INTRODUCTION

Since the first reports of the novel coronavirus disease, Covid-19, originated in Wuhan, in December 2019, the newly emerged coronavirus, SARS-CoV-2, has spread rapidly causing a worldwide pandemic with disastrous global consequences on health and economy.1-3 As of October 4th 2020, there have been 216 countries affected, more than 3 400 000 confirmed cases and over 1 000 000 deaths.4

Over the past two decades, SARS-CoV-2 is the third documented human pathogenic coronavirus to result in a pandemic and/or an epidemic, after the severe acute respiratory syndrome-related coronavirus (SARS-CoV) and the Middle East respiratory syndrome-related coronavirus (MERS-CoV) that have resulted in epidemics in 2003 and in 2014, respectively.5

Coronaviruses (CoVs) are pleomorphic, large, enveloped RNA viruses that belong to the Coronaviridae family. Phylogenetically, CoVs can be classified into the following genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus.6 Primarily, they infect many species (mammals and birds) causing respiratory, renal, gastrointestinal, and neurological diseases.7

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Abstract
Coronaviruses represent global health threat. In this century, they have already caused two epidemics and one serious pandemic. Although, at present, there are no approved drugs and therapies for the treatment and prevention of human coronaviruses, several agents, FDA-approved, and preclinical, have shown in vitro and/or in vivo antiviral activity. An in-depth analysis of the current situation leads to the identification of several potential drugs that could have an impact on the fight against coronaviruses infections. In this review, we discuss the virology of human coronaviruses highlighting the main biological targets and summarize the current state-of-the-art of possible therapeutic options to inhibit coronaviruses infections. We mostly focus on FDA-approved and preclinical drugs targeting viral conserved elements.

KEYWORDS
antivirals, Coronaviruses, Covid-19, entry inhibitors, MERS, MERS-CoV, monoclonal antibodies, nucleoside analogues, plasma therapy, SARS, SARS-CoV, SARS-CoV-2
immunosuppressed patients. The other three species, SARS-CoV, MERS-CoV, and SARS-CoV-2, are associated with severe respiratory infections and multiple organ failures, causing considerable global health emergencies. SARS-CoV is the causative agent of the severe acute respiratory syndrome epidemic in China from November 2002 to July 2003 that caused 15% mortality in infected patients. MERS-CoV is the etiologic agent of the severe acute respiratory syndrome outbreak that emerged in the Middle East in 2012 with a significant case fatality rate of ~34%. SARS-CoV-2, like MERS-CoV and SARS-CoV, attacks the respiratory tract but it seems to cause infections with different clinical manifestations ranging from mild respiratory diseases to interstitial pneumonia with a consequent lower fatality rate. Current data indicate that it has a higher transmissibility compared to SARS-CoV.

All the pathogenic human CoVs are thought to be emerged from animal reservoirs, probably from the spillover of bats to intermediate animal hosts leading then to animal-human cross-species transmission. Alarmingly, due to the presence of several CoVs strains in animal reservoirs and their frequent recombination, interspecies jumping and new potential outbreaks are likely to emerge from time to time in the future and, for these reasons, effective drugs and therapies are clearly needed in order to fight present and future pathogenic infections.

At present, there are no approved drugs and therapies for the treatment and prevention of human CoVs. Although several pharmaceutical industries and research groups are working on the discovery and the development of new drugs and vaccines, drug development is a slow process that requires several years. Given the current emergency, the main adopted strategy regards the repurposing of FDA-approved drugs like antivirals approved for treating infections caused by influenza virus, HIV, hepatitis virus, etc., immunomodulatory agents and so on.

In this review, we discuss first the biology of the human pathogenic MERS-CoV, SARS-CoV, and SARS-CoV-2 highlighting the different biological targets that can be exploited for the design and development of antiviral drugs. Then, we summarize the current state of the art of possible therapeutic options to inhibit viral infections, focusing on both FDA-approved and preclinical drugs, dividing them into three main classes on the basis of the biological target. Therapeutic approaches aimed to alleviate symptomatology of CoVs infections are out of the scope and will not be reviewed.

1.1 Coronaviruses: virology and key biological targets

SARS-CoV, MERS-CoV, and SARS-CoV-2 belong to the Betacoronavirus group and possess large single-stranded RNA genomes of about 29.7, 30.1, and 29.8 kilobases in length, respectively. SARS-CoV-2 shares 79% sequence identity at genomic level with SARS-CoV, whereas it is more distant from MERS-CoV (approximately 50% of sequence conservation). Despite their genomic diversity, all CoVs share the same genome organization (Figure 1). The 5' terminal encodes a polyprotein, pp1ab, that is then processed by two viral proteases, the 3C-like protease (3CLpro) and the papain viral protease (PLpro) into non-structural proteins involved in replication and transcription processes, like RNA-dependent RNA polymerase (RdRp) and helicase. On the other hand, within the 3' terminal are encoded four typical coronavirus structural proteins in the following order: the spike glycoprotein (S), the envelope protein (E), the membrane protein (M), and the nucleocapsid protein (N); moreover, from this terminal are encoded also accessory proteins that are thought to affect the host immune response.

Among the structural proteins, the spike glycoprotein is particularly interesting since it mediates host-cell entry utilizing specific host receptors: angiotensin-converting enzyme 2 (ACE2) for SARS-CoV and SARS-CoV-2, and dipeptidyl peptidase 4 (DPP4) for MERS-CoV.

The host receptors are the main determinant of the pathogenesis and the tissue and cellular tropism of viruses. Among the structural proteins, the spike glycoprotein is particularly interesting since it mediates host-cell entry utilizing specific host receptors: angiotensin-converting enzyme 2 (ACE2) for SARS-CoV and SARS-CoV-2, and dipeptidyl peptidase 4 (DPP4) for MERS-CoV.

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ACE2 is an ectoenzyme involved in the regulation of the renin-angiotensin system that is expressed at high levels in lungs, kidneys, heart, and gastrointestinal tract. A significant overexpression of ACE2 has been reported in different airway epithelial cell types, in particular in alveolar and nasal epithelial cells, consistent with the pathogenesis and viral transmissibility of SARS-CoV and SARS-CoV-2. However, although ACE2 is involved in viral entry for SARS-CoV and SARS-CoV-2, transgenic mouse models lacking ACE2 appear to suffer much worse disease pathogenesis when subjected to mechanical and chemical lung injury. In this regard, in vitro and in vivo studies have revealed that SARS-CoV-2 causes a downregulation of ACE2 expression that contributes to a worsening of lung injury severity.

DPP4 is a transmembrane ectoenzyme expressed in different tissues like kidney, gastrointestinal tract, and hematopoietic cells. Regarding the respiratory tract, DPP4 showed a lower level of expression in nasal epithelial cells compared to alveolar cells, consistent with the pathogenesis and lower transmissibility of MERS-CoV compared to SARS-CoV and SARS-CoV-2. Furthermore, an overexpression of DPP4 in alveolar cells seems to be in patients with chronic respiratory diseases.

2 | THERAPEUTIC STRATEGIES ACTING ON THE VIRUS

Despite their genomic diversity, CoVs lifecycle steps share key biological elements, with a certain homology between different CoVs strains, that can be exploited for the design and development of antiviral drugs; promising biological elements include: RNA-dependent RNA polymerase, the two viral proteases 3CLpro and PLpro, the spike glycoprotein and, finally, the cellular proteases involved in viral entry, such as TMPRSS2 and cathepsins.

Although, currently, there are no approved drugs and therapies for the treatment and prevention of human CoVs, several agents, FDA-approved and preclinical, that target key viral conserved elements, have shown in vitro (Table 1) and in vivo antiviral activity, becoming potential drugs to use to fight CoVs infections. However, some of them used clinically during SARS, MERS, and Covid-19 outbreaks did not show any beneficial results.

In the light of this, in this review we summarize the current state of possible therapeutic approaches to inhibit viral infections, investigating both FDA-approved and preclinical drugs, and depending on the biological target, we will focus on three main classes targeting the RNA-dependent RNA polymerase, the two viral proteases 3CLpro and PLpro, and viral entry pathways, respectively.

2.1 | RNA-dependent RNA polymerase

CoVs use a multisubunit complex for RNA replication and transcription. This machinery is formed by nonstructural proteins, such as RdRp, nsp7, nsp8, and nsp12, produced by the cleavage of viral polyproteins. Among them, RdRp (nsp12) is the key element since it has a prominent role in catalyzing RNA synthesis assisted by nsp7 and nsp8 that act as cofactors.

