Targeting the Dimerization of the Main Protease of Coronaviruses: A Potential Broad-Spectrum Therapeutic Strategy

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ABSTRACT: A new coronavirus (CoV) caused a pandemic named COVID-19, which has become a global health care emergency in the present time. The virus is referred to as SARS-CoV-2 (severe acute respiratory syndrome-coronavirus-2) and has a genome similar (∼82%) to that of the previously known SARS-CoV (SARS coronavirus). An attractive therapeutic target for CoVs is the main protease (Mpro) or 3-chymotrypsin-like cysteine protease (3CLpro), as this enzyme plays a key role in polyprotein processing and is active in a dimeric form. Further, Mpro is highly conserved among various CoVs, and a mutation in Mpro is often lethal to the virus. Thus, drugs targeting the Mpro enzyme significantly reduce the risk of mutation-mediated drug resistance and display broad-spectrum antiviral activity. The combinatorial design of peptide-based inhibitors targeting the dimerization of SARS-CoV Mpro represents a potential therapeutic strategy. In this regard, we have compiled the literature reports highlighting the effect of mutations and N-terminal deletion of residues of SARS-CoV Mpro on its dimerization and, thus, catalytic activity. We believe that the present review will stimulate research in this less explored yet quite significant area. The effect of the COVID-19 epidemic and the possibility of future CoV outbreaks strongly emphasize the urgent need for the design and development of potent antiviral agents against CoV infections.

KEYWORDS: 3CLpro, broad-spectrum antiviral agents, coronavirus, COVID-19, dimerization, homodimer, main protease (Mpro), mutation, SARS-CoV, SARS-CoV-2

INTRODUCTION

Coronaviruses (CoVs) have been known since 1947, when the first prototype murine strain JHM was reported. CoVs are enveloped viruses consisting of single positive-strand RNA, and they infect various vertebrates (bats, pets, livestock, poultry, and humans). Among humans, CoVs are responsible for respiratory, gastrointestinal, and neurological problems. CoVs belong to subfamily Coronavirinae of the family Coronaviridae. The Coronavirinae is further subdivided into four genera (α, β, γ, and δ). Each genus is further divided into four lineage subgroups.

A new coronavirus resulted in the outbreak of a pneumonia-like illness in Wuhan, China, in late December 2019, and has become a life-threatening concern worldwide in the present time. The virus has been termed SARS-CoV-2 (severe acute respiratory syndrome-coronavirus-2), as the RNA genome is ∼82% similar to that of the SARS coronavirus (SARS-CoV). SARS-CoV-2 belongs to the β-coronavirus group. The pneumonia-like illness caused by SARS-CoV-2 was named as COVID-19. Many patients infected with COVID-19 suffer from fever, dry cough, tiredness, and breathing difficulty under severe conditions; others may be just silent carriers of the virus. The World Health Organization (WHO) declared COVID-19 a pandemic on March 11, 2020. As of 2:00 am CEST, May 6, 2020, there were more than 3.5 million confirmed cases globally with 245,150 deaths due to the SARS-CoV-2. The figures clearly indicate that COVID-19 imposes a huge health care crisis globally. The scientific and medical fraternity across the world have been working tirelessly and at record-breaking speed to find a solution to bring this virus outbreak under control; however, no success has been achieved at the time of publication of this review.

Similar to SARS and MERS (Middle East respiratory syndrome), the genome of SARS-CoV-2 encodes non-structural proteins [SARS-CoV-2 Mpro (main protease), also known as 3-chymotrypsin-like cysteine protease (CCP or 3CLpro), papain-like protease, and RNA-dependent RNA polymerase (RdRp)], helicase, structural proteins (spike glycoprotein), and accessory proteins. The non-structural proteins play a key role during the

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The spike glycoprotein is necessary for the interactions of the virus with the host cell receptors during viral entry. The non-structural and structural proteins were recognized as promising targets for the design and development of antiviral agents against SARS and MERS. SARS-CoV-2Mpro plays a key role in polyprotein processing and is active in a dimeric form. The Mpro offers a promising target for the development of broad-spectrum anti-coronaviral therapeutic agents due to its highly conserved three-dimensional structure among various CoVs (Figures 1 and 2). The CoVs are subject to extensive mutagenesis; however, key proteins are highly conserved, as mutations in key proteins are often lethal to the virus. Thus, drugs targeting conserved Mpro are usually capable of preventing the replication and proliferation of the virus and display broad-spectrum antiviral activity. In addition, drugs targeting Mpro can reduce the risk of mutation-mediated drug resistance in future deadly viral strains.

