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Biochemical and Biophysical Research Communications 538 (2021) 137–144

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identifying and repurposing antiviral drugs against severe acute respiratory syndrome coronavirus 2 with *in silico* and *in vitro* approaches

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ARTICLE INFO

Article history:
Received 14 October 2020
Accepted 27 October 2020
Available online 20 November 2020

Keywords:
COVID-19
SARS-CoV-2
Approved drug
Repositioning
Screening
Infection

ABSTRACT

Coronavirus infectious diseases 2019 (COVID-19), a global pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been a serious public health threat worldwide. So far, there are no drugs and vaccines whose efficacy has been well-proven. After the outbreak, there has been a massive search for anti-SARS-CoV-2 medications, focusing on approved drugs because repurposing approved drugs will take less time to reach clinical usage than new drugs. This article summarizes the studies using *in silico* and *in vitro* approaches to identify therapeutic candidates among approved drugs that target the SARS-CoV-2 life cycle.

1. Introduction

Coronavirus infectious diseases 2019 (COVID-19) originated in Wuhan, China in Dec 2019. With over 30 million confirmed infections and 1 million death as of the end of Sep 2020, COVID-19 has been causing considerable public health, social, and economic damage [1–3]. Developing vaccine and antiviral drugs against COVID-19 is a high priority. The identification and development of new drugs generally require 10–20 years and high costs of around 800 million USD [4]. On the other hand, testing approved drugs may enable faster drug discovery and clinical use. Approved drugs' many advantages include commercial availability, proven safety and pharmacokinetics, and the reduced need for extensive preclinical or clinical testing for drug approval. The extensive focus on drug repurposing has led to a number of studies that have searched for candidates using different approaches, such as *in silico*, *in vitro*, cell culture, and *in vivo* models. This article summarizes the endeavors by researchers so far to identify anti-SARS-CoV-2 agents, especially those targeting the viral life cycle, from approved drugs.

2. *In silico* approach

In the early days after the outbreak of SARS-CoV-2, drug candidates were identified mainly by *in silico* docking. This virtual drug screening method targets the viral and host proteins essential for SARS-CoV-2 infection and replication. For example, the viral main protease (Mpro), papain-like protease (PLpro), and RNA-dependent RNA polymerase (RdRp) are essential for viral replication, and the viral Spike protein on the virion surface, angiotensin converting enzyme 2 (ACE2), a cellular receptor, and transmembrane protease, serine 2 (TMPRSS2), a cellular protease, are involved in viral entry into cells [5].

Before the SARS-CoV-2 proteins' structure are solved, homology modeling based on other related coronaviruses' structural information can help delineate these proteins' structures. Using the homology modeling of Mpro, PLpro, RdRp, helicase, Spike, ACE2, and TMPRSS2, a compound database was screened to predict a

Abbreviations: ACE2, angiotensin converting enzyme 2; COVID-19, coronavirus infectious diseases 2019; IC_{50}, a half maximal inhibitory concentration; Mpro, main protease; PLpro, papain-like protease; RBD, receptor-binding domain; RdRp, RNA-dependent RNA polymerase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TMPRSS2, transmembrane protease, serine 2.

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https://doi.org/10.1016/j.bbrc.2020.10.094
0006-291X/

series of drugs that bind to the target proteins, such as ribavirin to PLpro, montelukast to Mpro, remdesivir to RdRp and TMPRSS2, and hesperidin to Spike [6]. Another homology model-based study predicted that paritaprevir would bind to Mpro and furin, ritonavir to furin, and chloroquine to furin [7].

2.1. Main protease (Mpro)

The crystal structure of SARS-CoV-2 Mpro was firstly registered in the database on Feb 2020 (PDB: 6lu7) and later published by several groups [8–11]. Jin et al. used the structural information for the virtual screening of over 10,000 compounds and identified six candidate compounds, such as ebselen, which was shown to inhibit SARS-CoV-2 propagation in Vero cell-based infection assay with a half maximal inhibitory concentration (IC_{50}) of 4.67 μM [8]. Furthermore, the structural information of Mpro has enabled the broad use of molecular docking for virtual compound screening. Many papers report many approved drugs predicted to bind to Mpro, including ribavirin, telbivudine, zanamivir, indinavir, saquinavir, remdesivir, carfilzomib, eravacycline, disulfiram, captopril, ritonavir, viomycin, glecaprevir, maraviroc, telaprevir, boceprevir, argatroban, sitagliptin, vidarabine, lopinavir, tipranavir, raltegravir, daunorubicin, ergotamine, doxycycline, minocycline, cobicistat, simeprevir and pyronaridine [12–24].

