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REVIEW

Progress in CRISPR/Cas13-Mediated Suppression of Influenza A and SARS-CoV-2 Virus Infection in *in vitro* and *in vivo* Models

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Abstract—The worldwide number of deaths from complications caused by severe influenza and COVID-19 is about 1 million cases annually. Development of the effective antiviral therapy strategies for the disease treatment is one of the most important tasks. Use of the CRISPR/Cas13 system, which specifically degrades viral RNA and significantly reduces titer of the virus, could be a solution of this problem. Despite the fact that Cas13 nucleases have been discovered only recently, they already have shown high efficiency in suppressing viral transcripts in cell cultures. The recent advances in mRNA technology and improvements in non-viral delivery systems have made it possible to effectively use CRISPR/Cas13 in animal models as well. In this review, we analyzed experimental *in vitro* and *in vivo* studies on the use of CRISPR/Cas13 systems as an antiviral agent in cell cultures and animal models and discussed main directions for improving the CRISPR/Cas13 system. These data allow us to understand prospects and limitations of the further use of CRISPR/Cas13 in the treatment of viral diseases.

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INTRODUCTION

The CRISPR/Cas systems (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated nuclease) for targeted genome editing revolutionized modern molecular biology [1]. At present numerous types of such systems have been discovered, with majority of them using Cas-nuclease in complex with the guide RNAs to introduce double-strand breaks to the complementary DNA target [2]. A new class of Cas-nucleases has been discovered recently that targets single-stranded RNAs – Cas13 [3]. At present 6 types of Cas13 proteins have been identified (a-d, x, y) that differ in size [4, 5]. All Cas13 nucleases investigated so far exhibit ribonuclease activity mediated by two HEPN-domains. Cas13 protein in complex with guide CRISPR RNA (crRNA) binds to the complementary sequence of the RNA target followed by its cleavage by the activated Cas13. Unlike in the case of Cas9, the use of Cas13 does not cause any changes in the eukaryote genome and allows cleaving viral RNA inside the infected cells thus preventing translation of viral mRNA. Absence of the effects on genome provides advantage to this strategy in antiviral therapy.

The first studies on inactivation of the target RNA with the help of Cas13 were aiming at activation of apoptosis in pancreatic cancer cell line [6]. The studies on the use of nucleases of the Cas13 family

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in suppression of infection in mammalian cell culture were published in 2019 [7]. American scientists developed a CARVER platform (Cas13-assisted restriction of viral expression and readout), which combines the Cas13-mediated cleavage of viral RNA with rapid Cas13-based diagnostics (SHERLOCK) [7, 8]. The Cas13a nucleases of *Leptotrichia wadei* (LwaCas13a) and Cas13b of *Prevotella* sp. P5-125 (PspCas13b) were successfully used for inactivation of the following viral single-stranded RNA (ssRNA): Lymphocytic Choriomeningitis Virus (LCMV), influenza A virus (IAV), and vesicular stomatitis virus (VSV) in different cell cultures. Use of LwaCas13a and PspCas13b resulted in significant decrease of the level of viral RNA and titer of the LCMV, IAV, and VSV viruses; it was shown in the course of analysis of the virus genome after treatment with nucleases that there were no mutations in the target sequences of the guide RNAs [7]. These results opened new perspectives for the development of innovative preparations for antiviral therapy. As a result, many scientific groups worldwide joined the research including studies devoted to the search of new Cas13 orthologs capable of fighting infections. In 2020 Li et al. [9] discovered that cleavage of the *NS3* gene in Dengue virus by the LwaCas13a nuclease could effectively inhibit virus replication in the African green monkey kidney cells (Vero cells).

The coronavirus pandemic caused by the SARS-CoV-2 emphasized the need for improvement of antiviral strategies, which could arrest spread of infection as soon as possible and without additional vaccination of the population. For this purpose, Abbott et al. in 2020 [10] tested the possibility of using the RfxCas13d nuclease for suppressing development of IVA infection in the human lung adenocarcinoma cell line A549. Lentiviruses were used for nuclease gene delivery, while guide RNAs were transfected with the help of lipofectamine. This technique was termed PAC-MAN. Following this work in 2022 in the same laboratory the PAC-MAN technique was successfully used for suppression of different variants of coronaviruses, including SARS-CoV-2, in cell culture. Co-localization of nuclease and guide RNAs was shown to be important: nuclear localization in the case of added nuclear localization signal (NLS) to the RfxCas13d protein and lentivirus-mediated delivery of guide RNAs; and in the case of added nuclear export signal (NES) to the RfxCas13d protein and crRNA delivery mediated by lipid nanoparticles [11]. In 2021 Nguyen et al. [12] also used RfxCas13d with nuclear localization signal for suppression of development of the human immunodeficiency virus HIV-1 in the human cell culture.

In addition to the used delivery method, presence of modified nucleosides in the crRNA could significantly affect efficiency of the Cas13-mediated suppression of expression. In particular, it was shown by Chaves et al. [13] that the crRNAs containing four phosphorothioate modifications reduced the number of copies of PB2 (polymerase basic 2) of the influenza virus A to a greater extend in comparison with the non-modified crRNA. It was demonstrated in another study [14] that the presence of 2′-O-methylated bases or phosphorothioate modifications in the crRNA results in the enhanced knockdown of the endogenous transcripts in human cells, furthermore, modifications at the 3′-ends were preferable. It should be noted that the simultaneous presence of two types of modifications in crRNA results in a lesser extent of expression suppression [13, 14].

The results of the use of CRISPR/Cas13 in antiviral therapy of SARS-CoV-2 and influenza virus using cell culture demonstrated its high efficiency in suppression of viral transcripts. However, number of such studies using CRISPR/Cas13 in animal models is limited. In this review we discuss recent advances in this research area and provide detailed analysis of the results of experimental studies devoted to the development of CRISPR/Cas13-based preparations for treatment of influenza virus A and SARS-CoV-2 viral infections using cell cultures and animal models.

BIOLOGY OF INFLUENZA VIRUS A AND SARS-CoV-2

SARS-CoV-2 is a positive (+) single-stranded RNA-containing virus belonging to the family of coronaviruses (Coronaviridae). Coronaviruses have large genomes (~30 kb), which is 2-3-fold larger than the genomes of the majority of RNA viruses, and it encodes a large set of non-structural (nsp) and structural proteins as well as accessory proteins. Replication–transcription complex of this virus contains 16 nsp (1-16), which interact with each other and perform various functions required for effective transcription and replication [15] (Fig. 1). The key element of this complex is the RNA-dependent RNA polymerase (RdRp), which performs synthesis of the viral RNA genome, however, replication and transcription would be impossible without the presence of key non-structural proteins assisting these processes and, hence, representing effective targets for the Cas13-based antiviral therapy, same as RdRp, which catalyses RNA synthesis with the help of nsp7 and nsp8. Previous investigations of the SARS-CoV showed presence in the genome of nsp8 (which is primase and 3′-end adenylyl transferase [16]), nsp13 (which is RNA helicase containing Zn-binding domain [17] and RNA 5′-phosphatase [18]), nsp14 – (which is 3′→5′ editing exonuclease (proofreading) [19]), nsp15 (which is uridylyl-specific endonuclease [20]), and nsp16 (which is 2′-O-methyl transferase) [21]. These proteins are capable to perform