The individual monomers of SARS-CoV Mpro are enzymatically inactive, and two strategies have been employed to develop inhibitors against this enzyme: (i) molecules targeting the substrate binding pocket to block the catalytic activity, and (ii) dimerization inhibitors. Numerous reports on the inhibitor design against SARS-CoV Mpro are based on the substrate binding pocket. However, no inhibitor targeting the substrate binding pocket has reached clinical trials to date. An alternative potential therapeutic strategy is to inhibit the dimerization of Mpro, and there are a few reports on inhibitors targeting the dimerization of SARS-CoV Mpro.

In the present review, literature reports highlighting the effect of mutations and N-terminal deletion of residues of SARS-CoV Mpro on its dimerization and, thus, catalytic activity are compiled. To the best of our knowledge, this review is the first compilation of the various studies focusing on the dimerization of SARS-CoV Mpro. A number of inhibitors targeting the substrate binding pocket of SARS-CoV Mpro are reported in the literature, and they can be found discussed elsewhere.

### Structural and Functional Details of SARS-CoV-2 Mpro Enzyme

SARS-CoV-2 Mpro is a dimer consisting of two monomers that are arranged almost perpendicular to one another. Each monomer comprises three domains and possesses a catalytic dyad (His41 and Cys145) situated in a cleft between domains I and II (residues 10–99 and 100–182, respectively). The catalytic residues are situated in the chymotrypsin-like double β-barrel fold consisting of domain I and II. The catalytic domains are connected by a long loop region to the C-terminal domain III (residues 198–303) composed of five antiparallel α-helices. A contact interface (∼1394 Å²) was formed between domain II of monomer A and the NH2-terminal residues (“N-finger”) of monomer B in the dimeric structure of SARS-CoV-2 Mpro. The dimerization is necessary for enzymatic activity as the N-finger of each of the two monomers interact with Glu166 of the other monomer, which assist in the correct orientation of the S1 pocket of the substrate binding site. The C- and N-terminus of the monomers constitute the dimer interface and are closely held in the dimer than in the monomeric state where mobility of these termini are higher. The structural design of SARS-CoV-2 Mpro was found to be similar to the crystal structure of SARS-CoV Mpro (Figure 1a,b). Only 12 out of 306 residues are...
Table 1. List of Important Residues along with Their Key Roles in SARS-CoV Mpro

| residue | major role in SARS-CoV Mpro | references |
|---------|-----------------------------|------------|
| His41, Cys145 | catalytic dyad | Huang et al.,20 Shan et al.31 |
| His41, Met49, Gly143, Ser144, His163, His164, Met165, Glu166, Leu167, Asp187, Arg188, Gin189, Thr190, Ala191, Gin192 | substrate binding | Muramatsu et al.,22 Hsu et al.21 |
| Arg4, Ser10, Gly11, Glu14, Asn28, Ser139, Phe140, Ser147, Glu290, Arg298 | dimerization | Chou et al.,24 Barrila et al.,25 Chen et al.,26 Chen et al.,27 Lin et al.,24 Shiet al.,24 Hu et al.,30 Barrila et al.31 |

![Figure 3](image-url) - Three-dimensional crystal structure of SARS-CoV Mpro (PDB ID: 1UK4) is shown. SARS-CoV Mpro is a homodimer and the two monomers of the dimer are shown in light blue and orange. The three domains of the SARS-CoV Mpro monomer are labeled by Roman numbers. The catalytic dyad comprised of His41 and Cys145 are shown as blue, and yellow spheres, respectively. [An asterisk on His41 and Cys145 depict that these residues belong to monomer B (orange)]. The chain termini are labeled N and C for monomer A (light blue) and N* and C* for monomer B (orange). The magnified figure depict key residues of monomer A (Arg4, Ser10, Gly11, Glu14, Asn28, Ser139, Ser144, Ser147, Glu166, Glu290, Arg298, Gin299) in the stick representation that can be targeted to inhibit the dimerization of SARS-CoV Mpro. The other residues of monomer A were not shown in the magnified figure for the better clarity of the residues involved in the stabilization of the dimer structure of SARS-CoV Mpro. The figure was generated using PyMoL.