2.2. Papain-like protease (PLpro)

The first crystal structure of PLpro was registered in the database in April 2020 (PDB: 6W9C). The papers reporting the unliganded PLpro structure and the structure of PLpro in complex with its substrate, interferon-stimulated gene 15 (ISG15), were published in July and Sep 2020, respectively [25,26]. In the virtual docking screenings based on PLpro's structure, phenformin, quercetin, ritonavir, and tiracizine had the highest docking scores among the FDA-approved drugs examined [27,28].

2.3. RNA-dependent RNA polymerase (RdRp)

The structure of RdRp was reported by cryo-electron microscopy in April 2020 [29,30]. A structural analysis was performed to study the inhibition of RdRp by remdesivir's active metabolite, remdesivir-triphosphate [31,32]. The cryo-electron microscopic structure of the SARS-CoV-2 polymerase complex consisting of nsp12 (RdRp), nsp7, and nsp8 was reported in May 2020 [33]. Virtual screening has selected many approved drugs that were anticipated to bind to RdRp, including nilotinib, saquinavir, tipranavir, lonafarnib, tegobuvir, olysio, cepharanthine, filibuvir, ornipressin, lypressin, examorelin, polymyxin B1, nacortocin, cistinexine, cisatracurium, bedoradrine, palbociclib, quinupristin, dactinomycin, sirolimus, cetrorelix, rifampin, nebivolol, and sofosbuvir [34–39].

2.4. Spike

The cryo-electron microscopic structure of the SARS-CoV-2 Spike protein was solved as a trimer in the prefusion complex in February and March 2020 [40,41]. The crystal structures of the receptor-binding domain (RBD) of Spike protein in complex with its cellular receptor, ACE2, were also reported in March 2020 [42–44]. SARS-CoV-2 Spike's RBD had a more extensive binding interface and higher affinity with ACE2 than SARS-CoV Spike's RBD, suggesting that the SARS-CoV-2 Spike-receptor interaction can serve as a therapeutic target of antiviral agents. Spike protein's structure was also used for the *in silico* screening of approved drugs to identify Spike-binding candidates, such as phthalocyanine, hypericin, pirifibrate, talniflumate, cinacalcet, theaflavin, suramin, streptomycin, ciprofloxacin, glycyrrhizic acid, digitoxin, raltegravir, simeprevir, lumacaftor, pemirolast, sulfamethoxazole, valaciclovir, and pralatrexate [27,45–51]. Eltrombopag, an immune thrombocytopenia drug, was identified to target the S2 domain of the Spike protein; *in vitro* surface plasmon resonance analysis demonstrated that eltrombopag bound to the Spike protein weakly with μM order of K_D value [52].

2.5. Other viral or host proteins

The *in silico* screenings for drugs that target other viral and host proteins were also reported. Simeprevir, paritaprevir, and grazoprevir was estimated to bind to SARS-CoV-2 nsp13 helicase and nsp14 [53]. Eriodictyol was proposed to bind to the SARS-CoV-2 nsp10-nsp16 complex while lopinavir, eriodictyol, and pemirolast were selected as SARS-CoV-2 nsp3 binding compounds [54].

On the other hand, neratinib, dacomitinib, and domatinostat as cathepsin-binding drugs, and lodoxamide, boceprevir, and aceneuramic acid as TMPRSS2-targeting drugs were reported [55]. Another report selected approved drugs that could bind to TMPRSS2, including tanogitran, radotinib, and nafamostat [56]. Virtual screening with ACE2 as the target identified compounds, including lividomycin, burixafor, and quisinostat [57].

It is notable that, during *in silico* docking screenings, even when analyses used the same structural database and the same protein as a drug target, they often ended up identifying different compounds. Therefore, the activity of candidate compounds has to be confirmed *in vitro*, cell culture, or ideally *in vivo* experiments.

