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CRISPR Technology and Its Importance in SARS-CoV-2 Treatment

CRISPR Teknolojisi ve SARS-CoV-2 Tedavisindeki Önemi

Genç SARAÇAYDIN(İD), Özlem ÖZER ÇAKIR(İD)

Department of Gastroenterology and Hepatology, Demiroğlu Bilim University Faculty of Medicine, İstanbul, Türkiye

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ABSTRACT

Coronavirus disease-2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) predominantly affects the respiratory system. The COVID-19 pandemic has had devastating effects on the health system and the global economy worldwide. To reduce the worsening impact of the pandemic, various treatment options and vaccines have been developed. Despite these efforts the pandemic could not be stopped because of the single-stranded nature of the virus combined with the lack of proof-reading abilities of the RNA-dependent RNA polymerase (RdRp). This results in a high probability of error in the copying process and consequently, mutations occur. The increase in mutations in SARS-CoV-2 reduced the efficacy of antiviral medicines and vaccines. To fight this problem, studies were conducted on the efficacy and safety of using Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR) in the diagnosis and treatment of COVID-19. Initially, discovered in archaea, CRISPR is a gene-editing tool that works by altering specific parts of the genome. In this review, we focused on the efficacy and safety of CRISPR technology in the treatment of COVID-19.

Key Words: *CRISPR-Cas13d; SARS-CoV-2; Antiviral drugs; crRNA; Cytotoxicity*

ÖZ

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Genç SARAÇAYDIN, Özlem ÖZER ÇAKIR

Demiroğlu Bilim Üniversitesi Tıp Fakültesi, Gastroenteroloji ve Hepatoloji Anabilim Dalı, İstanbul, Türkiye

Şiddetli akut solunum yolu sendromu koronavirüs-2 (SARS-CoV-2) virüsünün neden olduğu Koronavirüs hastalığı-2019 (COVID-19), ağırlıklı olarak solunum yolunu etkilemektedir. COVID-19 pandemisi dünya çapında sağlık ve ekonomik sorunlara yol açmıştır. Pandeminin etkisini azaltmak için tedavi ve aşı çalışmaları yapılmıştır. Buna rağmen SARS-CoV-2 virüsünün RNA'ya bağımlı RNA polimerazının (RdRp) yetersiz kontrol mekanizmasının kopyalama işlemlerinde neden olduğu mutasyonlar yüzünden pandemi durdurulamamıştır. Yüksek mutasyon oranı, geliştirilen aşıların ve antiviral ilaçların etkinliğinin azalmasına neden olmuştur. Buna çözüm olarak, CRISPR sisteminin COVID-19 tedavi ve tanısındaki etkinliği ve güvenliği üzerine araştırmalar yapılmıştır. CRISPR sistemi, ilk olarak arkea bakterilerde keşfedilen, spesifik gen bölgelerini değiştirebilecek bir genom düzenleme aracıdır. Bu derlemede CRISPR teknolojisinin COVID-19 tedavisindeki güvenliği ve etkinliğine odaklanılmıştır.

Anahtar Kelimeler: *CRISPR-Cas13d; SARS-CoV-2, Antiviral ilaçlar; crRNA; Sitotoksisite*

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INTRODUCTION

Coronavirus disease-2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), is the third most lethal pandemic disease[1]. The World Health Organization (WHO) reports that there were approximately 602 million reported COVID-19 cases and 6.4 million deaths resulting from the COVID-19 pandemic between 11 March 2020 (date for WHO declaration as "pandemic") and 22 August 2022[2]. COVID-19 symptoms differ from person to person, but the most common symptoms are fever, cough, fatigue, and dyspnea. More severe cases may include respiratory failure, arrhythmias, shock, and multiorgan dysfunction syndrome (MODS)[3,4].

To prevent these detrimental conditions and further infection, different treatment options, such as vaccines and antiviral drugs, have been developed. Multiple types of vaccines are available against SARS-CoV-2. The WHO-recognized emergency use listing (EUL) vaccines have four main types: mRNA vaccines (BioNTech-Pfizer, Moderna), viral vector vaccines (Astra Zeneca, Janssen), protein subunit vaccines (Novavax, Covovax), and whole inactivated virus vaccines (Sinopharm, Coronavac, Covaxin)[5].

