals interactions in various dielectric environments form the "physicochemical language" of the cell.[1] Dynamical/conformational changes of large systems over long-time scales can be probed using Markov state models,[10,11] coarse-grained,[12,13] or ultra-coarse-grained[14] models, whereas those for smaller systems in the microsecond time scale are accessible by molecular dynamics (MD) simulations.[15,16] However, changes in the electron distribution during a chemical reaction, charge transfer, and/or polarization effects are best described by quantum theory.[17–19] Among the various quantum mechanical methods, density functional theory (DFT) has become the method of choice because of its good trade-off between accuracy and computational cost.[20,21] DFT combined with molecular mechanics[3] or continuum methods[22] have proven to be successful in revealing biophysical trends behind the secondary structure formation in proteins,[23] and predicting the ionization free energy (pK_a) of metal-bound water molecules[24] as well as amino acid (aa) residues and substrate/drug-like molecules in proteins.[25–27] They have helped to elucidate enzyme mechanisms,[4,6,28,29] and the physicochemical principles underlying metal-binding affinity/selectivity in metalloproteins,[30,31] including cation selectivity of ion channel selectivity filters.[32,33] They have also been used to investigate the effects of macromolecular crowding on protein aggregation and stability.[34]

In this focus article, we describe how studies of the physicochemical factors modulating cysteine (Cys) reactivity in proteins by combined DFT and continuum dielectric approach led to a novel strategy to battle the ongoing COVID-19 pandemic caused by SARS-CoV-2. We first present an overview of Cys: (i) its importance, (ii) its various states, (iii) its biological roles, and (iv) its applications in research and biotechnology. Next, we delineate the key factors governing the reactivity of free and metal-bound Cys in proteins. We then present a multi-targeting strategy exploiting the key factors controlling the reactivity of Zn^{2+}-bound Cys in combination with evolutionary principles to identify novel drug targets in the SARS-CoV-2. Finally, we discuss how this strategy may be beneficial for tackling new coronaviruses that cause future epidemics/pandemics.

1.1 | Importance of Cys

Despite being the second least abundant aa residue (only ∼1.9% of all aa residues in proteins),[35] Cys is very versatile with a myriad of functions. Cysteine reactivity has long been recognized as a key factor in the activity of many proteins.[36] Roughly 80% of Cys in proteins possess some functional importance.[1] Furthermore, Cys point mutations in proteins are associated with genetic diseases more often than expected, based on its occurrence frequency in protein sequences.[37,38] Conversely, point mutations to Cys in certain proteins can also lead to disease, as exemplified by the cancer-causing mutation of the native Gly-12 to Cys in the K-Ras enzyme that catalyzes the hydrolysis of guanosine triphosphate.[39]

1.2 | The diverse nature of cysteine: Free, bound, and derivatized

Cysteine is the most versatile aa building block in proteins, as it can exist as free, bound or derivatized in vivo.[40,41] Being a "soft" ligand, Cys prefers binding to "borderline" (e.g., Fe^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}) or "soft" (e.g., Cu^{+}, Cd^{2+}, Hg^{2+}) metal ions.[42,43] It can bind monodentately to a single cation or bridge two cations, as found in metallothioneins or FeS proteins.[44] The most common biogenic metal ion ligated by the Cys side chain is Zn^{2+},[45] the second most abundant transition metal ion in organisms.[46] Binding to Zn^{2+} lowers the Cys pK_a,[47] so Zn^{2+}-bound Cys is generally deprotonated under physiological conditions.[48] Because S has an electron configuration of $[Ne]3s^2 3p^4$ and *d*-orbitals for bonding, Cys can be readily oxidized to form derivatives with oxidation states ranging from −2 to +5 in vivo (Figure 1).[42] As evident

from Figure 1, the Cys S atom has a different oxidation state/number, depending on its surrounding atoms, unlike its charge, which only depends on the number of electrons and protons in the S atom.

1.3 | Biological functions of cysteine

The Cys thiol shows a vast range of measured pK_a values in proteins spanning from ~3 to 13,[49,50] reflecting its various biological functions, which fall into three main roles: (i) catalytic, (ii) regulatory, and (iii) structure-stabilizing (Figure 2).[35] In enzymatic catalysis, Cys plays an essential catalytic role in nucleophilic substitution reactions catalyzed by diverse enzymes including Cys proteases and thioredoxin reductase.[43] Cysteine also participates in electron transfer catalyzed by glutathione reductase as well as in oxygen-transfer, hydride-transfer, or thiol/thiyl hydrogen radical transfer catalyzed by enzymes such as human peroxiredoxins, glyceraldehyde 3-phosphate dehydrogenase, and ribonucleotide reductase, respectively.[43] In most of these reactions, the Cys thiolate serves as a catalytic nucleophile and can stabilize charges in the rate-limiting transition state of a reaction.[51,52] Zn^{2+}-bound Cys can facilitate alkyl transfer reactions in enzymes (e.g., the DNA repair protein ADA,[53] whose Zn^{2+}-bound Cys removes methyl groups from the DNA backbone) or promote conversion of ribonucleotides to deoxyribonucleotides catalyzed by ribonucleotide reductase.[54]