Comparing the different coronaviral RdRp structures, they share a certain structural conservation. In particular, SARS-CoV and SARS-CoV-2 show a remarkable sequence conservation (96%) with variations distant to the catalytic site highlighting the possibility to have broad-spectrum antivirals for different CoVs infections.

Ribavirin (R) is a broad-spectrum guanosine analogue active against several RNA viruses, approved for the treatment of HCV and RSV infections. Ribavirin showed a moderate inhibitory effect on the replication of human CoVs in vitro and contrasting results in animal models of MERS-CoV and SARS-CoV infections. In particular, ribavirin administration in a mouse model of SARS-CoV infection caused an increase in viral load and a prolonged viral replication, while, in association with interferon-alpha2b, an improvement of the clinical outcome in a nonhuman primate model (rhesus macaques) of MERS-CoV infection. Ribavirin has been widely used during SARS and MERS outbreaks, however, clinical data are inconclusive and contradictory. For example, a retrospective multicenter non-randomized study reported a remarkable (93.5%) 21-day survival in SARS patients treated with ribavirin, however, due to the retrospectivity of this study and the absence of a control group, it is impossible to understand if the treatment regimen had a real beneficial effect on the clinical outcome. In this context, another clinical study highlighted the beneficial effects of the use of ribavirin in combination with corticosteroids, with resolution of symptoms and radiographic improvement in the majority of the patients. In contrast to this, there are several clinical studies in which the use of ribavirin, alone or in combination with corticosteroids or interferon, did not show any beneficial effects in patients with
**TABLE 1** List of FDA-approved and preclinical agents effective in vitro against SARS-CoV, MERS-CoV, and SARS-CoV-2 infections. The half-maximal effective concentration (EC$_{50}$) is referred to the inhibition of viral replication or induced cytopathic effect. An alphanumerical reference has been introduced for elucidated or hypothesized target. C refers to 3CLpro; E to viral entry pathways, P to PLpro; R to RdRp and T to translation.

| Compound                        | Status                                | Target                  | Cell lines                                           | SARS-CoV EC$_{50}$ (µmol/L) | MERS-CoV EC$_{50}$ (µmol/L) | SARS-CoV-2 EC$_{50}$ (µmol/L) | References   |
|---------------------------------|---------------------------------------|-------------------------|-----------------------------------------------------|-----------------------------|-----------------------------|-------------------------------|--------------|
| Flusprilene E$_1$               | Approved as antipsychotic agent       | Viral entry             | Vero E6 cells                                       | 5.963                       | 7.477                        |                                | [131]        |
| Chloropromazine E$_2$           | Approved as antipsychotic agent       | Viral entry             | Huh7 cells (MERS-CoV); Vero E6 cells (SARS-CoV)    | 8.8                         | 4.9                          |                                | [83]         |
| Trifluromazine hydrochloride E$_3$ | Approved as antipsychotic agent     | Viral entry             | Vero E6 cells                                       | 6.398                       | 5.758                        |                                | [131]        |
| Phenazopyridine E$_4$           | Local analgiesic, now discontinued    | Viral entry             | BHK-21 cells                                        | 1.93                        |                              |                                | [132]        |
| Teicoplanin E$_5$               | Approved as glycopeptide antibiotic   | Viral entry             | HEK293T cells (pseudovirus)                         | 3.76 (pseudovirus)          | 0.63 (pseudovirus)            |                                | [133]        |
| Dalbavancin E$_6$               | Approved as glycopeptide antibiotic   | Viral entry             | HEK293T cells (pseudovirus)                         | 9.64 (pseudovirus)          | 2.99 (pseudovirus)            |                                | [133]        |
| Oritavancin E$_7$               | Approved as glycopeptide antibiotic   | Viral entry             | HEK293T cells (pseudovirus)                         | 4.96 (pseudovirus)          | 2.12 (pseudovirus)            |                                | [133]        |
| Telavancin E$_8$                | Approved as glycopeptide antibiotic   | Viral entry             | HEK293T cells (pseudovirus)                         | 3.45 (pseudovirus)          | 3.24 (pseudovirus)            |                                | [133]        |
| Chloroquine E$_9$               | Approved for malaria and chronic inflammatory diseases; Phase 2/3 for Covid-19 | Viral entry             | Vero E6 cells                                       | 4.4                         | 12.0                         | 2.71                          | [134,135]    |
| Hydroxychloroquine sulfate E$_{10}$ | Approved for malaria and chronic inflammatory diseases treatment; Phase 2/3 for Covid-19 | Viral entry             | Vero E6 cells                                       | 13.3                        | 4.06                         |                                | [134,135]    |
| Amodiaquine dihydrochloride E$_{11}$ | Approved for malaria treatment       | Viral entry             | Huh7 cells                                          | 2.4                         |                              |                                | [134]        |
| Nafamostat E$_{12}$             | Approved in Japan as anticoagulant and for pancreatitis treatment | Viral entry             | Vero E6 cells (SARS-CoV-2); Calu-3 cells (MERS-CoV) | Not known                  | 22.50                        |                                | [54,183]     |
| Vinyl sulfone-based inhibitor(K11777) E$_{13}$ | Preclinical                  | Viral entry             | Vero 76 cells                                       | <0.05                       |                              |                                | [184]        |
| Griffithsin E$_{14}$            | Phase 1 for HIV prevention           | Viral entry             | Vero 76 cells                                       | 0.048                       |                              |                                | [123]        |
| P9 E$_{15}$                     | Preclinical                          | Viral entry             | MDCK cells                                          | 5 µg/mL                     | 5 µg/mL                      |                                | [185]        |
| HR2P E$_{16}$                   | Preclinical                          | Viral entry             | Huh7 cells                                          | 0.6                         |                              |                                | [186]        |
| EK1 E$_{17}$                    | Preclinical                          | Viral entry             | HCT-8 cells (SARS-CoV-2); Vero E6 cells (MERS-CoV) | 2.23 (pseudovirus)          | 0.26 (pseudovirus)           | 2.375 (pseudovirus)            | [120,121]    |
| EK1C4 E$_{18}$                  | Preclinical                          | Viral entry             | Vero E6 cells                                       | 0.012 (pseudovirus)         | 0.004                        | 0.0365                        | [120]        |
| MERS-4 E$_{19}$                 | Preclinical                          | Viral entry             | Vero E6 cells                                       | 0.0033                      |                              |                                | [164]        |
| MERS-27 E$_{20}$                | Preclinical                          | Viral entry             | Vero E6 cells                                       | 0.0133                      |                              |                                | [164]        |
| 47D11 E$_{21}$                  | Preclinical                          | Viral entry             | Vero E6 cells                                       | 0.19                        | 0.57                         |                                | [169]        |