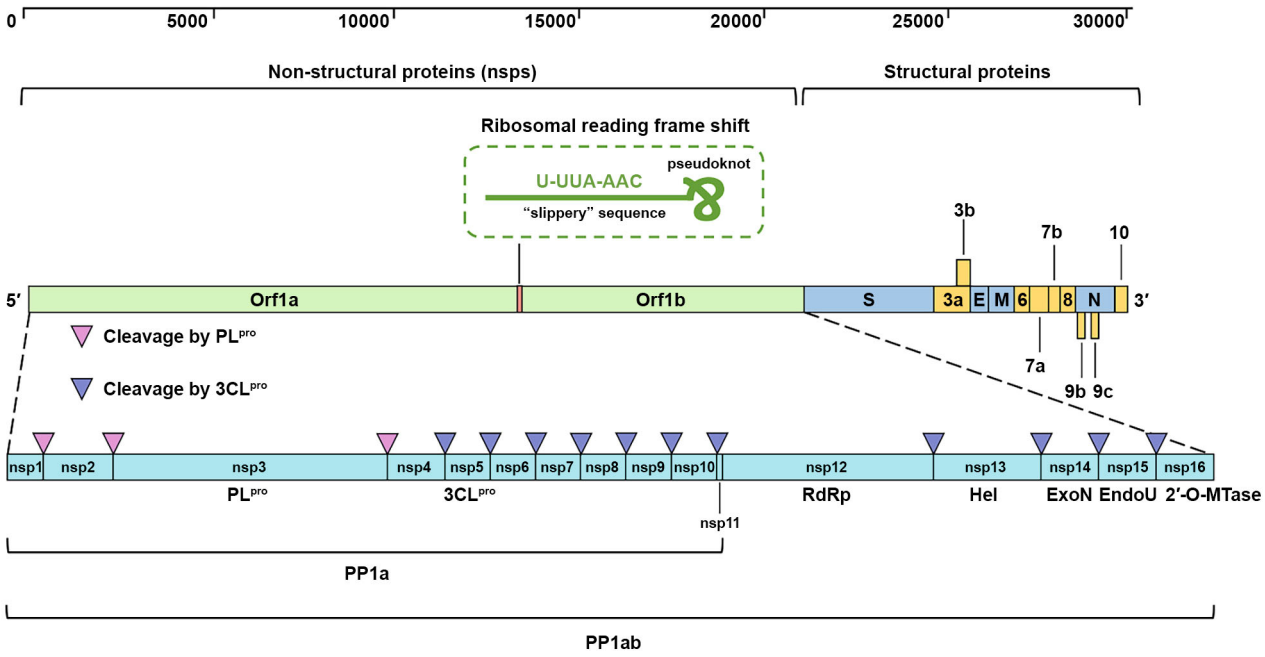

Fig. 1. Structural and non-structural proteins encoded in the genome of SARS-CoV. Non-structural proteins, nsp (1-16), perform important roles in virus replication and are encoded by the ORF1ab. Polyproteins PP1a and PP1ab are cleaved by the viral proteases PL^{pro} (papain-like protease) and $3CL^{pro}$ (3C-like basic protease) into active viral proteins. Structural and accessory proteins are encoded in the 3′-end part of the genome.

their functions only after the replicase polyproteins PP1a and PP1ab are proteolytically cleaved by the viral papain-like proteases PL^{pro} and $3CL^{pro}$ (3C-like protease) into individual active components. Synthesis of the PP1ab variant proceeds due to appearance of the programmed ribosomal shift of the open reading frame (ORF), which occurs at the end of ORF1a at the particular "slippery" sequence (5′-U UUA AAC-3′). As a result of the open reading frame shift, there could be the shift of the ribosome translating codons UUA and AAC back (−1) by one nucleotide, which is followed by the re-start of translation at the CGG codon in the ORF1b [22]. This process occurs due to the presence of the specific pseudoknot structure in the RNA located after the "slippery" sequence with tertiary structure preventing correct translation of the ribosome [23]. Efficiency of the open reading frame shift is 45-70%, hence, the level of expression of the protein encoded by the ORF1a is 1.5-2-fold higher in comparison with the protein encoded by the ORF1b [24].

Synthesis of RNA by the replication–translation complex from the viral (+) genomic RNA starts with the synthesis of the complete chain of antigenomic (−) RNA, which serves as a template for new genomic RNA and set of subgenomic mRNAs encoding four structural proteins required for the virion assembly, and accessory proteins exhibiting various functions such as modulation of innate immune responses of the host cells [25]. Replication produces full-size viral genomic (+) RNA, which could be translated into additional polyproteins of the replicative complex, as well as it serves as a template for additional synthesis of antigenomic (−) RNA or is packed into virions. Transcription produces a set of subgenomic mRNAs used for expression of structural and accessory proteins. Replication and transcription require specific templates of antigenomic (−) RNA.

The structural proteins encoded after the ORF1ab include the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. In addition to the above-mentioned proteins with investigated functions, small open reading frames have been identified overlapping with several described ORFs. For some SARS-CoV proteins presence of the leaky scanning mechanism has been reported, when the ribosome starts transcription at the second inner methionine rather than at the first terminal methionine [26, 27].

Influenza virus A belongs to the family of orthomyxoviruses (Orthomyxoviridae) and, similarly to SARS-CoV-2, causes seasonal respiratory diseases in humans. Influenza virus genome comprises a negative (−) genomic ssRNA. Unlike the (+) RNA virus SARS-CoV with the genomic RNA comprising functional mRNA, the genomic (−) RNA of these viral pathogens is not capable of initiating infection on its own when introduced into the permissive cells because the viral proteins are translated from the complementary mRNA (Fig. 2a). The transcriptionally active complex is a viral ribonucleoprotein (vRNP) consisting of the viral (−) genomic RNA in complex with nucleoprotein (NP)

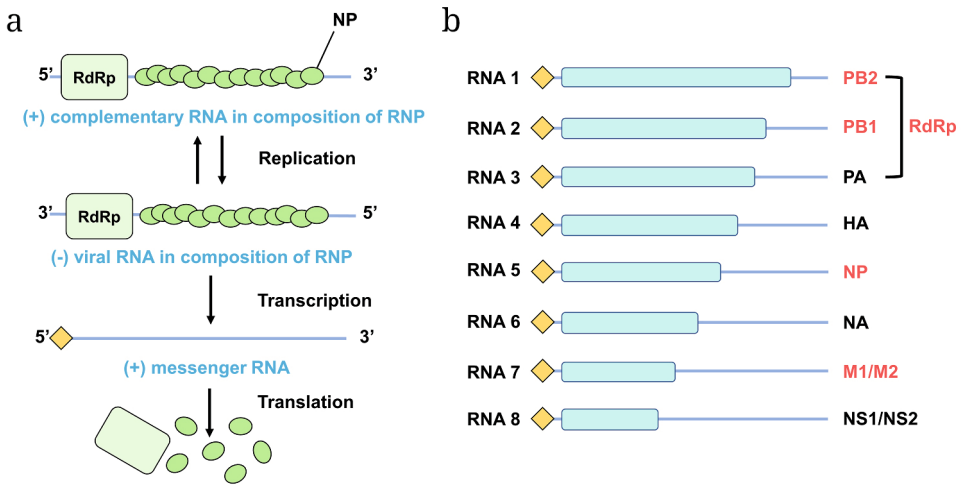

Fig. 2. Schematic representation of the influenza virus A genome. a) Replication of influenza virus and of other RNA viruses (–) RNA genome. Ribonucleoprotein complex (RNP) consisting of (–) genomic RNA in complex with NP cannot be translated and produce viral proteins. Viral polymerase RdRp interacting with RNP first transcribes (+) mRNA, from which viral proteins are translated that are required for the virus replication, and next participate in replication of a new genomic (–) RNA. b) 8 segments of RNA encode viral proteins. Cas13 targets are marked with red.

