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Double-Barreled CRISPR Technology as a Novel Treatment Strategy For COVID-19

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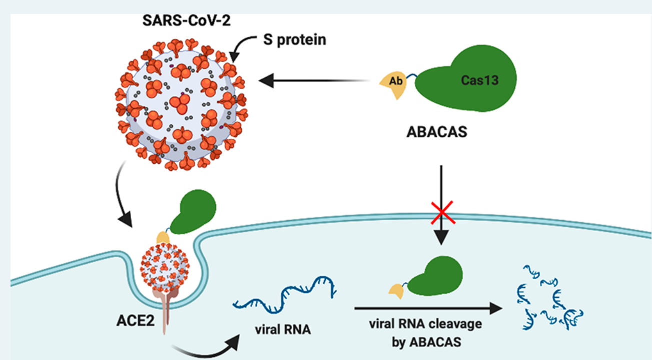
ABSTRACT: Coronavirus is one of the causative agents for multiple human respiratory illnesses. A novel coronavirus, similar to the one that caused severe acute respiratory syndrome (SARS) in 2003, was identified as the cause of the current pandemic of coronavirus disease (COVID-19), which was first reported in late December 2019 in Wuhan, China. Since then, this novel coronavirus has spread across the globe, with most identified COVID-19 cases and fatalities occurring in the United States. In this Perspective, we discuss coronavirus pathogenicity, conventional antiviral therapies, prophylactic strategies, and novel treatment strategies for COVID-19. We highlight the application of CRISPR technology as an emerging pan-antiviral therapy. We also discuss the challenges of *in vivo* delivery of CRISPR components and propose novel approaches to achieve selective delivery exclusively into SARS-CoV-2-infected cells with high efficiency by hijacking the surface proteins of SARS-CoV-2.



KEYWORDS: *SARS-CoV-2, COVID-19, CRISPR, Cas, ACE2, antiviral*

■ INTRODUCTION

In December 2019, a new form of deadly pneumonia was first reported in China, with a novel coronavirus later identified as the causative agent of the disease.[1,2] The novel coronavirus has infected over 89 000 people in China and caused 4723 deaths.[3] Since then, there has been an overwhelming spread of the virus across the world causing over 25.5 million cases with nearly 0.85 million fatalities to date. Over 6 million cases and over 183 000 deaths were reported in United States.[3] Structurally and genetically closely related to SARS-CoV-1, the cause of the 2003 Severe Acute Respiratory Syndrome (SARS), this new coronavirus as a result has been named as SARS-CoV-2, and the disease caused by SARS-CoV-2 is termed as COVID-19 by the World Health Organization.[4] Over the last two decades, three different coronaviruses were identified in the three major pandemics since 2003: SARS (in 2003), Middle East Respiratory Syndrome (MERS, in 2012), and COVID-19 (in 2019). All three coronaviruses are known to cause upper and lower respiratory illnesses in humans.[5,6]

Coronaviruses are the largest group in the order *Nidovirales* and belong to the family *Coronavirinae*.[7] Alpha- and betacoronaviruses are pathogenic to humans, while gamma- and deltacoronaviruses are often pathogenic to avian species.[8] SARS-CoV-2, SARS-CoV-1, and MERS-CoV are all betacoronaviruses. Coronaviruses are structurally distinct from other types of viruses due to a specific feature of the outer structural proteins. Spike protein (S protein) is one of the four main structural coronavirus proteins that are displayed on the surface of the virus particle.[9,10] Trimers of this S protein form a structural feature that resembles a solar corona from which the name of these viruses was derived.[11] In general, viruses contain DNA or RNA genetic material which wraps inside a protein bubble, and they always require a host cell to replicate their genomes and multiply the number of virus particles. Coronaviruses carry a positive (+)-sense RNA genome with ∼30 kb in size.[12,13] A large portion of the viral genome encodes 16 nonstructural proteins (nsp 1−16), 5−8 accessory proteins, and 4 structural proteins including spike (S) protein, membrane (M), envelope (E), and nucleocapsid (N) proteins (Figure 1A,B).[13] In addition to the protein-coding regions, viral RNA genome consists of a leader sequence and an untranslated region (UTR) at both 5′ and 3′ end allowing it to replicate and transcribe. Therefore, multiple copies of the viral genome can be generated by rapid and efficient replication to synthesize viral proteins and multiple virus particles inside the host cell.

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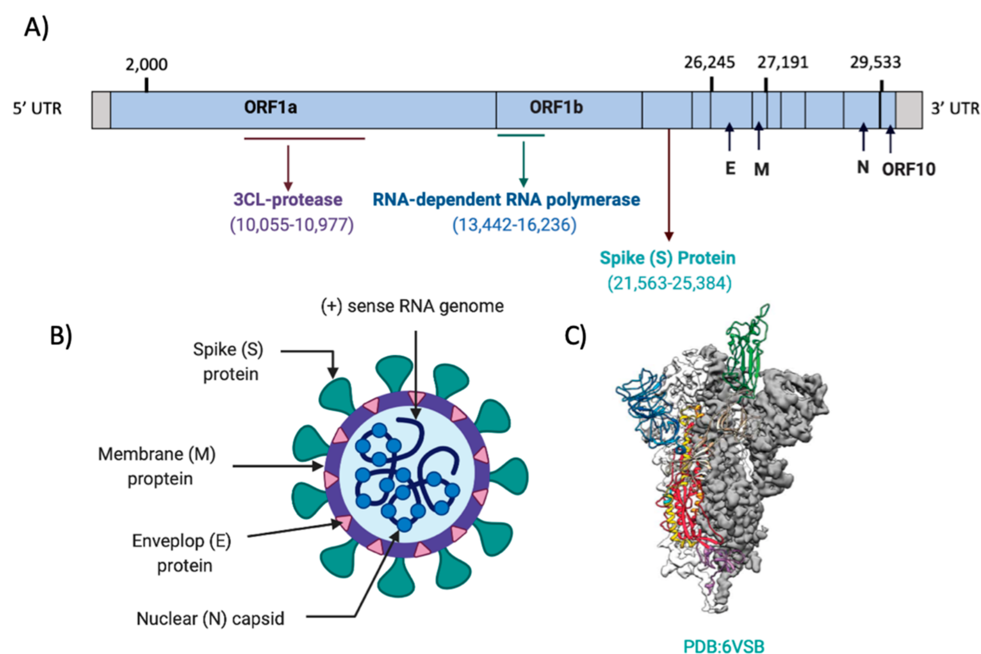