(Continues)
| Compound | Status | Target | Cell lines | SARS-CoV EC₅₀ (µmol/L) | MERS-CoV EC₅₀ (µmol/L) | SARS-CoV-2 EC₅₀ (µmol/L) | References |
|----------|--------|--------|------------|------------------------|------------------------|------------------------|-----------|
| Emetine dihydrochloride hydrate | Preclinical | Viral entry | Vero E6 cells | 0.051 | 0.014 | 0.50 | [112,131] |
| Toremifene citrate | Approved for the treatment of breast cancer | Viral entry | Vero E6 cells | 11.969 | 12.915 | | [131] |
| Remdesivir | Emergency use authorization for Covid-19; Phase 1 for EBOV disease | RdRp | Vero E6 cells(SARS-CoV) HAE cells (MERS-CoV,SARS-CoV) | 0.069 | 0.074 | 0.77 | [54,55] |
| Galidesivir | Phase 1 for yellow fever and Covid-19 | RdRp | HeLa cells | 57.7 | 68.4 | | [78] |
| Ribavirin | Approved for the treatment of HCV and RSV infection; Phase 2 for Covid-19 | RdRp | Vero E6 cells | 20-80 µg/mL | 10 µg/mL | 109.50 | [54,60,61] |
| Favipiravir | Approved in Japan for influenza; Phase 2 for Covid-19 | RdRp | Vero E6 cells | | | 61.88 | [54] |
| β-D-N⁴-hydroxycytidine | Preclinical | RdRp | Vero 76 cells(SARS-CoV) Calu-3 cells(MERS-CoV) | 10 | 0.15 | 0.30 | [56,57] |
| Lopinavir | Approved for HIV; Phase 3 for Covid-19 | 3CL⁰ | Calu-3 cells(MERS-CoV) Vero E6 cells line(SARS-CoV, SARS-CoV-2) | 17.11 | 11.6 | 26.63 | [72,83,112] |
| Ritonavir | Approved for HIV; Phase 3 for Covid-19 | 3CL⁰ | Calu-3 cells(MERS-CoV) Vero E6 cells(SARS-CoV) | 24.9[72] | >100[70] | | [72,112] |
| Lopinavir Ritonavir C₃ (4.6:1) | | Calu-3 cells | 8.5 | | | | [72] |
| Nelfinavir | Approved for HIV | 3CL⁰ | Vero cells | 0.048 | | | [118] |
| Ebselen | Preclinical | 3CL⁰ | Vero E6 cells | 4.67 | | | [106] |
| N3 | Preclinical | 3CL⁰ | Vero E6 cells | 16.77 | | | [106] |
| Carmofur | Approved as antineoplastic agent in Japan | 3CL⁰ | Vero E6 cells | 24.30 | | | [107] |
| a-ketoamide inhibitors(compound 11r) | Preclinical | 3CL⁰ | Huh7 cells | 0.0004 | | | [90] |
| a-ketoamide inhibitors(compound 13b) | Preclinical | 3CL⁰ | Calu-3 cells | 4.5 | | | [90] |
| Cinanserin | Preclinical | 3CL⁰ | Vero cells | 31 | | | [187] |
| Peptidomimetic inhibitor(TG-0105221) | Preclinical | 3CL⁰ | Vero E6 cells | 0.6 | | | [188] |
| Chloropyridinil ester-derived inhibitors (GRL-0496) | Preclinical | 3CL⁰ | Vero E6 cells | 6.9 | | | [100] |

(Continues)
| Compound                                      | Status                  | Target   | Cell lines               | SARS-CoV EC$_{50}$ (µmol/L) | MERS-CoV EC$_{50}$ (µmol/L) | SARS-CoV-2 EC$_{50}$ (µmol/L) | References |
|-----------------------------------------------|-------------------------|----------|--------------------------|------------------------------|----------------------------|-----------------------------|------------|
| Pyperidine-based inhibitor (GC813) P$_3$      | Preclinical             | 3CL$_{pro}$ | Vero E6 cells            | 0.5                          | 0.189                      | 2.10                        | [189]      |
| NSC158362 P$_1$                               | Preclinical             | PL$_{pro}$ | Vero E6 cells            | <1                           | 0.003                      |                             | [190]      |
| Naphtalene-based inhibitor (GRL0617) P$_2$     | Preclinical             | PL$_{pro}$ | Vero E6 cells            | 0.191                        | 0.084                      |                             | [191]      |
| Cycloheximide T$_1$                           | Preclinical             | Translation | Vero E6 cells          | 0.043                        | 0.189                      |                             | [131]      |
| Anisomycin T$_2$                               | Preclinical             | Translation | Vero E6 cells            | 1.63                         | 0.084                      |                             | [108,109] |
| Valinomycin T$_3$                              | Preclinical             | Translation | Vero E6 cells (SARS-CoV)|                             |                            |                             |            |
| Homoharringtonine T$_4$                       | Approved for the treatment of chronic myeloid leukemia | Translation | Vero E6 cells            | 0.0718                       | 2.10                       |                             | [112,131] |
| Aescin                                         | Preclinical             | Vero E6 cells | 6.0                      | 1.216                        |                            |                             | [192]      |
| Gemcitabine hydrochloride                      | Approved as antineoplastic agent | Vero E6 cells | 4.957                    | 1.216                        |                            |                             | [131]      |
| Abiraterone acetate                            | Approved for the treatment of prostatic cancer | Vero E6 cells | 1.94                      |                             |                             |                             | [193]      |
| Triparanol                                     | Withdrawn drug           | Vero E6 cells | 5.283                    |                             |                             |                             | [131]      |
| Cyclosporine                                   | Approved as immunosuppressant drug | Vero E6 cells | 3.3                      |                             |                             |                             | [194]      |
| Alisporivir                                    | Phase 3 for HCV        | Vero E6 cells | 8.3                      | 3.6                          |                             |                             | [195]      |
| Loperamide                                     | Approved as antidiarrheal agent | Huh7 cells (MERS-CoV) | 5.9                       | 4.8                          |                             |                             | [83]       |
| Cetilistat                                     | Phase 2 for the treatment of obesity | Vero E6 cells | 1.13                      |                             |                             |                             | [193]      |
| Diohydroxyquinoline                            | Approved as antiprotozoal agent | Vero E6 cells | 1.38                      |                             |                             |                             | [193]      |
| Pyrvinium pamoate                              | Approved in some countries as anthelmintic agent | BHK-21 cells | 1.84                      |                             |                             |                             | [132]      |
| Mycophenolate mofetil                          | Approved as immunosuppressive agent | BHK-21 cells | 1.54                      |                             |                             |                             | [132]      |
| Nidosamide                                     | Approved for the treatment of tapeworm infestations | Vero E6 cells (SARS-CoV) | 0.1                       | 0.32                         |                             |                             | [108,109] |
| Nitazoxanide                                   | Approved as antiprotozoal agent | Vero E6 cells (SARS-CoV 2) LLC-MK2 (MERS-CoV) | 0.92 µg/mL | 2.12                        |                             |                             | [54,196]  |
| Ivermectin                                     | Approved as anthelmintic agent | Vero cells | 2.5                      |                             |                             |                             | [197]      |
| Dasatinib                                      | Approved for cancer therapy | Vero E6 cells | 2.100                    | 5.468                        |                             |                             | [131]      |
| Saracatinib                                    | Phase 1 for Parkinson’s disease | Vero E6 cells | 2.9                       |                             |                             |                             | [134]      |
| Sotrastaurin                                   | Phase 2 as anticancer and immunosuppressive agent | Vero E6 cells | 9.7                       |                             |                             |                             | [134]      |
SARS or MERS and, in some cases, caused a worsening of the clinical situation.66-68

Moreover, most clinical studies report the frequent occurrence of adverse effects associated with the administration of high-dose ribavirin such as hemolytic anemia, hypomagnesemia, hypocalcemia, and hepatotoxicity.67,69 In this regard, in a reasonably sized clinical study involving 110 SARS patients treated with ribavirin, hemolytic anemia occurred in 61% of patients and 28% of these patients required blood transfusion.69 In conclusion, the contrasting clinical data and ribavirin hematologic and liver toxicity represent limiting factors for its clinical use in CoVs infections.

Recently, an experimental nucleoside analogue, remdesivir (R1) has been recognized as a potential broad-spectrum antiviral drug against CoVs infections. It is an adenosine analogue prodrug with broad-spectrum activity against several RNA viruses such as filoviruses, coronaviruses, and paramyxoviruses.55,70 It has been used in clinical trials for EBOV infections and recently has received emergency use authorization (EUA) for Covid-19.71

In vitro remdesivir showed potent inhibitory activity against several human and zoonotic CoVs with nanomolar IC50 values; in particular it inhibited SARS-CoV and MERS-CoV replication in HAE cells with IC50 values of 0.069 and 0.074 μmol/L (Figure 2), respectively, and SARS-CoV-2 replication in Vero E6 cells with an IC50 value of 0.77 μmol/L.54,55 Positive results emerged also from in vivo studies: in murine models with SARS-CoV and MERS-CoV, prophylactic administration of remdesivir reduced viral load and lung injury severity.55,72

Given the promising in vitro and in vivo results and the favorable safety and pharmacokinetic profile emerged from clinical trials for EBOV infection, the compassionate use of remdesivir has been authorized in patients with Covid-19, and, currently, it is tested in several clinical trials. At present, some encouraging case reports regarding the administration of remdesivir in patients with Covid-19 have been described. For example, a multicenter reasonably sized trial involving hospitalized patients with severe Covid-19, with more than half receiving mechanical ventilation, reported a clinical improvement in oxygen support class in 68% of patients and, approximately 60% of patients with mechanical ventilation was extubated.73 Positive clinical data emerged also from a large size randomized trial involving 1063 hospitalized patients with Covid-19 reporting a significant reduction in recovery time for patients treated with remdesivir compared to the placebo group.74 Although more clinical data are required, due to its therapeutic efficacy and safety, remdesivir seems to be one of the most promising candidate for the treatment of CoVs infections.

