REVIEW

THE PROTEIN SOCIETY WILEY

Characterization of the SARS-CoV-2 E Protein: Sequence, Structure, Viroporin, and Inhibitors

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Funding information
National Natural Science Foundation of China, Grant/Award Numbers: NSFC-31900894, NSFC-81872472: NSFC-31870843

Abstract

The COVID-19 epidemic is one of the most influential epidemics in history. Understanding the impact of coronaviruses (CoVs) on host cells is very important for disease treatment. The SARS-CoV-2 envelope (E) protein is a small structural protein involved in many aspects of the viral life cycle. The E protein promotes the packaging and reproduction of the virus, and deletion of this protein weakens or even abolishes the virulence. This review aims to establish new knowledge by combining recent advances in the study of the SARS-CoV-2 E protein and by comparing it with the SARS-CoV E protein. The E protein amino acid sequence, structure, self-assembly characteristics, viroporin mechanisms and inhibitors are summarized and analyzed herein. Although the mechanisms of the SARS-CoV-2 and SARS-CoV E proteins are similar in many respects, specific studies on the SARS-CoV-2 E protein, for both monomers and oligomers, are still lacking. A comprehensive understanding of this protein should prompt further studies on the design and characterization of effective targeted therapeutic measures.

KEYWORDS
coronavirus, COVID-19, E protein, inhibitors, viroporin

1 | INTRODUCTION

COVID-19 is a severe and highly contagious respiratory disease caused by a new coronavirus (CoV) called SARS-CoV-2. The World Health Organization (WHO) declared a global COVID-19 pandemic in March 2020. As of February 2021, hundreds of millions of patients have been diagnosed, and millions of patients have died. The mortality rate in different countries ranges from 1 to 10%, but this disease is more contagious than SARS and MERS.[1,2] At present, the virus with the spike (S) protein mutation D614G shows stronger infectiousness than wild-type virus.[3–6] Researchers speculate that the virus was transmitted to humans

through some type of wildlife. The symptoms include fever, general malaise, dry cough, shortness of breath, and respiratory distress,[7] as well as a weakened sense of taste and smell, gastrointestinal discomfort, and certain effects on the cardiovascular and nervous systems.[8–11]

The nucleic acid sequence of SARS-CoV-2, which includes a long single-stranded (30 kb) RNA, generally ranging in size from 26.4 to 31.7 kD, was identified as that of a β-CoV.[12] The viral genomic RNA strand attaches to the nucleocapsid protein to produce a nucleoprotein complex.[13] The SARS-CoV-2 genome shares approximately 82% sequence identity with SARS-CoV, while the identity of essential enzymes and structural proteins is greater than 90%.[14] The SARS-CoV-2 genome consists of 10 open reading frames (ORFs). Approximately two-thirds of the viral RNA in the ORFs encodes non-structural proteins (nsp), and one-third encodes structural proteins such as the S, membrane (M), envelope (E), and nucleocapsid (N) proteins.[15] The results of recent cryoelectron microscopy experiments showed that there were three size distributions of SARS-CoV-2 virus particles, and the diameters were mainly 64.8 ± 11.8, 85.9 ± 9.4, and 96.6 ± 11.8 nm. Viral structural proteins, including the S, E, M, and N proteins, are embedded in viral lipids.[16,17]

The S protein has an immune recognition site and binds to the surface receptor during the process of entering the host cell; it is the most important protein for viral entry into cells.[18,19] The S protein binds to the host receptor through the receptor-binding domain in the S1 subunit, and then, the S2 subunit is fused to the cell membrane. Different CoVs have different host entry mechanisms, indicating that changes in the amino acid residues of the S protein determine whether the virus enters the host cell, and this property is used to design vaccines.[20–22]

The main function of the N protein is to package the viral RNA into the helical ribonucleocapsid and interact with the other structural proteins during virion assembly.[23] The viral genome is filled with N proteins, which form a helical nucleocapsid protected by a lipid envelope.[24,25] The N protein is involved in the CoV replication cycle and the host cell's response to viral infection.[26] Therefore, it is also a potential target of SARS-CoV-2. In addition, the transient expression of N has been shown to substantially increase the production of virus-like particles (VLPs) in some CoVs, which indicates that the N protein is of great significance in the process of viral envelope conformation.[27,28]

The M protein defines the shape of the viral envelope and is the most abundant structural protein.[29] It is a 222-amino-acid transmembrane protein with N- and C-termini (exposed inside and outside the viral particle, respectively) and three transmembrane domains (TMD1–TMD3).[30] The C-terminus of the M protein can interact with the N and E proteins, and the TMD can bind with the S protein. The TMD of the M protein is also closely related to the homotypic interaction of the M protein itself.[31,32] Interactions with other structural proteins are essential for membrane bending and germination.[30,33] In summary, stable binding of the M and N, M and E, and M and S proteins leads to the formation of SARS-CoV-2's inner core of VLPs, which promotes the assembly of viruses.[34]

The E protein is the smallest of the major structural proteins, but it is also the most poorly understood. It is highly conserved in different viral subtypes. Studies have not fully elucidated the role of the E protein in viral invasion, replication and release. The E protein in the viral particle envelope functions by interacting with other structural proteins. The interaction of the E and M proteins maintains the shape of the viral particle and promotes release.[35,36] When E and M are coexpressed in host cells, the S protein relocates to the endoplasmic reticulum (ER)-Golgi intermediate region (ERGIC) or Golgi region.[37] Mutation of the gene encoding the E protein promotes apoptosis.[38]

Interestingly, previous studies have found that during the CoV replication cycle, the E protein is expressed at a high level in each infected cell, but only a small amount of this expressed E protein is inserted into the viral membrane.[39,40] Most of the protein is located at intracellular transport sites, namely, the ER, Golgi and ERGIC, which are involved in CoV assembly and budding.[41] Recombinant CoVs lacking the E protein show a significant reduction in viral titer and maturity or produce incompetent offspring, indicating the importance of the E protein in virus production and maturation.[42] The E protein can also bind to PALS1, which leads to enhanced destruction of the epithelial barrier, an enhanced inflammatory process and promotion of tissue remodeling, suggesting that the role of the E protein in viral infection may be important.[43]