and RdRp [28]. Viral RNA polymerase is required for transcription of both mRNA and complementary to the (–) genomic RNA antigenomic (+) RNA, because mammalian cells do not have such enzyme. Moreover, the viral mRNA is truncated at 3′-end and could serve only as a template for translation of viral proteins. The influenza virus A genome consists of eight RNA chains encoding the following proteins: PB1 (polymerase basic 1), PB2, PA (polymerase acidic), HA (hemagglutinin), NP, NA (neuraminidase), M (matrix proteins), and NS (non-structural proteins) [29] (Fig. 2b). Each segment of the viral RNA (1-6) encodes one protein: PB2, PB1, PA, HA, NP, and NA. The seventh RNA encodes 2 matrix proteins (M1 and M2), which are formed as a result of splicing of the initial RNA [30]. The eighth segment encodes interferon agonist NS1 [31]; this viral RNA also could be subjected to alternative splicing and could encode the NEP/NS2 protein [32], which participates in export of vRNP from the nucleus of the infected cells, where replication occurs, unlike in the case of the SARS-CoV virus, where replication occurs in cytoplasm. The active viral RdRp is composed of three subunits: proteins PB1, PB2, and PA [33]. Similar to the case of SARS-CoV-2, sequence of the *RdRp* RNA includes highly conserved regions in the majority of strains, which makes them very attractive targets for the CRISPR/Cas13 system [34]. On the contrary, the viral glycoproteins NA and HA are surface proteins with high degree of variability. Eighteen subtypes of HA and 11 of NA have been identified, however, only 3 HA subtypes (H1, H2, and H3) and 2 NA subtypes (N1 and N2) caused epidemics in humans [35]. Virion assembly requires interaction of three integral membrane proteins, HA, NA, and M2, with M1, which encloses virion core. Nuclear export protein (NEP, also known as non-structural protein 2, NS2) and RNP are located inside the M1 matrix. It is worth mentioning that, unlike the SARS-CoV-2, the influenza virus does not have exonuclease with 3′→5′ editing activity, which is the cause of emergence of a large number of mutations in the virus genome and, hence, reduced efficiency of vaccines.

CLASSIFICATION OF CRISPR/Cas13 SYSTEMS

Systems of ‘adaptive immunity’ in bacteria, CRISPR/Cas, are widely used in laboratory practice, and, recently, started to be introduced into therapy. Cas-nucleases are commonly divided into class I nucleases (in which cleavage of the target nucleic acids is mediated by multisubunit protein complex) and class 2 nucleases (in which a full multidomain protein functions as an effector nuclease). In the class 2 nucleases 3 main nuclease families have been identified: Cas9, Cas12, and Cas13. The first two families include mainly DNA-specific nucleases, while the family of Cas13 nuclease include predominantly RNA-specific enzymes [36].

The following variants have been recognized in the family of Cas13 nucleases: Cas13a (previously known as C2c2), Cas13b (previously known as C2c6), Cas13c (previously known as C2c7), Cas13ct, Cas13d (synonymic name CasRx), Cas13X, and Cas13Y (previously assigned to the group Cas13bt) [37, 38]. The so-called ‘ancestral’ Cas13an nuclease has been recently discovered [39] (Fig. 3a). Furthermore, metagenomic analysis predicts existence of at least 5 more groups of Cas13 enzymes, which have not been found yet [40].

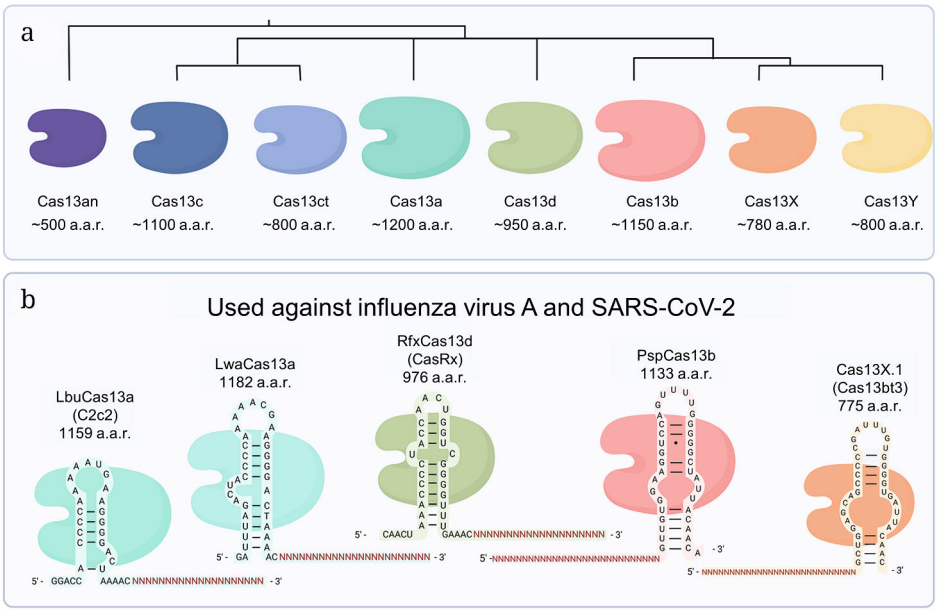

Fig. 3. Diversity of Cas13 nucleases. a) Discovered and characterized enzymes of the Cas13 family with the degree of phylogenetic relatedness (based on the information reported in [37-39]). b) Enzymes of the Cas13 family used for inactivation of viruses in cell culture and under *in vivo* conditions (based on the information reported in [3, 37, 38, 41, 42]).

The Cas13 nucleases are capable of cleaving RNA in complex with crRNA consisting of a spacer complementary to the target RNA and direct repeat forming a hairpin through which the guide RNA binds to nuclease and activates it [41]. Depending on the nuclease type spacer could be located at the 5′-end relative the direct repeat (such as in the case of PspCas13b nuclease), or at the 3′-end of the direct repeat (such as in the case of LbuCas13a) (Fig. 3b).

As has been mentioned above, Cas13 nucleases perform cleavage of the target RNA at two HEPN sites [42]. In addition to the target RNA, these sites are responsible for cleaving pre-crRNA in the process of maturation of guide RNA [43]. Unlike the enzymes of the Cas12 or Cas9 families, the PAM sequence (Protospacer adjacent motif) is not required for cleaving the target sequence, however, the enzymes of the Cas13 family exhibit loose preference for 5′- or 3′-flanking protospacer sequence (PFS): for example, the enzyme Cas13X.1 is more active towards the sequences with 5′-flanking guanine [38].

Characteristic feature of the Cas13 nucleases is the so-called collateral activity – non-specific cleavage of non-target RNA after introduction of the targeted break; the SHERLOCK system for detection of nucleic acids is based on this property [8]. It has been also demonstrated that this activity could cause arrest of the host cell growth to prevent spread of the viral infection in the culture [44].