In addition to remdesivir, other two investigational nucleoside analogues, favipiravir and galidesivir, have shown in vitro antiviral activity against CoVs and are currently tested in clinical trials for Covid-19. Favipiravir (R2) is a guanine nucleoside analogue prodrug active against several RNA viruses such as EBOV, influenza virus, RSV, etc.75 Regarding CoVs, a recent study reported a moderate inhibitory activity of favipiravir on SARS-CoV-2 replication in Vero E6 cells with an EC50 value of 61.88 μmol/L.54 Given its toxicity and

| Compound | Microbicide | Status | Target | Cell lines | References |
|----------|-------------|--------|--------|------------|------------|
| Mizoribine | Approved as immunosuppressive agent in Japan | Approved as immunosuppressive agent | Mycoplasma cell | Vero E6 cells | [60] |
| Mycophenolic acid | Approved as immunosuppressive agent | Approved for HSV and VZV infections | Preclinical | Vero E6 cells | [84] |
| Penciclovir | Approved for HSV and VZV infections | Approved in China and Russia for influenza treatment | Preclinical | FRhK-4 cells | [54] |
| Ranitidine bismuth citrate | Approved in China and Russia for influenza treatment | Phase 4 for Covid-19 | Preclinical | Vero E6 cells | [98] |
| Umifenavir | Approved for influenza treatment | Approved in Japan for influenza treatment | Treatment | Vero E6 cells | [88] |
| Baloxavir | Approved as immunosuppressive agent | Approved as immunosuppressive agent | Treatment | Vero E6 cells | [88] |
| Laninamivir | Approved in Japan for influenza treatment | Approved in Japan for influenza treatment | Treatment | Vero E6 cells | [88] |
teratogenicity, it is only approved for the treatment of influenza in Japan. However, due to the current emergency, favipiravir compassionate use has been authorized for patients with Covid-19 and there are several clinical trials ongoing. Positive results emerge from a non randomized open-label study involving 80 patients: a shorter viral clearance (4 days vs 11 days) and a radiographic improvement (91.43% vs 62.22%) was observed in patients treated with favipiravir and interferon alfa compared to patients treated with lopinavir-ritonavir and interferon alfa. However, at present, few clinical data are available and further randomized studies are needed in order to evaluate the real therapeutic efficacy of favipiravir.

Galidesivir (R2) is an adenosine analogue active against several RNA viruses such as coronaviruses, filoviruses, flaviviruses, etc. In vitro studies show a moderate inhibitory activity against SARS-CoV and MERS-CoV in HeLa cells with EC50 values of 57.7 and 68.4 \( \mu \text{mol/L} \), respectively. In vitro studies have not been performed on SARS-CoV-2, however, a recent molecular docking study suggests the potential effectiveness of galidesivir since it can bind tightly to SARS-CoV-2 RdRp. At present, it is tested in clinical studies for yellow fever and Covid-19.

Recently, a ribonucleoside analogue has demonstrated potent antiviral activity against CoVs. It is N4-hydroxycytidine (R4), a cytidine analogue, active against a wide range of RNA viruses such as EBOV, RSV, influenza virus, etc. Moreover, an orally bioavailable prodrug has been developed in order to improve its pharmacokinetic properties. N4-hydroxycytidine activity has been tested in vitro against SARS-CoV, MERS-CoV and SARS-CoV-2 showing EC50 values of 0.15 and 0.30 \( \mu \text{mol/L} \) for MERS-CoV and SARS-CoV-2, respectively. Moreover, positive data emerged also from in vivo studies: in murine model (C57BL/6) with SARS-CoV, prophylactic (−2 hours) and post-exposure (+12 hours, 24 hours or 48 hours) oral administration of N4-hydroxycytidine determined a significative reduction in viral load and lung hemorrhage (Figure 3B and C) and an improvement of lung function, as can been seen from ATS acute lung injury and diffuse alveolar damage scores (Figure 3D).

Given the potent broad-spectrum antiviral activity and favorable pharmacokinetic profile, N4-hydroxycytidine represents one of the most promising preclinical drug to investigate for the treatment of CoVs infection.

2.2 Viral proteases: 3CL\textsuperscript{pro} and PL\textsuperscript{pro}

The two main coronaviral proteases, 3CL\textsuperscript{pro} and PL\textsuperscript{pro}, represent potential drug target since they are essential for viral replication and seem to contribute to viral infection.

3CL\textsuperscript{pro} and PL\textsuperscript{pro} are cysteine proteases responsible for the proteolytic processing of viral polyproteins into nonstructural proteins part of the RNA transcription complex. Moreover, PL\textsuperscript{pro} exhibits deubiquitinating and delSGylating activity with consequent significant
implications in host immune response. In particular, PL pro was found to affect the activation of several transcription factors such as interferon regulatory factor 3 and NF-κB causing a reduction in the production of proinflammatory cytokines and chemokines like interferon beta, CCL5, and CXCL10; the reduction in the endogenous levels of proinflammatory mediators modulates the host immune response and promotes the progression of viral infections.80-82

Comparing the different CoVs PL pro structures, it was found that SARS-CoV and SARS-CoV-2 share a moderate sequence identity (83%), whereas MERS-CoV and SARS-CoV exhibit significant structural differences of the blocking loop 2, domain that has been proven to be a key element in inhibitor binding.83,84 On the other hand, regarding 3CL pro, SARS-CoV, and SARS-CoV-2 show a remarkable sequence conservation (96%).53

At present, several pharmacophores have been identified and based on this, new compounds have been designed and tested, some of them showing promising results (Table 2). However, most of them have not been tested in vitro and in animal models.

SARS-CoV PL pro can be targeted by different types of protease inhibitors like zinc ion, zinc derivatives, thiopurine analogues, and naphthalene inhibitors.85-87 Thiopurine analogues, such as 6-thioguanine and 6-mercaptopurine, have shown to inhibit also the deubiquitinating and proteolytic activity of MERS-CoV PL pro; the reduction in the endogenous levels of proinflammatory mediators modulates the host immune response and promotes the progression of viral infections.80-82

FIGURE 3  Prophylactic and therapeutic efficacy of N4-hydroxycytidine prodrug in a murine model with SARS-CoV on the basis of the following parameters: weight loss (a), lung hemorrhage (b), viral lung titer (c), pulmonary function (d) and histopathological features of the lungs (e) [57]. C57BL/6 mice were orally administrated vehicle (10% PEG and 2.5% Cremophor RH 40 in water) or N4-hydroxycytidine prodrug at -2h pre-exposure and +12h, 24h, 48h post-exposure every 12h. The histological features of acute lung injury (ALI) were blindly scored using an American Thoracic Society lung injury scoring system and a DAD scoring system. Reproduced with permission from The American Association for the Advancement of Science.
Surprisingly, among them we find disulfiram, an FDA-approved drug for use in alcohol dependence. It is an irreversible inhibitor of hepatic aldehyde dehydrogenase and recent studies report also an inhibitory activity against other enzymes, like, urease, hydroxylase, kinase, transferase, and dehydrogenase, attributable to interactions with cysteine residues.\textsuperscript{101-104} Moreover, it showed in vitro antiviral activity against HCV by ejecting zinc from NS5A protein.\textsuperscript{105}

Recently, disulfiram has been reported to inhibit the PL\textsuperscript{pro} of both SARS-CoV and MERS-CoV assuming two different mechanisms of action: allosteric inhibition for MERS-CoV and competitive/mixed inhibition for SARS-CoV. In particular, it has been hypothesized that it forms a covalent bond with the catalytic Cys-112 on the active site for SARS-CoV, and, for both SARS-CoV and MERS-CoV, may also bind to the zinc-binding site with consequent zinc ejection.\textsuperscript{76} Remarkably, disulfiram not only showed inhibitory activity against PL pro but also against the main protease: it inhibited the proteolytic activity of SARS-CoV-2 3CL\textsuperscript{pro} with an IC\textsubscript{50} of 9.35 \(\mu\text{mol/L}\), a lower value compared to the IC\textsubscript{50} values for the SARS-CoV and MERS-CoV PL\textsuperscript{pro} (14.22 and 22.7 \(\mu\text{mol/L}\), respectively).\textsuperscript{106}

Given the favorable safety profile and promising results, disulfiram turns out to be one of the most interesting drugs to further investigate for potential off-label use.

