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Assessing the capacity of CRISPR systems as anti-viral therapies against SARS-CoV-2 variants of concerns

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SUMMARY Throughout the pandemic, COVID-19 has evolved into a disease of multiplicity; the rapid emergence of SARS-CoV-2 variants of concerns (VoCs) have revealed the necessity of broad-ranged, anti-viral treatments as a potential pandemic countermeasure. CRISPR systems are being explored as a promising venture to solve this issue. Current research on CRISPR anti-viral research has utilized the suitability of the CRISPR effector Cas13 due to the substantial amounts of potential Cas13 target sites on the SARS-CoV-2 genome as well as its ability to specifically cleave RNAs. Using bioinformatics pipelines, a wide variety of crRNAs can be generated and pooled to easily target a large range of viral RNA sequences. Moreover, proof-of-concepts such as Cas13-assisted restriction of viral expression and readout (CARVER), and Prophylactic Antiviral CRISPR in huMAN cells (PAC-MAN) promote the feasibility of this idea. Despite this, the development of CRISPR systems as an anti-viral therapeutic is still in its infancy, and many limitations must be overcome before they can reach the clinical stage. This article will strive to objectively assess 1) the mechanisms of the antiviral CRISPR platforms and their potential as a pandemic countermeasure and 2) current limitations plaguing CRISPR Cas13 anti-viral development and a few viable solutions. It is important to understand the potential of CRISPR and how it could benefit Sars-CoV-2 research in ways that are lacking in available/traditional antiviral therapies. Moreover, employing these CRISPR systems would require the creation of novel solutions for overcoming the current obstacles against clinical testing. By further understanding the modularity of CRISPR systems, additional forays into countermeasures can be explored against not only the rapidly evolving strains of Sars-CoV-2, but any RNA virus.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has emerged as a disease that has been reported to have killed more than six million people across the world as of March 2022 (1). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the driving factor behind COVID-19. SARS-CoV-2 is a positive single-stranded RNA virus (+ssRNA), possessing an envelope and a non-segmented genome (2). Due to the absence of RNA-dependent RNA polymerase (RdRP) proofreading, the highly error prone replication of RNA viruses has given SARS-CoV-2 the ability to rapidly acquire mutations in short periods of time (3). This in turn has caused a surge in the number of genetically unique viral strains, termed variants of concerns (VoCs). VoCs may gain mutations that affect the infectivity, transmissibility, pathogenicity, and antigenicity of the virus (4), impeding the global effort against the damage caused by the virus. Furthermore, researchers have firmly established that the emergence of VoCs introduce tangible clinical impacts. This may include changes in transmissibility/virulence, decreased therapeutic and diagnostic capabilities, and lower vaccine effectiveness (5). For example, the L452R spike mutation in the Epsilon variant brought transmissibility up 24% compared to the wild type strains (5).

The COVID-19 pandemic has shown us how woefully unprepared we are for global pandemics. In particular, the development of therapeutics lags behind other viral research developments. First, with each new virus, a novel anti-viral may need to be developed for

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treatment. Moreover, it takes time to perform large scale studies on new treatments (6), even with emergency use authorizations (EUAs). Second, this is further complicated with the presence of VoCs. Due to the unpredictability of additional mutations on viral phenotype, anti-viral effectiveness may vary, especially when the mutations manifest in the anti-viral target sites (7). For example, public health agencies have recommended to not treat patients with monoclonal antibodies due to the presence of concurrent variants (8). It is also not out of the question for SARS-CoV-2 variants to develop resistance to anti-viral therapies (3, 9), but more research must be done. Third, there is an absence of programmability for current anti-viral therapies. Due to the target specificity of current antivirals, e.g. the targeting of the conserved viral RdRP by Remdesivir (10), these treatments cannot be used against dissimilar viruses. Overall, despite the advances achieved against SARS-CoV-2, there is still a lack of a proactive, modular therapeutic platform with the capability to pre-emptively combat future pandemics.