**Figure 3**

Different in SARS-CoV-2 Mpro as compared to SARS-CoV Mpro (96% sequence identity). Further, none of the 12 variant residues (T35V, A46S, S65N, L86V, R88K, S94A, H134F, K180N, L202V, A267S, T285A, I286L) are involved in any major roles in the enzymatic activity of SARS-CoV-2 Mpro. The overall structure of SARS-CoV-2 Mpro was not affected by mutations, and the structure fully superimposed on the SARS-CoV Mpro structure (Figure 2).

The homology models of SARS-CoV-2 Mpro were found to be very much similar to SARS-CoV Mpro.16 Thus, inhibitors targeting SARS-CoV Mpro may also block the enzymatic activity of SARS-CoV-2 Mpro. Previous studies highlighted that many SARS-CoV Mpro inhibitors displayed efficacy against MERS-CoV.17,18 The SARS-CoV Mpro exists as a homodimer in the crystal structure,19 and the important residues along with their key roles are listed in Table 1. The key residues that stabilize the dimeric structure of SARS-CoV Mpro are shown in Figure 3.

The Mpro play a vital role in cleaving the polyproteins translated by the virus RNA.32 The Mpro cleave the large polyprotein 1ab (replicase 1ab, ~790 kDa) at 11 different sites and the recognition sequence at most sites was found to be Leu−Gln↓(Ser/Ala/Gly) (↓ shows the cleavage site). The replication of the virus can be efficiently blocked by inhibiting Mpro activity. As no human proteases with an analogous cleavage specificity were reported, the inhibitors against Mpro are not likely to be toxic.

## EFFECT OF MUTATIONS AND N-TERMINAL TRUNCATION ON THE DIMERIZATION AND CATALYTIC ACTIVITY OF SARS-CoV Mpro

The various mutation analyses, N-terminal truncation studies, and MD simulation studies that highlighted key residues of SARS-CoV Mpro involved in the stabilization of the catalytically active dimeric structure of the enzyme are listed in Table 2 and are arranged in chronological order.

In 2004, Bacha et al. identified a cluster of conserved serine residues (Ser139, Ser144, and Ser147) situated in the close proximity of the active site of SARS-CoV Mpro that can be targeted to inhibit the protease activity.33 The alamine substitution of Ser139, Ser144, and Ser147 had a devastating impact on the SARS-CoV Mpro catalytic activity. The serine cluster (Ser139, Ser144, and Ser147) is highly conserved in proteases among various CoVs, which, in turn, highlight that targeting this site will provide broad-spectrum therapeutic agents against CoV protease. In a later report, Barrila et al. highlighted that Ser147 of SARS-CoV Mpro play a key role in stabilizing the dimeric structure and mutation of conserved Ser147 to Ala lead to dimer instability.35 The backbone of Ser147 forms hydrogen bonds with the backbones of Ser144 and His163 residues. A 150-fold reduction in the catalytic efficiency and complete loss of dimerization was observed in S147A mutant as compared to wild-type (wt) enzyme.

In another study, Chou et al. reported that high salt concentration and low pH led to a decrease in the Mpro dimerization and activity.34 The observed decrease in activity of Mpro was attributed to the salt bridge interaction between Arg4 and Glu290 residues. The study highlighted that E290A mutation led to a complete loss of catalytic activity and...
Table 2. List of Various Mutation Analyses, N-Terminal Truncation Studies, and MD Simulation Studies of SARS-CoV Mpro