FDA-approved SARS-CoV-2 small-molecule antiviral drugs as of August 2022 are remdesivir, molnupiravir, and nirmatrelvir. They act by targeting various viral replication mechanisms. Despite the emergence of antiviral treatments and vaccination efforts, the pandemic could not be taken under control, and the fast spread of SARS-CoV-2 has continued[6].

There was a decrease in the efficacy of vaccines and antiviral drugs due to mutations, resulting in the emergence of new SARS-CoV-2 variants[7]. Consequently, CRISPR technology was considered to control the virus more effectively. CRISPR technology with CRISPR-associated protein (Cas) endonuclease, especially Cas13d (CRISPR-associated protein 13d), was used in combination with CRISPR RNA (crRNA) to target the specific gene loci of the SARS-CoV-2 genome[8].

The CRISPR-Cas system originally constituted the adaptive immune system of archaea and bacteria against bacteriophages[9]. This system is found in 84% of bacteria and 45% of archaea[10]. CRISPR-Cas systems were first identified in 1987 by Yozshizumi Ishino while conducting research on *Escherichia coli*, but the function of the CRISPR genes was not yet known[11]. The connection between the adaptive immune defense of bacteria and the CRISPR-Cas system was established later in 2007 by Rodolphe Barrangou during his study on *Streptococcus thermophilus*[9]. When he infected the bacteria with bacteriophages, he found that the bacteria gained spacer DNA in the CRISPR1 locus. Moreover, a positive correlation between spacer DNA and bacteriophage resistance was reported. Barrangou also reported that the RNA transcribed from these DNA spacers was cut into small pieces by related Cas proteins to form crRNAs that are now used to target specific genes[9]. The first genetic engineering application was performed in 2012 by Emmanuel Charpentier along with Jennifer Doudna. They created a single-guide RNA (sgRNA) composed of trans-activating CRISPR RNA (tracrRNA) and crRNA to guide Cas9 in the deletion of target double-stranded DNA[12]. In 2016, Abudayyeh et al. discovered a new CRISPR system called the type VI CRISPR Cas system, which includes a new Cas13 endonuclease that can detect and cleave RNA[13]. This review provides an overview of the CRISPR technologies used in treating COVID-19, their efficacy, and safety.

Small-Molecule Antiviral Drugs in COVID-19 Treatment

The Food and Drug Administration (FDA) approved antiviral therapies that focus on blocking viral replication processes in various ways (Figure 1). Pfizer's Paxlovid works by blocking specific enzymatic activity for the proper functioning of the virus. There are two different medications in Paxlovid's three-pill dosage. While two of the pills were nirmatrelvir, the other pill was ritonavir. Nirmatrelvir's main function is to block proteases from cutting proteins into functional viral particles, which in turn disables

the virus from infecting unaffected healthy cells. The latter, ritonavir, works by preventing Nirmatrelvir's metabolism in the liver, thus, increasing its duration so that it can have a longer time to act and stop infection[14]. Another antiviral is Merck & CO.'s Lagevrio, which contains an RdRp inhibitor called 'molnupiravir'. It disrupts the virus' RdRp production by inserting itself into the viral instruction and essentially makes RdRp completely useless via RNA mutagenesis[15]. Similarly, remdesivir, which was initially developed against the Ebola virus in the 2014 Ebola pandemic, works by blocking RdRp[16]. While it can be effective in treating COVID-19, there are some concerns about the cardiovascular complications caused by remdesivir. Moreover, remdesivir can create cytotoxic effects in cardiomyocytes, and the adverse effects were found to be more pronounced in patients with a history of cardiovascular disease[17].

Remdesivir was found to be effective in reducing mortality estimates by 5.25% by day 15[18]. Molnupiravir treatment showed a 6.8% reduction in hospitalization risk through day 29 and a 3.0% reduction at hospitalization or death[19]. Ritonavir-boosted Nirmatrelvir treatment demonstrated a 6.32% reduction in hospitalization or death[20].