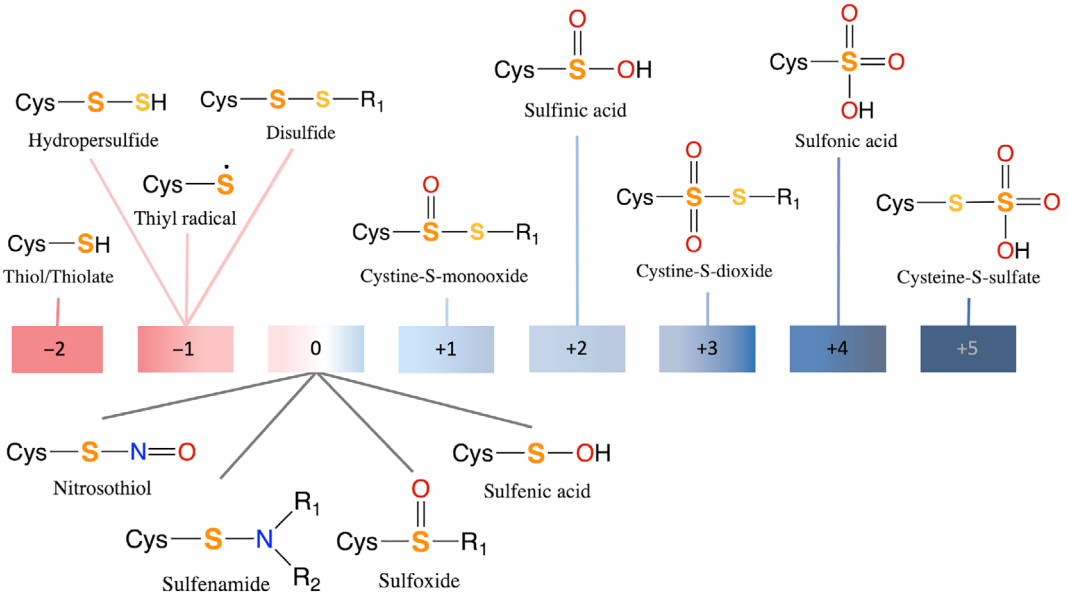


FIGURE 1 The various Cys(S) oxidation states found in vivo. The S corresponding to the indicated oxidation state is in bold (the oxidation state of the second S, if present, is −1)

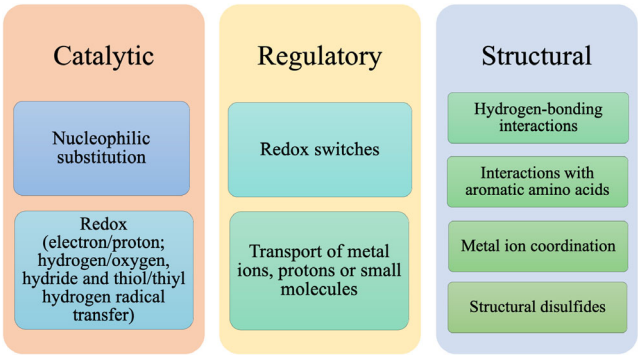


FIGURE 2 Catalytic, regulatory, or structural role of free, metal-bound, or modified Cys

Apart from serving a *direct* catalytic role, Cys also plays a regulatory role in enzyme catalysis by changing its S oxidation state (Figure 1). For example, Cys oxidation, resulting in disulfide bond formation, can modulate protein structure and function,[41] and serve as regulatory functional switches.[55] It deactivates redox-sensitive enzymes including caspases-3/9, glyceraldehyde 3-phosphate dehydrogenase, or protein tyrosine phosphatase that employ a catalytic Cys.[40] Zn^{2+}-bound Cys oxidation *inhibits* alcohol dehydrogenase by releasing the catalytic Zn^{2+}, but *activates* matrix metalloproteases by freeing a coordination site for a nucleophilic water molecule/substrate to bind.[40]

Cysteines, as part of nonprotein redox pairs such as glutathione disulfide/glutathione, regulate the reduction potential of the cell.[50,56,57] Post-translationally modified Cys can regulate cellular signaling pathways: Cys palmitoylation regulates protein localization and trafficking.[58] Cysteine lipoxidation (modification by lipid-derived electrophiles) triggers signaling pathways leading to stress adaptation.[59] Methylation of Zn^{2+}-bound Cys in the zinc finger domains of human TAB2 and TAB3 during bacterial infection disrupts the NF-κB pathway, which mediates innate immune defense against microbial infection.[60] Another regulatory role of Cys is in ligand or cation transport: Cysteine thiols present on the cell surface mediate cell entry of molecules or proteins via thiol/disulfide exchange reactions followed by membrane fusion.[61–63] In metallothioneins, Cys thiolates regulate intracellular Zn^{2+} homeostasis, as their oxidation triggers release and transfer of Zn^{2+} ions to other proteins.[64,65]