3. *In vitro* approach

The virtually predicted activities of a drug against viral or host enzymes can be demonstrated by *in vitro* enzymatic assays. For example, an active SARS-CoV-2 RdRp composed of nsp12 and nsp8 could be expressed in Sf9 insect cells and purified [58]. Enzymatic assays using the recombinant proteins demonstrated that remdesivir-triphosphate was incorporated into RNA by RdRp, thus terminating RNA synthesis with high efficiency. On the other hand, the active triphosphate form of sofosbuvir and favipiravir showed over 1000-fold lower efficiencies. Collectively, these findings were consistent with a significant anti-SARS-CoV-2 activity observed for remdesivir, but not sofosbuvir and favipiravir in cell culture (see below).

An enzymatic assay using a recombinant Mpro prepared in *E. coli* demonstrated the inhibitory activity of boceprevir as well as other preclinical compounds with IC_{50} of μM ranges or less [59,60]. The *in vitro* assays are significant for demonstrating the mode of action of drugs; however, the recombinant protein purifications take longer than cell culture infection assays. Therefore, high-throughput drug screenings for SARS-CoV-2 have been performed mostly with cell culture infection assay rather than *in vitro* assays.

4. Cell culture approach

Evaluating and screening drugs can be performed using cell culture assays that support SARS-CoV-2 infection. SARS-CoV-2 infection can be reproduced in cell lines such as Vero, VeroE6, Vero-based cells overexpressing TMPRSS2, Caco2, Huh-7, and Calu-3 cells; Vero and VeroE6 cells have been frequently used for evaluation of drugs [61–65]. High-throughput screening is performed by infecting these cell lines with SARS-CoV-2, then detecting the viral RNA by real-time RT-PCR or viral proteins by immunofluorescence. In addition, a specific combination of cell lines and viral strains exhibits cytopathic effects, which is another marker of viral

propagation useful for large-scale screening. Other virus-free cell models can be used to reproduce a part of the viral life cycle, such as virus entry, membrane fusion, and replication; these models can also be used for compound screening at a lower biosafety level [66,67]. The drugs identified to inhibit SARS-CoV-2 infection/replication in cell culture assays were summarized in Table 1.

4.1. Drug candidates of other related viruses

The cell-based assays were also used to rapidly examine a small number of drugs that have been reported to inhibit the other related coronaviruses, such as SARS-CoV or other viruses. In the early days after the SARS-CoV-2 outbreak, this approach was used to identify remdesivir, chloroquine, hydroxychloroquine, and arbidol, which inhibited SARS-CoV-2 propagation with IC_{50} of uM range or less using VeroE6 cells [65,68,69]. Chloroquine, hydroxychloroquine, and arbidol were suggested to inhibit viral entry, whereas remdesivir was suggested to inhibit viral replication. On the other hand, the antiviral activity of ribavirin and favipiravir was found to be minimal. A known TMPRSS2 inhibitor, camostat, inhibited SARS-CoV-2 infection in Vero cells overexpressing *TMPRSS2* [66]. Other approved antiviral drugs, including

Table 1
A list of approved drugs that have anti-SARS-CoV-2 activities (<5 μM of IC_{50}) in cell culture.