Vaccines Used to Prevent Severe SARS-CoV-2 Infections

RNA Vaccines

Among the WHO-recognized EUL vaccines, mRNA vaccines developed by BioNTech-Pfizer and Moderna were the most recent type of vaccines. These vaccines deliver the genetic material of the virus's spike (S) proteins into the body. When the immune system detects newly synthesized spike proteins, the body is prompted to produce monoclonal antibodies. When the body is faced with a real infection, the body will

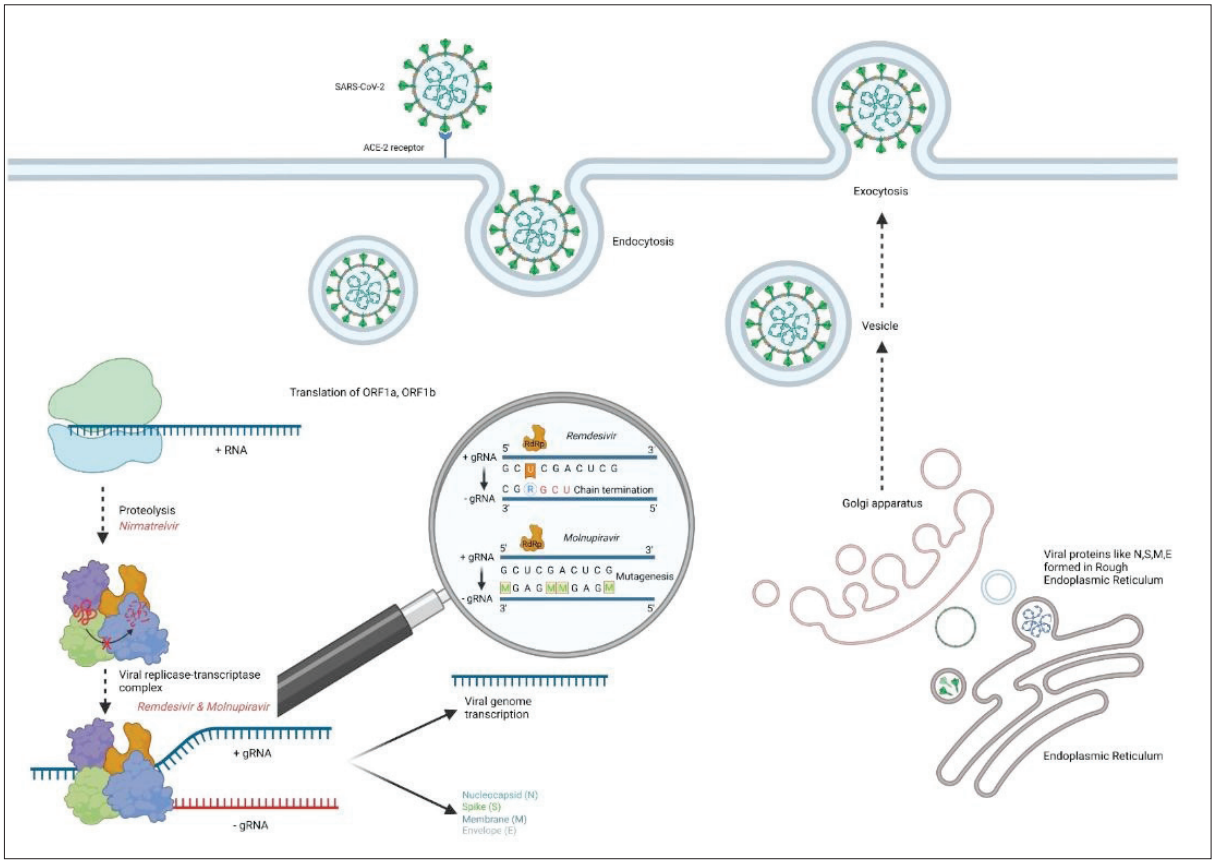

Figure 1. The viral replication mechanism of the SARS-CoV-2 virus, and the effect of antiviral drugs: Remdesivir, Molnupiravir and Nirmatrelvir.