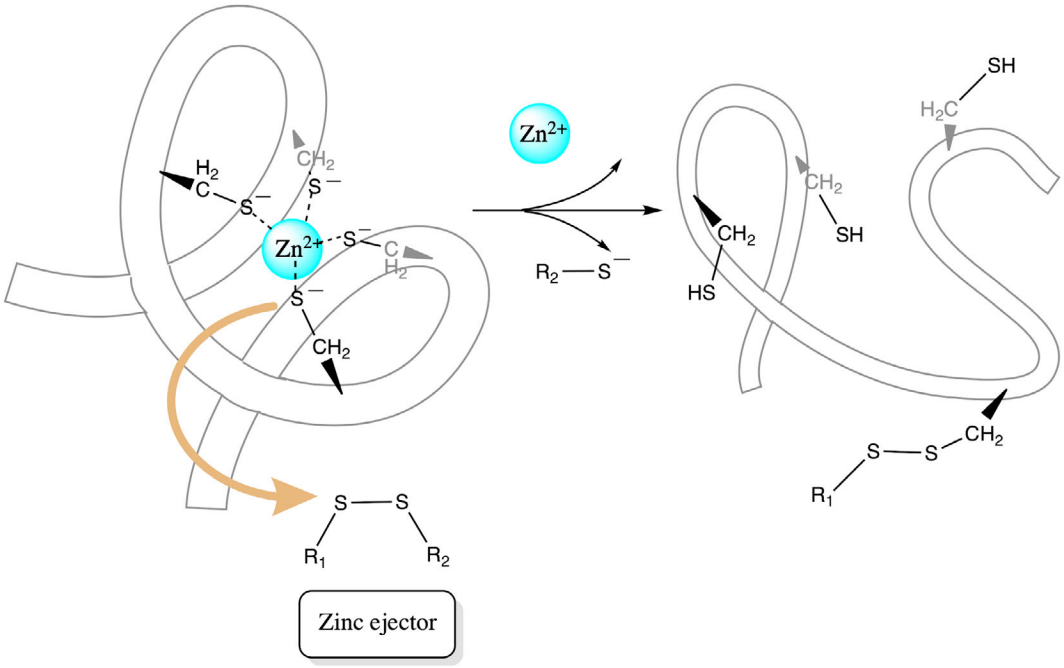
Cysteines are often employed to stabilize protein structure by forming disulfide bonds.[66–69] Notably, cysteine-rich peptide toxins from animal venom secretions possess multiple disulfide bonds, resulting in exceptional thermal and chemical stability.[70] Thermophilic prokaryotes also employ structural disulfides in their proteins to protect them from thermal denaturation in high-temperature environments.[71,72] Apart from forming covalent disulfide bonds, cysteines can form bonds with metal cations and stabilize specific folds in proteins such as Zn•Cys_4, Zn•Cys_3His, Zn•Cys_2His_2, and Zn_2•Cys_6 zinc fingers, zinc-sensing proteins, zinc transport proteins, and metallothioneins.[73–75] By coordinating the catalytic metal cofactor in enzymes such as oxidoreductases[76] and zinc-β-lactamases II,[77] Cys helps to ensure the correct positioning of the active-site residues and maintain enzyme catalytic efficiency/stability. By itself, Cys can also stabilize protein structure by interactions with aromatic amino acids, as evidenced by the Cys52–Phe65 interaction in SUMO-1 where mutation of Cys52 to Ala significantly perturbed the SUMO-1 secondary structure and thermal stability.[78]

1.4 | Applications of reactive cysteines

Due to its unique physicochemical properties, Cys is utilized in biochemical research. Based on the Cys thiolate reactivity, Cys scanning mutagenesis has been used to probe the structure and function of ion channels.[79] Cys thiol reactivity has also been exploited to design specific probes to investigate protein structure and functionality.[80,81] Cys, incorporated into lysine to form γ-thialysine, has been proposed for studying methylation processes on intact histones and the nucleosome assembly,[82] as histone peptides containing native lysine and the unnatural γ-thialysine are equally good substrates for methylation catalyzed by histone lysine methyltransferases. The ability of thiols to undergo via thiol/disulfide exchange on the cell surface (Section 1.3) has been exploited to enhance cellular uptake of a wide variety of cargos (e.g., small molecules, oligonucleotides, peptides/proteins, and synthetic constructs).[61] Structural motifs containing two Cys; for example, CXC where X denotes any aa, can enhance cellular uptake efficiency of cationic peptides, as the two Cys in the motif can form disulfide bonds with cell-surface components.[83,84]