Recent studies have indicated that several SARS-CoV-2 proteins, including E, ORF3a, and ORF8a, can self-assemble into oligomers and generate ion channels (ICs).[44,45] The SARS-CoV E protein has higher ion permeability than the 3a and 8a channels, and blockade of the E protein channel can significantly reduce viral pathogenicity.[46] The transmembrane voltage (TMV) can promote E protein pentamer IC activity,[47] and the mutations N15A and V25F may disrupt IC activity.[48–50] Therefore, the E protein may play an important role in regulating the ion balance and microenvironment of host cells (Figure 1).
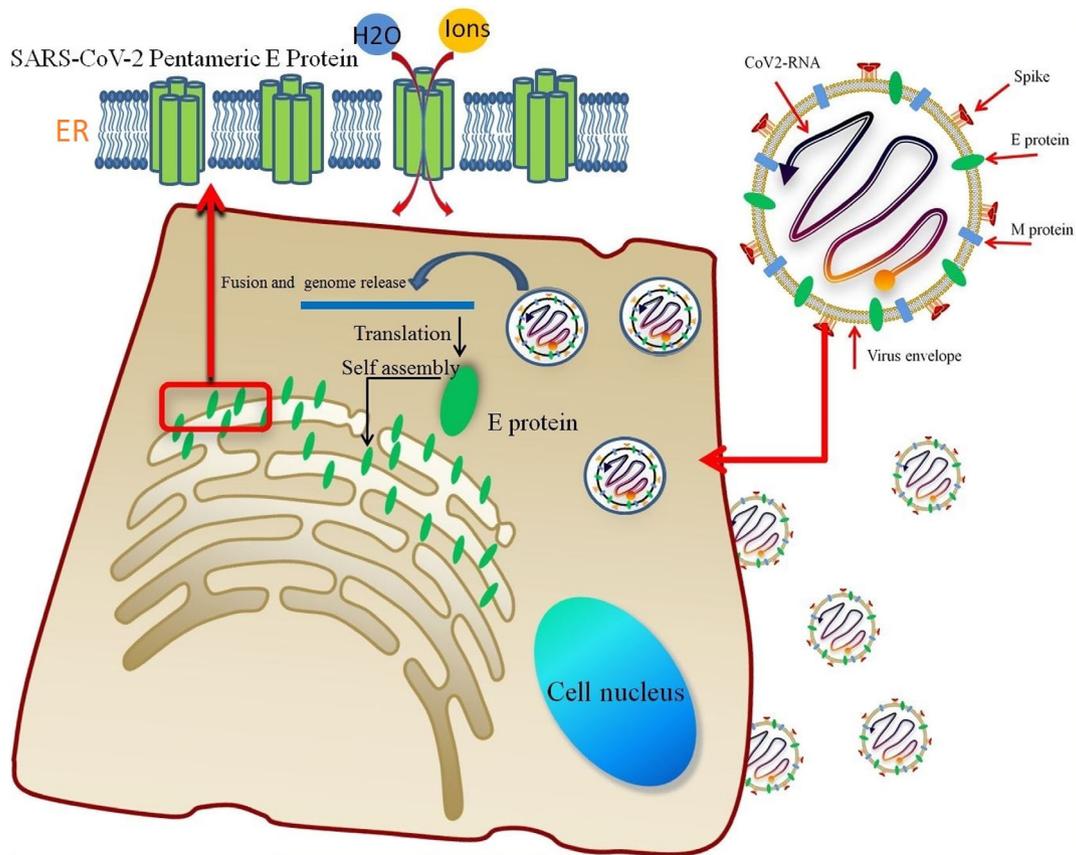
FIGURE 1 The lipid envelope encloses the virus and facilitates the entry of the SARS-coronavirus (CoV)-2 E protein into the host cell. The E protein is translated in the endoplasmic reticulum (ER) and accumulates in the Golgi. Then, the E protein monomer self-assembles into an oligomer that functions as an ion channel

2 | DIFFERENCES IN AMINO ACID SEQUENCE AND STRUCTURE BETWEEN THE SARS-COV AND SARS-COV-2 E PROTEINS

The size of CoV E proteins ranges from 8.4 to 12 kDa.[51] NMR analysis of the SARS-CoV-2 E protein showed that there was a single TMD. This result was consistent with the fragment L18-L39 found in sodium dodecyl sulfate (SDS) micelles of SARS-CoV.[52] The conformation of the SARS-CoV-2 E protein TMD pentamer has been identified, while other domains have not yet been identified. SARS-CoV and SARS-CoV-2 are composed of 76 and 75 amino acids, respectively. Both of them consist of three main domains: one TMD (residues 17–37), an intermediate helical domain, and N- and C-terminal domains. The N-terminus of the CoV E protein has a negatively charged hydrophilic amino terminal. In addition, the protein has an uncharged (nonpolar) hydrophobic TMD (Figure 2). Compared with the TMD, the C-terminus shows low hydrophobicity, but it also has 20 hydrophobic residues out of a total of 37 amino acids that are positively charged. The total charge on the E protein is zero.[46,53] The E protein of SARS-CoV-2 differs in only three amino acid substitutions and one deletion: T55S, V56F, E69R, and G70-GAP (red box in Figure 2).[54] The amino acid sequences of the TMDs of the two CoVs were identical. Two nonpolar, neutral amino acids, namely, valine and leucine, confer strong hydrophobicity to the E protein TMD.[55] The E protein may not exhibit a uniform membrane topology, and its orientation depends on the level of protein expression or oligomerization.[56]

The exact function of the N-terminus of the E protein has not been clearly identified. This region is located inside the Golgi/ER membrane, and Asn15 in the domain forms an H-bond, which maintains the stability of the N-terminus.[57,58] Studies have shown that homotypic interactions mediated by the N-terminus of alternating E protein molecules may interact with each other through the luminal loops between the TMD of the proteins to initiate the rearrangement of ER membranes and induce membrane curvature. Similar mechanisms have been found for the interactions of the CoV nonstructural proteins nsp3 and nsp4, which mediate membrane bending.[59,60] The E protein also has a
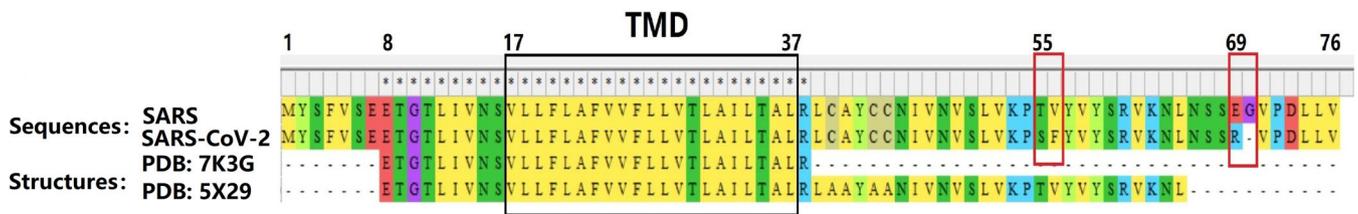
FIGURE 2 Multiple sequence alignment of SARS-coronavirus (CoV) and SARS-CoV-2. Sequences 1 and 2 represent whole E proteins, and 3 and 4 represent 3D structures with identified amino acid regions (black box: transmembrane domain (TMD), red box: substituted residue or deletion)

predicted signal peptide cleavage site and two glycosylation sites, which are involved in the interaction between the E protein and other membrane proteins. This protein aids in the proper folding and trafficking of cellular and viral proteins.[61,62] Other targeting information can be found at the N-terminus.[63]