be primed to fight the virus when it encounters the same spike protein. The BNT162b2 mRNA (BioNTech-Pfizer) vaccine utilizes ionizable ALC-0315 for a lipid nanoparticle system (LNP) and a nucleoside-modified mRNA where all uridine residues are replaced by N1-methylpseudouridine to enhance mRNA translation. The mRNA encodes a full-length SARS-CoV-2 spike glycoprotein that includes two proline substitutions in the S2 subunit to lock the protein in its prefusion combination[21]. BNT162b2 generates strong immunogenicity by inducing both CD4+ and CD8+ T cells. Dendritic cells that are transfected with the mRNA vaccine present the class II MHC complex to immune cells[22]. According to the Centers for Disease Control and Prevention (CDC), mRNA vaccines are the most effective type of vaccine against SARS-CoV-2 and their use is recommended. The vaccine efficiency (VE) against B.1.351 (Beta) was 96.4% for mRNA-1273 (Moderna) and 72.1% for the BNT162b2 vaccine at two doses[23,24].

DNA Vaccines

INO-4800 is a DNA vaccine that has fragmented DNA encoding foreign proteins in bacterial plasmids[25]. This vaccine has 70% efficacy in preventing symptomatic COVID-19 cases[26].

Vector Non-replicating Vaccines

The ChAdOx1 vaccine is a replication-deficient adenovirus vector vaccine expressing the full-length MERS-CoV spike (S) protein optimized for protein translation. ChAdOx1 was found to be successful at inducing CD8+ T cells to create cytotoxic responses and B cells to synthesize neutralizing antibodies[27]. The VE of the viral vector, adenovirus, and vaccine ChAdOx1 was 10.4%, and the VE of the whole inactivated Vero cell virus vaccine NVX-CoV2373 was 51.0% for two doses against the B.1351 (beta) variant[28,29]. AD5-nCOV (CanSino) is a non-replicating viral vector vaccine that uses AD5-nCOV as a vector. This vaccine was shown to be 48% effective. Ad26.COV2. S and Gam-COVID-Vac are also other non-replicating viral vector vaccines with recombinant adenovirus.

Protein Subunit Vaccines

The VNVX-CoV2373 (Novavax) vaccine is a subunit vaccine from the full-length S protein that is stable in the prefusion conformation. The vaccines form 27.2 nm thermostable subunit nanoparticles that bind to the human angiotensin-converting enzyme two (hACE2) receptor with high affinity[30].

Whole Inactivated Virus Vaccine

Inactivated vaccines enhance humoral immunity and cell-mediated responses to inactivate the arrangement of the whole virus. They are non-live vaccines. While its safety in immunocompromised patients is an advantage, its low efficacy in comparison to live vaccines is a disadvantage. CoronaVac had 50.7% efficacy. Sinopharm also had 79% efficacy and 91% effectiveness[31]. The BBIBP-CorV (Sinopharm) vaccine is a whole inactivated virus vaccine that is grown in Vero cells and inactivated by beta-propiolactone. The vaccine uses aluminum hydroxide as an adjuvant to increase its efficacy[32].

Types of CRISPR-Cas Systems

Cas1 and Cas2 genes are present in all CRISPR/Cas systems since they constitute the adaptation process of the adaptive immune system, but other Cas protein-encoding genes can be found in addition to Cas1 and Cas2[33]. There are three main types of CRISPR: Type I, Type II, and Type III. Type II systems are only found in bacteria, whereas Type I and Type III systems can be found in both bacteria and archaea[34]. Types I and III both utilize Cas6 as an endonuclease to obtain crRNA from pre-crRNA (Figure 2). In contrast to Type III systems, Type I systems use Cas3 to degrade the intrusive foreign genome[35].