Cysteines have also been used in biotechnology and in drug design. They have been used to conjugate antigen-specific antibodies to potent small-molecule drugs, creating antibody–drug conjugates.[85] Importantly, reactive Cys have become important drug targets for developing specific covalent inhibitors to treat various human diseases including cancers, arthritis, and bacterial/viral infections.[86–89] This is exemplified by one of the most frequent cancer-causing mutations of the GTPase KRas enzyme, whereby the wild-type G12 is mutated to Cys (G12C). By designing an inhibitor that covalently bonded specifically to the mutant C12 sulfur, KRasG12C is locked in the inactive GDP-bound state and cannot switch to the active GTP-bound state, hence oncogenic KRasG12C cell proliferation is blocked.[90] This strategy for inhibiting KRasG12C has been used successfully to develop the new anti-cancer drug, Sotorasib (brand name: Lumakras), which was approved in May 2021 to treat metastatic nonsmall cell lung cancer patients with the KRas G12C mutation. As another example, reactive cysteines have also been chosen as targets in the battle against the COVID-19 pandemic. Specific covalent inhibitors targeting the catalytic cysteines of the SARS-CoV-2 proteases (M^{pro} or PL^{pro}) have been developed in silico[91] and in vitro,[92–94] and the inhibition mechanisms of several inhibitors have been investigated.[95,96]

Apart from free Cys, reactive Cys bound to Zn^{2+} ions that play a structural role in stabilizing the protein (termed *labile* Zn^{2+}-site) can also serve as drug targets for retroviral or cancer therapy.[97–100] They react with electrophilic agents,



SCHEME 1 Proposed reaction mechanism for a reactive Zn^{2+}-bound Cys in a *labile* Zn^{2+}-finger to react with a Zn-ejecting agent, causing loss of Zn^{2+} and protein structure

resulting in the loss of structural Zn^{2+} cations and thus protein structure and function (Scheme 1). Such reactive Zn^{2+}-bound Cys has been found in the Zn fingers of several viruses including the HIV nucleocapsid p7,[101] herpes simplex virus,[102] and Junín virus,[103] as well as the human estrogen receptor DNA-binding domain, which is essential for breast cancer growth.[104]

2 | PHYSICAL PRINCIPLES UNDERLYING REACTIVE CYSTEINES

How can a simple amino acid such as Cys fulfill such diverse functional roles in proteins? The answer lies in the high reactivity of its side chain and its ability to interconvert between various oxidation states in vivo (Section 1.2).[43] Whether a Cys in a protein is reactive or inert depends on its environment, which modulates its pK_a and consequently its reactivity.[105] For example, hydrogen bonds to metal-bound Cys help to stabilize/protect the metal complex and enhance metal-binding affinity/specificity, enzyme–substrate recognition, and enzyme activation.[106] Since anionic thiolates (S^-) are more reactive/nucleophilic than neutral thiols (SH), a protein microenvironment that reduces the Cys pK_a would enhance its reactivity.[49,107] Below, we summarize the key factors determining the reactivity of free or metal-bound Cys in proteins from our previous studies, relying on the original references to provide details of the methodology.[35,51]

2.1 | Factors governing reactivity of free cysteine

Free Cys can be reactive as a catalytic or noncatalytic nucleophile in the Cys-dependent enzymes. The reactivity of the free Cys side chain is dictated by its (i) solvent accessibility and (ii) hydrogen-bonding interactions.[35] This is because the Cys thiolate is stabilized/destabilized to varying degrees depending on its hydrogen-bonding partner and dielectric environment.[35,49,107,108] In peroxiredoxin enzymes, for example, Cys is stabilized in its reactive thiolate form by hydrogen-bonding interactions with conserved Thr and Arg residues.[109] In thioredoxins, the number of hydrogen bonds to the catalytic Cys correlates with the decrease in the Cys pK_a.[49] However, hydrogen-bonding contacts to Cys alone do not suffice to determine the reactivity of free Cys. Protein conformational changes that position different hydrogen-bonding partners to the Cys side chain and/or alter solvent access can modulate the nucleophilicity/reactivity of free Cys. Even two slightly different conformations of the same protein may differ greatly in their Cys reactivities due to

differences in the Cys hydrogen-bonding partners.[108] Along the same vein, mutations that alter the Cys hydrogen-bonding partner and dielectric environment could change the degree of thiolate stabilization and thus Cys pK_a. For example, in the disulfide-binding protein A, mutation of His-32 to Gly results in a loss of a strong hydrogen bond with the catalytic Cys-30, whose unusually low pK_a of 3.5 becomes elevated to 4.9, thus decreasing its reactivity.[110]