| entry | residues | key findings | references |
|-------|----------|--------------|------------|
| 1     | S139A, S144A, S147A | S139A, S144A, and S147A mutations have a devastating effect on the catalytic activity of SARS-CoV Mpro. | Bacha et al.33 |
| 2     | S147A    | A 150-fold reduction in the catalytic efficiency and complete loss of dimerization were observed in S147A mutant as compared to wild-type (wt) enzyme. | Barrila et al.25 |
| 3     | R4A, E290A | E290A mutation led to a complete loss of catalytic activity and dimerization, whereas R4A mutation resulted in an approximately 5-fold decrease in the dimerization and a modest loss in the enzymatic activity. | Chou et al.24 |
| 4     | N-terminal truncated (residues 1–7) SARS-CoV Mpro | N-terminal truncated protease dimer adopts a different state as compared to the full-length protease dimer; MD simulations depicted that the angle between the two monomers increased and the dimension of the substrate binding pocket was reduced in the N-terminal truncated protease dimer, which is not appropriate for the substrate binding. | Chen et al.35 |
| 5     | N-terminal truncated SARS-CoV Mpro | N-terminal truncated (residues 1–31) SARS-CoV Mpro exists predominantly as a dimer with 76% enzymatic activity; however, N-terminal truncated (residues 1–4) SARS-CoV Mpro exists mostly as a monomer with very little enzymatic activity. Both N- and C-terminal regions affect the dimerization and enzymatic activity of the SARS-CoV Mpro. | Hsu et al.23 |
| 6     | SARS-CoV Mpro | Size and conformation of the substrate binding pocket S1 are linked to the protonation states of the histidine residues (His163 and His172) comprising the pocket. The N-terminus of another monomer in the protease dimer plays a critical role in the catalytic activity by sustaining the correct conformation of the oxyanion loop and substrate binding pocket S1 through hydrogen bonds. | Tan et al.36 |
| 7     | C145A    | Analytical ultracentrifugation experiments depicted that a dimer was formed in the mature enzyme (Kd = 0.35 nM) as compared to the C145A mutant possessing 10 additional N-terminal (Kd = 17.2 nM) or C-terminal residues (Kd = 5.6 nM). The inhibitors targeting the dimer interface may block the maturation of protease, as both N and C termini are near to the SARS-CoV Mpro active site in the product-bound C145A structure. | Hsu et al.23 |
| 8     | dimerization inhibitor (SGFRKMAF) | Thermodynamic analysis highlighted that hydrophobic contacts along with electrostatic force play major roles in the binding of dimerization inhibitor with Mpro. | Ding et al.34 |
| 9     | N-terminal octapeptide (N8) as dimerization inhibitor of SARS-CoV Mpro | N-terminal octapeptide (SGFRKMAF, or N8) inhibited dimerization of the Mpro with a Ki of 2.20 mM; hydrophobic contact of Met6 and electrostatic interaction of Arg4 of N8 contributed significantly in its binding with the enzyme. | Wei et al.15 |
| 10    | hybrid SARS-CoV Mpro between the wt enzyme and the inactive mutant C145A | The simulation and experimental results concluded that (i) dimerization was a mandatory requirement for the enzymatic activity of the protease, and (ii) only one monomer in the protease dimer displayed catalytic activity. | Chen et al.22 |
| 11    | SARS-CoV Mpro | SARS-CoV Mpro exists as a homodimer in its active form. The biochemical and biophysical data depicted a monomer–dimer equilibrium with a dissociation constant Kd ≈ 6 μM. | Graziano et al.30 |
| 12    | MD simulations of dimeric and monomeric forms of SARS-CoV Mpro | MD simulations highlighted that the interactions between the N-terminus of one monomer and another monomer of the protease helped to maintain the dimer’s enzymatic activity. | Zheng et al.21 |
| 13    | S1A, F2A, R4A, S10A, E14A, S139A, F140A | The Ser10 and Gln14 residues located in the α-helix A’ of domain I of SARS-CoV Mpro are highly conserved among various CoV proteases and contribute significantly in the monomer–monomer interactions. The individual mutations of Ser10 and Gln14 to Ala resulted in weak dimerization and no enzymatic activity. | Chen et al.26 |
| 14    | G11A     | G11A mutation led to a complete loss in the enzymatic activity of SARS-CoV Mpro. The G11A mutant structure was the first reported crystal structure of the monomeric SARS-CoV Mpro, and the structure provided a better understanding of the dimerization and catalytic mechanism of the protease. | Chen et al.27 |
| 15    | S123A, S123C, S139A, and double mutants S123A/R298A, S139A/Q299A | Deletion of Gln299 or Arg298 significantly decreased the catalytic activity to only 1–2% of wt enzyme, and the enzyme existed predominantly in the monomeric form. The point mutants of Gln299 and Arg298 depict that these residues are involved in dimerization and play a key role in fixing the catalytically active conformation of the enzyme. | Lin et al.28 |
| 16    | R298A    | R298A mutation leads to disruption of the dimeric structure as well as irreversible inhibition of the catalytic activity of the enzyme. | Shi et al.29 |
| 17    | R298A in the presence of peptide substrate | Crystal structure of the R298A mutant of SARS-CoV Mpro in the presence of peptide substrate indicated the dimeric structure is the functional form of the R298A; however, minute changes were observed in the relative position of domain III of each monomer. | Wu et al.10 |
| 18    | Mpro-C and N-finger deleted SARS-CoV Mpro | C-terminal domain (Mpro-C [residues 187–306]) of SARS-CoV Mpro exists in monomer and dimer forms, and Mpro-C dimer possesses a novel dimerization interface. The N-finger of SARS-CoV Mpro plays a critical role in the formation of the catalytically active dimer of SARS-CoV Mpro. | Zhong et al.31 |
| 19    | S139A and F140A | S139A and F140A on the dimer interface of SARS-CoV Mpro resulted in different conformational changes in the crystal structure of the enzyme. Ser139 of monomer A was involved in the hydrogen-bond interaction with Gln299 of monomer B, and S139A mutation resulted in the complete loss of dimerization. | Hu et al.30 |
| 20    | SARS-CoV Mpro | Substrate-induced dimerization is necessary for the enzymatic activity of SARS-CoV Mpro in the polyprotein. | Li et al.40 |
| 21    | SARS-CoV Mpro | The mutagenesis studies highlighted that Gln166 plays a linking role between the dimer interface and substrate binding site. | Cheng et al.40 |
| 22    | N28A     | N28A mutation led to a complete inactivation of the enzyme and a decrease of 19.2-fold in the dimerization Kd. | Barrila et al.35 |
dimerization, whereas R4A mutation results in approximately 5-fold decrease in the dimerization and modest loss in the enzymatic activity.