The family of Cas13 proteins is very diverse and includes enzymes with size from ~500 aa (ancestral Cas13) to ~1250 aa (Cas13a enzymes). Efficiency and specificity of the targeted RNA cleavage for different enzymes also vary: it was shown that the cleavage by miniature nucleases (Cas13X.1 or RfxCas13d) is more effective and specific than by the larger LwaCas13a or PspCas13b [37].

APPLICATION OF CRISPR/CaS13 IN SARS-CoV-2 THERAPY

The SARS-CoV-2 coronavirus attracts the highest interest in the context of using the CRISPR/Cas13 system against viral infection: the first study evaluating antiviral efficiency of the CRISPR/Cas13 system was published in 2020 (Table 1) [10]. The highly conserved genes *RdRp* and *N* were used as targets, and the RfxCas13d system was selected as an effector. The study was carried out using the human lung adenocarcinoma cell line A549, which was transduced with lentivirus to generate a transgenic cell line Cas13d-A549 stably expressing Cas13d. It was shown that the use of one from the five pools of crRNA specifically recognizing sequence of the RdRP gene resulted in the decrease of expression of the reporter GFP protein fused with RdRp and N by 86%. The results of quantitative PCR (qPCR) demonstrated that the therapy with different pools of crRNA resulted in the decrease of the amount of viral mRNA by more than 80%. In other studies, with crRNA targeting mRNA of nucleocapsid, the researchers also demonstrated significant success in the cell cultures [11, 37, 45, 46]. In particular, the use of crRNA together with Cas13d effectively inhibited

Table 1. Use of the CRISPR/Cas13 system for protection against viral infection *in vitro*

Pathogen	Target sequence	Enzyme	Delivery method	Experimental model	Main results	References
SARS-CoV-2	*RdRp*, *N*	RfxCas13d-NLS	Cas13 – lentiviral transduction; crRNA – lipofection	A549	pool of crRNAs targeting *RdRp* ↓ expression of viral proteins by 86%, reporter proteins – by 83%; pool of crRNAs targeting *N* ↓ expression of viral protein by 71%, reporter proteins – by 79%	[10]
IAV H1N1	highly conserved regions in *RdRp*, *HA*, *NA*, *NP*, *M1*, *M2*, *NS1*, *NEP* genes				crRNA targeting *NA* ↓ expression of the reporter by 72% at MOI 2.5; crRNA targeting *NA* ↓ expression of the reporter by 52% at MOI 5; crRNA targeting *HA* ↓ expression of the reporter by 26% at MOI 2.5; crRNA targeting *HA* ↓ expression of the reporter by 55% at MOI 2.5	
SARS-CoV-2	*S*, *NCP*	pspCas13b27-NES	lipofection	HEK293T	crRNA targeting *S* ↓ level of *S*-transcripts by 99%; crRNA targeting *NCP* ↓ level of nucleocapsid transcripts by 99%	[49]
				Vero	crRNA targeting *S* ↓ level of *S*-transcripts by 90%; crRNA targeting *NCP* ↓ level of nucleocapsid transcript by 90%, ↓ virus load by ~90% and by 60% 24 and 48 h after infection	
				Calu-3	crRNA targeting *NCP* ↓ viral titer by 98%	
IAV WSN/33	*PB1*, *PB2*	LbuCas13a/ LbuCas13a-NLS	lipofection	A549	crRNA targeting *PB1* + LbuCas13a ↓ number of copies of *PB1* gene by 83%; crRNA targeting *PB1* + LbuCas13a-NLS ↓ number of copies of *PB1* gene by 78%; crRNA targeting *PB2* + LbuCas13a/LbuCas13a-NLS ↓ number of copies of *PB2* gene by 75%; crRNA targeting *PB1* + crRNA targeting *PB2* + LbuCas13a-NLS ↓ number of copies of *PB1* and *PB2* genes by 10% in comparison with monotherapy	[53]
SARS-CoV-2	*RdRp*, *N*			Vero E6	pool of crRNAs targeting *N* + LbuCas13a ↓ cell death by 72%; crRNA targeting *RdRp* + LbuCas13a ↓ number of copies of *RdRp* genes by 93.7%; crRNA targeting *N* + LbuCas13a ↓ number of copies of *N* genes by 94.1%.	

Table 1 (*cont.*)

Pathogen	Target sequence	Enzyme	Delivery method	Experimental model	Main results	References
SARS-CoV-2	*RdRp*, *N*	LbuCas13a/ LbuCas13a-NLS	lipofection	Huh7	crRNA targeting *RdRp* + LbuCas13a ↓ number of copies of *RdRp* genes by 94.5%; crRNA targeting *N* + LbuCas13a ↓ number of copies of *N* genes by 99.1%	[53]
SARS-CoV-2	*N*	RfxCas13d-NLS	lentiviral transduction	Vero E6	crRNA targeting *N* ↓ viral titer by 96% during 72 h	[11]
		Cas13d-NES	lentiviral transduction and LNP with crRNA	hPBECs	↓ viral titer by 54% after 24 h and by 97% – after 48 h	
HCoV-229E	*N*, *RdRp*	Cas13d-NLS	lentiviral transduction	MRC-5	crRNA targeting *N* ↓ virus replication by 95%; crRNA targeting *N* ↓ viral titer by 99%	
	N	Cas13d-NES	lentiviral transduction and LNP with crRNA	hPBECs	↓ of viral titer by 78% after 48 h and by 92% – after 72 h	
HCoV-OC43	*N*, *RdRp*	Cas13d-NLS	lentiviral transduction	MRC-5	crRNA targeting *N* ↓ viral titer by 99%	
HCoV-OC43	*N*, *RdRp*	CasRx	transfection with calcium phosphate method	Vero	crRNA targeting *RdRp* ↓ content of viral RNA by ~80% during 96 h with peak at 72 h (90%)	[50]
HCoV-OC43	*N*, *RdRp*	CasRx	transfection with calcium phosphate method	Vero	↓ of viral titer by 99.97% after 2 days	[45]
HCoV-229E					↓ of viral titer from 78.44% to 99.39% after 2 days	
SARS-CoV-2					↓ of viral titer from 69.65% to 94.07% after 4 days	
SARS-CoV-2	pseudoknot in ORF1b, *RdRp*	Cas13b-NES	transfection	Vero E6	crRNA targeting ORF1b ↓ number of copies of SARS-CoV-2 genes and viral titer by 99%; crRNA targeting *RdRp* ↓ number of copies of SARS-CoV-2 genes by 98%, and viral titer by 90%	[55]
				Calu-3	crRNA targeting ORF1b ↓ number of copies of SARS-CoV-2 genes by 99.9%	

Table 1 (*cont.*)