2.2 | Factors governing reactivity of Zn-bound cysteines

In contrast to free Cys, which can exist as a neutral thiol (SH) or anionic thiolate depending on its pK_a in the protein, Cys is deprotonated when bound to Zn^{2+},[48] thus Zn^{2+}-bound Cys serves as a nucleophile. The reactivity of a Zn^{2+}-bound thiolate towards an electrophile that can access the Zn^{2+}-site depends on whether (1) the thiolate S^- can keep its negative charge, and (2) the positive charge on the cation is attenuated to free the Cys to undergo reaction. The negative charge on the Zn^{2+}-bound thiolate would be maintained if it is (i) *not* shared with a second Zn^{2+} or (ii) *not* withdrawn to the more electronegative carbonyl O via backbone hydrogen bonds to the Zn^{2+}-bound thiolate. Thus, whereas hydrogen bonds to free Cys stabilize the thiolate form, enabling it to serve as a nucleophile, hydrogen bonds to the Zn^{2+}-bound thiolate suppress nucleophilicity of the metal-bound Cys (Figure 3). As for free Cys, conformational changes can affect the reactivity of the Zn^{2+}-bound thiolate due to changes in the hydrogen-bonding interactions and/or solvent exposure of the structural Zn^{2+}-site. As negatively charged Cys^- transfers much more charge to Zn^{2+} than neutral histidine ligands,[111] the positive charge on Zn^{2+} in Zn-finger cores with >2 Cys would be reduced compared to that in Zn•Cys_2His_2 sites, where the higher positive charge on the cation would prohibit the two Cys from undergoing reaction.[51]

3 | APPLICATION OF PHYSICAL PRINCIPLES IN THE COVID-19 PANDEMIC

3.1 | Guidelines to identify labile Zn^{2+}-sites given the protein structure

The above factors controlling the Zn^{2+}-bound Cys reactivity in structural Zn^{2+}-sites have helped to establish guidelines to identify *labile* (druggable) Zn^{2+}-sites given the protein structure. Since neutral Zn^{2+}-bound ligands, or a second Zn^{2+}, or hydrogen bonds to Zn^{2+}-bound Cys would suppress the reactivity of the Zn^{2+}-bound Cys, structural Zn-Cys_4 or Zn-Cys_3His, denoted collectively as Zn-Cys_4/(Cys_3His) sites, with no hydrogen bond to any of the Zn^{2+}-bound Cys were predicted to be labile.[51] These guidelines were first used to identify putative labile Zn-sites in human proteins that are promising drug targets, but whose Zn^{2+}-sites have not been considered to be drug targets.[112] Subsequently, they were used to predict labile Zn-sites in the hepatitis C virus—the structural Zn^{2+}-Cys_4 sites in NS5A, a multifunctional nonstructural protein (nsp), was predicted and subsequently verified to be labile.[113]

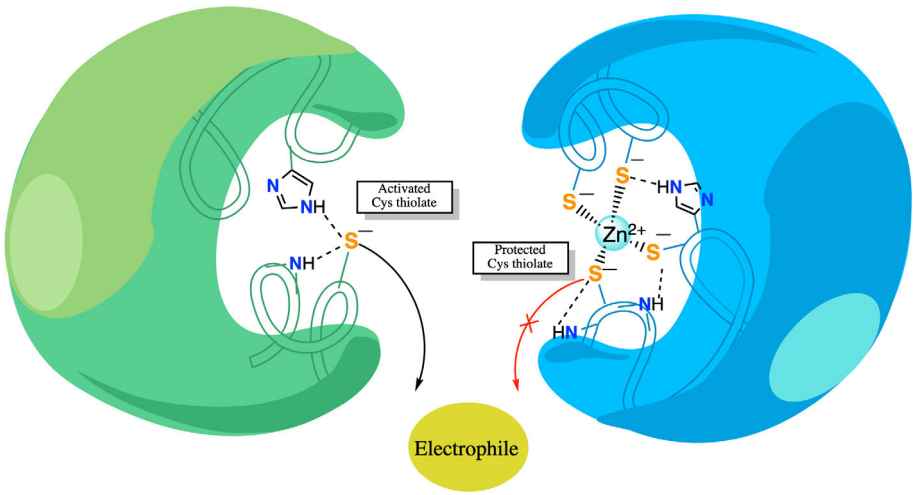


FIGURE 3 NH—S hydrogen bonds enhance nucleophilicity of free Cys (left), but suppress the nucleophilicity of Zn^{2+}-bound Cys (right)

3.2 | How to target a viral labile Zn^{2+}-site without deadly cytotoxic effects

Since putative labile Zn-sites are found in both human and viral Zn-finger proteins, how can a Zn-ejector selectively target the *labile* Zn^{2+}-site of a viral protein without affecting cellular Zn-finger proteins? Instead of screening/designing a Zn-ejecting compound that ejects Zn^{2+} from only the drug target viral protein, but not from essential human proteins, we had proposed using Zn-ejecting agents that have passed safety tests in clinical trials or have been approved by the Food and Drug Administration. Although such Zn-ejecting agents are generally not highly specific unlike antibody drugs that target a specific protein, they have been found to be clinically safe when used according to their recommended dosage.[114] The non-specificity of clinically safe Zn-ejector drugs can be exploited to target multiple viral proteins containing reactive Cys (see below).