In addition to disulfiram, in a recent study, five compounds, approved or pharmacologically active, including Ebselen, Tideglusib, Carmofur, Shikonin, and PX-12 showed inhibitory activity against

| TABLE 2 | List of FDA-approved and preclinical agents active against the two viral proteases, 3CL\textsuperscript{pro} and PL\textsuperscript{pro}, of SARS-CoV, MERS-CoV, and SARS-CoV-2. Data summarized are the results from in vitro experiments using recombinant proteins as well as structure-assisted drug design and virtual screening. The inhibitory activity is expressed as IC\textsubscript{50} and referred to the proteolytic or deubiquitinating activity |
| Compound | Target | SARS-CoV IC\textsubscript{50} (\(\mu\text{mol/L}\)) | MERS-CoV IC\textsubscript{50} (\(\mu\text{mol/L}\)) | SARS-CoV 2 IC\textsubscript{50} (\(\mu\text{mol/L}\)) | References |
|----------|--------|----------------|----------------|----------------|----------------|
| Disulfiram | PL\textsuperscript{pro} | 14.2 | 22.7 | | [199] |
| Disulfiram | 3CL\textsuperscript{pro} | 222.5 | 9.35 | | [106] |
| Mycophenolic acid | PL\textsuperscript{pro} | 12.4 | | | [85] |
| 6-Thioguanine | PL\textsuperscript{pro} | 5.0 | 25.8 | | [85,86] |
| 6-Mercaptopurine | PL\textsuperscript{pro} | 21.6 | 45.0 | | [85,86] |
| N-Ethylmaleimide | PL\textsuperscript{pro} | 4.4 | | | [86] |
| Cinanserin | 3CL\textsuperscript{pro} | 4.92 | | | [187] |
| Naphtalene-based inhibitors (GRL0617) | PL\textsuperscript{pro} | 0.6 | | | [191] |
| Benzotriazole-based inhibitors (XP-59) | 3CL\textsuperscript{pro} | 0.1 | | | [92] |
| Zinc ion | PL\textsuperscript{pro} | 1.3 | | | | |
| Zinc conjugates (N-ethyl-N-phenylthio carbamic acid) | PL\textsuperscript{pro} | 3.3 | | | [87] |
| Isatin derivatives(compound 8k) | 3CL\textsuperscript{pro} | 1.04 | 5.8 | | [93] |
| Neuraminidase inhibitors derivatives (compound 3i) | 3CL\textsuperscript{pro} | 7.4 | | | [94] |
| Pyrazoline derivatives (compound 2t) | 3CL\textsuperscript{pro} | 6.8 | | | [95] |
| Pyrithiobac derivatives (compound 6-4) | 3CL\textsuperscript{pro} | 3.30 | | | [96] |
| Triazole-based inhibitors (compound 14d) | 3CL\textsuperscript{pro} | 8.95 | | | [97] |
| Diphenyl sulfone-based inhibitors (compound 3) | 3CL\textsuperscript{pro} | 0.3 | | | [98] |
| Pyrimidine derivatives (compound 6m) | 3CL\textsuperscript{pro} | 6.1 | | | [99] |
| Chloropyridinil ester-derived inhibitors (compound 10) | 3CL\textsuperscript{pro} | 0.003 | | | [100] |
| Nitrile-based peptidomimetic inhibitors (Cbz-AVLQ-CN) | 3CL\textsuperscript{pro} | 4.6 | | | [200] |
| Aldehyde-based peptidomimetic inhibitors (TG-0204998) | 3CL\textsuperscript{pro} | 0.038 | | | [201] |
| \(\alpha\)-ketoamide inhibitors (compound 11s) | 3CL\textsuperscript{pro} | 0.24 | 0.6 | | [91] |
| Pyperidine-based inhibitors (compound 9a) | 3CL\textsuperscript{pro} | 2.1 | 1.7 | | [189] |
| \(\alpha,\beta\)-unsaturated peptidomimetic inhibitors (compound 6d) | 3CL\textsuperscript{pro} | 0.2 | | | [202] |
| Ebselen | 3CL\textsuperscript{pro} | 0.67 | | | [106] |
| Tideglusib | 3CL\textsuperscript{pro} | 1.55 | | | [106] |
| Carmofur | 3CL\textsuperscript{pro} | 1.82 | | | [106] |
| Shikonin | 3CL\textsuperscript{pro} | 15.75 | | | [106] |
| PX-12 | 3CL\textsuperscript{pro} | 21.39 | | | [106] |
| \(\alpha\)-ketoamide inhibitors (compound 11r) | 3CL\textsuperscript{pro} | 0.58 | 0.18 | | [90] |
| \(\alpha\)-ketoamide inhibitors (compound 13b) | 3CL\textsuperscript{pro} | 0.90 | 0.67 | | [90] |
SARS-CoV-2 main protease with IC_{50} values ranging from 0.67-21.39 μmol/L (Figure 5).106

Among these compounds, Ebselen (C_{5}) exerted the strongest antiviral activity in cell culture with an EC_{50} of 4.67 μmol/L. It is an organoselenium pharmacologically active molecule and its antiviral mechanism of action is attributable to the formation of a covalent adduct with the catalytic Cys-145. The same mechanism of action has been assumed for Carmofur (C_{7}), an approved antineoplastic agent; it showed a lower in vitro antiviral activity compared to Ebselen, with an EC_{50} value of 24.30 μmol/L, and an advantageous selectivity index of 5.36, proving to be a potential lead compound for the design of new effective antiviral drugs.107

Niclosamide, an FDA-approved anthelmintic medication, has been reported to be active against a wide range of viruses such as filoviruses and coronaviruses. In vitro, it exhibited potent antiviral activity against SARS-CoV and MERS-CoV with EC_{50} values of 0.10 and 0.32 μmol/L, respectively.108,109 Since a series of niclosamide chloro anilide derivatives was found to be an effective inhibitor of the SARS-CoV main protease, it was hypothesized the same mechanism of action for niclosamide that, however, surprisingly, did not show any enzyme inhibitory activity.110

At present, the only FDA-approved drug that has been tested and authorized during SARS, MERS, and Covid-19 outbreaks is lopinavir.

Lopinavir (C_{1}) is a peptidomimetic HIV protease inhibitor commercially available in combination with another protease inhibitor, ritonavir, under the brand name Kaletra; Ritonavir improves the pharmacokinetic profile of lopinavir and increases its serum concentration by inhibiting the cytochrome P450.111

Although the HIV protease is an aspartic protease, whereas 3CL{\textsuperscript{pro}} and PL{\textsuperscript{pro}} belong to the cysteine protease family, surprisingly lopinavir has shown in vitro antiviral activity against CoVs.57,72,112 In this regard, in an in vitro study, lopinavir used in combination with ritonavir (4.6:1) showed a greater inhibition of MERS-CoV replication in Calu-3 cells compared to lopinavir and ritonavir alone with EC_{50} values of 8.5, 11.6, and 24.9 μmol/L, respectively.72 Recently, a moderate inhibitory effect has been observed also on SARS-CoV-2 replication in Vero cells with an EC_{50} of 26.63 μmol/L.112 Regarding SARS-CoV, lopinavir inhibited SARS-CoV induced cytopathic effect in Vero cell line with an EC_{50} of 17.11 μmol/L.56

The therapeutic efficacy of lopinavir has been observed also in a MERS-CoV infected nonhuman primate model: marmosets treated with lopinavir/ritonavir had a better clinical outcome, with radiological improvement, reduction in viral load and fatality rate compared to untreated animals.113

Most available clinical studies regard the use of lopinavir/ritonavir in SARS patients; in a reasonably sized, nonrandomized open study involving 152 patients, a significative reduction in adverse clinical outcome (death and acute respiratory distress syndrome) has been observed in patients treated with lopinavir/ritonavir and ribavirin compared to the control group treated with ribavirin alone (2.8% vs 28.8%).114

**FIGURE 4** Dose response curve of C9 against SARS-CoV-2 replication in Calu-3 cells [90]. Reproduced with permission from The American Association for the Advancement of Science

**FIGURE 5** Inhibition of SARS-CoV 3CL{\textsuperscript{pro}} hydrolytic activity by: Ebselen (a), Disulfiram (b), Tideglusib (c), Carmofur (d), Shikonin (e) and PX-12(f) [106]. IC_{50} values were measured using 0.2 μM protein, 20 μM substrate and 11 different drug concentrations. Reprinted with permission from Springer Nature
Positive clinical data emerge also from a multicenter retrospective clinical study that, however, highlights the beneficial effect of this therapy only if used at the early stages of the infection and not as rescue therapy. The ineffectiveness of the use of lopinavir/ritonavir as rescue therapy has been also reported in a reasonably sized, randomized, open-label study involving hospitalized patients with severe Covid-19: no significant difference in recovery time, fatality rate (19.2% vs 25.0% at 28 days) and viral clearance was observed in patients treated with lopinavir/ritonavir compared to patients who received standard care.