Pathogen	Target sequence	Enzyme	Delivery method	Experimental model	Main results	References
SARS-CoV-2	ORF1ab, *nsp13*, *nsp14*, *N*	Cas13d	transfection	HeLa-ACE2	crRNA targeting *N* ↓ expression of *N* by 99%; crRNA targeting ORF1ab ↓ amount of RNA by 99%	[47]
SARS-CoV-2	*Ctsl*	RfxCas13d	lipofection	HEK293FT	crRNA targeting *Ctsl* ↓ expression of *Ctsl* by 75%	[54]
				Vero-ACE2-TMPRSS2	63% blocking of pseudotyped infection; ↓ expression of *N* by 60%; ↓ infection 10-fold	
				Caco-2	65% blocking of pseudotyped infection	
SARS-CoV-1				Caco-2	45% blocking of pseudotyped infection	
SARS-CoV-2	*RdRp*, *E*	Cas13X.1	lipofection	HEK293T	↓ expression of reporter by 70%	[37]
H1N1	*NP*			MDCK	↓ expression of viral proteins by 70%; ↓ viral load 4-fold	
IAV H1N1 WSN	*PB*, *NP*, *M*	Cas13a-NLS	lipofection	DF1	↓ 4-fold of WSN titer after 24 h	[62]
IAV H1N1 PR8					↓ 2-fold titer of PR8 after 24 h	
IAV	mRNA and complementary viral RNA	PspCas13b	electroporation	MDCK	↓ of viral RNA 7–22-fold	[7]
		PspCas13b-NLS			crRNAs does not decrease level of viral RNA; pool of crRNA ↓ viral RNA 8-fold	
		PspCas13b-NES			infectivity of IAV ↓ 300-fold; ↓ of viral RNA 5-fold	
SARS-CoV-2	*S*	Cas13a	lentiviral transduction for Cas13a; lipofection for crRNA	HepG2	↓ expression of *S* by 99.9% after 48 h;	[48]
				AT2	↓ expression of *S* by 93% after 48 h; ↓ total expression by 90%	

Table 1 (*cont.*)

Pathogen	Target sequence	Enzyme	Delivery method	Experimental model	Main results	References
SARS-CoV-2	*M*, *N*, *E*, *S*, *nsp3*, *nsp5*, *RdRp*	Cas13b-NES	transfection	HEK293-ACE2	crRNA targeting *RdRp* ↓ infectivity of SARS-CoV-2 by 63% crRNA targeting *RdRp* + crRNA targeting *nsp15* ↓ amount of viral RNA 1.25–1.5-fold in comparison with monotherapy; crRNA targeting *RdRp* + crRNA targeting *S* ↓ viral RNA 1.8-fold in comparison with crRNA targeting *RdRp*; crRNA targeting *RdRp* + crRNA targeting *N* ↓ viral RNA 2.2-fold in comparison with crRNA targeting *N*; testing efficiency of crRNA targeting *RdRp* against SARS-CoV demonstrated decrease of SARS-CoV RNA level by 35%, while the decrease in the case of crRNA targeting *N* was 22%	[46]
SARS-CoV					crRNA targeting *RdRp* ↓ the level of SARS-CoV RNA by 35%; crRNA targeting *N*↓ the level of SARS-CoV RNA by 22%	
SARS-CoV-2	*5′-UTR*, *nsp3*, *nsp4*, *nsp6*, *RdRp*, *N*, *nsp13*, pseudoknot in ORF1b, “slippery” sequence, s2m	Cas13d-NLS	lipofection	HEK293T	crRNA targeting s2m ↓ expression of the reporter by 83%; crRNA targeting s2m + crRNA targeting *nsp3* ↓ expression of the reporter by 92%; crRNA targeting 5′-UTR ↓ replication of the virus by 82%	[52]
SARS-CoV					crRNA targeting 5′-UTR ↓ replication of the virus by 90%	
IAV/H1N1 Cal04/09	*PB2*	LbuCas13a + LbuCas13a-NLS	transfection	A549	90% of viral knockdown; ↓ of viral titer by 90%; ↓ of the level of *PB2* by 90%	[46]
IAV H3N2				A549	↓ of viral titer 10–50-fold	
				HBEC3-KT	↓ of viral titer 5–25-fold	

replication of different variants of *N*-transcript in the SARS-CoV-2, thus reducing the virus titer by 96% [11]. Decrease in inhibition of expression of viral proteins with time has been demonstrated for some crRNAs, however, individual combinations of crRNAs retain their efficiency of inhibition from 24 to 72 h. The authors emphasize that targeting the *N*-transcript allows inhibiting different strains of SARS-CoV-2 including D614G, Alpha, Zeta, Epsilon (B.1.427), and Epsilon (B.1.429) with close efficiency of more than 90%. Moreover, increase of the degree of SARS-CoV-2 inhibition has been observed on addition of low molecular weight preparations (camostat mesylate, EIDD-1931, remdesivir, clofazimine, E-64d, elbasvir, and velpatasvir) in combination with Cas13d and crRNA. Using of the low molecular weight compounds EIDD-1931 or of the Cas13d + crRNA-N preparation separately ensured 5.9- and 2.9-fold decrease of the virus titer, respectively, while the use of their combination resulted in 32.2-fold decrease of the virus titer. Hence, the CRISPR/Cas13-based antiviral therapy could be used in combination therapy [11].

First successes in the SARS-CoV-2 therapy demonstrated with cell cultures served as a starting point for initiation of investigation of the use of different variants of Cas13 in the fight against the SARS-CoV-2 coronavirus. Main attention of the studies was focused on selection of nuclease, delivery methods, selection of the crRNA target, design of crRNAs and their multiplexing, as well as development of optimal regime of therapy.

In their study Xu et al. [37] selected transcripts of the *RdRp* and *E* genes as targets for crRNAs, which are conserved among the SARS-CoV viruses. The HEK293T cells were co-transfected with the reporter vector containing a hybrid gene combining sequence of the *GFP* gene and fragments of the *RdRp* and *E* genes, and the vector with *Cas13X.1*/crRNA. It was shown that 48 h after co-transfection almost all tested crRNAs (27 of 30) inhibited GFP fluorescence in the cells by ~70%. One other study devoted to the use of Cas13d against the SARS-CoV-2 used the most conserved regions of the ORF1ab, *nsp13* and *nsp14*, which perform important functions in all SARS-CoV-2 strains, as targets [47]. The transgenic HeLa-ACE2 cells line producing ACE2 receptor was used to evaluate efficiency of the system. This cell line was co-transfected with the plasmid containing *Cas13d*, and pool of the crRNA plasmids targeting non-structural proteins nsp13 and nsp14. Twenty-four hours after co-transfection the cells were infected with the SARS-CoV-2, and one day after infection the amount of N protein was assessed with the help of Western blotting. The obtained result showed that the crRNA/Cas13d system indeed was capable of dealing with high virus load [MOI (multiplicity of infection) of 2] in the course of SARS-CoV-2 infection, and all tested crRNAs decreased significantly the levels of viral proteins and RNA. The Cas13d + crRNA system was capable of suppressing the emerging epidemic strains of SARS-CoV-2 (including strains B.1, B.1.351, B.1.1.7, and B.1.617) due to targeting the highly conserved regions [47].

In 2021 Wang et al. [48] used LwaCas13a in their study with crRNA targeting the sequence of receptor-binding domain in the S-protein, which plays a key role in interaction with ACE2. This study did not involve infection of the cells with virus, and efficiency of the CRISPR/Cas13 system was determined in the model cell line stably expressing the coronavirus S-protein. Human alveolar type 2 cells (AT2) and human hepatocellular carcinoma cells (HepG2) with high level of ACE2 expression were selected as targets. The results of qPCR demonstrated that the efficiency of suppression of the expression of S-protein mRNA reached >99.9% in the HepG2 and AT2 cells. In another study using S-protein mRNA as a target the variant of PspCas13b with NES was used with increased spacer sequence in the crRNA, which, supposedly, increases specificity of this system [49]. High efficiency of suppression of the S-protein expression was demonstrated for all tested crRNAs. Similar results were obtained using the Vero cell line with the best crRNA demonstrating >90% decrease of the level of S-protein transcripts in the HEK293T and Vero cells. Dose-dependent effect was observed indicating that the availability of crRNA in the cell is a key factor for degradation of viral RNA, moreover, it has been noted that presence of only few copies of the plasmid encoding crRNA is sufficient for effective suppression of the S-protein expression.