3.3 | Multi-targeting of conserved, vital SARS-CoV-2 nonstructural proteins

The SARS-CoV-2 that is responsible for the current pandemic employs multiple conserved Cys and Zn^{2+}, which play crucial roles in the virus life cycle. Its main protease (M^{pro}) and papain-like protease (PL^{pro}) employ catalytic Cys to cleave the large viral polyproteins into its constituent nonstructural proteins. In addition to the catalytic Cys, structures of SARS-CoV-2 and the closely related SARS-CoV viral proteins reveal conserved Zn^{2+}-bound Cys in (i) the PL^{pro} enzyme of nsp3, (ii) the nsp10 zinc-finger domain, (iii) the nsp12 RNA-dependent RNA polymerase (RdRp), (iv) the nsp13 helicase, and (v) the nsp14 N-terminal 3′→5′ exoribonuclease (ExoN) domain (see Table 1). Using the guidelines outlined in Section 3.1, each structure was checked to see if the Zn-Cys_4/(Cys_3His) site lacks hydrogen bonds to the Zn-bound thiolates. However, the cryo-electron microscopy structure of SARS-CoV-2 nsp12 and the ≥3.2 Å crystal structures of SARS-CoV nsp14 (5c8s, 5c8t, and 5c8u) have poor resolution, which prohibited reliable hydrogen-bond analyses of these Zn-sites. The other SARS-CoV or SARS-CoV-2 structures in Table 1 show no hydrogen bonds to the Zn-Cys_4/(Cys_3His) sites. Subsequently, the Zn-sites in the SARS-CoV-2 PL^{pro}, the nsp10 zinc-finger, the nsp13 helicase, and the nsp14 ExoN domains were experimentally verified to be labile: clinically safe Zn-ejector drugs, disulfiram/ebselen, can release Zn^{2+} from these four viral proteins and decrease their functional activities in vitro as well as inhibit SARS-CoV-2 replication in Vero E6 cells.[115,116]

These *labile* Zn^{2+}-sites are attractive drug targets, as they play important functional roles in the SARS-CoV-2 life cycle: The Zn-Cys_4/(Cys_3His) sites play important structural roles in nsp3 PL^{pro} domain,[117] nsp10 zinc-finger,[118] nsp12,[119] and nsp14,[120] whereas they play vital catalytic roles in the nsp13 helicase activity,[121] and the nsp14 3′→5′ exoribonuclease activity.[120] Furthermore, the viral proteins hosting these Zn^{2+}-sites are constituents of a large replication-transcription complex that plays a critical role in viral (i) RNA synthesis, (ii) RNA proofreading, and (iii) RNA modification to evade the human immune response, as follows:[122,123] First, the nsp12 C-terminal RdRp domain catalyzes viral RNA synthesis with the help of nsp7 and nsp8 cofactors.[119,124,125] Next, the nsp14 N-terminal ExoN domain proofreads the viral RNA by recognizing erroneous nucleotides and catalyzing their excision, thereby maintaining the integrity of the SARS-CoV-2 genome.[122] Subsequently, the nsp13 helicase, as well as the nsp14 and nsp16 methyltransferase domains, are involved in modifying the newly synthesized viral RNA, enabling its efficient translation by host cell ribosomes. Without this modification, the viral RNA molecules would be degraded and may be detected as foreign, triggering innate immune responses.[126] The nsp10 zinc-finger protein activates the nsp14 and nsp16 methyltransferase enzymatic activities and boosts the nsp14 ExoN nucleolytic activity.[118] Furthermore, it stabilizes the conserved domains involved in RNA proofreading (nsp14) and modification (nsp13–16).[122,126]

3.4 | Advantage of using Zn^{2+}-ejecting drugs to target cysteines

Because the large nsp12–nsp13–nsp14–nsp10–nsp16 complex is indispensable for SARS-CoV-2 replication, using clinically safe Zn-ejecting drugs such as disulfiram/ebselen to target *labile* Zn^{2+}-sites in the constituent proteins would reduce viral load, as shown in Vero E6 cells. By reacting with Zn^{2+}-bound Cys and ejecting "structural" Zn^{2+} cations from the multi-functional nsp10 cofactor,[115] disulfiram/ebselen can destabilize the nsp10 zinc-finger itself as well as its partner proteins, nsp14 and nsp16. The same Zn-ejecting drug can not only affect protein stability, but also inhibit the enzyme activities of nsp3 PL^{pro},[115] nsp13, and nsp14, and probably nsp12 RdRp. In addition to Zn^{2+}-bound Cys, disulfiram/ebselen can also target catalytic Cys,[115] and thereby inhibit SARS-CoV-2 M^{pro},[127] which does not possess a Zn^{2+}-