Relatively new research has pinpointed the use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) protein systems as a promising, innovative mechanism for viral therapeutics. While the concept of CRISPR mediated anti-viral activity has been present in bacteria for millennia (11, 12, 13), the research for its use against mammalian viruses is still in its infancy. Due to its high specificity, CRISPR systems can be utilized to disrupt viral replication by either cleaving the viral genome or inhibiting critical host factors required by the virus (14). Over the past few years, CRISPR systems have been used to target at different viruses, such as herpesviruses (15), HIV (16, 17), and now SARS-CoV-2 (18, 19, 20, 21, 22). CRISPR Cas13 has been singled out for anti-viral research due to its unique ability to cleave ssRNA molecules and its simple structure (requires only one protein and a crRNA strand to work) (16, 17). There are four distinct Cas13 subtypes being studied including VI-A (C2c20), VI-B (Cas13b/C2c6), and VI-D (Cas13d) (23). The Cas13 orthologs vary in size, optimal target sites, and catalytic ability (24). Overall, CRISPR Cas13 works by 1) transcribing a long pre-CRISPR RNA (crRNA) from a CRISPR array, 2) processing the pre-crRNA into mature crRNA by the Cas13 protein, 3) target recognition through complementary base pairing between the crRNA and 4) collateral (non-specific) RNA cleavage (23). Not only can Cas13 be programmed to cleave distinct ssRNA, which is appropriate for the RNA genome of SARS-CoV-2, it also does not depend on the protospacer adjacent motif (PAM), increasing its versatility (16, 17). Furthermore, due to its strong catalytic activity in human cells, high level of specificity, and smaller proportions (18), it is very suitable for gene editing therapies against viral infection.

PROPOSED RESEARCH QUESTIONS

COVID-19 has been shown to be a globally destructive disease and thus, finding a rapidly developed, bespoke viral therapy could save millions of lives. Further research is needed to uncover the potential of CRISPR Cas13 as a broad-range, pan-viral pandemic countermeasure. To better understand the use of CRISPR platforms towards this use, an objective assessment of the mechanisms, potential, and current limitations of this technology is crucial. However, the current research on this topic is sparse. To overcome the current lack of research on pre-emptive pandemic therapeutics, this article will summarize and explore the capability of CRISPR Cas13 systems as a pandemic countermeasure within the context of the SARS-CoV-2 and its VoCs. First, the paper will discuss the mechanisms of the antiviral CRISPR systems and their potential as a pandemic countermeasure. Second, the paper will examine current limitations plaguing CRISPR Cas13 anti-viral development and several promising solutions.

PROPOSED PROJECT NARRATIVE

What are the mechanisms behind CRISPR Cas13 platforms and their suitability towards utilization as a pandemic countermeasure?

Emergent viruses are still a looming threat and with predicted increase in the number of pandemics for the years to come (25), it is worrying that we still do not have a rapid, flexible

method to develop anti-viral therapies. Conventional methods require months if not years of design, optimization, and testing for the drug to have widespread use. The COVID-19 pandemic and the associated research regarding CRISPR Cas13 platforms is an excellent opportunity to study the potential of a broad-range, one-size-fits-all strategy for current SARS-CoV-2 VoCs and future pandemics.

The initial step to developing a CRISPR Cas13 anti-viral platform is rational design of the crRNA using a bioinformatics pipeline. First, the sequences of the viral target and its associated strains are aligned. The pipeline then analyses the alignment and, depending on the parameters, return the most effective target sites available. Oftentimes, this would be the most conserved regions across the virus strains. With future iterations, viral genome binding availability can be integrated into these calculations. The bioinformatics pipeline would return a tiled crRNA library, screened for undesirable characteristics such as off-targets or polynucleotide repeats, which can be synthesized and experimentally validated (18, 19, 20, 21, 22). This screening platform exploits the modularity of CRISPR Cas13 to automate the rapid generation of high-quality target sites for cleavage. In theory, Cas13 platforms could be used to comprehensively target any RNA virus genome just by changing the crRNA sequence. Researchers have determined that only 22 crRNAs would be needed to target all sequenced coronaviruses with no mismatches (18).