Type III systems branch into Type III-A, which degrades DNA molecules, and Type III-B, which degrades RNA molecules[36]. Type II systems need fewer molecules to obtain crRNA from pre-crRNA using Cas9 to recognize tracrRNA complementary to the repeated sequences of pre-crRNA and the pre-crRNA/tracrRNA hybrid complex[37]. The nature of crRNA-guided Cas9 DNA degradation allowed

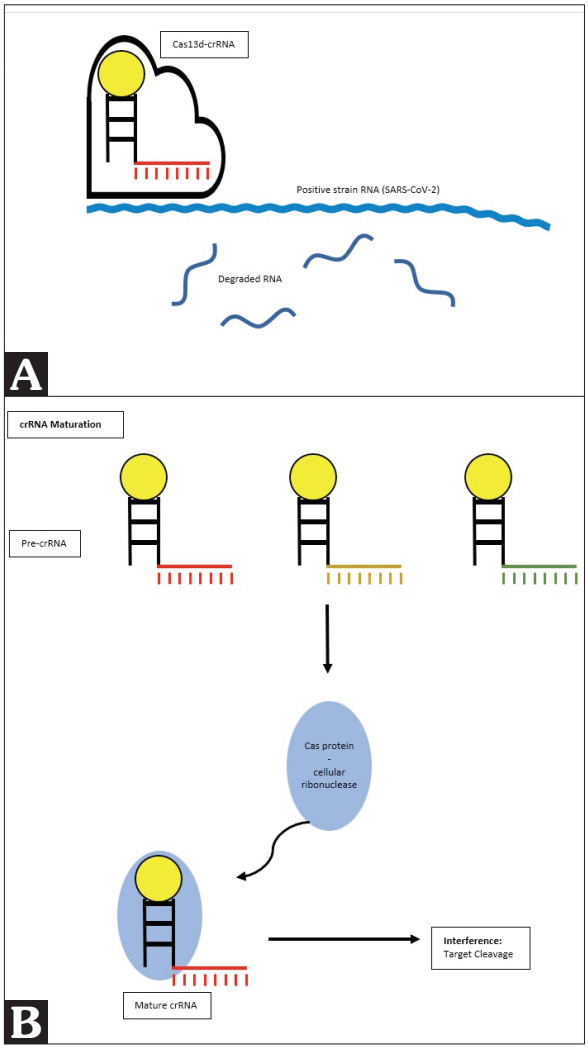

Figure 2. Three stages of CRISPR/Cas13d (A) Cas13d/CRISPR RNA; Cas13d/crRNA complex binds and degrades viral RNA genome (B) Pre-CRISPR RNA; Pre-crRNA is matured by the Cas protein to later apply target cleavage in Interference.

Type II systems to be used as a genome editing tool. Thought to be evolved from Type III systems, Type IV CRISPR-Cas systems have the ability to encode Cas-like proteins that have the potential to combine with small RNAs[38]. A subunit of the Type IV system, Type IV-d, was utilized against SARS-CoV-2. Other types of CRISPR-Cas systems require specific adjacent sequences to guide the Cas protein to the target cleavage site. On the other hand, RNA-guided RNA targeting Cas13d is the endonuclease of Type IV-d CRISPR-Cas systems that can target cleavage-specific RNA without the use of these specific adjacent sequences. This feature allows for the development of more flexible guide RNAs (gRNAs) with speed and efficiency, enabling the rapid development of gRNAs that can compete with the pace of mutations[8].

CRISPR/Cas13d in COVID-19 Treatment

Multiple studies have used CRISPR/Cas13d to develop a broad-spectrum treatment for COVID-19. The flexible nature of the gRNAs of the Cas13d endonuclease has made Cas13d an ideal option to be used against SARS-CoV-2. Nguyen et al. engineered 10.333 gRNAs to simultaneously target 10 peptide-coding regions of the ORF1ab (replicase-transcriptase) and Spike (S) genes of SARS-CoV-2. To safely deliver the Cas 13d effector, they used adeno-associated viruses (AAVs). The micro nature of the complex enables the delivery of up to three gRNAs targeting various peptide-encoding genes in a single AAV vector[8]. Abbot et al. examined 47 SARS-CoV-2 strains and found two highly conserved areas: the nucleocapsid (N) gene encoding the capsid protein for viral packaging and the ORF1ab gene. They then designed 20 crRNAs for each conserved gene to make a total of 40 crRNAs. They infected the lung epithelial A549 cell line with lentiviruses followed by an mCherry marker that was coexpressed with Cas13d. They used green fluorescent protein (GFP) to test the expression of RdRp-(ORF1ab) targeting crRNAs and nucleocapsid- (N gene) targeting crRNAs. The results were 86% repression of GFP in one pool of crRNAs in the central region of the RdRp fragment and 71% repression in one pool of N-gene targeting crRNAs. The employment of dual gene targeting enables a better reduction in the expression of essential viral proteins and mitigates the loss of efficacy induced by rapid mutations of SARS-CoV-2[39]. SARS-CoV-2-Vero 6 cells expressing Cas13d with SN1 or SN11 crRNA targeting the N gene tested separately inhibited the viral genomic titer by 96% and 94%, respectively, at 24 hours post-infection (hpi)[40]. The combination of SN1 and SN11 together resulted in a 97% inhibition rate. Cas13d was also found to be effective at inhibiting the endemic human coronavirus HCoV-229E. The designed crRNAs