Figure 1. (A) SARS-CoV-2 carries a 29 903 bp long (+) sense RNA genome with 10 open reading frames (ORF). ORF1 codes for two large polyproteins, and their subsequent cleavage by proteases forms several individual nonstructural proteins including RNA-dependent RNA polymerase, which is essential for replication of the viral genome. ORFs 2, 4, 5, and 9 contain genetic information for structural proteins including S, E, M, and N proteins, respectively. (B) Representation of key structural proteins and RNA genome of SARS-CoV-2. (C) Crystal structure (Protein Data Bank: 6VSB) of S protein of SARS-CoV-2. The ACE2 binding domain is shown in green color in the open conformation of S protein.[14] S protein is the key structural protein in SARS-CoV-2 that mediates ACE2-dependent virus infection into host cells. The graphic was created using BioRender.com.

Along with other essential structural viral proteins, S protein mediates viral entry into the host cell.[15,16] The S proteins are heavily *N*-glycosylated in the endoplasm reticulum of host cells, and these host-cell-derived N-linked glycan signatures allow the virus to circulate undetected by the host immune system. Furthermore, glycosylation also provides specific binding to their cognate receptors on the host cell membrane, and subsequent cleavage by proteases facilitates membrane fusion and virus endocytosis. Sequence and structural features of S protein are important to determine the cognate receptor of the host cell. For example, S protein in SARS-CoV-1 binds to angiotensin-converting enzyme 2 (ACE2), while S protein in MERS-CoV binds to dipeptidyl-peptidase 4 (DPP4).[17,18] Given the high sequence and structural similarity of the S protein of SARS-CoV-2 to that of SARS-CoV-1, the former also binds to ACE2 on the host cell surface to facilitate virion entry into host cells.[19] The S protein is composed of a S1 subunit with a receptor binding domain (RBD) and a S2 subunit with the membrane fusion domain.[20,21] ACE2 is ubiquitously expressed in numerous tissues in humans, with high expression levels on the surface of the epithelia of the lung, heart, and kidney,[22] such that these and other organs with abundant ACE2 expression are at high risk of SARS-CoV-2 infection resulting in multiple organ failures and even death. ACE2, with its carboxypeptidase activity, is a type I integral membrane glycoprotein.[23] The octapeptide angiotensin II is the natural substrate of ACE2, which mediates the production of a heptapeptide vasodilator, angiotensin (1−7). Thus, ACE2 plays a critical role in regulating blood pressure and blood volume by inducing vasodilation.[24] Even though SARS-CoV-2 depends on ACE2 expression to enter the host cell, given its wide expression and essential role, targeting ACE2 as a therapeutic strategy is not straightforward. In contrast, many antiviral therapies are being developed and currently under investigation.

Due to the high infection rate of SARS-CoV-2 among individuals, one key mitigation strategy that has been taken to avoid spread is social distancing. Even though social distancing has proven efficacious, there is an urgent need to develop novel antiviral therapies to prevent infection and/or to suppress virus multiplication within patients.

Conventional Therapeutic Strategies. Multiple strategies have actively been put forward to identify possible treatment options for COVID-19. Over 300 different treatment strategies and over 200 vaccines are being tested in preclinical stage or in different stages of clinical trials worldwide. One key factor that scientists must take into consideration is the time limitation during an active pandemic like COVID-19: With the rapid spread of the virus and the increasing rate of fatalities, there is an interest in novel strategies like drug repurposing to identify effective and potentially safer drugs that are already in the market or currently in clinical trials.

Drug Repurposing Strategy: Antivirals. To this end, Wu et al. used homology modeling and target-based virtual ligand screening to predict potential drug candidates for the treatment of COVID-19.[25] One advantage of this strategy is the rapid screening of drugs that are known to have a high safety profile and can be used in humans within a very short period of time. However, identified drug candidates need to be evaluated using *in vitro* and *in vivo* settings to confirm their antiviral activity and potential efficacy in humans against the SARS-CoV-2. A recent and elegant study has predicted a number of Food and Drug Administration (FDA)-approved drugs as potential antiviral candidates for the treatment of COVID-19 patients.[26] In this study, 26 SARS-CoV-2 proteins were cloned into mammalian vectors and expressed in mammalian cells. Subsequent enrichment of ectopically expressed viral proteins identified 332 host cell proteins as potential binding partners. Among the identified host cell interactome, several identified proteins were known to

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Table 1. Summary of the Key Treatment Strategies and Clinical Candidates for COVID-19[a]