In 2005, Chen et al. utilized glutaraldehyde cross-linking SDS-PAGE, isothermal titration calorimetry, and size-exclusion chromatography techniques to characterize the dimerization ability of the full-length and N-terminal truncated SARS-CoV Mpro. MD and docking simulations highlighted that N-terminal truncated protease dimer adopts a different state as compared to the full-length protease dimer. MD simulations depicted that angle between the two monomers increased and the dimension of the substrate binding pocket reduced in the N-terminal truncated protease dimer, which is not appropriate for the substrate binding. Additionally, surface plasmon resonance highlighted that N-terminal truncated protease does not bind with the model substrate.

In 2005, Hsu et al. reported that N-terminal truncated (residues 1–3) protease exists predominantly as dimer with 76% enzymatic activity; however, N-terminal truncated (residues 1–4) protease exists mostly as monomer with very little enzymatic activity. The study indicated that Arg4 have an influential effect on the catalytic activity and dimeric structure of the protease. The last C-terminal helically truncated protease also displayed a higher tendency to exist as a monomer and displayed little activity. These observations highlighted that both N- and C-terminal regions affect the dimerization and enzymatic activity of the SARS-CoV Mpro. The study provided key insights for the novel design of inhibitors targeting the dimer interface of SARS-CoV Mpro.

In another study, the conformational flexibility of the SARS-CoV Mpro was investigated by analyzing several crystal structures and MD simulations. The size and conformation of the substrate binding pocket S1 are linked to the protonation state of His163 and His172 comprising the pocket. The study highlighted that the N-terminus of another monomer in the protease dimer plays a critical role in the catalytic activity in sustaining the correct conformation of the oxyanion loop and substrate binding pocket S1 through hydrogen bonds.

In 2005, Hsu et al. reported the crystal structure of the product-bound C145A mutant protease and suggested the maturation mechanism of the enzyme. The analytical ultracentrifugation experiments depicted that a tight dimer was formed in the mature enzyme (Kd = 0.35 nM) as compared to C145A mutant possessing 10 additional N-terminal (Kd = 17.2 nM) or C-terminal residues (Kd = 5.6 nM). The inhibitors targeting the dimer interface may block the maturation of protease as both N and C termini are near to the SARS-CoV Mpro active site in the product-bound C145A structure.

In 2005, Ding et al. studied the interaction between SARS-CoV Mpro and a dimerization inhibitor N8 (SGFKRMADF) by affinity capillary electrophoresis. The thermodynamic analysis highlighted that hydrophobic contacts and electrostatic interactions play major roles in the binding of dimerization inhibitor with SARS-CoV Mpro. In a later report by the same research group, N8 and its mutants were evaluated for their ability to act as dimerization inhibitors of SARS-CoV Mpro. The peptide cleavage assay highlighted that N8 inhibited the dimerization of protease enzyme with a dimerization inhibition constant (Ki) of 2.20 mM. The comparison between the inhibitory activities of N8 and its mutants indicated that hydrophobic contact of Met6 and electrostatic interaction of Arg4 of N8 contributed significantly in its binding with the enzyme.

In 2006, Chen et al. employed MD simulations and mutational studies to investigate that why dimer is catalytically active as compared to the monomer and whether both monomers in the dimer are active. The MD simulations depicted that the monomers are always catalytically inactive, two monomers comprising the dimer are asymmetric, and only one monomer display catalytic activity at a time. MD simulations also highlighted that the correct conformation required for the catalytic activity in one monomer can be induced by the formation of dimer. The simulation and experimental results concluded that (i) dimerization was a mandatory requirement for the enzymatic activity of the protease, and (ii) only one monomer in the protease dimer displayed catalytic activity.