not only target the N gene of 229E but also target RdRp. N20, the best-performing crRNA, inhibited 97% of the viral genomic titer at 48 hpi. 229E coronavirus inhibition in MRC-5 fibroblast cells delivered via lentiviruses with subcellular localization tag NLS-Cas13d and N crRNA at 24 hpi showed 83% and 64% antiviral inhibition, respectively (Table 1).

Combined Treatment of CRISPR/Cas13d and Small-Molecule Antiviral Drugs in COVID-19

Leiping Zeng et al. performed combined therapy of Cas13d together with drugs that target RNA replication (EIDD-1931, remdesivir, clofazimine), drugs that are in synergy with remdesivir (elbasvir and velpatasvir), and drugs that target viral entry (camostat mesylate, E-64, and clofazimine). The cytotoxic dosage of these drugs was tested, and half-maximal responses (EC50) were determined in Medical Research Council cell strain five (MRC-5) lung fibroblast cells against human coronavirus 229E (HCoV-229E). These drugs were combined with Cas13d, and all the combinations showed great performance boosts. EIDD-1931 and Cas13d with crRNA SN1, which showed the best targeting against the N gene, reduced the virus titer by 5.9-fold and 2.9-fold, respectively. On the other hand, the combinational use resulted in a 32.2-fold reduction in SARS-CoV-2. In recent experiments on the 229E virus, a reduction of more than 5000-fold was observed in combination therapy. Cas13d/SN1 and EIDD-1931 alone showed

Table 1. Effect of CRISPR-Cas13d and respective crRNA, tag, and delivery method on virus titer

Endemic coronavirus	crRNA used	Inhibited viral genome titer
SARS-CoV-2 Cas13d variant 24 hpi	SN1	96%
	SN11	94%
	SN16	63%
	SN9	54%
	SN1 + SN11	97%
HCOV-229E 48 hpi	N1	77%
	N4	90%
	N9	96%
	N20	97%
HCOV-229E-RdRp 48 hpi	N1	75%
	N4	87%
	N9	95%
	N20	96%
HCOV-229E-N 48 hpi	N1	70%
	N4	82%
	N9	94%
	N20	95%
MRC-5 cells challenged with 229E 24 hpi	**Cas13d with NES tag using N1 crRNA**	16%
	Cas13d with NLS tag using N1 crRNA	83%
	Lentivirus delivery of crRNA	
MRC-5 cells challenged with 229E 24 hpi	NES-Cas13d-N20	29%
	NLS-Cas13d-N20	64%
	LNP delivery of crRNA	
MRC-5 cells challenged with 229E 24 hpi	NES-Cas13d-N20	66%
	NLS-Cas13d-N20	31%