drug name	classification	anti-SARS-CoV-2 activity (cell type used)	references
abiraterone	anti-tumor	IC_{50}=1.94 μM, IC_{90}=8.40 μM (VeroE6 cells)	81
amodiaquine	anti-parasitic	IC_{50}=4.2-5.15 μM (Vero, VeroE6 cells)	74, 80
anidulafungin	anti-fungal	IC_{50}=4.64 μM (Vero cells)	80
arbidol	anti-viral	IC_{50}=3.537-4.11 μM (VeroE6 cells)	69, 76
astemizole	anti-allergic	IC_{50}=1.2 μM (VeroE6 cells) IC_{50}=0.87 μM (293T-ACE2 cells) IC_{50}=1.3 μM (Huh7-ACE2 cells)	84
atazanavir	anti-viral	IC_{50}=2.0 μM (Vero cells)	73
auranofin	anti-inflammatory	IC_{50}=1.4 μM (Huh-7 cells)	63
azithromycin	anti-biotic	IC_{50}=2.12 μM, IC_{90}=8.65 μM (VeroE6 cells)	82
bazedoxifene	anti-osteoporosis	IC_{50}=3.44 μM (Vero cells)	80
bexarotene	anti-tumor	IC_{50}=2.10 μM, IC_{90}=9.40 μM (VeroE6 cells)	81
camostat	anti-pancreatitis	IC_{50}<1 μM, IC_{90}=2-5 μM (Calu-3 cells)	66
cepharanthine	anti-inflammatory	IC_{50}=0.98-4.47 μM (Vero, VeroE6 cells) IC_{50}=0.35 μM, IC_{90}=0.91 μM (VeroE6/TMPRSS2 cells)	79, 80, 83
cetilistat	anti-obesity	IC_{50}=1.13 μM (VeroE6 cells) IC_{90}=2.90 μM (VeroE6 cells)	81
chloroquine	anti-parasitic	IC_{50}=1.0-7.36 μM (Vero, VeroE6 cells) IC_{50} =1.31 μM, IC_{90} = 3.97 μM (VeroE6/TMPRSS2 cells)	65, 68, 73, 76, 77, 80, 83
ciclesonide	anti-asthmatic	IC_{50}=4.33 μM (Vero cells)	80
cyclosporine A	immunosuppressive	IC_{50}=3.048-5.82 μM (Vero, VeroE6 cells)	76, 80
digitoxin	cardiac	IC_{50}=0.23 μM (Vero cells)	80
digoxin	cardiac	IC_{50}=0.19 μM (Vero cells)	80
diiodohydroxyquinoline	anti-parasitic	IC_{50}=1.38 μM (VeroE6 cells) IC_{90}=4.50 μM (VeroE6 cells)	81
dronedarone	cardiac	IC_{50}=3.92 μM (Vero cells)	80
emetine	anti-protozoal	IC_{50}= < 0.01-0.46 μM (VeroE6 cells)	72, 74
gemcitabine	anti-tumor	IC_{50}=1.24 μM (Vero cells)	77
hexachlorophene	disinfectant	IC_{50}=0.90 μM (Vero cells)	80
homoharringtonine	anti-tumor	IC_{50}=0.03-2.55 μM (VeroE6 cells)	72, 74
hydroxychloroquine	anti-parasitic	IC_{50}=4.06-17.31 μM, IC_{90}=25.49 μM (VeroE6 cells)	68, 82
ivermectin	anti-parasitic	IC_{50}=2.2-2.8 μM (Vero/hSLAM cells)	70
LDK378 (ceritinib)	anti-tumor	IC_{50}=2.86 μM (Vero cells)	80
lopinavir	anti-viral	IC_{50}=5.246-9.12 μM (Vero, VeroE6 cells)	76, 80
lusutrombopag	anti-thrombocytopenia	IC_{50}=3.78 μM (Vero cells)	80
mefloquine	anti-parasitic	IC_{50}=4.33 μM (Vero cells)	80
mycophenolic acid	immunosuppressive	IC_{50}=0.87 μM (VeroE6/TMPRSS2 cells)	75
nafamostat	anti-pancreatitis	IC_{50}=31.6 μM (VeroE6/TMPRSS2 cells) IC_{50}=0.0068-0.0115 μM (Calu-3 cells)	67
nelfinavir	anti-viral	IC_{50}=2.1-3.1 μM (VeroE6 cells) IC_{50}=0.77 μM, IC_{90}=1.18 μM (VeroE6/TMPRSS2 cells)	74, 78, 83
niclosamide	anti-parasitic	IC_{50}=0.28 μM (Vero cells)	80
nitazoxanide	anti-parasitic	IC_{50}=2.12 μM (VeroE6 cells)	65
obatoclax	anti-tumor	IC_{50}=0.3-0.5 μM (VeroE6 cells)	74
osimertinib	anti-tumor	IC_{50}=3.26 μM (Vero cells)	80
ouabain	cardiac	IC_{50}<0.097 μM (Vero cells)	80
oxyclozanide	anti-parasitic	IC_{50}=3.71 μM (Vero cells)	80
proscillaridin	cardiac	IC_{50}=2.04 μM (Vero cells)	80
remdesivir	anti-viral	IC_{50}=0.5-11.41 μM (Vero, VeroE6 cells) IC_{50}=0.0072 μM (293T-ACE2 cells) IC_{50}=0.0026 μM (Huh7-ACE2 cells)	65, 73, 76, 80, 84
salinomycin	anti-protozoal	IC_{50}=0.2-0.4 μM (Vero, VeroE6 cells)	74, 80
spiperone	anti-psychotics	IC_{50}=2.49 μM, IC_{90}=13.10 μM (VeroE6 cells)	82
suramin	anti-parasitic	IC_{50}<20 μM (VeroE6 cells) IC_{90}=9 μM (Calu-3 cells)	64
tetrandrine	anti-inflammatory	IC_{50}=3.00 μM (Vero cells)	80
tilorone	anti-viral	IC_{50}=4.09 μM (Vero cells)	80
toremifene	anti-tumor	IC_{50}=3.58 μM (Vero cells)	80