The therapeutic potential of a triple antiviral therapy with lopinavir/ritonavir, interferon beta-1b, and ribavirin has been reported in a recent clinical study. This is a randomized, open-label, controlled, phase 2 trial involving 127 patients with mild/moderate Covid-19 at early stage: a shorter viral clearance (7 vs 14 days) and recovery time (4 vs 8 days) and a reduction in IL-6 levels were observed in patients treated with the triple antiviral therapy compared to the control group treated with lopinavir/ritonavir alone.

Common adverse effects of lopinavir/ritonavir observed in clinical studies include nausea, diarrhea, and hepatotoxicity. In this regard, darunavir, a HIV protease inhibitor, sold as colibicistat boosted form, that has an improved intestinal tolerability, is currently tested in several clinical trials for Covid-19, despite the lack of efficacy against CoVs infections.

Another FDA-approved HIV protease inhibitor, nelfinavir (C₄), has shown in vitro a great antiviral activity against SARS-CoV: it inhibited SARS-CoV induced cytopathic effect in Vero cells with an EC₅₀ of 0.048 μmol/L. However, despite the positive in vitro results, no in vivo and clinical studies have been performed.

2.3 Viral entry inhibitors: spike glycoprotein and viral entry pathways

Among the structural proteins, the spike glycoprotein is particularly interesting since it mediates host-cell entry of CoVs representing an important drug target site. It is a type I transmembrane protein that shows the same structural organization for several CoVs: a N-terminal domain, called S1, containing the receptor binding-domain (RBD), responsible for cellular receptor binding, and a C-terminal domain, called S2, that mediates viral fusion process. Comparing the different structures, SARS-CoV and SARS-CoV-2 share a moderate sequence identity (76%) with a higher similarity (89.8%) in their S2 subunit.

Remarkably, a highly conserved region named the heptad repeat (HR) is located in the S2 domain and represents an appealing target for the development of broad-spectrum antiviral drugs. In this regard, in a recent study, a potent broad-spectrum peptidic entry inhibitor named EK1 (E₄₃) that targets the HR domain was developed. It was designed by optimizing a peptide derived from the HR2 region of HCoV-OC43, named OC43-HR2P, that was found to be active against different human CoVs. In vitro EK1 showed significative inhibitory activity against several HCoVs, including MERS-CoV, OC43, 229E, and NL63, with IC₅₀ values ranging from 0.11 to 0.69 μmol/L; moreover, in a recent study, EK1 proved to be effective also against SARS-CoV-2 pseudovirus with an IC₅₀ of 2.375 μmol/L. Positive data emerged also from in vivo studies: EK1 exhibited potent therapeutic and prophylactic effect with a significative reduction of mortality in a mouse model of MERS-CoV infection.

Moreover, in vivo studies reported a prolonged permanence in lung tissue and a significative distribution in extrapulmonary organs, features that turn out to be advantageous since CoVs primarily attack the respiratory tract but in severe cases can contribute to multiple organs failure.

Interestingly, a potent EK1 derivative named EK1C4 (E₄₄) was obtained by functionalizing the peptide with a cholesterol molecule. This lipopeptide showed potent inhibitory activity against protein-mediated cell fusion of SARS-CoV, MERS-CoV, and SARS-CoV-2 with IC₅₀ values of 4.3, 1.5, and 1.3 nmol/L, respectively, that are about 100-fold more active than those of EK1 (Figure 6). Greater inhibitory activity was observed also in vitro against live infections.

Given the potent broad-spectrum antiviral activity and favorable pharmacokinetic profile, EK1 and its derivatives represent one of the most interesting preclinical compounds to investigate for the treatment of CoVs infections.

**Figure 6** Inhibitory activity of EK1 and EK1C4 against cell-cell fusion of SARS-CoV (a), MERS-CoV (b) and SARS-CoV-2 (c). Huh-7 cells were used for testing all CoVs except for SARS-CoV-2 (293T ACE2 cells) [120]. Reprinted with permission from Springer Nature
Another interesting feature of the viral spike protein that can be exploited as drug target is its extensive glycosylation. In this regard, lectins turn out to be potential antiviral candidates and, in particular, a lectin named Griffithsin (E₁₄), extracted from a red marine alga, shows broad-spectrum antiviral activity against SARS-CoV, MERS-CoV, HIV, and HCV. It has been tested in phase I clinical trial for HIV.²² In vitro it inhibited SARS-CoV and MERS-CoV replication in a dose-dependent way, with an EC₅₀ value of 0.048 μmol/L for SARS-CoV.²²,²³ The potent antiviral activity of Griffithsin was confirmed in vivo: in a murine model with SARS-CoV, the intranasal administration of Griffithsin reduced viral load, lung injury severity, and fatality rate.²³

Since CoVs spike proteins are class I viral fusion proteins, in their natural state are inactive and protease cleavage is essential for their activation and consequently for viral entry. Depending on virus strain, several cellular proteases are implicated in this process becoming possible targets for antivirals development. Common element for the activation of SARS-CoV, MERS-CoV, and SARS-CoV-2 spike protein is a transmembrane serine protease, TMPRSS2, that seems to promote direct viral entry through plasma membrane bypassing endocytosis.⁴⁵,⁴⁶,¹²⁵

Two synthetic serine protease inhibitors, camostat mesylate and nafamostat mesylate appear to be potential viral entry inhibitors. They are approved drugs for pancreatitis treatment in Japan and currently are tested in clinical trials for Covid-19 (Rancona study and CamaCO-19). Recently, nafamostat and camostat mesylate were found to inhibit SARS-5, MERS-5 SARS-2-S driven entry into Calu-3 cells; notably, comparing the different EC₅₀ values, nafamostat was proven to be 17-, 140-, and 75-fold more active than camostat mesylate against SARS-CoV-2, SARS-CoV, and MERS-CoV entry, respectively.¹²⁶ Moreover, these drugs exhibit also anticoagulant activity, an important pharmacological feature since thromboembolic events seem to be among the main causes of death in patients with Covid-19.¹²⁷ For these reasons, in addition to their antiviral activity, the use of nafamostat or camostat in CoVs infections could be also useful to prevent or treat severe complications that generally occur in critical ill patients such as coagulopathies and thrombosis.¹²⁸ However, at present, there is no clinical evidence about their therapeutic efficacy.