Treatment strategy/candidate			Target	Status			Advantages and disadvantages
				Phase 1	Phase 2	Phase 3	
Antiviral therapy	Remdesivir		Nucleotide analogue/RdRp				**Pros:** 1. Target multiple stages of the virus cycle 2. Multidrug administration 3. Less-time consuming 4. Potential to repurpose **Cons:** 1. Emergence of resistant mutants 2. Potential adverse effects 3. Selectivity issues/cross react with human enzymes or pathways
	Ribavirin		Nucleoside analogue				
	Brensocatib		DPP1				
	Sofusbuvir		HCV-NS5B				
	ASC09		HIV protease				
	Emetine hydrochloride		NS5 polymerase				
	Clevudine		DNA synthesis				
	EIDD-2801		Ribonucleoside analogue /Viral RNA				
	Truvada		HIV-1 reverse transcriptase				
	Darunavir		HIV-1 protease				
Antibody therapy	REGN-COV2		SARS-CoV-2 Spike protein				**Pros:** 1. Inactivate the virus before the infection 2. Can be produce in large scale within a short time 3. High specificity 4. Instant immunity **Cons:** 1. Short half-life/multiple admirations may require 2. Expensive 3. Potential immunogenicity 4. Antibody response may vary patient to patient
	LY-CoV555		SARS-CoV-2 Spike protein				
	VIR-7831/VIR-7832		SARS-CoV-2 Spike protein				
	Bevacizumab (Avastin)		VEGF				
	Nivolumab (Opdivo)		PD-1				
	Leronlimab (PRO140)		CCR5				
	Tocilizumab (Actemra)		IL-6 receptor				
	Gimsilumab		Macrophage colony stimulating factor				
	BRII-196/BRII-198		SARS-CoV-2 Spike protein				
	CT-P59		D614G variant (Spike protein)				
Vaccine	RNA-based	mRNA 1273	SARS-CoV-2 Spike protein				**Pros:** 1. Long lasting immunity 2. Safe 3. Cost effective 4. Very low doses of antigen is enough to activate host immunity **Cons:** 1. Time consuming; not readily available during an active pandemic 2. Animal models are not readily available for testing antigens of novel viruses 3. Potential health risk in individuals participate in human challenge studies
		LUNAR-COV19	SARS-CoV-2 Spike protein				
		LNP-nCoVsaRNA	SARS-CoV-2 Spike protein				
		3 LNP-mRNAs	SARS-CoV-2 Spike protein				
	DNA-based	INO-4800	MERS Spike protein				
		ZyCoV-D	SARS-CoV-2				
		GX-19	SARS-CoV-2				
	Inactivated virus	CoronaVac	N/A				
		COVAXIN	N/A				
	Other	Ad5-nCoV	SARS-CoV-2 Spike protein				
		AZD 1222	SARS-CoV-2 Spike protein				
		Trimeric S protein	N/A				

[a]Here, clinical candidates for the treatment of COVID-19 are summarized with its molecular targets, the stage of clinical trials, and potential advantages and disadvantages of each strategy. Data were collected from ClinicalTrials.gov and the Milken Institute COVID-19 Tracker.[44]

play key roles in innate immunity, epigenetic regulation, and the proteasomal degradation pathway. Interestingly, 62 of the identified host cell proteins already have FDA-approved or preclinical small-molecule modulators that are predicted to have an antiviral effect.

There are several known antiviral drugs being tested in clinical trials such as the nucleotide analog, remdesivir, in COVID-19 patients. Remdesivir, which inhibits RNA-dependent RNA polymerase (RdRP), has been tested in patients with COVID-19 in 75 centers worldwide.[27] The crystal structure for RdPR of SARS-CoV-2 has also been resolved in a complex with remdesivir at high resolution,[28] and the resultant structural data will provide insightful information to develop more potent inhibitors in the future. Despite having shown promising antiviral properties *in vitro* and in small human studies,[27] potential COVID-19 drug candidates must show better efficacy in humans before their approval for use in COVID-19 patients. However, a drug repurposing strategy applied to medicines like remdesivir can bypass the lengthy and expensive processes that are involved in establishment of synthetic strategies for new drugs, construction of dedicated facilities, and synthesis of raw materials according to current good manufacturing practices.[29]

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Once already FDA-approved antiviral drugs are proven to be effective in COVID-19 patients, the available resources can be rapidly implemented for large-scale production within a short period of time. Recently, on the basis of the global phase 3 trial data, FDA has allowed remdesivir to be used in COVID-19 patients under the condition of "emergency use authorization" within the United States. In addition, the Japanese Ministry of Health has also been granted a regulatory approval for the use of remdesivir in critically ill COVID-19 patients.[30,31] Although remdesivir has proven to be effective to some extent, due to the short supply and high cost, there is an urgent need for alternative strategies to control current pandemic. Recent data also suggested that dexamethasone, a steroid, has a potential benefit in critically ill patients.[32] However, dexamethasone treatment has not shown any benefit in patients who did not require oxygen supplementation. The antimalarial drug hydroxychloroquine was another potential candidate drug to treat patients infected with SARS-CoV-2, which started phase 3 clinical trials in early April 2020. However, recent data suggested that hydroxychloroquine treatment is ineffective in COVID-19 patients.[33]

Development of Vaccines. Vaccination is one of the most established preventive strategies against many infectious diseases and has played a key role in increasing the lifespan of modern people.[34] Development of a vaccine for an infectious disease ordinarily takes years to complete prior to its use in the clinic. Several different vaccine development platforms are available worldwide. In addition to traditional live-attenuated or inactivated virus particles, recombinant proteins, nucleic acids, and viral-vector-based platforms are widely used in COVID-19 vaccine development. While vaccines offer durable immunity against infections, development of vaccines during an active pandemic is challenging because of the time-consuming processes. Also, several vaccine strategies are associated with safety issues. For instance, use of live-attenuated viruses has a risk of inducing the disease in some patients, or in very rare cases, the virus can transmit to the people who live with the vaccinee.[35] DNA- and mRNA-based vaccines suffer from stability issues as they are inherently susceptible to DNases and RNases.[36] However, some challenges have been successfully addressed by combining nanotechnology to improve stability and delivery efficiencies.