Potential flexibility of the template and its efficiency even in the case of presence of mutations in the viral RNA target could play an important role in the overall efficiency of the CRISPR/Cas13 system. The study by Fareh et al. [49] evaluated efficiency of using PspCas13b together with crRNAs carrying 3, 6, and 9 substitutions. The 3-nt mismatch in the central part of the crRNA spacer at positions 14-16 or at the 3′-end of the spacer (positions 28-30) affected only insignificantly cleavage of the target RNA, while the 3-nt mismatch at the 5′-end of crRNA resulted in the decrease of cleavage of the target transcript by ~50%. The 6-nt mismatch introduced at different positions of the crRNA decreased significantly efficiency of inhibition, and the 9-nt mismatch resulted in the absence of degradation of the S-protein transcript. It is important to note that the ability of PspCas13b to recognize the target sequence even in the presence of unpaired nucleotides, especially in the inner regions of the spacer, indicates its potential efficiency against majority of the variants of the target sequences with single-nucleotide polymorphisms, thus providing protection

against mutant strains. Similar results were obtained in the Vero cell line in the experiments with coronavirus HCoV-OC43, with highly conserved *RdRp* and *N* genes used as targets [50]. Single-substitutions or triplet substitutions in the crRNA sequence only slightly affected the decrease of the viral RNA in comparison with the crRNA fully complementary to the *RdRp* mRNA.

Efficiency of crRNAs multiplexing during infection with SARS-CoV-2 was also evaluated [49]. Vero cells were transfected using different crRNA pools with each of them including 4 different crRNAs targeting either structural proteins S and N, or non-structural proteins nsp7 and nsp8 [51]. All 4 pools of crRNAs decreased significantly the level of viral RNA and titer of the virus in the supernatant at MOI both 0.1 and 0.01; however, the efficiency was higher at lower dose and it was retained over the entire observation period, while at higher dose of the virus the inhibition effect noticeably and gradually decreased with time. In the process of infection of Vero cells with SARS-CoV-2 at MOI 0.1 and 0.01 and 72 h after transfection with PspCas13b and crRNA, expression of viral RNA was suppressed by ~80% and ~90%, respectively [49].

The degree of the virus titer decrease also depends on the type of the target RNA chain – (+) or (−) [52]. Efficiency of cleavage of the target RNA in the HEK293T cells was tested using PfxCas13d and crRNA targeting different sequences of the SARS-CoV-2 genome in both RNA chains. It was shown in the experiments with HEK293T cells using SARS-CoV-2 replicons that the most efficient cleavage of the target RNA was observed when the crRNA targeting (+)-chain was used, furthermore, all crRNAs targeting (−)-chain of RNA were ineffective except the crRNA targeting the “slippery” sequence.

The studies evaluating efficiency of Cas13a both in *in vitro* system and under *in vivo* conditions are of particular interest (Table 2) [53-55]. In the study by Blanchard et al. [53] crRNAs targeting highly conserved areas in the replicase and nucleocapsid regions of the SARS-CoV-2 genome were tested. For this purpose, Vero E6 and Huh7 cells were transfected with the *LbuCas13a* mRNA and various combinations of crRNAs. It was found out that combination of the crRNAs targeting both *N* and *RdRp* used together with the *LbuCas13a* mRNA decreases cell death by more than 50%. The crRNA targeting *N* or combination of two best crRNAs targeting *N* ensured decrease of the Vero E6 cells by more than 72%; moreover, the similar result was observed for the Huh7 cell line [53]. Therapy based on the use of Cas13a + crRNA targeting *RdRp* resulted in the decrease of the number of copies of the *RdRp* gene by 93.7% and 94.5% in the Vero E6 and Huh7 cells, respectively; and the system based on Cas13a + crRNA targeting *N* decreased the number of *N* transcript by 94.1% and 99.1% in the Vero E6 and Huh7 cells, respectively. Golden hamsters (*Mesocricetus auratus*) were used as an *in vivo* model in the study by Blanchard et al. [53]: 125 μg of mRNA encoding *Cas13a* and crRNA targeting *N* together with the PBAE polymer were administered intranasally to 4-week-old male hamsters. After 20 h hamsters were intranasally infected with 10^3 plaque forming units of SARS-CoV-2 (USA-WA1/2020). It was shown that the Cas13a/crRNA-based therapy resulted in the decrease of the number of copies of the viral *N* RNA in the hamster lungs by 57%.

The CRISPR/Cas13-based strategy for fighting virus infection in the case of coronavirus could be also not associated with suppression of viral transcripts. In their elegant study, Cui et al. [54] suggested a non-typical approach to CRISPR/Cas-based antiviral therapy, which targets not the viral protein, but the lung protease cathepsin L (Ctsl). Ctsl is an important endosomal cysteine protease, which facilitates priming of S-protein and penetration of the virus into the cell via the virus–host cell endosome membrane fusion. Ctsl inhibitors block penetration of coronaviruses (such as SARS-CoV-1 and SARS-CoV-2) *in vitro* and development of pseudotyped SARS-CoV-2 infection *in vivo* [56-59].

Investigation on suppression of *Ctsl* expression in the cell culture was performed using the HEK293FT cells with the help of co-transfection of different crRNAs and expression plasmid carrying the *RfxCas13d* gene [54]. The most effective crRNA facilitated 75%-suppression of the *Ctsl* expression; it was shown in further experiments that this effect not only resulted in inhibition of the of pseudotyped SARS-CoV-2 infection, but also of the of pseudotyped SARS-CoV-1 infection in the Vero-ACE2-TMPRSS2 and Caco-2 cells. In addition, the RfxCas13d-mediated specific knockdown of the *Ctsl* gene resulted in the effective inhibition of different virus strains, such as, for example, B.1.617.2 Delta. These results on successful suppression of the *Ctsl* gene were confirmed in the experiments with animal models [54]. Delivery of the CasRx mRNA and *Ctsl*-targeting crRNA to the mouse lungs was carried out with the help of LNP-particles based on MC3 with addition of supplementary cationic lipids. Upon infection with SARS-CoV-2 (USA-WA1/2020) the K18-hACE2 mice in three control groups demonstrated graduate weight loss and 100% mortality within 8 days after virus administration. On the contrary, administration of LNP-CasRx-Ctsl-crRNA to the mice 2 days prior and 1 day after infection with SARS-CoV-2, resulted in the delayed manifestation of the disease, and survival level reached 50%. It was also detected that the amount of mRNAs of the Ctsl protein, and of the viral proteins N and E decreased in the animals after the LNP-CasRx-Ctsl-crRNA injection. In addition to evaluation of the level of expression of the *Ctsl* gene

Table 2. Use of the CRISPR/Cas13 system for protection against viral infection *in vivo*