Experimentally, the Cas13 platform has been established to work effectively in cell culture. Cas13-assisted restriction of viral expression and readout (CARVER), described in 2019, demonstrates the effectiveness of programmed Cas13 against lymphocytic choriomeningitis virus (LCMV), influenza A virus (IAV), and vesicular stomatitis virus (VSV) (26). Recombinant viruses were transfected into human cells containing the CRISPR constructs. The results showed obvious and significant decreases in viral levels, effective against the broad range of viruses. The first exploration as a COVID-19 therapeutic was the Prophylactic Antiviral CRISPR in Human cells (PAC-MAN) platform. The researchers confirmed the efficacy of the Cas13 platform against two viruses in the coronavirus family (SARS-CoV-2 and influenza), targeting the highly conserved RdRP and N proteins (18). They also determined that different pools of crRNA (e.g. different recognition sites) have discrete levels of viral inhibition, indicating preferable target sites in specific regions (18). Another group sought to counteract the potential for SARS-CoV-2 to escape therapy by determining the mismatch tolerance of the Cas13 system and the effectiveness of multiplexing (19). Multiplexing, or the employment of crRNA pools with distinct target sites, was found to be as effective as the most efficient single crRNA. Additionally, the Cas13 system was shown to have a high mismatch tolerance against single nucleotide mutations, maintaining efficacy up to six mismatches. Both results demonstrate the endurance of the Cas13 system against spontaneous mutations and viral escape (19). Moreover, this flexibility reinforces the notion that by targeting the parental strains, corresponding VoCs will also be functionally inhibited.

What are the current limitations, and some promising solutions, of CRISPR Cas13 anti-viral development?

Despite the potential of CRISPR Cas13 platforms through the proof of concepts, there are still a plethora of hurdles against its advancements into clinical trials. This article will be addressing three of the most pressing issues around CRISPR technologies: off-target effects, a safe and efficient delivery system, and the *in vivo* efficacy of the system.

Off-target effects are the Achilles heel of health-related CRISPR technologies. It is critical to ensure experimentally that there are not any adverse effects caused by the catalytic ability of the Cas13 protein. Multiple papers have touted little to no transcriptomic off-target effects caused by Cas13 catalysis (27, 28, 29, 30), especially in comparison to shRNA results. Abudayyeh, et. al 2017 (31) determined the presence of off-target effects of Cas13a knockdown by utilizing transcriptome wide sequencing. The researchers found high levels of knockdown with no off-target effects when performing differential analysis with shRNA knockdowns. In contrast, there also have been reports of off-target effects from Cas13 orthologs (32, 33, 34). Due to the foreign RNA targets of the anti-viral Cas13, these considerations may be less relevant, but more research must be done. There are currently a few methods of decreasing off-target rates. The first method would be in-silico rational

design. Off-target models and mismatch searching can analyse the designed crRNAs and decrease the probability of incorrect cleavage (18, 35, 36). Another method would be to optimize the CRISPR platform. Cas13 ortholog choice, target transcript concentration, target transcript structure, and cell type all may contribute to different levels of off-target effects (33).

An efficient delivery system is crucial for the safety and efficacy of the CRISPR platform *in vivo*. First, it must be established whether the therapeutic platform will be delivered as a protein or mRNA (37). Proteins are more transient, acting rapidly with lower-off target effects. It is also more expensive and may have a higher chance of being immunogenic. On the other hand, mRNA is easier and cheaper to deliver, but comes with the cost of time and stability (37). Adeno-associated virus (AAVs) is a gene therapy vector that has been thoroughly explored and is currently the most common research tool used (38). Methods of AAV utilization have obtained positive clinical results in medical applications such as treating vision loss and hemophilia (38). Furthermore, due to the small size of Cas13 proteins, the entire construct may be deposited into the virus for delivery (39). Some challenges include the possibility of pre-existing immunity to AAVs, undesired editing events, and less transient expression of vectors (40). Another delivery method is hyperbranched poly(beta amino esters) (hPBAEs), a method to transport drugs (e.g. mRNA) through the lungs in a temporal and dose dependent manner (41). Because the entry point for COVID-19 is the lungs, the localization and translation of the mRNA construct in the lungs via hPBAEs is highly appropriate as a delivery method. Recently, research group successfully used hPBAEs to inhibit SARS-CoV-2 pathogenesis in infected mice (29). One of the biggest issues this method faces is possible toxicity and accumulation due to the extremely stable nature of hPBAEs (41). Lastly, the victories we have achieved in delivering mRNA vaccines through lipid nanoparticles may be applied to the delivery of Cas13 mRNA and crRNA (42). Multiple types of lipid nanoparticles have been commercialized and have seen widespread efficacy across the world (42). Hitching on to this presiding technology may be the quickest and most promising way that CRISPR Cas13 anti-viral platforms reach the clinical phase.