Current COVID-19 pandemic has triggered multiple biotech companies and government agencies to actively experiment on COVID-19 vaccines and will be available to use in patients in the near future. One vaccine candidate has already progressed to phase 3 clinical trials on July 27, 2020. The candidate vaccine under consideration (mRNA-1273 manufactured by ModernaTX, Inc.) is based on a mRNA that encodes the full-length S protein of SARS-CoV-2, consisting of a transmembrane anchor and an intact S1−S2 cleavage site. mRNA-1273 is administered as lipid nanoparticles and is composed of four lipid molecules with a fixed ratio of mRNA molecules to lipid content.[37] The current phase 3 trial is randomized, observer-blind, and placebo-controlled and included ∼30 000 individuals to evaluate the efficacy, safety, and immunogenicity (NCT04470427). In addition, recombinant S protein based vaccines such as Ad5-nCoV (encoded in an adenovirus type 5 vector, NCT04313127) from CanSino Biologics and AZD-1222 nCoV-19 (encoded in a chimpanzee adenovirus Oxford 1 vector, NCT04324606) from University of Oxford are also being evaluated in phase 1/2 clinical trials (Table 1).[38,39] Live-attenuated vaccines, inactivated vaccines, and DNA vaccines are a few of the many vaccine candidates that are still in preclinical or early clinical stages.

Antibody Therapy. The development of neutralizing antibodies (nAb) against SARS-CoV-2 is also an active area of investigation. Antibody treatment can be carried out in two different ways. First, convalescent plasma containing nAb (polyclonal) that have been naturally produced in response to infection is harvested from COVID-19-recovered individuals and then administered to COVID-19 patients (passive-antibody therapy). Convalescent plasma therapy based on the combined effect of a complex mixture of antibodies and other molecules which were generated in recovered patients as an immune response to the invading pathogen.[40] This strategy has been used during previous pandemics including SARS. Recently, convalescent plasma therapy was tested in a small group and showed potential benefits in COVID-19 patients.[41,42] Convalescent plasma therapy has received approval from the FDA to test on front-line medical staff as well as on moderately ill COVID-19 patients. However, there several limitations and potential health risks such as limited supply, variability of neutralizing capacity, and adverse effects due to plasma transfusion are associated with convalescent plasma therapy. As an alternative approach, developing specific monoclonal nAb against SARS-CoV-2 using recombinant technologies is becoming more popular and predicted to be more efficient than plasma transfusion.[43]

Monoclonal antibodies are synthetic versions of naturally derived nAb that are found in recovered patients. Naturally derived antibodies are isolated from recovered patient plasma via affinity-based approaches and are evaluated for their neutralizing activity against SARS-CoV-2.[45] Once identified, nAb will be produced recombinantly and evaluated *in vitro* and *in vivo* to investigate their prophylactic and therapeutic efficacy followed by clinical trials. Although development of nAb seems promising, application of this method is challenging due to time limits during an active pandemic and high cost. However, similar to the repurposing of FDA-approved antiviral drugs, antibodies that have been developed against other coronaviruses such as SARS-CoV-1 can be repurposed to treat COVID-19 patients.[46] Due to the high sequence and structural similarity of SARS-CoV-2 S protein to the SARS-CoV-1 homologue, the potential cross-neutralizing activity of antibodies developed against the latter can be predicted and should be rapidly evaluated.[47]

According to publicly available resources, over 80 antibodies are being tested in preclinical or clinical trials, 12 of which are FDA-approved or currently being tested for other indications, such as CCR5 antagonist (leronlimab-PRO 140).[44] Intriguingly two antibodies are being progressed to phase 3 clinical trials including REGN-COV-2, a cocktail of two monoclonal antibodies targeting two regions of S protein (NCT04452318), and LY-CoV555, a monoclonal antibody derived from a recovered COVID-19 patient (NCT004497987) (Table 1). Similar to the mechanism of action of nAb, another approach has been developed using engineered phages that bind to hemeagglutinin, the spike protein of the influenza A virus.[48] Bacteriophage capsids modified with a hemeagglutinin ligand that binds to the surface of the influenza virus in a multivalent fashion to inhibit the virus's interaction with the host cell's receptors, resulting in inhibition of virus infection. Potentially, this new technology could be modified to neutralize the S protein of SARS-CoV-2 as a therapeutic strategy for COVID-19 in the future.

Antibody therapies are an essential component of the battle against a pandemic until a successful vaccine is developed to establish a long-lasting immunity. While development of nAb is a promising approach to circumvent the issues associated with vaccine development, the efficacy of these antibody therapies could be investigated while hoping for a better outcome when used in combination with other antiviral therapies. Also, it is important to know how long these antibodies remain effective once administered into patients, when it should be administered, how to define the appropriate patient population for antibody therapy, and how can these therapies be manufactured at a large scale and in a cost-effective way. These questions are yet to be answered, but it remains indispensable to find potential solutions for these outstanding questions if COVID-19 antibody therapies need to be pushed from the bench to the bedside on a global scale. On account of the high cost burden and time-consuming processes involved in antibody and vaccine development and as a collective effort to achieve this goal, there is a growing interest in developing alternative, cost-effective, efficient ,and rapidly adaptable and deployable antiviral therapies. In the next section, development of novel antiviral therapies using clustered regularly interspaced short palindromic repeats (CRISPR) technology will be discussed.