TABLE 1 Labile Zn-sites in SARS-CoV and/or SARS-CoV-2 nsp proteins

	SARS-CoV		SARS-CoV-2		
Protein name	**Structures[a]**	**Zn-ligands**	**Structures[a]**	**Zn-ligands**	**Experimentally confirmed labile Zn-sites**
PL^{pro} subdomain of nsp3	4m0w, 3e9s, 5tl7	C190, C193, C225, C227	6wrh, 6wrz, 6wuu, 6wx4, 6wzu, 7e35, 7jit, 7jn2, 7lbr, 7lbs, 7llf, 7llz, 7los	C189, C192, C224, C226	Lin, 2018[117] Sargsyan, 2020[115]
nsp10	2fyg, 2ga6, 2xyq, 2xyr, 2xyv, 3r24 5c8s, 5c8t, 5c8u, 5nfy	C74, C77, C90, H83 C117, C120, C128, C130	6xkm, 6wjt, 6wvn, 6wrz, 6zpe, 7c2i, 7jib, 7jpe, 7jyy, 7jz0, 7krx	C74, C77, C90, H83 C117, C120, C128, C130	Sargsyan, 2020[115]
nsp12	6nus, 6nur (EM)	C487, C645, C646, H642	6xez, 6yyt, 7aap, 7b3b, 7b3c, 7b3d, 7btf, 7bv1, 7bv2, 7bw4, 7bzf, 7c2k, 7ctt, 7cxm, 7cyq (EM)	C301, C306, C310 H295 C487, C645, C646, H642	None
nsp13	6jyt	C50, C55, C72, H75	6zsl, 5rl7, 5rlb, 5rlc, 5rlg, 5rlz, 5rm1, 5rm4, 5rmc, 5rmd, 5rme, 5rml, 5rmm, 5rob, 7nio, 7nng	C5, C8, C26, C29 C50, C55, C72, H75	Chen, 2021[116]
nsp14	5c8s, 5c8t, 5c8u, 5nfy	C207, C210, C226, H229 C477, C452, C484, H487	7diy, 7mc5, 7mc6 (nsp14 ExoN domain)	C207, C210, C226, H229	Chen, 2021[116]

[a]The Zn-ligand residues are taken from the first PDB highlighted in bold; e.g., the Zn-ligand numbers for SARS-CoV-2 nsp10 were taken from PDB 6xkm.

site. By impeding M^{pro}- and PL^{pro}-catalyzed viral proteolysis, disulfiram/ebselen can prevent efficient cleavage of the replicase polyproteins into components.

Hence, these Zn-ejecting drugs work against coronaviruses at various stages: First, they inhibit viral polypeptide proteolysis; then, they cripple the functions of several proteins that are crucial for viral RNA synthesis, proofreading, and modification. Targeting multiple viral proteins at different stages would create a high barrier to drug resistance. In contrast, drugs targeting a specific viral protein may lose their effectiveness if a lineage appears with mutations leading to drug resistance. For example, a popular SARS-Cov-2 drug target is the spike protein that mediates entry of the virus into the host cell. Numerous mutations are found in the genomic region corresponding to the spike protein that alters host cell entry. Some mutations lead to enhanced transmission,[128] and may, as a by-product, evade neutralizing antibodies targeting the spike protein.[129] On the other hand, the number of mutations observed in the regions containing catalytic or reactive Zn^{2+}-bound Cys is much lower than that for the rest of the genome (https://nextstrain.org/ncov/global). Hence, the spontaneous occurrence of lineages with mutations conferring resistance to disulfiram/ebselen would be unlikely. Furthermore, mutations of the conserved catalytic or reactive Zn^{2+}-bound Cys would likely disrupt their vital catalytic/structural roles in the respective viral protein functions, incurring a destructive cost for the virus.

Combining disulfiram/ebselen with other broad-spectrum anti-viral drugs that target other viral regions/pathways could further enhance the barrier to drug resistance. It can also enhance antiviral effect compared to each drug alone. For example, disulfiram/ebselen combined with remdesivir exhibited synergistic inhibition of SARS-CoV-2 in cell-based assays.[116] This is because remdesivir A stops viral RNA synthesis by the nsp12 RdRp domain, and can escape removal by the proofreading nsp14 ExoN, as disulfiram/ebselen inhibits nsp14 exoribonuclease activity and destabilizes its allosteric activator nsp10. Disulfiram/ebselen combined with the zinc ionophore, hydroxychloroquine, could also synergistically inhibit SARS-Cov-2,[115] as these drugs may increase the local Zn^{2+} concentration and inhibit nsp12 RNA-dependent RNA polymerase.[130]

Finally, the SARS-CoV-2 caused the current pandemic in less than two decades after the SARS-CoV caused outbreaks in several countries in 2003. Hence, we should prepare for new emergent coronaviruses even after the current pandemic is over. Analysis of genomes for different bat and human coronaviruses belonging to the same family as