Lastly, the efficiency of the CRISPR Cas13 system may come into question when applied to an *in vivo* context. This is an extremely important consideration when considering the therapeutic value of the platform. However, there is currently a shortage of good data. While preliminary results and the proof of concepts are auspicious, further advancements, such as off-target effects and robust delivery systems, must be made before this question can be answered.

POTENTIAL IMPACT/CONCLUSIONS

The COVID-19 pandemic has devastated the world since early 2020. Even though prophylactic methods (e.g. vaccines) of inhibiting viral infection have been relatively successful, there is still a void in the side of therapeutics. Over the past few years, research surrounding CRISPR Cas13 has delved into its potential as a programmable, broad-range anti-viral platform (18, 19, 20, 21, 22, 29). This article explores the possibility for Cas13 system as not only a promising therapeutic against SARS-CoV-2 and its VoCs, but as a primed platform for the rapid development of anti-virals against emerging pandemics. The CRISPR Cas13 platforms have a chance to completely shift the current perspective on anti-viral development and treatment. However, this technology is immature and faces numerous obstacles before it can enter its clinical phase.

CRISPR Cas13 platforms have three main characteristics that make it suitable as a programmable, broad-range anti-viral platform. First, the CRISPR Cas13 catalytic activity is highly specific. Complementary base pairing between the crRNA and the target RNA facilitates recognition and highly effective cleavage (30, 31) of the target RNA. Second, Cas13 platforms are highly modular and programmable. This can be done by simply modifying the crRNA sequence to the chosen target. This level of simplicity enables bioinformatic approaches to design and generate lists of optimal crRNAs that can potently inhibit the selected virus (18, 19, 20, 21, 22). The combined specificity and programmability of Cas13 allows for the targeting of any RNA virus (18, 19, 20, 21, 22); this is the foundation for the rapid deployment of bespoke anti-viral therapy. Third, multiplexing and mismatch

tolerance impedes viral escape and VoC resistance (19). It is crucial for viral therapies to constrain the virus despite possible spontaneous mutations. Multiplexing and mismatch tolerance does not only overcome genomic variation in VoCs, but it enables the targeting of a broad spectrum of viruses only from one set of crRNAs (18).

The advancement of CRISPR Cas13 technology is crucial to overcome current bottlenecks to clinical usage. Three of the most significant issues include off-target effects, lack of delivery mechanisms, and the issue of in vivo efficacy. For off-target effects, there have been conflicting results of the presence of off-target activity for Cas13 (27, 28, 29, 30, 32, 33, 34). However, this article surmises that off-target effects may not be as relevant due to the direct targeting of viral factors (not host RNA). More research and experimental data are required to fully elucidate the impact of off-target activity. Currently, there is not a gold standard for delivery methods for the platform. Some delivery methods explored are AAVs (40), hPBAEs (29, 41), and lipid nanoparticles (42). The rapid development and deployment of lipid nanoparticles during the pandemic may have created available infrastructure for the delivery of CRISPR systems. Thus, lipid nanoparticles may be the solution to fast tracking CRISPR Cas13 anti-viral into clinical use. Finally, the efficacy of the Cas13 platforms is still a mystery. Other than the one study looking into Cas13 anti-viral efficiency in rodents (29), there is an unsurmountable lack of information regarding use in mammals. While the experiments in cell lines have been tremendously encouraging, much more research is needed before the Cas13 platforms can be employed in the real world.

CRISPR technology has revolutionized genetic engineering and anti-viral research has also felt the full force of its impact. Developing a system that could target any currently established or emerging virus would be monumental, both for the field and for public health. Additional research into these topics would aid the progress required for these concepts to become fully realized and usher in a genetic revolution within viral therapeutics.

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