Four mutations (including one deletion mutation) in the C-terminus of the SARS-CoV-2 E protein, at positions 56, 57, 69, and 70, have an additional amino acid with an alkaline R group compared to the E protein of SARS-CoV.[64] Many functional properties of the protein can be predicted from the amino acid sequence of the C-terminus of the E protein. According to reports, the C-terminus of both SARS-CoV and SARS-CoV-2 contains a conserved DLLV motif (the PDZ domain of the viral E protein), which can bind to PALS1 in the host cell to promote infection.[65,66] This region also includes the conserved "FYXY" motif, which could be associated with the high propensity for amyloid formation.[67,68] Another highly conserved cysteine motif, "CxxC," can isomerize disulfide bonds. The oxidized CxxC motif can maintain the structural topology of the TMD and its adjacent cytoplasmic domain, including glycosylation sites, and participate in signal transduction and protein–protein interactions.[69] In addition, the activation of thiol in this motif could trigger other Cys residues to participate in disulfide bond formation between subunits to promote the directional conformation of the membrane.[70]

Hassan, Khan, et al. also performed multiple sequence alignments of various SARS-CoV-2 mutations before June 18, 2020. They found that in the NCBI database, there are only 16 different E proteins among the 4,917 SARS-CoV-2 genomes, and most variations and mutations occur at the C-terminus.[71,72] Mutation at the C-terminus of the SARS-CoV-2 E protein results in the nonsynonymous R group, which may affect the interaction of the E protein with the host protein. In CoV and CoV-2, there are two cysteine residues at the C-terminus next to the TMD, which are essential for oligomerization and for the membrane-directed amyloidogenic propensity.[40,73,74]

3 | E PROTEIN VIROPORIN PORE FORMATION AND ION PERMEATION

The formation of homo-oligomers by the E protein depends on the TMD. Synthetic peptides corresponding to SARS-CoV and SARS-CoV-2 E protein TMDs can form dimers, trimers, and pentamers, proving the importance of the TMD in homotypic interactions[75,76] of the mutations F26L, L39M, A36V, and L37H in the TMD were found in different countries. These mutations or deletions and mutations have been shown to attenuate the virus in the body and reduce the viral titer.[77] The substitution of charged residues for some hydrophobic residues in the TMD can significantly change the electrophoretic migration rate.[78] So far, research to identify the TMD residues that are required for the CoV E homotypic interactions has proven inconclusive. Many studies have suggested that the E protein can self-assemble into oligomers through the interaction between the GxxxG motif and leucine–isoleucine zipper.[79–81] The TMD sequences of both the SARS-CoV and SARS-CoV-2 E proteins contain repeated Leu–Ile sequences, which may be closely related to their homotypic interactions. Wang et al. also indicated that the glycosylation site of the E protein might affect its oligomerization ability, and glycosylated amino acids might prevent oligomerization of the E protein.[82] Asparagine 15 (N15) is mutated to alanine (N15A) and valine 25 (V25) is mutated to phenylalanine F (V25F) in the TMD. The mutations seem to abolish, or at least reduce to some extent, CoV-E oligomerization. However, the V25F mutant showed apparent monomer formation, in contrast to the N15A mutant. V25F plays a more critical role in oligomerization, while N15A only reduces pentamerization of the E protein.[83]

To control the transport of intracellular ions through lipid membranes, the optimal ion conditions of subcellular compartments can differ from those of extracellular media, and different organelles present specific ion components. These asymmetrical distributions of ions between produce electrochemical gradients, which are

essential for maintaining normal cellular functions.[84,85] The key functions of cells are controlled by the membrane potential, Ca^{2+} storage in the ER/Golgi, and different pH conditions in the secretory pathway organelles, mainly due to the ion concentration gradient. The coordinated actions of multiple ICs and transporters produce and control the intracellular ionic environment. Recent studies have reported that most viral viroporins can induce inflammasome activity and the production of interleukin-1β (IL-1β).[86]

It is well known that viruses utilize and modify host cell ion homeostasis in different ways to facilitate viral infection. One of the important ways is via the activity of viroporins. Various viruses encode viroporins, which constitute a broad family of multifunctional proteins, are widely distributed in different virus families, and are mainly found in RNA viruses.[87,88] Highly pathogenic human viruses, such as influenza A virus, human immunodeficiency virus 1, hepatitis C virus, and CoVs (SARS-CoV, MERS virus, and SARS-CoV-2), encode at least one viroporin. Viroporins are generally composed of small hydrophobins encoded by viral RNA. These transmembrane proteins can stimulate key aspects of the viral lifecycle through various mechanisms, such as viral entry, trafficking, morphogenesis, maturation, and even virulence. When proteins oligomerize in the ER/Golgi of host cells, they form a channel-like topology and disrupt a number of physiological properties of the cell by improving the membrane permeability of the host cell, membrane remodeling, and interactions with other normal membrane proteins.[87–89]

Deleting the E protein of SARS-CoV and SARS-CoV-2 causes the attenuation of viral virulence, suggesting that the E protein is a potential antiviral and vaccine target. Although it is important for the pathogenesis of viruses, the structure of the SARS-CoV-2 E protein was unclear until September 2020, when Mandala et al. analyzed the SARS-CoV-2 TMD (PDB: 7K3G), but the structures of the N- and C-termini have remained elusive.[90] Sedimentation equilibrium and gel electrophoresis data indicate that the TMD of the E protein assembles into a homopentamer in detergents such as SDS and perfluorooctanoic acid, but the topology is controversial. NMR analysis of the SARS-CoV-2 E protein showed that residues 14–34 form the α-helical core of the E protein pentamer.[75,90–92] Comparison of the spectra of the E protein at different temperatures showed that the N-terminus is dynamic (flexible) at high temperatures, while the C-terminus is more rigid but exhibits a temperature-dependent conformation.[52,67] The self-assembly and oligomer formation mediated by the E protein on the host membrane can also affect the integrity of the lipid bilayer, which is conducive to viral reproduction.[93] The TMD of the CoV E protein can form

stable dimers, trimers, and pentamers through self-assembly via the Leu–Ile zipper motif,[75] and the homopentameric E protein was identified as the CoV viroporin. The viroporin is involved in many functions of CoVs, including facilitating the release of viral particles from host cells.[94]

Viroporins regulate the host cell microenvironment (including ion concentration and pH) through the pentameric hydrophilic pores of the cell membrane. The channels allow water and ions to pass through the cell membrane, leading to the disruption of membrane potential, collapse of ionic gradients and release of essential compounds from the cell; this condition facilitates viral dissemination and continuous virion production.[47,78] They affect host cell functions, including membrane vesiculation, glycoprotein tracking, and membrane permeability.[95] These characteristics suggest that the viroporin is important for regulation of the viral replication circle.