238.9- and 100.8-fold reductions, respectively (Table 2). The same effect was observed when an alternative crRNA (N20) was used[40]. One limitation of small-molecule antiviral drugs is their cytotoxicity at high levels, but their combination with Cas13d/SN1 allowed the use of a lower dosage of the antiviral drug while maintaining high efficacy[41]. E-64, EIDD-1931, and remdesivir showed minimal antiviral activity at low dosages, but combination therapy created a synergy that allowed the therapy to show greater inhibition than the individual effects of Cas13d/crRNA and antiviral drugs combined[40]. The researchers used human primary bronchial epithelial cells (PBEC) transduced with Nuclear Export Sequence (NES)-Cas13d antiviral treatment cultures, and infected them with human coronaviruses 229E, and many SARS-CoV-2 variants, including Omicron. At 6 hpi, the culture was transfected with crRNA using lipid nanoparticles (LNPs). At 48 and 72 hpi, viral genome copies of 229E were reduced by 78% and 92%, respectively, and the viral genome copies of SARS-CoV-2 were reduced by 54% at 24 hpi and 97% at 48 hpi. Finally, crRNA-SN1 effectively reduced the Omicron variant viral genome copy number by 70% at 24

Table 2. Effect of CRISPR-Cas13 and combined antiviral therapy on virus titer with respect to different crRNAs on SARS-CoV-2 variants

Endemic coronaviruses	Cas13d virus titer reduction in folds (x)	Drug treatment virus titer reduction in folds (x)	Cas13d and drug treatment virus titer reduction in folds (x)
	Using SN1 crRNA		
SARS-CoV-2 treatment 48 hpi	2.9x		
EIDD-1931		5.9x	32.2x
Remdesivir		10.5x	40.7x
Clofazamine		15.7x	64.1x
E-64 d		1.9x	7.7x
Elbasavir		1.6x	5.7x
Velpatasvir		1.4x	9.5x
	Using N1 crRNA		
HCOV-229E treatment 48 hpi	239x		
EIDD-1931		100.8x	5001.3x
E-64		3.6x	927.8x
229E treatment with Cas13d and drug 1 hpi	4.7x		
EIDD-1931		1.6x	20.6x
Remdesivir		1.1x	7.2x
E-64 d		1.2x	8.6x
229E treatment with Cas13d and drug 3 hpi	7.5x		
EIDD-1931		2.6x	56.5x
Remdesivir		1.9x	39.9x
E-64 d		1.4x	27.3x
229E treatment with Cas13d and drug 6 hpi	2.3x		
EIDD-1931		2.4x	10.6x
Remdesivir		2.3x	11.1x
E-64 d		2.3x	5.8x

Table 3. Inhibited viral genome titers of SARS-CoV-2 variants with respect to NES-Cas13d-LNP delivery of crRNA

		Inhibited viral genome titer		
Endemic coronaviruses	**NES-Cas13d-LNP delivery of crRNA**	6 hpi	24 hpi	48 hpi
SARS-CoV-2-Vero 6 cells	SN1	93%		88%
	SN11	92%		83%
HCOV-229E	N1			78%
SARS-CoV-2 WA1	SN1		54%	97%
SARS-CoV-2 Omicron	SN1		70%	85%

hpi and 85% at 48 hpi (Table 3). This suggests that this treatment will be effective in targeting future variants as well, essentially providing a broad-spectrum therapy[40].

CONCLUSION

In conclusion, there is a decrease in produced monoclonal antibodies in response to vaccines and antiviral drugs. Small-molecule antiviral drugs work by targeting multiple viral replication mechanisms. However, viruses can easily evade small-molecule antiviral drugs through mutations in their targeted regions. As a result, a need for broad-spectrum combination therapy emerged. The contemporary method engineered for this problem is the use of CRISPR-Cas13d/crRNA to target the conserved gene of SARS-CoV-2 that is not altered throughout different variations. This will provide a broad-spectrum solution that will remain effective against all known, and potential future variants. In the future, the ability to more precisely target crRNAs and gRNAs may allow for the administration of lower doses of antiviral drugs while still providing relatively high inhibition. This will also help to reduce antiviral cytotoxicity. Further studies are needed to establish the efficacy and safety of CRISPR/Cas13d treatment in COVID-19.

CONFLICT OF INTEREST

The authors declare no competing interest. All the figures and tables in our article are originally created by the authors.

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Address for Correspondence/Yazışma Adresi

Dr. Özlem ÖZER ÇAKIR

Department of Gastroenterology and Hepatology, Demiroğlu Bilim University Faculty of Medicine, İstanbul-Türkiye

E-posta: tansozlem@yahoo.com