CoVs enter host cells through the combination of endocytic and nonendocytic pathways.⁴²

Whilst TMPRSS2 is involved in viral entry via direct membrane fusion, cathepsins, a group of cysteine proteases, were proven to be implicated in the cleavage and activation of the viral spike glycoprotein for endocytic pathways.¹²⁹,¹³⁰ In confirmation of this, a recent study investigating SARS-CoV-2 cell entry, reported that camostat mesylate, a TMPRSS2 inhibitor, only partially inhibited viral entry in Caco-2 cell line and full inhibition was observed by adding it in combination with E-64d, a cathepsin B/L inhibitor.⁴³ A plausible model for endocytic SARS-CoV entry has been proposed by Simmons et al: firstly, SARS-CoV binds to ACE2, this in turn induces protein conformational changes that are followed by pH-dependent cathepsin B/L activation into endosomes.⁴⁷

Based on these findings, FDA-approved drugs that exhibit cathepsins inhibitory activity such as antimicrobials (E₅-E₈), antimalarials (E₇-E₁₄), and antidepressants (E₁-E₄) were proven to inhibit viral entry and infection in cell culture.⁸³,¹³¹-¹³⁵ Among them, we find two much-debated drugs that have attracted most of the attention in Covid-19 pandemic: chloroquine (E₄) and its less toxic derivative hydroxychloroquine (E₂₀).⁴²

They are well-known antimalarial agents used also for the treatment of autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. In addition to these pharmacological effects, chloroquine shows also broad-spectrum antiviral activity against a wide range of viruses such as orthomyxoviruses, retroviruses, and coronaviruses.¹²⁶ Chloroquine can exert its antiviral activity through different mechanisms of action: inhibition of the glycosylation of cellular receptors, endosomal alkalinization, and alteration of viral proteins post-translational modifications.¹³⁷ Moreover, it explicates immunomodulatory effects reducing the production and secretion of proinflammatory mediators such as TNF alpha and IL-6 that, in their turn, promote inflammatory and thrombotic complications in CoVs infections.¹³⁸

In February 2020, one of the first in vitro study investigating potential drugs against SARS-CoV-2 infection came out identifying, among several FDA-approved drugs, chloroquine, and remdesivir as most effective drugs against viral infection.⁴⁴ Moreover, several studies report an inhibitory effect of chloroquine on the replication of SARS-CoV and MERS-CoV in vitro.¹³⁴,¹³⁹ However, at present, chloroquine is basically not used in clinical practice due to its high toxicity and has been replaced by its derivatives like hydroxychloroquine. Hydroxychloroquine, like chloroquine, showed inhibitory effect on SARS-CoV-2 replication in Vero cells and, comparing their EC₅₀ values, chloroquine seemed to be a little more active than hydroxychloroquine (2.71 and 4.51 μmol/L, respectively).¹³⁵

No clinical studies regarding the use of chloroquine and hydroxychloroquine in SARS or MERS patients exist. However, at the beginning of Covid-19 pandemic, a report of clinical trials investigating the use of chloroquine in hospitalized patients with Covid-19 in China came out reporting that a radiological improvement and a shorter viral clearance were associated with the administration of chloroquine.¹⁴⁰ These positive results, given the emergency situation, led to the start of numerous clinical trials investigating the therapeutic effects of chloroquine/hydroxychloroquine in patients with Covid-19. However, clinical data which have arisen recently are contradictory and inconclusive. Positive data emerged from one of the first published clinical studies, a nonrandomized study involving 42 patients: the administration of hydroxychloroquine determined a shorter viral clearance compared to the control group (70.0% vs 12.5%).¹⁴¹ However, several methodological issues are associated with this clinical study: first of all, it is a small-sized nonrandomized trial involving only 36 patients of which 20 received hydroxychloroquine and 6 were asymptomatic; several concerns on selectivity bias since six patients of the experimental arm were excluded after transfer to intensive care unit (3 patients), death (1 patient), and intolerance of the drugs (1 patient); a short duration (6 days); viral clearance...
as the sole end-point and no report on patients clinical situation and side effects.\textsuperscript{142,143}

Despite the initial encouraging results, the latest data show that these drugs are unlikely to be effective against SARS-CoV-2 and, moreover, serious cardiac side effects such as QT interval prolongation and ventricular arrhythmias have been reported. In this regard, an observational large size study involving 1376 hospitalized patients with Covid-19 reported that the use of hydroxychloroquine was not associated with a lower risk of adverse clinical outcome (death or intubation).\textsuperscript{144} In support of this, another reasonably sized observational study involving 173 hospitalized patients with Covid-19 did not show any beneficial effect of hydroxychloroquine on the clinical outcome but rather reported electrocardiographic modifications in 10% of patients.\textsuperscript{145}

Cardiac side effects associated with the administration of chloroquine and hydroxychloroquine like ventricular arrhythmias were reported in a multinational registry analysis that led to the suspension of clinical trials of chloroquine and hydroxychloroquine.\textsuperscript{146} However, at present, this analysis has been retracted and clinical trials have resumed but FDA revoked the emergency use authorization for chloroquine phosphate and hydroxychloroquine phosphate in Covid patients. Moreover, in patients with comorbidities or taking QT-prolonging drugs, the risk of QT-interval prolongation and, consequently, torsade de pointes, increases dramatically.

In this regard, in a clinical study involving 90 hospitalized Covid-19 patients, most of them having cardiovascular diseases and/or taking QT-prolonging drugs, the administration of hydroxychloroquine, alone or in combination with azithromycin, was associated with the frequent occurrence of QT-prolongation events; in particular, in the hydroxychloroquine group, 27% of patients had prolonged QTc, and, moreover, in the experimental arm, 10 patients had to discontinue hydroxychloroquine administration due to QT-prolongation and one case of torsade de pointes occurred.\textsuperscript{147}

In addition to these strategies, another interesting approach to inhibit viral entry has recently emerged and regards the use of cellular receptors in order to block and prevent viral host uptake. This strategy shows, compared to the others, the main advantage of potentially being effective also in case of virus mutations, since it acts on host cells instead of on viruses. Interestingly, in this context, a recent study highlighting the therapeutic potential of human recombinant soluble ACE2 came out; in particular, it reported that human ACE2 significantly blocks SARS-CoV-2 entry and consequently infection in a dose-dependent way in Vero cells.\textsuperscript{148}

Furthermore, in this regard, a novel approach has been proposed by Zhang et al; in particular, they designed cellular nanosponges consisting of a polymeric core of PLGA coated with human lung cells or macrophages-derived membranes displaying cellular receptors used for SARS-CoV-2 entry such as ACE2 and basigin. Interestingly, these nanosponges inhibited SARS-CoV-2 infection in Vero E6 cells with IC\textsubscript{50} values of 827.1 and 882.7 µg/mL, respectively.\textsuperscript{149}

### 2.3.1 Monoclonal antibodies and plasma therapy

The viral spike glycoprotein, in addition to its role in receptor binding and cell fusion, plays a key role in the induction of host immune responses and neutralizing antibodies turning out to be an important biological target of vaccines and antibodies.\textsuperscript{150} In this context, we find one of the most effective therapy to date: the use of convalescent plasma. It is an adaptive immunotherapy that has been used as emergency therapy in several epidemics such as SARS, influenza A H1N1, MERS, Ebola, etc.\textsuperscript{151}

Encouraging reports for convalescent plasma use have been reported during SARS, MERS, and Covid-19 outbreaks; however, since several clinical studies performed were uncontrolled, more stringent clinical trials are clearly needed in order to evaluate its real therapeutic efficacy.\textsuperscript{152-155} For example, in a nonrandomized study involving 80 SARS patients, a significative reduction in mortality and hospitalization has been observed in patients who received convalescent plasma especially at the early stage of the infection (58.3% vs 15.6%).\textsuperscript{155} Moreover, recent clinical studies highlight the potential effectiveness of convalescent plasma as rescue therapy for Covid-19; a multicenter, small size, nonrandomized pilot study involving 10 severe Covid-19 patients reported a radiological and clinical improvement in the majority of the patients that received convalescent plasma transfusion.\textsuperscript{156} A possible rationale for the efficacy of this therapy is that antibodies from recovered donors may inhibit viral entry and promote viral uptake into immune cells neutralizing viral infections.\textsuperscript{157,158} However, despite the several advantages such as efficacy, safety, easy scalability and low cost, unfortunately, plasma therapy shows some downsides since it can occasionally cause the occurrence of immune-mediated side reactions (i.e., hemolysis, transfusion-related acute injury, and anaphylactic reactions) that can actually exacerbate the immune response aggravating patients clinical situation.\textsuperscript{159}

In the light of this, an alternative and safer therapeutic approach to plasma therapy is represented by the passive administration of monoclonal antibodies. In the last years, this strategy has attracted growing interest since it shows the potential to be effective in both prophylaxis and treatment of CoVs infections without leading to immune-mediated side reactions associated with plasma therapy.\textsuperscript{160-162}

All the developed anti-CoVs monoclonal antibodies to date target the spike glycoprotein and, depending on the targeted domain, they neutralize viral infections by blocking the receptor binding or interfering with viral fusion process. Mostly, they target different epitopes of the RBD and only a few the S2 region.\textsuperscript{163}

In this regard, among the most potent RBD inhibitors, we find MERS-4, MERS-27, m396, and S2015.\textsuperscript{164,165} MERS-4 (E\textsubscript{10}) and MERS-27 (E\textsubscript{20}) are two human monoclonal antibodies isolated from nonimmune human antibody libraries; in vitro they showed potent neutralizing activity against live MERS-CoV infection with nanomolar IC\textsubscript{50} values of 3.33 and 13.33 nmol/L, respectively, and, moreover, inhibition of syncytia formation was observed with MERS-4 (Figure 7). Their mechanism of action was elucidated by biochemical studies attributing their neutralizing activity to the RBD binding and,
in particular, MERS-4 was found to be more active than MERS-27 with an excellent Kd of 0.95 nmol/L.