Development of Novel Antiviral Therapeutics. In addition to the above-listed conventional antiviral strategies, new treatment modalities were recently proposed as potential treatment options for the current COVID-19 pandemic. One such strategy is the application of CRISPR-Cas associated gene-editing technology. The CRISPR-Cas system was identified in bacteria where it provides innate protection to the host cell against invading viruses.[49,50] When the bacteria encounter foreign genomic sequences, such as viral DNA or RNA, they generate a specific CRISPR RNA (crRNA) with a target sequence that binds and cleaves one or multiple regions in the invading viral genetic material, thereby providing the host protection against virus.[50] CRISPR-Cas bacterial immune system is broadly divided into classes 1 and 2: Class 1 is further divided into three types including types I, III, and IV. The class 1 CRISPR system is composed of multiple Cas proteins where they mediate different functions such as processing of pre-crRNA, binding to crRNA, and interference by cleavage. With recent advances in computational programs, novel variants or subtypes of CRISPR system have been identified. For instance, 33 subtypes have been identified in class 1 category.[51]

On the contrary, class 2 is considerably simple and only includes single, multidomain crRNA binding Cas proteins. The class 2 CRISPR system consists of both DNA- and RNA-targeting Cas proteins and is further subdivided into types II, V, and VI. Among these, RNA-targeting CRISPR-Cas13 is categorized under type VI, and it is the only known RNA-targeting-resistant mechanism in bacteria in addition to the type III CRISPR system in class 1.[52,53] Intriguingly, researchers have successfully adapted the CRISPR-Cas mechanism to correct genetic abnormalities in human cells with the hope of using CRISPR technology as a therapeutic strategy to treat human diseases as well as a valuable chemical biology tool.[54−57] The CRISPR-Cas system includes both DNA- and RNA-targeting nucleases such as Cas9 and Cas13, respectively. Inspired by the CRISPR-Cas system that mediates protective immunity against invading pathogens such as bacteriophages, the CRISPR-Cas system has been co-opted to use as a potential antiviral therapy.[58−61] In addition to DNA-targeting Cas9, the RNA-targeting CRISPR-Cas13 has also been successfully demonstrated as an antiviral strategy against single-strand RNA viruses such as lymphocytic choriomeningitis virus (LCMV), influenza A virus (IAV), and vesicular stomatitis virus (VSV) in human cells.[62]

In a recent study, Abbott et al. have established that the CRISPR technology can be used to cleave SARS-CoV-2 genetic material using Cas13d and CRISPR-associated RNA (crRNA).[63,64] The authors coined the new technology of Prophylactic Antiviral CRISPR in huMAN cells (PAC-MAN). In this study, the authors utilized Cas13d, which is an RNA-guided RNA endonuclease. By building a bioinformatic pipeline to identify conserved regions in SARS-CoV-2, they designed multiple crRNAs that can target different coronaviruses identified and sequenced to date. First, the authors evaluated different coronavirus genomes and identified two highly conserved regions that code for essential proteins such as RdRP and N protein. A library of crRNA was generated *in silico*, from which were chosen 40 crRNA sequences with less off-target binding to the human genome, but that did target RdRP and N protein and other conserved regions of SARS-CoV-2. Transfection of GFP fused with the aforementioned SARS-CoV-2 proteins as infection reporters into lung epithelial cells (A549 cell line) expressing Cas13d and a pool of targeting crRNAs resulted in a significant reduction of the reporter signal compared to control cells. A pool of crRNAs targeting different regions of SARS-CoV-2 RdRP gene resulted in more than 85% repression of the GFP reporter signal, and a pool of crRNAs targeting conserved regions of SARS-Cov-2 N protein gene resulted in more than 70% repression of the GFP signal. To more closely mimic the viral infection of human lung epithelial cells, the authors encoded these conserved SARS-CoV-2-GFP fusions into lentiviral vectors and infected A549 cells. Afterward, the authors evaluated the efficiency of SARS-CoV-2 RNA degradation by measuring reporter signal: Intriguingly, a pool of crRNA could repress approximately 70% of reporter signal, showcasing the potential of CRISPR PAC-MAN technology to degrade viral genetic materials. Further, multiple crRNAs targeting the whole conserved region of the RdRP and N protein genes of SARS-CoV-2 induced over 80 and 90% RNA degradation, respectively.

One key advantage of PAC-MAN is that this technology can be adapted to treat multiple coronaviruses using a single cocktail containing different crRNAs targeting conserved regions across different coronaviruses. In this study, the authors suggested a potential pan-coronavirus inhibition strategy by just few crRNAs using PAC-MAN technology. Strikingly, the computational analysis has predicted that just three crRNAs are enough to target all betacoronaviruses implicated in SARS, MERS, and COVID-19.

Lessons Learned from PAC-MAN. The lessons include the following: (1) A single crRNA is not enough to achieve successful viral inhibition. A pool of crRNA can be used. (2) Combinations of crRNAs should be carefully selected to obtain high efficiency. (3) PAC-MAN technology can successfully target and degrade SARS-CoV-2 genetic information to inhibit viral replication in human lung cells. (4) PAC-MAN can be used as a pan-coronavirus treatment strategy. (5) PAC-MAN technology can target both DNA and (±) RNA viruses. (6) Given the possibility of targeting conserved sequences and the possibility of using multiple crRNAs, evolution of the virus (mutations) can be easily tackled. (7) PAC-MAN technology can be successfully applied to other viruses found in the animal reservoir that pose a future threat to humans and to prepare

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potential treatment regimens. (8) Once the CRISPR technology proves safe in humans, this strategy can be rapidly deployed at very early stages of the next pandemic with only minor changes to the existing system.