Pathogen	Target sequence	Enzyme	Delivery method	Experimental model	Main results	References
IAV WSN/33	*PB1*	LbuCas13a	PBAE + mRNA	BALB/c mice	↓ of viral RNA by 96.2%	[53]
SARS-CoV-2	*N*			LVG Syrian gold hamsters	↓ number of copies of *N* by 57%	
SARS-CoV-2	pseudoknot in ORF1b, *RdRp*	Cas13b	intranasal administration *in vivo*-jetRNA with siGLO	K18-hACE2 mice	crRNA targeting ORF1b ↓ viral titer by 99%	[55]
SARS-CoV-2	*Ctsl*	RfxCas13d	injection into tail vein of LNP-mRNA	K18-hACE2 mice	↑ survival by 50%; ↓ weight loss, level of *Ccl5*, *Ccl2.* and *Isg15* cytokines, inflammation in lungs; ↓ expression of *Ctsl* 100-fold; ↓ expression of proteins N and E; ↓ infectivity of the virus by 2log	[54]
IAV/H1N1 Cal04/09	*PB2*	LbuCas13a	intranasal administration of mRNA	Syrian gold hamster	prophylactics: ↓ of number of copies of *PB2* and of viral titer in lungs by 1log; therapy: ↓ of number of copies of *PB2* by 93%, viral titer – by 88%; use of two-dose regime: ↓ of number of copies of *PB2* and viral titer in lungs by 1log and 2log, respectively	[46]

the authors also assessed the levels of expression of proinflammatory cytokines and chemokines associated with the development of cytokine storm. Expression of *Cxcl10*, *Tnf*, *Ccl5*, *Ccl2*, and *Isg15* decreased in response to the LNP-CasRx-Ctsl-crRNA therapy. The similar results were demonstrated when the LNP-CasRx-Ctsl-crRNA preparation was administered 2 h and 1 day after infection with 10^5 plaque forming units of SARS-CoV-2.

Biodistribution of the preparation and its safety were also examined in the study by Cui et al. [54]. Expression of the CasRx mRNA in lungs reached maximum 4 h after infection followed by significant decreased after 24 h. Remarkably, the *Ctsl* expression did not change in liver or spleen, and expression of other representatives of the cathepsin family, *Ctsd*, *Ctsb*, and *Ctss*, did not change in lungs after therapy. These results demonstrate high specificity of the used system. Moreover, there were no significant changes in the expression of cytokines/chemokines in the lungs following treatment with the LNP-CasRx-Ctsl-crRNA. And finally, the authors did not observe significant changes in the liver and kidney functions of the mice, as well as in hematological parameters. Taken together these data demonstrate that the targeted delivery of the CasRx-Ctsl-crRNA to lungs with the help of lipid nanoparticles allows for effective, specific, and safe suppression of the targeted transcripts. Moreover, customized targeting and fast biodistribution of lipid nanoparticles with mRNA over the entire organism within a few hours after administration of the preparation opens up new possibilities for emergency therapy of the severe cases of viral infection [60, 61].

In another study with animals the PspCas13b-NES system targeting the *RdRp* gene in SARS-CoV-2 that included the pseudoknot region of the ORF1b was used [55]. Cas13b and crRNA were delivered as mRNAs, Cas13b expression was detected already 2 h after transfection, degradation of both *RdRp* mRNA and of the RdRp protein was also observed 2 h after transfection; this effect increased 6 h after transfection. Analysis of the viral load with the TCID50 assay showed that the titer of SARS-CoV-2 in the lungs of mice treated with targeting crRNA decreased by 99% in comparison with the virus titer in the lung of non-treated mice. Similarly, the levels of viral RNA of the *RdRp* gene and *N* protein were also significantly reduced in the lungs. These results could indicate that targeting the region of pseudoknot in ORF1b followed by the Cas13b-mediated knockdown is a promising strategy for treatment of the SARS-CoV-2 infection [55].

USE OF CRISPR/Cas13 IN THERAPY OF INFLUENZA A VIRUS

CRISPR/Cas13 system is used for inhibition of a wide spectrum of viruses including influenza A virus with single-stranded negative-sense RNA. In one of the first studies devoted to this issue the PspCas13b nuclease was used with the Madin–Darby canine kidney cells (MDCK); next, efficiency of five different crRNAs targeting genes encoding proteins NP and M was examined (Table 1) [7]. The cells were transfected using electroporation with two plasmids, with one of them containing sequence of crRNA, and another – sequence of the *PspCas13b* gene, and 24 h after infection of the cell culture with the IAV amount of viral RNA in the supernatant was determined using PCR. It was show that the amount of viral RNA decreased 7-22-fold in comparison with the control group [7]. In another study the Cas13X.1 was used in the MDCK cells with crRNAs targeting mRNA of nucleoprotein required for IAV replication and transcription [37]. For this purpose, the cells were transfected with the vector containing *Cas13X.1* and crRNA, which was followed by infection with the influenza strain H1N1 A/Puerto Rico/8/1934. Three of the four tested crRNAs demonstrated high efficiency of the nucleoprotein transcript knockdown and significantly decreased titer of the virus in the mammalian cells.

Detailed investigation of the antiviral effect in cell cultures also included experiments on the effects of localization of PspCas13b, type of cells, and multiplexing [7]. It was revealed that the Cas13b localization indeed could affect efficiency of crRNA, because one of the crRNAs targeting the gene encoding NP significantly decreased the level of viral RNA only in the cases, when Cas13b was localized in cytoplasm and not in the nucleus. At the same, change of the cell line did not affect significantly efficiency of the system – amount of the viral RNA decreased 20.6-fold in the A549 cells, which was comparable with the results obtained in the MDCK cells. Multiplexing was also shown to be efficient: using simultaneously 4 crRNAs targeting the gene encoding NP protein resulted in the 8-fold decrease of viral RNA in comparison with the effects of mono-therapy. Similar results of multiplexing of crRNAs targeting transcripts of the *PB*, *NP*, and *M* genes in the virus genome were obtained with the chicken embryo fibroblasts cell line (DF1) [62].

Another study performed with the help of RfxCas13d in the A549 cell line used crRNAs targeting highly conserved regions, which affect the virus assembly [10]. Selection of this type of targets could potentially facilitate inhibition of a wide spectrum of IVA strains using the CRISPR/Cas13d system. In total 48 crRNAs were used in the study, 6 for each of the 8 segments of the influenza virus A genome. The Cas13d A549 cells stably expressing Cas13d nuclease were transfected with the pool of 6 crRNAs targeting highly conserved regions of the viral genome. Two days after transfection the cells were infected with the virus at MOI 2.5 or 5.0; 18 h after infection the cells were analyzed for IVA infection using flowcytometry and microscopy. Among all tested crRNAs, the pool of crRNAs targeting the segment 6, encoding the NA protein, demonstrated most consistent and reproducible results at both MOI levels (decrease by 72% for MOI = 2.5 and decrease by 52% for MOI = 5). Furthermore, the pool of crRNAs targeting the segment 4, which encodes the HA protein, also demonstrated moderate inhibition of the viral infection. Interestingly enough, it was shown that expression of the Cas13d nuclease affected the levels of RNAs of those regions of IVA that were not directly targeted. In total, the results of this study demonstrated that Cas13d is capable of affecting highly conserved regions in the viral genome and inhibit IVA replication in the human lung epithelial cells.