Indeed, these data are very promising and highlight the therapeutic potential of these two monoclonal antibodies, however, no investigations regarding their efficacy, safety, and pharmacokinetic profile in vivo have been performed. In this regard, encouraging in vivo results have been reported for another anti-MERS-CoV human monoclonal antibody named LCA60, obtained from memory B cells of a MERS patient. In vitro LCA60 neutralized several MERS-CoV isolates with IC_{50} values ranging from 110 to 279 ng/mL showing an excellent binding affinity (Kd = 0.12 nmol/L). The administration, intranasal or systemic, of LCA60 into MERS-CoV infected mouse models exerted great prophylactic and therapeutic efficacy with a significant reduction in viral load, improvement of the clinical situation and, interestingly, no interstitial pneumonia occurred.166

Additionally, m396 and S320.15 are two potent anti-SARS-CoV human monoclonal antibodies obtained from a human antibody library and memory B cells of a SARS patient, respectively. Remarkably, they showed in vitro broad-spectrum neutralizing activity against several human and zoonotic SARS isolates with IC_{50} values ranging from 0.01 to 2 μg/mL in pseudoviral infections. In vitro results were confirmed by in vivo studies: the administration of m396 and S320.15 in murine models with different SARS-CoV isolates determined a full or significant protection from viral infections.

Currently, few anti-SARS-CoV-2 monoclonal antibodies have been reported and since SARS-CoV spike glycoprotein shares a moderate sequence identity with that of SARS-CoV-2, some of them have shown cross-neutralizing activity highlighting the possibility to have broad-spectrum anti-CoVs monoclonal antibodies.

In this regard, a highly conserved epitope in the RBD of Sarbecovirus genus was found becoming an attractive target for the design of broad-spectrum monoclonal antibodies and vaccines. Remarkably, a human monoclonal antibody obtained from B cells of a SARS patient, named S309, targeting this epitope was identified and showed potent cross-neutralizing activity against SARS-CoV and SARS-CoV-2 with IC_{50} values of 120-180 and 79 ng/mL, respectively. Furthermore, it was found to promote viral phagocytosis into immune cells turning out to be one of the most promising preclinical candidates for the treatment of CoVs infections and the design of broad-spectrum vaccines.167 Moreover, according to this finding, around 200 human monoclonal antibodies obtained from B cells of SARS patients were reported to mainly target the S1 region and to be active against several human and zoonotic CoVs such as SARS-CoV, SARS-CoV-2, and WIV1.168

Another potential cross-neutralizing monoclonal antibody targeting the RBD was recently identified. It is 47D11 (E21), a human monoclonal antibody obtained from transgenic mice. In vitro it showed cross-neutralizing activity inhibiting both SARS-CoV and SARS-CoV-2 infections with IC_{50} values of 0.19 and 0.57 μg/mL, respectively. Its cross-neutralizing activity has been attributed to its binding to a conserved epitope of the RBD hypothesizing a mechanism that goes beyond the receptor-binding block.169 However, in contrast to this, C3022, a monoclonal human antibody obtained from B cells of a SARS patient targeting a conserved epitope of the RBD of SARS-CoV and SARS-CoV-2 spike glycoprotein with excellent binding affinity, surprisingly, showed neutralizing activity against SARS-CoV but not against SARS-CoV-2.170 Nevertheless, since coronaviral spike glycoproteins have also significative structural differences, unfortunately, not all the monoclonal antibodies show cross-reactivity. For example, a recent study investigating around 200 RBD targeting human monoclonal antibodies isolated from memory B cells of SARS-CoV-2 patients, reported a potent neutralizing activity against SARS-CoV-2 but a complete absence of cross-reactivity with SARS-CoV and MERS-CoV.171

Although the RBD is the main target for anti-CoVs monoclonal antibodies, recently, the N-terminal domain of the S1 has emerged as critical epitope for some potent human monoclonal antibodies, such as 4A8 and 7D10.172,173

In the light of this, monoclonal antibodies can be considered potential candidates for prophylaxis and treatment of viral infections especially when no specific vaccines are available. However, the use of monoclonal antibodies for the treatment of viral infections has some additional drawbacks such as high cost of production, and,
consequently, impossibility to use in large scale for the worldwide population and possible inefficacy in case of virus mutations.

Moreover, despite their undoubted importance in this field, monoclonal antibodies still have a small risk of inducing the anti-body-dependent enhancement (ADE) of disease. It is a phenomenon that leads to a worsening of disease severity as a result of an enhancement of viral-cell entry and hyperimmune response via Fab domains-Fc receptors interactions.\(^\text{174}\)

ADE has been observed with flaviviruses such as Dengue virus, EBOV, and Zika virus, but for CoVs no defined correlation has been established.\(^\text{175}\)

Although data from several in vitro studies have highlighted an enhanced antibody-mediated viral uptake of MERS-CoV and SARS-CoV into immune cells, only MERS-CoV was found to increase pro-inflammatory cytokines levels and, moreover, current data indicate that SARS-CoV-2 is unlikely to infect immune cells.\(^\text{175-178}\) On the other hand, in animal models, most available studies report the protective and therapeutic efficacy of antibodies, and just a few the evidence of ADE.\(^\text{174,179}\) However, since is not clear the correlation between ADE and human CoVs, it should be given full consideration to adopt strategies in order to reduce ADE risks for antibody therapies such as modifications of the Fab domain in order to minimize virus-antibody complex internalization into immune cells, administration of high concentrations of neutralizing antibodies and so on.\(^\text{176,180}\)

In the light of this, monoclonal antibodies efficacy, safety, and pharmacokinetic profiles should be further investigated in animal models and clinical studies are clearly needed in order to evaluate their real prophylactic/therapeutic efficacy.

### 3 | CONCLUSION AND FUTURE PERSPECTIVES

Coronaviruses represent global health threat since they caused serious epidemics and pandemics leading to global health emergencies in the last decade.

At present, there are no approved drugs and therapies for the treatment and prevention of human CoVs and, given the state of emergency, several FDA-approved medications such as antiviral drugs active against RNA viruses, immunomodulatory, and anti-inflammatory agents have been used and tested in clinical trials.

This review highlights coronaviruses key biological targets and provides an overview of the current state of therapeutic strategies to inhibit viral infections focusing on both FDA-approved and preclinical agents that have shown to be effective in vitro and in vivo.

Although the large number of identified drugs with potent in vitro antiviral activity, some of them including ribavirin, chloroquine, and hydroxychloroquine have shown several limitations in clinical studies such as lack of efficacy and severe side effects.

Regarding other FDA-approved drugs showing in vitro antiviral activity, like, antimalarials, antidepressants, antimicrobials, etc., there is a lack of in vivo and clinical studies and, since they are not specific against viral infections and, consequently, their use can determine the occurrence of side effects, further investigations are clearly needed in order to evaluate a potential off-label use. Moreover, these drugs can be also exploited as potential lead compounds for the design of new antivirals.

Based on current clinical data, some preclinical and approved antivirals active against RNA viruses, such as remdesivir and lopinavir, as well as plasma therapy appear to be the most effective therapeutic options against CoVs infections to date. However, at present, numerous clinical trials investigating the therapeutic efficacy of several FDA-approved drugs against SARS-CoV-2 are still ongoing or recruiting and the results are not known yet.

Due to the presence of several CoVs strains in animal reservoirs and their frequent recombination, interspecies jumping and new potential outbreaks are likely to emerge from time to time in the future and, for these reasons, the development of broad-spectrum antiviral drugs can offer a flexible and fruitful strategy to fight future pandemics.

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### DISCLOSURE

None of the authors has any potential conflicts of interest

### NOMENCLATURE OF TARGETS AND LIGANDS

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20.\(^\text{181,182}\)

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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