Limitations of CRISPR-Mediated Antiviral Therapies and Potential Solutions. The proposed CRISPR-based antiviral therapeutics hold a great promise in treatment of the COVID-19 pandemic. However, the rationale for successful human application of antiviral CRISPR therapies are entirely based on an *in vitro* study. Preclinical and clinical data to prove the safety of the technology for human use is lacking, and as a result, the technology will not be readily available to use during the current pandemic due to the need for lengthy clinical studies. However, the delivery of CRISPR components into patients is the major hurdle impeding clinical applications. There are three possible treatment strategies that can be employed to get the CRISPR system to work inside animals or humans: (1) The system (Cas13 and crRNA) could be transiently expressed inside cells by introducing CRISPR components in the form of DNA. (2) Moreover, Cas13 and crRNA could be introduced in the form of RNA and translated inside host cells. (3) Finally, CRISPR components could be introduced as a ribonucleoprotein complex which is known to have a high editing efficiency within a short period of time, with limited off-target effects.[65,66] However, each of these strategies need to be coupled with an efficient delivery mechanism to ensure the delivery of CRISPR components into the cells. Even though existing delivery strategies such as liposomal, polymer-based, and adeno-associated virus (AAV) vectors appear to be feasible, these methods are of limited efficiency.[67] Furthermore, they lack selective delivery of the CRISPR components exclusively into the infected cells; instead, the existing delivery methods nonspecifically deliver CRISPR components into both healthy and infected cells. In addition to the lower transfection efficiency of existing delivery methods, nonspecific delivery of CRISPR components can lead to increased off-target effects when no viral RNA is present; therefore, selective and efficient delivery strategies must be engineered before testing of CRISPR antiviral therapies in humans.

Selective and Efficient Delivery of CRISPR-Cas13 in Antiviral Therapy. One potential strategy to achieve selective and efficient delivery is to engineer Cas13 nuclease in a way it can adhere onto viral particles, thereby delivering the CRISPR components along with the virus into the host cell; high specificity of delivery is achieved because the delivery mechanism and coronavirus infection are one and the same. Once the virus, carrying its own shredding machinery (CRISPR-Cas), enters the cell and its RNA released into host cell cytoplasm, the CRISPR system can rapidly access viral RNA and cleave it before the viral RNA is replicated or translated. Ironically, selective and efficient delivery could be accomplished with the support of virus itself.

Cas13 is the RNA-guided RNA endonuclease used in the antiviral CRISPR therapy to mediate the cleavage of target viral RNA.[63] To colocalize the CRISPR components with the virus itself, Cas13 can be tethered to one of the main structural proteins on the virus surface, such as S protein (Figure 1B,C). S protein is the key protein that mediates virus infection through interaction with its receptor, ACE2, present on the host cell surface. Tethering of CRISPR components could be accomplished by fusing Cas13 nuclease to an antibody fragment specific to the S protein of SARS-CoV-2. The proposed approach is termed AntiBody And CAS fusion (**ABACAS**) (Figure 2). Thus, **ABACAS** would serve as a heterobifunctional macromolecule which composed with Cas13 and an antibody fragment specific to the S protein of SARS-CoV-2. Antibody fragment of the **ABACAS** would recognize S protein on SARS-CoV-2 and facilitates the selective delivery of the Cas13 into the infected cell along with the virus (Figure 3, right panel, step 1). Once **ABACAS** is delivered to the infected cell, it will recognize and cleave viral RNA. This will result in a potent viral inhibition and potentially reduce off-tissue effects due to the selective delivery by virus itself. Modification of Cas endonucleases, such as Cas9 fusion proteins, has been successfully employed for gene editing and transcriptional control in numerous studies, suggesting the feasibility of Cas13 fusion proteins in antiviral therapy with minimum effects on their catalytic activity.[68−70]

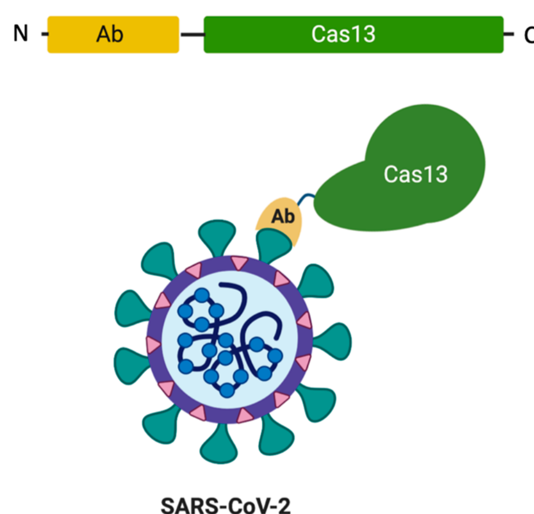

Figure 2. Schematic representation of the **ABACAS** construct and its SARS-CoV-2-bound form. An antibody fragment could be fused to either side of the Cas13 protein. The antibody fragment of the **ABACAS** would recognize and bind to S protein of SARS-CoV-2. If the virus infects a host cell via endocytosis, then **ABACAS** would enter the infected cell along with the virus. Once **ABACAS** is released from the endosome via an inducible endosomal escape mechanism, the Cas13 portion of the **ABACAS** would recognize the viral RNA and mediate its cleavage. The graphic was created using BioRender.com.