Approved drugs having <5 μM of IC_{50} are shown.

ivermectin, lopinavir, emetine, homoharringtonine, auranofin, gemcitabine, lycorine, oxysophoridine, suramin, nafamostat, obatoclax, salinomycin, amodiaquine, nelfinavir, mycophenolic acid, umifenovir, berberine, cyclosporine A, atazanavir, and artemisinin, were shown to inhibit SARS-CoV-2 propagation [63,64,67,70–77]. Nelfinavir was also shown to inhibit SARS-CoV-2-induced membrane fusion in a virus-free cell model, using Vero cells overexpressing the Spike protein [78].

4.2. Large scale cell-based screening

Several groups have reported more wide-scale screenings of approved drug libraries. An early study identified cepharanthine, selamectin, and mefloquine as having anti-SARS-CoV-2 potency [79]. Jeon et al. confirmed the anti-SARS-CoV-2 activity of the expected drug candidates, such as remdesivir, chloroquine, and lopinavir. Additionally, from approximately 3000 FDA-approved/pre-approved drugs, they identified 24 anti-SARS-CoV-2 drugs, including niclosamide, digitoxin, digoxin, hexachlorophene, salinomycin, and ouabain, which had submicromolar IC_{50} values in Vero cells [80]. Yuan et al. screened a compound library of 1528 FDA-approved drugs. They identified four candidates, cetilistat, diiodohydroxyquinoline, abiraterone acetate, and bexarotene, that inhibited viral propagation with IC_{50} values in the μM range [81]. Touret et al. screened 1520 approved drugs and found nine having an IC_{50} under 10 μM in VeroE6 cells; sulfadoxine, exemestane, dyclonine, and arbidol showed the highest anti-SARS-CoV-2 activities in Caco2 cells [82]. However, these reports demonstrated the antiviral activity of drugs without an in-depth analysis of their mechanism.

From a library of approved drugs, we identified nelfinavir and cepharanthine, which, with their submicromolar IC_{50}, showed higher antiviral potential than remdesivir, chloroquine, and lopinavir in VeroE6 cells overexpressing *TMPRSS2* [83]. *In silico* and *in vitro* assay combined with cell culture infection demonstrated that nelfinavir bound to SARS-CoV-2 protein Mpro and inhibited its enzymatic activity with an IC_{50} equivalent to that observed in the cell-based infection assay, while cepharanthine was shown to inhibit virus-cell attachment, possibly blocking Spike protein's interaction with its cellular receptor ACE2. Mathematical modeling predicted each of these two drugs, at the clinical drug concentration, will decrease the viral load; the combination treatment with both drugs will further shorten the time needed to achieve virus elimination.

A combination of *in silico*, *in vitro*, cell culture, and mathematical analysis would be useful to test anti-SARS-CoV-2 drug candidates after drug screening. Riva et al. provided another example with a screening of approximately 12,000 FDA-approved or clinical-stage drugs. They selected 21 drugs showing dose-dependent antiviral activity; most of these drugs were pre-approved compounds, such as those classified as PIKfyve inhibitor and cysteine protease inhibitors also included were clofazimide, astemizole, and remdesivir [84]. They examined the expression level of possible host targets of hit compounds in human airway samples, and measured the enzymatic activity of viral proteases *in vitro* to predict the drug efficacy and mode of action. They also tested the candidate compounds' antiviral activities in induced pluripotent stem cell-derived human pneumocyte-like cells and a primary human lung explant model, which were more physiologically relevant models. These models enabled them to identify a series of promising compounds, although all of the candidates are still in the developmental phase.