In another study, Graziano et al. employed chemical cross-linking, enzyme kinetics and small-angle X-ray scattering techniques to investigate the oligomeric state of SARS-CoV Mpro. The SARS-CoV Mpro exists as a homodimer in its active form. The biochemical and biophysical data depicted a monomer–dimer equilibrium with a dissociation constant, Kd, of ~6 μM.

In 2007, Zheng et al. performed MD simulations of the dimeric and monomeric form of a SARS-CoV Mpro to get insight into the activity of the enzyme. The key interactions between the two monomers in the dimer were investigated and how these interactions help in maintaining the function of the dimer was studied. The study highlighted that the interactions between the N-terminus of one monomer with another monomer of the protease helped to maintain the dimer enzymatic activity. The key insights obtained from MD simulations will be beneficial in the design of specific protease inhibitors targeting the dimer interface of SARS-CoV Mpro enzyme.

Chen et al. identified critical residues involved in the SARS-CoV Mpro dimerization and activity by systematic mutation analysis. A total of seven residues on the dimer interface of the enzyme were selected to assess their influence on the catalytic activity and dimer stability by employing biophysical and biochemical techniques. The Ser10 and Glu14 residues located in the α-helix A’ of domain I of SARS-CoV Mpro are highly conserved among various CoVs proteases and contribute significantly in the monomer–monomer interactions. The individual mutations of Ser10 and Glu14 to Ala resulted in weak dimerization and no enzymatic activity. The results of the study will be beneficial in the better understanding of the dimerization activity relationship of SARS-CoV Mpro and will provide key insights for the design of antiviral compounds targeting the dimer interface of the SARS-CoV Mpro. Further Chen et al. reported that mutation of Gly11 residue situated at the dimer interface to Ala led to a complete loss in the enzymatic activity of SARS-CoV Mpro. A complete dimer dissociation in the crystal structure of G11A mutant was observed. The G11A mutation might shorten the α-helix A’ (Ser10→Gly15) of domain I, which led to the misorientation of the N-finger of the enzyme. As a result, N-finger could not properly squeeze into another monomer pocket during dimerization; thus resulting in the destabilization of the dimer structure. The hydrogen bond interactions between two helices A’[Ser10A⋯Ser10B and Gly11A/B⋯Glu14B/A (Ser10A and Ser10B indicate Ser10 of monomer A and B, respectively)] play a major role in the stability of the dimer interface. The G11A mutant structure was the first reported crystal structure of the monomeric SARS-CoV Mpro and provided a better understanding of the dimerization and catalytic mechanism of the protease.
In 2008, Grum-Tokars et al. reviewed the literature related to different SARS-CoV M\textsuperscript{pro} expression constructs and assays used to calculate the enzymatic activity.\textsuperscript{45} The enzymatic activity of SARS-CoV M\textsuperscript{pro} was significantly reduced in two cases: (i) on adding affinity-tags or non-native sequences to the N- or C-terminus of the protease enzyme, and (ii) when the concentration of the enzyme used in assays was below the equilibrium dissociation constant of the SARS-CoV M\textsuperscript{pro} dimer.

In 2008, Lin et al. analyzed the quaternary structure of the C-terminal truncated mutants of SARS-CoV-M\textsuperscript{pro} enzyme by employing sedimentation velocity and sedimentation equilbrium analytical ultracentrifugation techniques.\textsuperscript{28} The deletion of C-terminus from 306 to 300 does not affect the structure and catalytic activity of the enzyme. However, deletion of Gln299 or Arg298 significantly decreased the catalytic activity to only 1−2\% of wt enzyme, and the enzyme existed predominantly in the monomeric form. The point mutants of Gln299 and Arg298 depicted that these residues are involved in dimerization and played a key role in fixing the catalytically active conformation of the enzyme.