Previous works have indicated that most viroporins have specific ion selectivity, including the viroporins of human papillomavirus, rotavirus, hepatitis E virus, influenza virus, and CoVs.[96–100] Viroporins can be formed by the E, 3a, and 8a proteins in SARS-CoV, and the IC activity of only the E protein causes viral pathogenicity.[101] The E protein of SARS-CoV and avian infectious bronchitis virus (IBV) viroporin function as cation-selective channels in a planar lipid bilayer.[102,103] Surya et al. showed that the SARS-CoV E protein could form pentameric ICs in LMPG micelles.[52] The model included contraction of a >0.2 nm radius formed by the side chains of residues V25 and V28. This can be considered a gate that can expand the central pore with a diameter of less than 0.6 nm.[104] It has been found that the V25F mutation abolish channel permeability, suggesting that the channel's IC activity depends on the homopentameric conformation.[48,105] Pervushin et al. suggested that N15 plays an important role in IC activity, and the side chain of the TMD pore forms a pore space with a diameter of approximately 0.4 to 0.5 nm, which can accommodate a single dehydrated Na^+ or K^+ ion and form a cation selectivity filter for the SARS-CoV viroporin.[106] The cation permeability of the E protein viroporin is different in high-concentration KCl and NaCl solutions, the order of selective permeability is $Na^+ > K^+ > Cl^-$, and is the permeability of Na^+ is 10 times higher than that of K^+.[104] Cao et al. indicated that the gating amino acids of the SARS-CoV-2 E protein viroporin are L10 and F19. The changes in pore radius at L10 and F19 range from 0.2 to 0.4 nm and 0.1 to 0.3 nm, respectively, under different TMVs. The results demonstrated that the SARS-CoV-2 E protein viroporin is a voltage-dependent channel with a certain ion selectivity.[47] The ion-selective permeability of the

viroporin also depends on the composition of the cell membrane. The E protein is less likely to form a viroporin in membranes containing lipid components with a negative intrinsic curvature (such as DOPE), but membranes with a positive intrinsic curvature provide a more favorable environment.[105,107] Electrophysiological experiments in the DPhPC/DPhPS membrane further confirmed that the charge contained in lipids may affect IC activity. For example, the channel conductance in completely negatively charged lipids (DPhPS) is much lower than that in neutral lipids (DPhPC).[108]

The conductivity and ion selectivity of the E protein viroporin are also influenced by different pH values. Investigation of the SARS-CoV E protein pentamer inserted into the ERGIC showed that the carboxyl group of the Glu8 side chain in TMD was deprotonated at neutral pH but protonated at acidic pH. The proton balance of Glu8 and the charged lipids in the ERGIC membrane may adjust the ion selectivity at the entrance of the channel.[90] An intriguing study recently reported that the influenza B virus M2 proton channel is pH gated and is activated by acidic pH to mediate virus uncoating.[109,110] This suggests that viroporins are sensitive to changes in cellular pH and can have therapeutic value. Furthermore, the E protein viroporins of both SARS-CoV and SARS-CoV-2 are capable of Ca^{2+} permeation. This may be due to the large Ca^{2+} concentration gradient between the two sides of the membrane and because a negative charge interaction occurs between Ca^{2+} and the protein-lipid pore, which affects the IC activity of the channel.[111] A computational model revealed the mechanisms of Ca^{2+} permeation of CoV-2 E protein pores. At a high TMV ($\geq$0.4 V), the pore radius greatly expands, making it sufficiently large to accommodate Ca^{2+}. However, a higher TMV may cause loss of the gating ability of the viroporin.[47] This is also consistent with other viral viroporins.[98,112–114] The leakage of Ca^{2+}, K^+, and H^+ via the viroporin induces the activation of NLRP3 inflammasomes, leading to the overproduction of IL-1β, which affects the occurrence and development of respiratory inflammation.[86] Ca^{2+} homeostasis plays a key role in the pathogenicity of SARS-CoV and SARS-CoV-2.[115–117]

The IC activity of the SARS-CoV-2 E protein causes injury to numerous vital organs. Xia et al. injected purified SARS-CoV-2 E protein into mice intravenously and produced lung and spleen ARDS-like pathological damage.[118] This indicates that the cation channel formed by the overexpressed E protein can eventually rupture the host cell membrane. Polina et al. suggested that the K^+ IC activity of SARS-CoV-2 may abnormally regulate the transmission of AP and Ca^{2+} in cardiomyocytes, promoting a decrease in cardiac contractility and an increase in sensitivity to arrhythmia.[119] Therefore, targeted inhibition of the SARS-CoV-2 E viroporin can reduce the risk of sudden cardiac death and heart damage in COVID-19 patients with cardiovascular disease.

Although the residues affect IC activity, they also affect different aspects of the virus life cycle. The relationship between CoV IC activity and pathogenesis remains to be further explored.

4 | SARS-COV-2 E PROTEIN VIROPORIN INHIBITORS

Specific inhibition of the CoV E protein's IC activity causes virus attenuation.[46] Mandala et al. identified the SARS-CoV-2 E protein pentamer structure by using NMR spectroscopy.[90] The SARS-CoV-2 E protein pentamer is a CoV viroporin, and some inhibitors of viroporins are considered to be equally effective in inhibiting the SARS-CoV-2 E protein viroporin.[120]

4.1 | Hexamethylene amiloride and amantadine

One potential therapeutic approach is to target the pentameric E protein channels through hexamethylene amiloride (HMA) and amantadine (AMT) and their combinations. Importantly, HMA and AMT represent a broad range of viroporin inhibitors, and their chemical formula and 3D structure are shown in Figure 3a,b. In addition to their effect on CoVs, they have good inhibitory effects on HIV, IBV, and MHV.[121–123] AMT and its derivatives can inhibit the IC activity of influenza virus M2 ICs and have been used in the clinical treatment of influenza A infection.[124,125]