In the later study, in addition to *in vitro* experiments, *in vivo* experiments were also conducted (Table 2) [53]. The used crRNAs targeted the sequences of the *PB1* and *PB2* genes. The A549 cells were infected with the influenza virus A/WSN/33 at MOI 0.01; 24 h after infection the cells were transfected with the mixture of mRNAs of *LbuCas13a* with or without NLS and crRNA targeting *PB1*, and 48 h after infection the levels of *PB1* RNA were assessed with the help of PCR. One of the crRNAs demonstrated high efficiency decreasing expression of the viral RNA within 24 h by 83% when the cytosolic form of Cas13a was used, and by 78%, when Cas13a with nucleus localization was used. In addition, efficiency of the crRNA targeting the conserved region of the *PB2* gene was evaluated.

The crRNA was introduced 24 h after infection, the degree of viral infection was evaluated 48 h after infection. Three of six tested sequences of crRNAs caused decrease of the level of viral RNA by more than 50%, however, efficiency of the antiviral effect varied depending on the variant of the used nuclease – with or without NLS.

In the *in vivo* study with mice, RNA was introduced intranasally with the help of nebulizer using the PBAE polymer [53]. The mice were infected with 3 LD50 of the influenza virus A/WSN/33 via intranasal administration, and 6 h after mRNA encoding *LbuCas13a* without NLS was administrated, as well as crRNA targeting *PB1*. The CRISPR/Cas13-based therapy resulted in the decrease of amount of the viral RNA 96.2% 3 days after infection.

In the experiments involving Syrian golden hamster [46], the LbuCas13a nuclease mRNA was administered intranasally together with the crRNA targeting *PB2* with incorporated 2′-O-methyl-nucleotides. The animals were infected with the influenza virus A/H1N1/California/04/09 (Cal04/09). In the case of prophylactic approach administration of one dose of the preparation decreased the level of RNA and of the infectious virus (PFU/ml) in lungs by approximately one order of magnitude. Therapeutic administration with the help of Cas13 resulted in 93%-reduction of the number of the *PB2* gene copies and 88%-reduction of the virus titer. In the case of two-dose regime (one injection prior to infection and one – after) the level of RNA and of the infectious virus (PFU/ml) in the lungs decreased by almost 2 orders of magnitude. Hence, the results of experiments with mice and hamsters that used the CRISPR/Cas13-based treatment against the influenza virus indicate that this system causes efficient suppression of the viral RNA and decrease of the virus titer.

DISCUSSION

Viral infections represent a serious problem for health care, primarily due to the fact that the specific therapeutic approaches exist for only few types of viruses. Recent coronavirus pandemics and influenza virus A pandemics (H1N1) in 2009 demonstrated that the development of innovative antiviral treatments is a very urgent task. The idea for using bacterial system of adaptive immunity, CRISPR/Cas13, that specifically targets viral RNA as an antiviral therapy is currently realized in the experiments with cell cultures; however, promising data on the efficiency of this system *in vivo* in model animals start to emerge.

One of the advantages of the CRISPR/Cas13 system is its activity towards the viral RNA, hence, its use does not cause any changes in the cell genome. This system is capable of recognizing RNA target even in the presence of unpaired bases in the crRNA and target, hence, high mutation rate in the viral genomes does not prevent effective cleavage of the viral RNA. For therapeutic purposes it is advantageous to use Cas13 nucleases with the smallest size, as it facilitates their delivery to the cell, hence, the Cas13d and Cas13X nucleases, as well as the recently discovered Cas13an nuclease seems to be most promising for treatment of influenza and SARS-CoV-2 infections. In the experiments with cell cultures all used Cas13 nucleases demonstrates their efficiency by significantly decreasing the level of viral RNA and titer of the virus (Table 1). The results of recent studies with model animals on the use of the CRISPR/Cas13 system in antiviral therapy of SARS-CoV-2 and influenza virus infection are encouraging (Table 2). Despite the fact that the number of such studies is limited, they generally demonstrate level of efficiency similar to the results obtained in cell cultures. Small number of studies with model animals is likely related to the fact that the use of CRISPR/Cas13-based preparations for antiviral therapy is associated with two problems: selection of the effective delivery method and possible excessive activation of the innate and adaptive immune response to the individual components of the CRISPR/Cas13 system. In 2022 Tang et al. [63] examined the levels of IgG-antibodies specific to RfxCas13d and evaluated the RfxCas13d-induced proliferation of the $CD4^+$ and $CD8^+$ T-lymphocytes in healthy donors. It was revealed that majority of the donors have IgG-antibodies to RfxCas13d- and RfxCas13d-reacting T-cells capable of producing proinflammatory cytokines IFN-γ, TNF-α, and IL-17. Development of such immune response could be explained by colonization of the human gut by the *Ruminococcus bicirculans* bacteria producing proteins of the Cas13 family, which are similar to the RfxCas13d nuclease. Potential for the development of unwanted side reactions limits possibilities of clinical application of the preparations based on CRISPR/Cas13, but development of new administration formats (such as in form of mRNA) and delivery systems, including systems for targeted delivery based on lipid nanoparticles, opens wide perspectives to minimize potential side effects and increase efficiency of the future CRISPR/Cas13-based preparations [54, 61].

The results of presented studies also indicate that localization of the Cas13 nuclease and selection of the optimal crRNA sequences, as well as the use of multiplexing involving several crRNAs allowed to increase significantly efficiency of the system. Moreover, effective system for the delivery of the components of CRISPR/Cas13 system to the infection foci also has not been fully developed. In the recent study on the use of CRISPR/Cas13 the delivery systems based on lipid

nanoparticles are considered most promising, they have been shown to ensure effective and selective delivery with sufficiently high safety level [54]. Delivery of mRNA with the help of lipid nanoparticles has been proven to be reliable in the case of the known and approved mRNA SARS-CoV-2 vaccines: RNA-1273 vaccine (Moderna) and BNT162b2 vaccine (Comirnaty®, BioNTech and Pfizer). Considering that the mRNA platform allows to accelerate significantly development of preparations with changed sequences of the target antigen/crRNA, this platform is highly suitable for prophylaxis and therapy of seasonal viral diseases. In this context, the idea of complex development of two mRNA preparations with delivery system in the form of lipid nanoparticles against the seasonal strains of the influenza virus A and SARS-CoV-2 including mRNA vaccine encoding sequence of viral antigens as a prophylactic preparation and mRNA–CRISPR/Cas13 as an emergency therapy in the case of severe disease seems very interesting. As has been mentioned above, mRNA packed into the lipid nanoparticles ensured fast biodistribution in the tissues and translation of the target protein already in the first hours after administration of the preparation [60].

Another strategy to increase efficiency of antiviral therapy is successful use of combination of the CRISPR/Cas13-based therapy with treatment with small molecules [37]. Conducting complex studies on the use of different combination of Cas13 variants, RNA targets, and delivery methods, as well as search for the compounds without antiviral activity, but capable of potentiation of the CRISPR/Cas13 activity, seems as a promising direction for future studies on the strategies for fighting viral diseases.

Abbreviations. Structural proteins: E – envelope protein, N – nucleocapsid protein, S – spike protein; crRNA, CRISPR RNA, portion of CRISPR guide RNA; Ctsl, lung cathepsin protease L; HA, hemagglutinin; IAV, influenza A virus; MDCK, Madin–Darby canine kidney cells; MOI, multiplicity of infection; A, neuraminidase; NES, nuclear export signal; NLS, nuclear localization signal; NP, nucleoprotein; nsp, non structural proteins; ORF, open reading frame; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2; RdRp, RNA dependent RNA polymerase.

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