The monomeric crystal structure of the SARS-CoV M\textsuperscript{pro} R298A mutant was reported by Shi et al.\textsuperscript{29} The study highlighted that Arg298 play an important role in maintaining the dimer structure of the enzyme. The authors tried to solve two puzzles: (i) how the dimer–monomer switch was controlled, and (ii) why dimerization was necessary for the enzymatic activity. The results highlighted that R298A mutation leads to disruption of the dimeric structure as well as irreversible inhibition of the catalytic activity of the enzyme. In 2013, Wu et al. presented the crystal structure of R298A mutant of SARS-CoV-M\textsuperscript{pro} in the presence of a peptide substrate.\textsuperscript{30} The R298A mutant undergoes a reversible substrate induced dimerization with minute changes in the relative position of the domain III of each monomer as compared to wt M\textsuperscript{pro}. As indicated by active enzyme centrifugation (AEC) experiments, the kinetic parameters of the R298A mutant were identical with that of wt M\textsuperscript{pro}. The study provided key insights into the mechanisms that governed monomer–dimer switch during M\textsuperscript{pro} maturation process.

In 2008, Zhong et al. reported that C-terminal domain [M\textsuperscript{pro}, C (residues 187−306)] of SARS-CoV-M\textsuperscript{pro} exist as a monomer and dimer, and M\textsuperscript{pro}C dimer possess a novel dimerization interface.\textsuperscript{41} The N-finger deleted SARS-CoV-M\textsuperscript{pro} does not maintain the active dimer structure; however, form a new dimer that is not active. Thus, the N-finger of SARS-CoV M\textsuperscript{pro} play a critical role in the formation of catalytically active dimer of SARS-CoV M\textsuperscript{pro}. Later in 2009, the authors reported stable M\textsuperscript{pro}C dimer as the 3D domain-swapped dimer.\textsuperscript{42} The N-finger deleted M\textsuperscript{pro} also undergo 3D domain swapping of the C-terminal domains and form a stable dimer.

Next, Hu et al. reported that two adjacent mutations (S139A and F140A) on the dimer interface of SARS-CoV M\textsuperscript{pro} resulted in the different conformational changes in crystal structure of the enzyme.\textsuperscript{43} The S139A mutation resulted in the complete loss of dimerization. The Ser139 of monomer A was involved in the hydrogen bond interaction with Gln299 of monomer B. The study suggested that the cooperativity among all the key elements control the dimerization of SARS-CoV M\textsuperscript{pro} and the stability of the dimer greatly depends on the integrity of the dimer interface.

In 2010, Li et al. presented the maturation mechanism of SARS-CoV M\textsuperscript{pro} and concluded that substrate-induced dimerization is essential for the enzymatic activity of SARS-CoV M\textsuperscript{pro} in the polyprotein.\textsuperscript{44} A modified model for the M\textsuperscript{pro} maturation process was proposed in the study. In 2010, Cheng et al. demonstrated the significance of substrate-induced dimerization of M\textsuperscript{pro} to its catalytic mechanism.\textsuperscript{45} The results of the experimental studies highlighted that dimerization of M\textsuperscript{pro} was necessary for the protease activity. The mutagenesis studies highlighted that Glu166 plays a linking role between dimer interface and substrate binding site. The authors mentioned that the connection between dimer interface and substrate binding site by Glu166 may be universal in all proteases among various CoVs.

The another study highlighted that Asn28 was essential for the enzymatic activity and dimerization of SARS-CoV M\textsuperscript{pro}.\textsuperscript{46} The N28A mutation led to a complete inactivation of the enzyme and a decrease of 19.2-fold in the dimerization K\textsubscript{d}. The interactions between Asn28 and Cys117 play a key role in the dimer stability and enzymatic activity of SARS-CoV-M\textsuperscript{pro}. The residue Asn28, a buried residue, display interactions with catalytic loop and β-sheet region of SARS-CoV M\textsuperscript{pro} where Cys117 is present. The conformational switch of residues (Ser139, Phe140, and Leu141) in the catalytic loop region from standard loop conformation to a short 3\textsubscript{10} helical conformation lead to a diminished dimerization of M\textsuperscript{pro}. The N28A mutant crystal structure revealed about the critical role of Asn28 in preserving the structural integrity of the active site and in positioning critical residues that are involved in binding at the dimer interface and substrate catalysis.