Specifically, HMA is exchanged between the helices of the pentameric SARS-CoV-2 E protein and disturbs 5 residues at the N-terminus (namely, Thr9, Gly10, Thr11, Ile13, and Ser16, shown in Figure 3c), blocking IC conductance. It has high affinity for both acidic Glu8 and polar Asn15 to seal the N-terminal entrance of the protein.[90,92] AMT can block the conductance of the SARS-CoV wild-type (WT) and F23A mutant proteins and exhibits significant binding only with F23A, showing nonspecific agglutination with N15A, V25F, and A32F. The conductance of E protein double mutants (N15A-V25F and V25A-A32F) is not affected by AMT.[48,77] Moreover, the combination of HMA and AMT inhibited SARS-CoV-2 E protein channel conductance, and HMA had a stronger affinity than AMT. Some clinical reports have indicated that HMA and AMT have some preventive and therapeutic effects on SARS-CoV-2.[126–128]
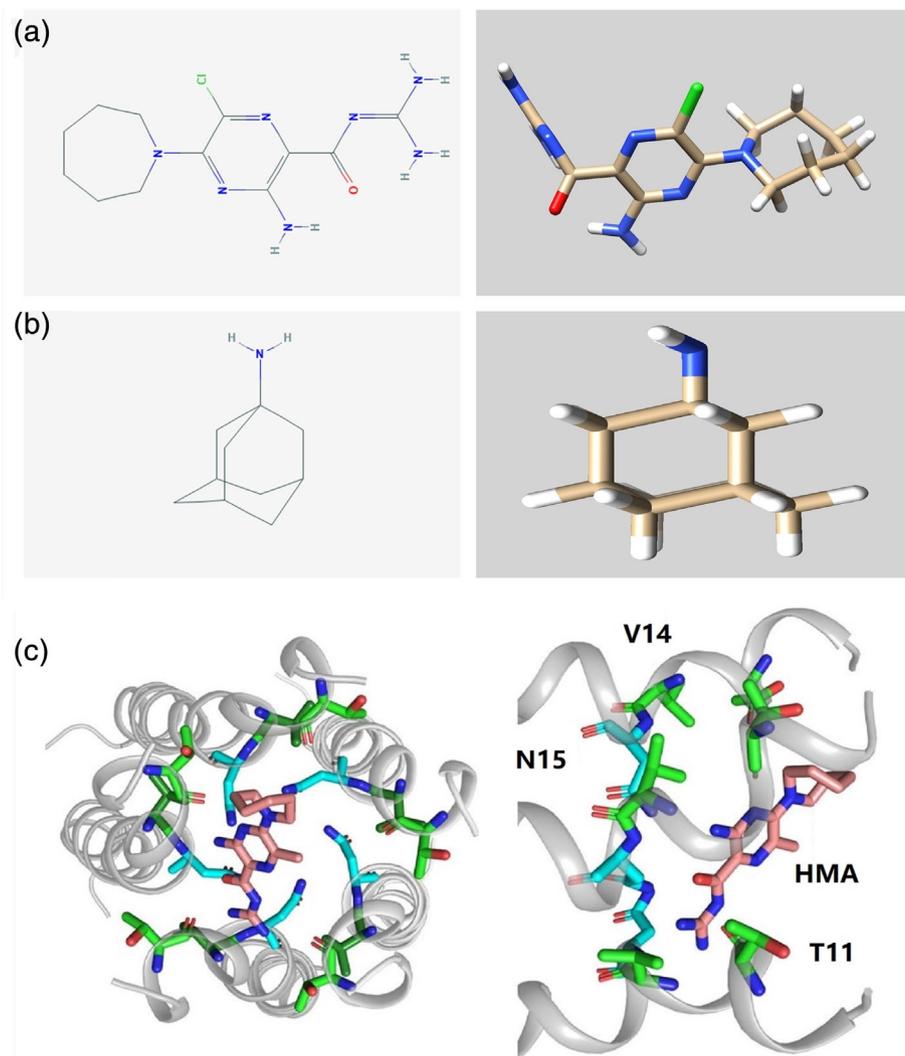
FIGURE 3 Molecular formula and 3D chemical structure of hexamethylene amiloride (HMA) and amantadine (AMT) (a and b). Docking region for HMA in the coronavirus (CoV)-2 transmembrane domain (TMD) (c)
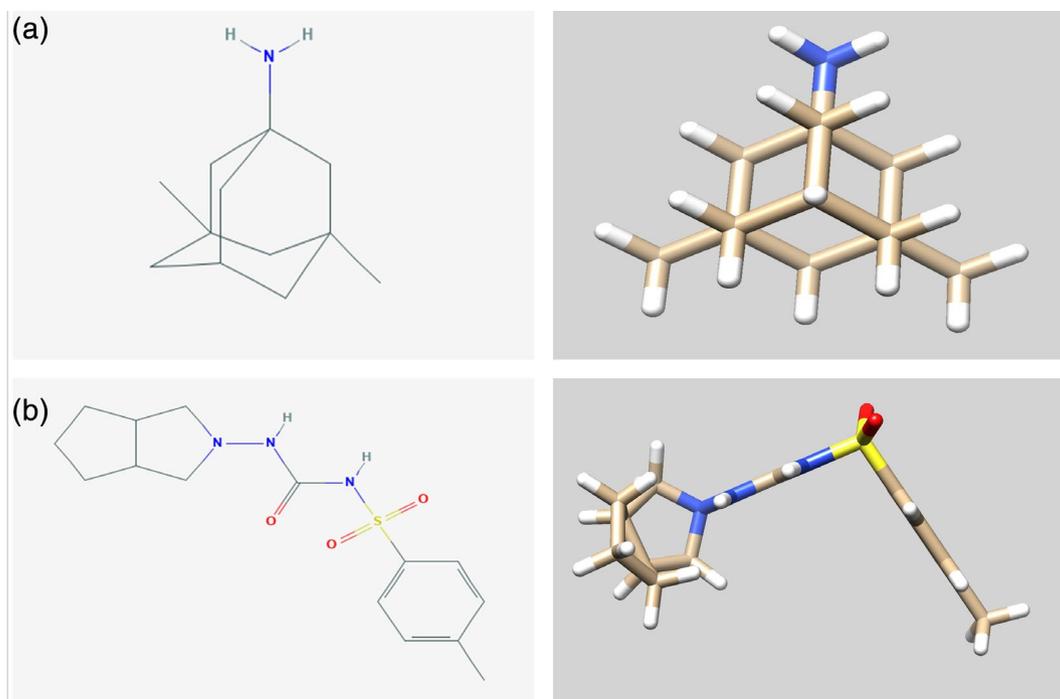
4.2 | Memantine and gliclazide

Memantine is a derivative of AMT (Figure 4a), which can inhibit the virus by blocking the M2 proton channel of influenza virus. It is the earliest antiviral drug used to inhibit influenza virus.[125] It is also inhibits the NMDA receptor, similar to the inhibitory effect of AMT on the virus.[129] Memantine has anti-inflammatory effects. It reduces the release of cytokines (TNF-α, IL-6, and IFN) through several mechanisms, inhibits the expression of NMDA receptor 1, and has a regulatory effect on Ca^{2+}.[130] It prevents and reverses the expression of some inflammatory factors, restores them to normal levels, and alleviates inflammation.[131]

Gliclazide, the molecular formula of which is $C_{15}H_{21}N_3O_3S$ (Figure 4b), is a sulfonylurea oral antidiabetic drug that is widely used in the treatment of Type 2 diabetes.[132] There is evidence that gliclazide and some diabetes drugs have a potential therapeutic effect on neocoronary pneumonia, but the exact mechanism remains unknown.[133,134]

Tomar et al. screened 372 compounds in the MedChemExpress library and found that memantine and gliclazide are potential E protein channel inhibitors. Tipton et al. suggested that the inexpensive drug memantine could be useful in COVID-19 therapy.[135] The negative/positive comparison experiment showed that the blockade of channel conductance by the compound had a significant effect on the growth of bacteria expressing the SARS-CoV-2 E protein. However, while the E protein is essential for bacteria, gliclazide and memantine are harmful to bacterial growth. This result suggested that the two compounds are potential channel inhibitors.