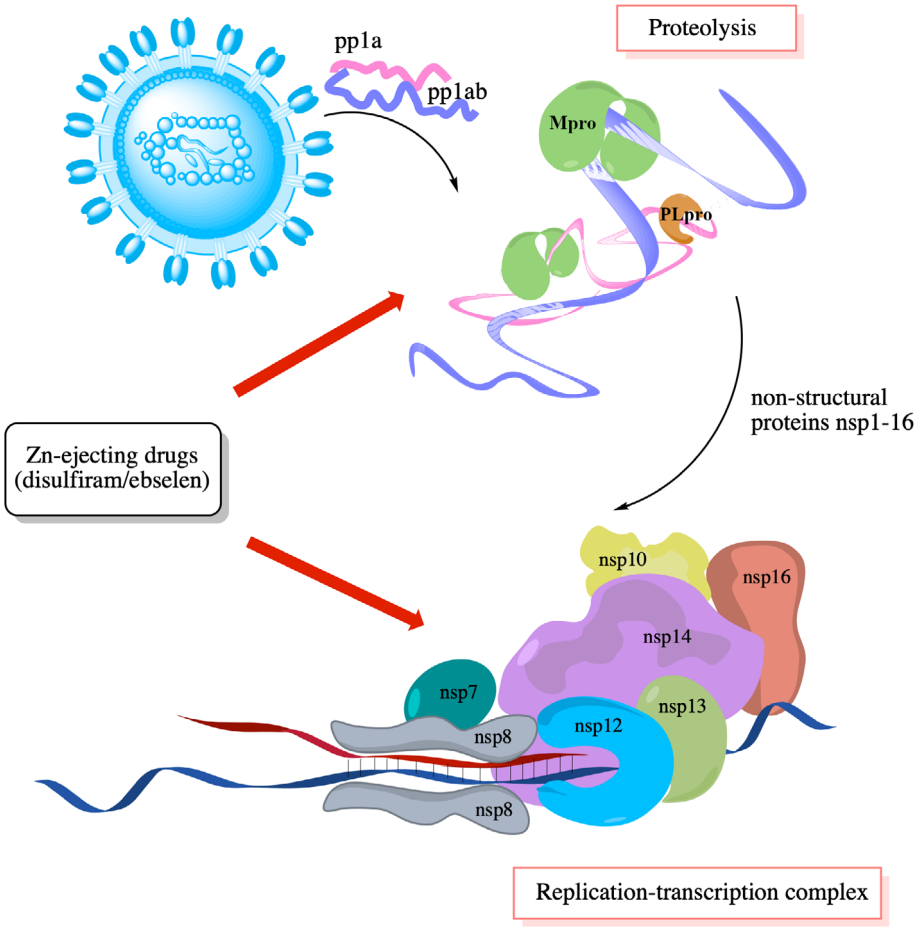


FIGURE 4 Viral drug targets and their functions

SARS-CoV-2 shows the highest conservation in regions encoding the viral targets revealed herein.[131] This suggests the possibility of targeting the multiple conserved reactive free/Zn^{2+}-bound Cys in emergent coronavirus pathogens indispensable for the coronavirus replication using a cocktail of Zn^{2+}-ejecting drugs and other broad-spectrum antivirals.

4 | CONCLUSION AND FUTURE OUTLOOK

Reactive Cys is of widespread interest, as they can serve as important drug targets among many other applications (Section 1.4). Herein, we have delineated the key physicochemical principles underlying their reactivity in proteins: The strength of the hydrogen bond stabilizing the Cys thiolate, which depends on the solvent accessibility and hydrogen-bonding partner of the Cys, dictate the reactivity of *free* Cys. On the other hand, the negative charge on the thiolate S^- and the positive charge on the cation dictate the reactivity of Zn^{2+}-bound Cys. These principles have provided guidelines to identify *labile* Zn^{2+}-sites given the protein structure, which have been used to reveal novel druggable Zn^{2+}-sites in multiple SARS-CoV-2 proteins. These predicted SARS-CoV-2 druggable sites have been validated in in vitro and *cell-based* experiments using clinically safe Zn^{2+}-ejector drugs, which target not only reactive Zn^{2+}-bound Cys, but also catalytic Cys. The importance of the multiple SARS-CoV-2 targets revealed herein is underscored by their evolutionary conservation and crucial functions in viral polypeptide proteolysis as well as viral RNA synthesis, proofreading, and modification (Figure 4).

Because of the threat of another pandemic caused by a novel coronavirus, studying the best combination of Zn-ejecting drugs and other broad-spectrum antivirals targeting the multiple conserved viral regions/pathways in coronaviruses would be useful. Furthermore, since it takes time to solve structures of proteins for new infections, predicting which Cys are reactive and which are inert from sequence alone would also be useful.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Karine Mazmanian: Visualization (lead); writing – original draft (equal). **Ting Chen:** Visualization (equal); writing – original draft (equal). **Karen Sargsyan:** Writing – original draft (equal). **Carmay Lim:** Conceptualization (equal); funding acquisition (equal); resources (equal); supervision (equal); writing – review and editing (equal).

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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