Drug candidates having anti-SARS-CoV-2 activity in cell culture assay have been identified, and some of them are in a clinical trial. However, many reports only showed the drugs' anti-SARS-CoV-2 activity without a detailed analysis of their mechanism. In the future, further mechanistic analysis and the drug resistance profile would be important for improving treatment outcomes. Also, it should be noted that the drug activities, especially those targeting cellular factors, can depend on the type of cells used in the assay. For example, host-targeting entry inhibitors, chloroquine and its derivatives and camostat, showed diverse antiviral activities in cell types with different expression levels of *TMPRSS2* [85]. In line with this argument, examining drug activity in physiologically relevant cells is essential for prospecting *in vivo* antiviral efficacy. Human airway epithelial cells in an air-liquid interface culture [86] and human induced pluripotent stem cell-derived lung epithelial cells [87,88] will provide more physiological relevance to the study of SARS-CoV-2 infections. In addition, human organoids of a variety of tissues such as lung, intestine, blood vessel, kidney, liver, and brain were reported to be susceptible to infection by SARS-CoV-2 or its pseudovirus [89–96].

5. *In vivo* approach

So far, the animal models used for evaluating SARS-CoV-2 infection and the resultant diseases include Syrian hamster, ferrets, hACE2-transgenic mice, and nonhuman primates such as cynomolgus macaques and rhesus macaques [97–108]. On the other hand, the wild type mice cannot be infected with SARS-CoV-2 due to a lack of efficient interaction between the Spike protein and mouse ACE2; however, an adaptive strain of SARS-CoV-2 was generated that infects the wild type mice [109–111].

These animal models have been shown to be useful for evaluating the efficacy of vaccine candidates and neutralizing antibodies [109–120]; however, there are only a few examples that demonstrated the antiviral effect of drugs using these models. Remdesivir was examined in the rhesus macaque model. Although remdesivir did not reduce the viral load in the upper respiratory tract, it significantly decreased the virus infectious titer in the lower respiratory tract from 12 h after administration. The percentage of virus-negative in the lung lobe at seven days after infection was higher in remdesivir-treated animals than the untreated animals [121]. Remdesivir-treated animals also showed a low clinical score of respiratory disease and a reduction in lung damage, suggesting that remdesivir inhibits SARS-CoV-2 replication and prevents the progression of pneumonia *in vivo*. A commentary reported no significant antiviral effect of hydroxychloroquine in infected hamsters, cynomolgus macaques, or rhesus macaques, or in mice infected with mouse-adapted SARS-CoV-2, despite its anti-SARS-CoV-2 activity in cell culture [122]. Mice infected with adapted SARS-CoV-2 strains were also used to evaluate chloroquine and chlorpromazine, which resulted in no apparent reduction in viral load but improvement of clinical symptoms [123]. The infection model using ferrets showed that treatment with lopinavir-ritonavir, hydroxychloroquine, or emtricitabine-tenofovir did not show an overall reduction in the viral load in nasal, stool, or respiratory tissues; however, these drugs lowered the clinical scores in ferrets infected with SARS-CoV-2 [124]. In contrast, azathioprine, an immunosuppressant, delayed viral clearance, and prolonged clinical symptoms. These papers show that changes in clinical symptoms are likely more visible than those in the viral load in these animal models after drug treatment. It would be demanded to develop or optimize infection animal models that can evaluate antiviral activity by measuring viral load with higher sensitivity.

6. Conclusion

The screening of approved drugs enables the rapid identification of drug candidates with lower costs. Many approved drugs have been identified to have anti-SARS-CoV-2 activity. Given the urgent

demand for COVID-19 drugs and the known adverse effect profile of approved drugs, some of the identified drugs were moved directly to clinical evaluation without detailed experimental analysis. More detailed analysis, including the mode of action and the drug resistance profile as well as the examination of drug efficacy with *in vivo* infection models, are needed to enable the effective treatment of SARS-CoV-2 infection over the long term.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The author would like to thank Enago for the English language review. The author's work was supported by the Japan Society for the Promotion of Science KAKENHI Grants JP20H03499; the Japan Science and Technology Agency (JST) MIRAI program; the Japan Agency for Medical Research and Development (AMED) Grants JP19fk0108111j0001, JP19fk0108156j0101, JP20fk0108274j0201, JP20fk0108179j0101, JP20fk0310114j0004, JP20fk0310101j1004, JP20fk0310103j0204, JP20fk0210036j0003, JP20wm0325007j0101, and JP20jm0210068j0002; the Takeda Science Foundation; the Smoking Research Foundation; the Taiju Life Social Welfare Foundation; and the Mochida Memorial Foundation for Medical and Pharmaceutical Research. The authors declare that they have no conflicts of interest with the contents of this article.

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