4.3 | ZINC23221929 and ZINC06220062

Tomar et al. used virtual screening methods to analyze 7,000 different compounds in the ZINC database[136] and obtained 10 possible inhibitors of the E protein viroporin. Then, they identified two compounds (ZINC ids

FIGURE 4 Molecular formulas and 3D chemical structures of memantine and gliclazide (a and b)

ZINC23221929 and ZINC06220062) by using saturation transfer difference (STD) NMR spectroscopy.[137] The chemical names of the two potential inhibitors are [2-[(3S)-2,3-dihydro-1,4-benzodioxin-3-yl]methylamino]-2-oxo-ethyl](2S)-2-(1,3-dioxoisoindolin-2-yl)-4-methylsulfanyl-butanoate (ZINC23221929) and 2-(2-amino-2-oxo-ethoxy)-*N*-benzyl-benzamide (ZINC06220062) (Table 1).

The molecular formula of ZINC23221929 is $C_{24}H_{24}N_2O_7S$. There are two loop systems of this inhibitor that can bind with the I46, L51, and P54 residues of the E protein, found to be engaged in hydrophobic interactions and with S60, C44, and V47 via H-bonding interactions. The 4-methylthiobutyrate fragment and L31, L34, and C40 maintain the hydrophobic interaction of the alkyl group. The molecular formula of ZINC06220062 is $C_{16}H_{16}N_2O_3$, in which the 2-oxo functional group in the 2-amino-2-oxo-ethoxy fragment is engaged in a crucial H-bonding interaction with the S60 residue of the C-terminal domain of the E protein. The phenyl moiety anchoring the ethoxy amine fragment and the *N*-phenyl moiety are hydrophobic interactions with the V47 and C40 residues and L28, L31, and Y57 residues, respectively. STD NMR showed that ZINC06220062 has a stronger binding affinity than ZINC23221929, and this difference may be related to the structural orientation of aromatic groups. The stable binding of these two inhibitors to the C-terminus of the E protein may inhibit its function, which also shows that the C-terminus can be potential targeted by inhibitors, thereby preventing viral assembly and replication.

4.4 | In silico prediction of SARS-CoV-2 viroporin inhibitors

The SARS-CoV-2 viroporin inhibitors were predicted by computational methods. Many studies predicted SARS-CoV-2 E protein viroporin inhibitors based on high-throughput computer screening and computational modeling methods.[138] Although the inhibitors have not been experimentally verified, they can save a large amount of money and investment required by laboratory methods to identify new drugs.[139]

4.4.1 | Tretinoin

Tretinoin, also known as retinoic acid, is a metabolic intermediate product of vitamin A in the body that mainly affects bone growth and promotes epithelial cell proliferation, differentiation, and keratolysis. It is generally used to treat skin diseases.[140] Tretinoin is also considered an effective HBV inhibitor. In the RAR agonist family, the biological activity of retinoic acid is demonstrated through different RAR receptors. All-trans retinoic acid can restore the HBV core-specific response of T cells by targeting myeloid-derived suppressor cells in isolated peripheral blood mononuclear cells in patients and mice.[141] Based on experimental evidence, tretinoin is an inhibitor of influenza virus A.[142]

The composition of SARS-CoV-2 E protein TMD residues (from 18 to 26) is characterized by hydrophobic

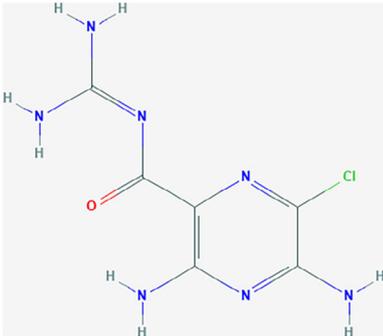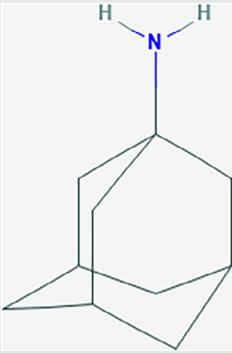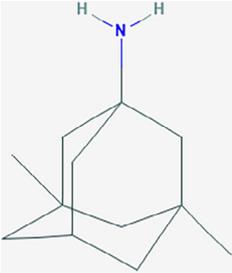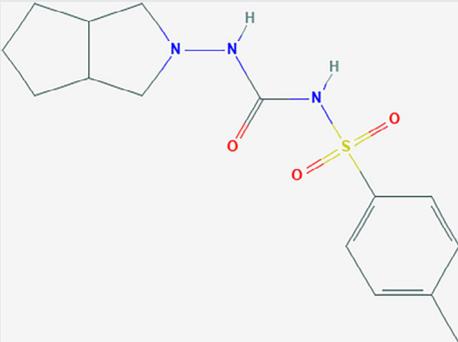**TABLE 1** Potential inhibitors of the SARS-CoV-2 E protein