Although a handful of clinical studies are underway to identify safe and efficacious antibodies against SARS-CoV-2 S protein, no specific monoclonal antibodies have yet to be approved to treat COVID-19. However, a recent study suggested that polyclonal antibodies and antigen-binding fragments (Fabs) purified from convalescent plasma from infected or vaccinated individuals are capable of binding to certain regions of SARS-CoV-2 S protein and elicit neutralizing activity.[71] Intriguingly, REGN-COV2 and LY-CoV555 have been identified as tight binders of S protein of SARS-CoV-2 and would be ideal candidates for the development of **ABACAS**. Several studies have also suggested that antibodies developed against SARS-CoV-1 homologue could be used to neutralize SARS-CoV-2 due to its cross-reactivity or cross-neutralizing activity.[46,72,73] Among these, the SARS-CoV-1 S protein RBD-specific human monoclonal antibodies, CR3022 and S309, are potential candidates with which to develop fusion biologics with Cas13 and to use in CRISPR-mediated antiviral treatments. Even though the proposed antibodies do not directly interfere with the ACE2 binding, they still bind to the viral S protein tightly.[72] Hence, essential fragments of REGN-COV2, LY-CoV555, or

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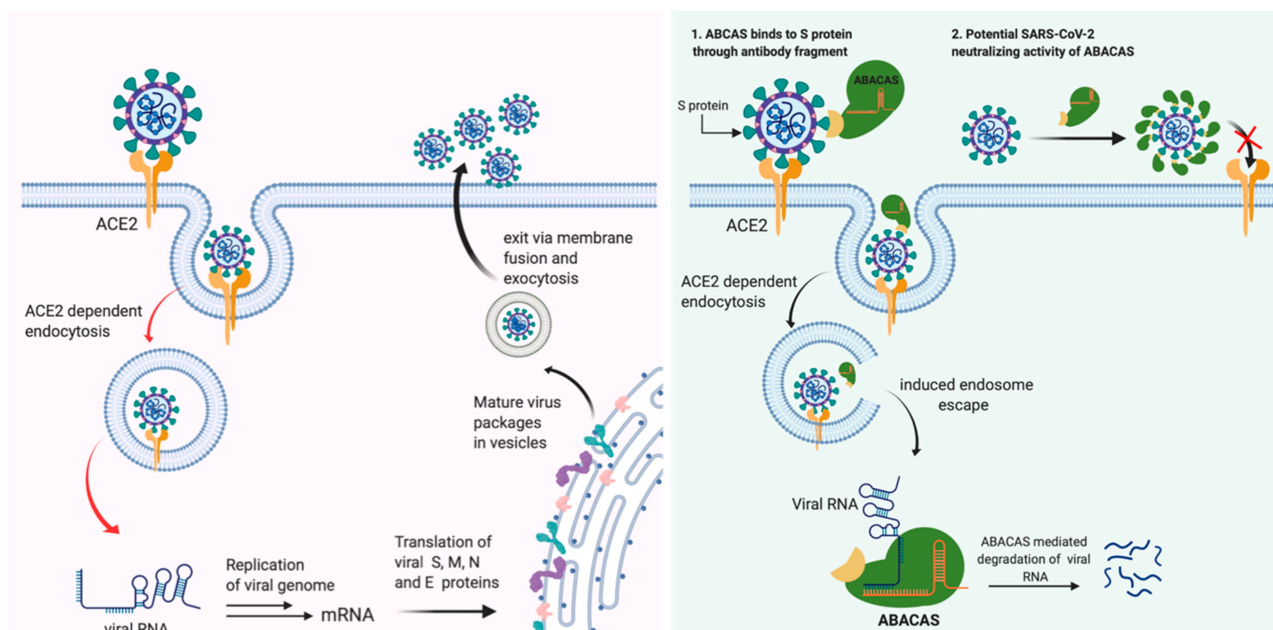

Figure 3. Schematic visualization of proposed **ABACAS** technology. The S protein of SARS-CoV-2 interacts with ACE2 receptor of lung epithelial cells and enters through endocytosis to mediate its replication by hijacking the translational machinery of the host cell (left panel). **ABACAS** technology hijacks the viral S protein to selectively deliver CRISPR components to infected cells. Upon delivery, CRISPR components can readily access viral RNA even before it translates to RdRP and replication of its genome. In addition to the selective delivery of CRISPR by **ABACAS** technology, the neutralization activity of antibody fragments could lead to interfere with S protein and ACE2 receptor interaction and minimizes endocytosis-mediated viral entry into host cells, providing a secondary role as a neutralizing agent. Thus, **ABACAS** could provide a dual mechanism by inhibiting both viral replication and minimizing viral infectivity by interfering with the interaction between the S protein of SARS-CoV-2 and ACE2 on host cells. The graphic was created using BioRender.com.

CR3022 antibodies could be fused to Cas13 to achieve high efficiency and selective delivery of CRISPR components exclusively into infected cells. Potentially, by combination with an induced endosomal escape mechanism, Cas13 in **ABACAS** will be released into the cytoplasm and access viral RNA.[74] Thus, shredding of the viral RNA inside the host cell by Cas13 and virus inhibition by **ABACAS** could be achieved. Endosomal escape exposes viral proteins to host cell defense mechanisms, thereby inducing a secondary immune effector role by the host cell to fight against the infected virus is also envisioned.

Different combinations of such monoclonal antibody fragments, such as the antigen-binding fragment (Fab), single-chain variable region fragment (scFv), and single-domain antibodies (nanobodies), can be attached to obtain optimum **ABACAS** biologics with minimum effect on its catalytic activity and with improved safety for use in humans.[75] By utilizing such antibody fragments specific to the ACE2-binding region of S protein in **ABACAS** design, it would elicit a "double-barreled" effect: First, it functions as a prophylactic by interfering with SARS-CoV-2 binding to its receptor ACE2, and second, the viral RNA will be cleaved by **ABACAS** inside the infected cell. Thus, the proposed **ABACAS** technology will provide a bifunctional role by interfering with virus infection and by destroying the viral genome, resulting in the inhibition of viral multiplication inside the host cells (Figure 3, right panel).