### CONCLUSIONS AND FUTURE DIRECTIONS

COVID-19, a novel infectious disease caused by a single-stranded positive-sense RNA virus, has presented a serious worldwide public health care emergency. The whole world remains unprepared to efficiently control this infectious disease, despite the lessons learned from the previous coronavirus (CoV) infections that caused SARS and MERS. Scientists around the world are looking for effective and promising therapeutic antiviral agents as well as vaccine candidates for combating COVID-19. However, no specific antiviral drugs or effective vaccines for COVID-19 have been discovered to date. The genetic reshuffling, mutations, and interspecies transmission of the RNA viruses highlight the urgent need for the design and development of broad-spectrum antiviral drugs. Thus, a coherent effort is required to develop effective drugs and vaccines against CoV infections and other highly pathogenic viruses to decrease the devastating impact on human life. As the clinical drug discovery and development process is costly, time extensive, and difficult, the design and development of broad-spectrum antiviral drugs are of paramount importance. Thus, drugs targeting highly conserved proteins such as main protease (M\textsuperscript{pro}) among various CoVs will provide two advantages: (i) the potential for broad-spectrum antiviral activity, and (ii) reduced risk of mutation-mediated drug resistance.

The CAS data highlighted that SARS-CoV M\textsuperscript{pro} has drawn significantly more attention than other targets, and a large number of compounds with therapeutic potential have been identified against SARS-CoV M\textsuperscript{pro}. A total number of 49 patents and 2178 potential drug candidates have been listed in the CAS Registry of Chemical Substances for M\textsuperscript{pro}. CoV-infected patients administered with the HIV drug combination of lopinavir/ritonavir (Kaletra), a M\textsuperscript{pro} inhibitor, have shown considerable improvement (NCT04307693 and NCT04255017), highlighting M\textsuperscript{pro} as a high-value target for the development of drug candidates against CoVs.\textsuperscript{3,46} Another clinical trial study on
different combinations of protease inhibitors such as oseltamivir, favipiravir, and hydroxychloroquine (HCQ) is presently underway for treatment of COVID-19. Twenty-seven clinical trial studies registered with the U.S. National Library of Medicine database were underway on the protease inhibitors in CoV infections as of May 6, 2020. A number of reports have been published on the design of small-molecule and peptidomimetic inhibitors targeting the substrate binding pocket of SARS-CoV M\textsuperscript{pro}. However, no such inhibitor has advanced to clinical trials to date. An alternative approach includes the combinatorial design of peptide-based inhibitors that target the dimerization of SARS-CoV M\textsuperscript{pro} as a potential therapeutic strategy. Dimerization inhibitors have been successfully employed against HIV protease and other viral enzymes.

The various mutation analyses listed in the present review highlight the key residues of SARS-CoV M\textsuperscript{pro} that are crucial for the dimerization and thus catalytic activity of the enzyme. The studies provide future directions for the design of potential dimerization inhibitors against M\textsuperscript{pro}. The MD studies of M\textsuperscript{pro} compiled in the review will act as molecular guide for the structure-based design of potent dimerization inhibitors. In addition, the already reported dimerization inhibitors of M\textsuperscript{pro} will provide the framework for further modifications to design potent antiviral agents. The peptide-based interface inhibitors may provide better therapeutic options than small molecules, as the large surface area of the dimer interface of M\textsuperscript{pro} will be better targeted with peptide inhibitors. The peptide-based inhibitors have additional advantages over small molecules as drug candidates due to their greater chemical diversity, high specificity, low toxicity, possibility of rational design, low accumulation in tissues, and stability toward proteolytic cleavage (peptidomimetics). In parallel with the drug development, scientists around the globe are actively involved in developing rapid point-of-care diagnostic methods for SARS-CoV-2.

We believe that the combinational design of peptide-based dimerization inhibitors of M\textsuperscript{pro} provide an attractive approach to combat CoVs and that this review will stimulate research in this less explored yet highly relevant area. The previous research efforts toward the design of potent antiviral agents against SARS and MERS should be used to draw the line of defense more quickly against the novel deadly SARS-CoV-2. The present review provides strong groundwork for the design and development of novel dimerization inhibitors of SARS-CoV-2 M\textsuperscript{pro} for combating this mysterious and rapidly evolving virus on an invisible battlefield.

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**Notes**

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**ABBREVIATIONS**

AEC, active enzyme centrifugation; CAS, Chemical Abstracts Service; CCP, 3-chymotrypsin-like cysteine protease; CEST, Central European Summer Time; CoV, coronavirus; COVID-19, coronavirus disease 2019; HIV, human immunodeficiency virus; K\textsubscript{D}, dissociation constant; K\textsubscript{i}, dimerization inhibition constant; MERS-CoV, Middle East respiratory syndrome coronavirus; MD, molecular dynamics; M\textsuperscript{pro}, main protease; mM, millimolar; nM, nanomolar; RdRp, RNA-dependent RNA polymerase; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; WHO, World Health Organization; wt, wild-type

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