Compound name	ZINC id	PubChem id	Chemical formula	Structure	Rings	Binding site
Amiloride (HMA)	ZINC4340269	16231	$C_6H_8C_1N_7O$		1	Thr9, Glu8, Gly10, Thr11, Ile13, Asn15, Ser16
Amantadine (AMT)	ZINC968256	2130	$C_{10}H_{17}N$		4	Thr9, Gly10, Thr11, Ile13
Memantine	ZINC3812933	4054	$C_{12}H_{21}N$		4	/
Gliclazide	ZINC523925	3475	$C_{15}H_{21}N_3O_3S$		3	/
Tretinoin	ZINC22066351	444795	$C_{20}H_{28}O_2$		1	Leu18, Leu21, Ala22, Val25, Phe26, Ala22, Val25, Phe26, Phe4
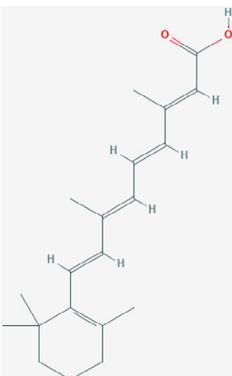
TABLE 1 (Continued)
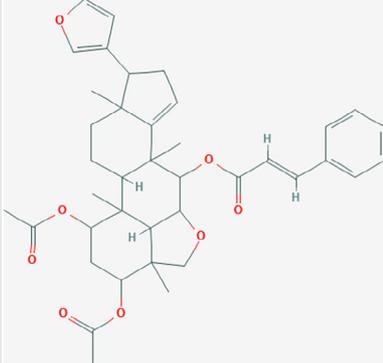
Compound name	ZINC id	PubChem id	Chemical formula	Structure	Rings	Binding site
Rutin	ZINC4096846	5280805	$C_{27}H_{30}O_{16}$		5	Leu31, Thr35, Ser55, Arg69, Pro71
Doxycycline	ZINC16052277	54671203	$C_{22}H_{24}N_2O_8$		4	Leu31, Thr35, Val52, Ser55
Nimbolin A	/	6443004	$C_{39}H_{46}O_8$		6	Leu18, Leu19, Leu21, Ala22, Val25, Phe26
/	ZINC23221929	30927842	$C_{24}H_{24}N_2O_7S$		4	Ile46, Leu51, P54 S60, C44 Val47, Leu31, Leu34, Cys40
/	ZINC06220062	7917802	$C_{16}H_{16}N_2O_3$		2	Leu28, Leu31, Pro51, Tyr57 Val42, Cys40, Cys44, Thr35
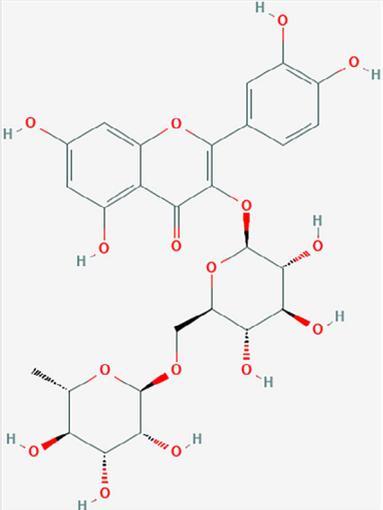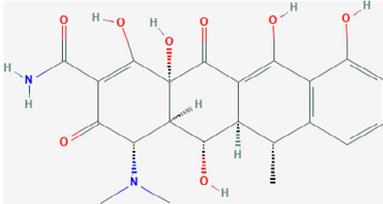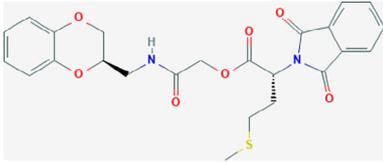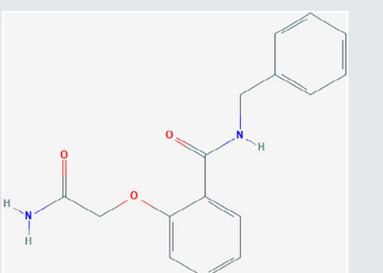
(a)

(b)

(c)
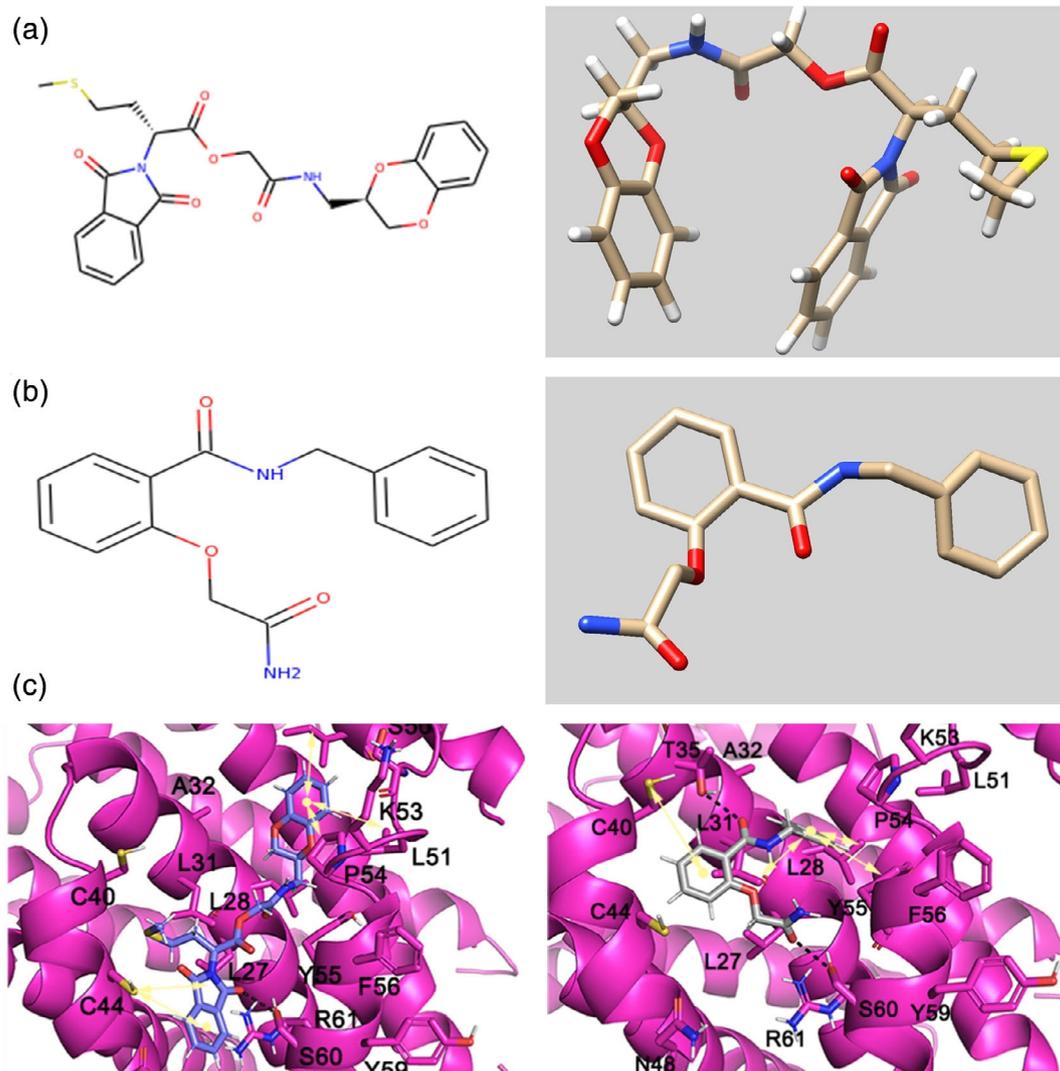
FIGURE 5 Molecular formulas and 3D chemical structures of ZINC23221929 (a) and ZINC06220062 (b). Cartoon representation of the binding mode of two ligands with the E protein (c)

amino acids. Dey et al. applied a combination of all-atom molecular dynamics simulations, including H-bond and binding energy analysis (MM-PBSA), to evaluate binding affinity, and tretinoin showed the strongest hydrophobic interactions in the viroporin pore. Tretinoin exhibits the ability to form extensive H-bond interactions and shows high binding energy (−412.8 kJ/mol) with the hydrophobic side chains of the E protein pores; it has been selected as the best candidate viroporin inhibitor.[143]