Fusion Biologics Comprising the Peptidase Domain of ACE2 and Cas13 (PDCas13): An Alternative Approach to ***ABACAS***. Given the technical challenges in the development of monoclonal antibodies, the high cost associated with the manufacturing process, and potential resistant mechanisms, alternative biologics that can bind to the S protein could also be envisioned to achieve selective delivery of Cas13 and viral inhibition. Since SARS-CoV-2 S protein interact with ACE2 receptor, when recombinant human ACE2 protein (rhACE2) is administered in a soluble form, it has the potential to competitively inhibit the interaction between S protein and ACE2 receptor of the host cell.[76,77] Therefore, rhACE2 acts as a masking agent for SARS-CoV-2. Recently, Lei et al. demonstrated that both the catalytically active and inactive extracellular domains of ACE2 fused to the Fc region of the human immunoglobulin IgG1 were able to successfully bind and neutralize SARS-CoV-2.[76]

If resistant mutations evolve to **ABACAS** treatment, then an alternative approach to achieve selective and efficient delivery of CRISPR components can be envisioned. As an alternative to **ABACAS**, the antibody fragment of **ABACAS** could be replaced with the peptidase domain (PD) of ACE2 (PDCas13 biologics) or with a simple peptide of ACE2 that was identified to bind to the S protein of SARS-CoV-2.[78] The PD of human ACE2 is known to interact with the RBD of S protein of SARS-CoV-2. Thus, PDCas13 biologics interact with SARS-CoV-2 via its S protein. Therefore, it would facilitate selective and efficient delivery of CRISPR components into infected cells, and potential prophylactic activity is also envisioned due to the possible neutralizing activity of rhACE2. However, despite previous studies on rhACE2 that showed promising neutralizing activity, a complete inhibition of virus infection was not achieved.[79] This can be attributed to the incomplete masking of S protein on the virus surface and sparing enough freely available S protein to induce endocytosis of SARS-CoV-2 via ACE2 interaction and infection of healthy cells. However, regardless of the weak neutralizing activity, if rhACE is fused with Cas13, then it would still promote the binding of Cas13 onto the virus particle via S protein. Thus, PDCas13 would enter

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infected cells along with the virus and elicit antiviral activity via internalized PDCas13. Although undesirable systemic effects are a safety concern of using rhACE2, recent evidence supports catalytically inactive rhACE2 still binding to and neutralizing SARS-CoV-2, suggesting the feasibility of this approach.[76,80] Even though there are no FDA-approved specific monoclonal antibodies currently available against the S protein of SARS-CoV-2, **ABACAS** using SARS-CoV-1 nAb as well as PDCas13 using rhACE2 or ACE2 peptide are worth investigating to evaluate the efficacy of the proposed systems for selective and efficient delivery of CRISPR components to achieve potent antiviral activity against SARS-CoV-2.

OUTLOOK

SARS-CoV-2 infection has caused unprecedented damage to human society, the world economy, and the entire healthcare system. COVID-19 resulting from SARS-CoV-2 infection through the respiratory system has caused over 0.85 million global deaths. Despite many identified global cases and deaths due to COVID-19, multiple organizations including public, academic, private, and industry sectors are being actively researched to develop antivirals and vaccines for SARS-CoV-2. There are globally over 100 projects currently underway to find an effective and safe COVID-19 vaccine. Although it has been proven historically that vaccines are effective and safest in preventing future pandemics, given the time-consuming process of vaccine development, alternative treatment strategies must be developed to control an active pandemic. These alternative treatment strategies should satisfy several factors in order to be used in patients. The studies on new antiviral strategies must have a defined mechanism of action which includes but is not limited to targeting a single protein, genomic DNA or RNA, or any biological pathway in the virus or in the host cell. A well-defined mechanism of action will be helpful to develop new therapies or to modify existing approaches to overcome any resistance mechanism. Also, this evidence might provide a basis to predict any adverse effects associated with it. Furthermore, antiviral strategies must establish *in vitro* antiviral activity against the virus of interest. The studies on cytotoxicity profiles, serum binding, and therapeutic indexes should also be executed prior to clinical investigation of the antivirals. More importantly, any proposed novel antiviral strategies must be readily adaptable to target new viruses during future pandemics. Furthermore, the design, development, and preclinical evaluation of the antiviral should be rapid and less time-consuming.

Here we have proposed potential new antiviral strategies to target viruses such as SARS-CoV-2. These strategies harness the power of the CRISPR-Cas13 system to cleave the RNA virus genome by introducing itself exclusively into the infected host cells by co-opting the endocytic mechanism of the virus. Once introduced to patients, **ABACAS** would tether itself on to the outer surface of the virus via the antibody fragment of **ABACAS** and is endocytosed together with the virus. Thus, the **ABACAS** approach has the potential to deliver the CRISPR-Cas system selectively into the infected cells. Therefore, this approach would allow researchers to effectively and safely deliver CRISPR machinery without using additional delivery agents such as nanoparticles or electroporation techniques. Furthermore, **ABACAS** would be an ideal delivery strategy compared to existing delivery strategies which are associated with adverse effects due to nonspecific delivery.[81]

Since a short RNA sequence on CRISPR gRNA targets predetermined sequences in the viral genome to initiate RNA cleavage, this system can be readily adaptable to target any new invading viruses. Once the virus is identified, thanks to the power of new bioinformatic technologies, genome sequencing data will be available within a short time. The genome sequencing data will provide the basis for the modification of the gRNA portion of **ABACAS** to target and cleave the new viral genome to induce a viral inhibition. If successful *in vitro*, the the optimized CRISPR-Cas13 system can quickly be progressed to preclinical and clinical studies. Once **ABACAS** proves safe and efficient in humans as an antiviral therapy, this technology could be rapidly deployable during future coronavirus pandemics with minor changes to the crRNAs complement to target novel virus RNA. Thus, CRISPR technology holds tremendous potential not only as a valuable therapeutic tool to correct lethal mutations in human diseases such as cancer but also as an extraordinary candidate for antiviral therapy to save hundreds of thousands of people from dying during future pandemics.

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Notes

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