4.4.2 | Rutin and doxycycline

Bhowmik et al. used molecular docking to screen rutin and the antibiotic doxycycline as the strongest inhibitors of the SARS-CoV-2 E protein viroporin among 548 antiviral compounds (Figures 5 and 6).[144] Rutin is a natural flavonoid glycoside. Studies have shown that it can

inhibit many viruses.[145–147] Doxycycline is a tetracycline antibiotic that can be used to treat *Mycoplasma* and *Chlamydia* infections and is also an antiviral.[148,149] Rutin binds to the five active-site residues of the E protein TMD via H-bonds, while doxycycline interacts with the other four residues, which can effectively bind to the E protein to inhibit the formation of viroporin.[150]

4.4.3 | Nimbolin A and others

The computational model suggested that nimbolin A, nimocin, 7-deacetyl-7-benzoylgedunin, 24-methylenecycloartanol, and cycloeucalenone are potential E protein viroporin inhibitors. Among them, the binding energy of nimbolin A with viroporin is the highest.[151]

An in silico method can predict SARS-CoV-2 E protein pentamer inhibitors quickly and at low cost.
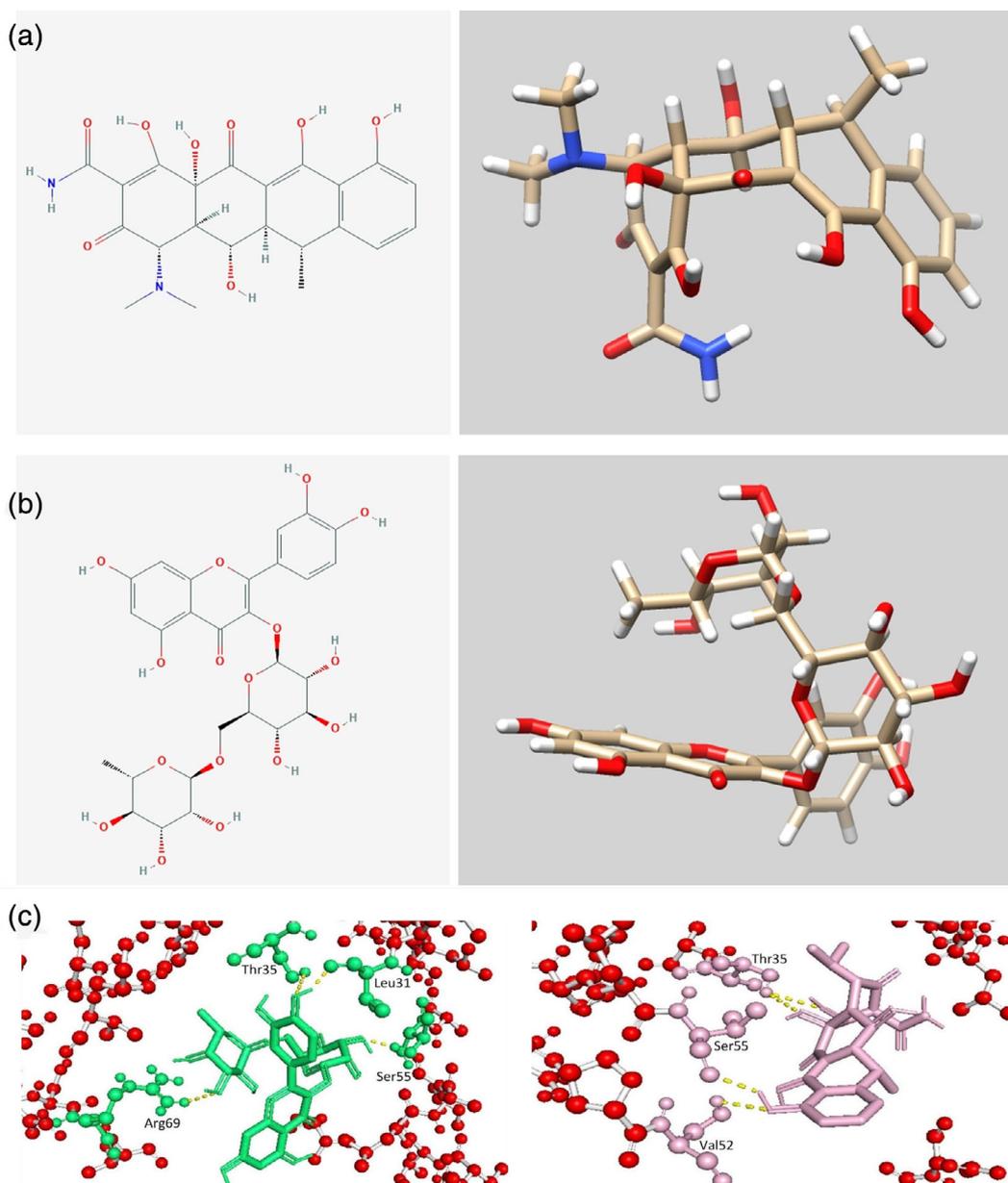
FIGURE 6 Molecular formulas and 3D chemical structures of rutin and doxycycline (a and b). Docking region for rutin and doxycycline in the coronavirus (CoV)-2 E protein (c)

However, candidate inhibitors based on in silico approaches must be subjected to further experimental investigations.

5 | CONCLUSION

Studies on the SARS-CoV and SARS-CoV-2 E proteins indicate that the E protein could be involved in multiple important aspects, from the assembly and induction of membrane curvature to division or budding and release to apoptosis, inflammation and even autophagy.[152] Because the amino acid sequence and protein structure

of the SARS-CoV and SARS-CoV-2 E proteins are highly homologous, their multimer formation mechanisms could be similar, which is reflected in their characteristics. The E protein is involved in many aspects of the viral replication cycle through the formation of oligomers and viroporins. The ion selectivity of the viroporin in the ER/Golgi membrane strongly affects the maintenance of the ion balance of the host cell microenvironment, pH and TMV. There are limited studies on the mechanism of the E protein viroporin in virus-infected cells according to the current understanding. Although in vitro experiments have shown that some inhibitors can effectively block the E protein viroporin of SARS-CoV-2 and weaken

or abolish the virulence of the virus, their exact therapeutic effect remains to be further explored.

AUTHOR CONTRIBUTIONS

Yipeng Cao: Conceptualization; funding acquisition; writing-original draft. **Rui Yang**: Data curation; investigation. **Imshik Lee**: Funding acquisition; investigation; writing-review and editing. **Wenwen Zhang**: Data curation; investigation. **Jiana Sun**: Investigation; writing-original draft. **Wei Wang**: Conceptualization; project administration. **Xiangfei Meng**: Investigation; project administration.

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