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Review

Small nucleic acids and the path to the clinic for anti-CRISPR

Christopher L. Barkau [a], Daniel O'Reilly [b], Seth B. Eddington [a], Masad J. Damha [c], Keith T. Gagnon [a,d,*]

[a] Department of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale, IL 62901, USA
[b] RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA 01605, USA
[c] Department of Chemistry, McGill University, Montreal, Quebec H3A 0B8, Canada
[d] Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, IL 62901, USA

A B S T R A C T

CRISPR-based therapeutics have entered clinical trials but no methods to inhibit Cas enzymes have been demonstrated in a clinical setting. The ability to inhibit CRISPR-based gene editing or gene targeting drugs should be considered a critical step in establishing safety standards for many CRISPR-Cas therapeutics. Inhibitors can act as a failsafe or as an adjuvant to reduce off-target effects in patients. In this review we discuss the need for clinical inhibition of CRISPR-Cas systems and three existing inhibitor technologies: anti-CRISPR (Acr) proteins, small molecule Cas inhibitors, and small nucleic acid-based CRISPR inhibitors, CRISPR SNuBs. Due to their unique properties and the recent successes of other nucleic acid-based therapeutics, CRISPR SNuBs appear poised for clinical application in the near-term.

1. Introduction

Clustered, regularly interspaced short palindromic repeats (CRISPR) and their associated (Cas) proteins constitute a nuclease-mediated acquired immune system against phage infection in non-eukaryotes. These mechanisms, particularly the RNA-guided endonucleases responsible for digesting invading phage DNA, have been co-opted over the past several years as biochemical tools [1–10]. After a double-stranded break is induced in a living cell by a Cas endonuclease, it can primarily be repaired in two ways [11]. Non-homologous end-joining (NHEJ), the dominant repair pathway, ligates the ends of the break together, often causing short indels that can result in frameshift mutations and consequently knockout of protein-coding genes. The other pathway, homology-directed repair (HDR), uses a homologous DNA sequence for precision repair. Thus, precise insertion or deletion of sequences into the genome can be achieved by providing homologous DNA donor sequences during gene editing. More recently, it has been shown that a number of mutations following cleavage by Cas endonucleases can also be attributed to microhomology-mediated end-joining [12]. This pathway can be exploited in a manner similar to HDR but incorporates sequences with short homologies to the target DNA [13]. Owing to their easily programmable nature, Cas enzymes, particularly *Streptococcus pyogenes* (Sp)Cas9, have made an impact on food production [14], pathogen management [15], development of model systems for research [16], and genome engineering technologies [3].

More recently, CRISPR has made its way into the clinic. It is being developed for *ex vivo* and *in vivo* gene therapies to treat genetic disorders of the eye [17], HIV [17], sickle cell disease [18], and cancer [19–28]. The very first clinical trial testing the safety of CRISPR-based therapy in humans modified patient T-cells to create double knockouts of PD-1, an immune checkpoint protein often exploited by cancer cells to evade the immune system [29]. The *ex vivo* modified T-cells were then infused into the patients to be observed for adverse events. It was recently reported that CRISPR-Cas9 had been safely used to edit patient T-cells *ex vivo* in order to knockout endogenous T-cell receptor proteins and PD-1 [30]. Edited cells were found to be persistent in each patient for up to nine months after infusion. The persistence, low off-target editing, and absence of any significant negative response to treatment in this early clinical study provides some confidence for clinical CRISPR applications moving forward. Another trial modified hematopoietic stem and progenitor cells from a patient with HIV and lymphocytic leukemia to knock out the *CCR5* gene, which encodes a receptor important for HIV entry into cells [31]. A CRISPR-based treatment for sickle cell disease being developed by CRISPR Therapeutics, CTX001, began trials in 2018 [32]. Although each of these is an example of early-phase clinical trials, they present a clear indication that CRISPR-based medicines are on the horizon.

2. Why inhibit CRISPR-Cas?

Amid the excitement and progress in CRISPR research and therapeutic development it may not be immediately obvious why inhibiting

* Corresponding author at: Department of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale, IL 62901, USA.
E-mail address: ktgagnon@siu.edu (K.T. Gagnon).

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Cas proteins is desirable. However, inhibition is vital to the responsible use of CRISPR. It is becoming increasingly clear that CRISPR will impact disparate and possibly unforeseen aspects of our day-to-day life, including our environment [33], our food [34], and our health [35–39]. Thus, there emerges a potential need for kill-switch inhibitors that can directly and completely disable CRISPR-Cas systems in a variety of contexts. For therapeutic development, possibly even FDA approval of certain CRISPR-based drugs, the development of an easily deliverable inhibitor to stop activity may become essential. Many approved drugs have an antidote that can be administered in the event of accidental misuse or to alleviate side effects, such as vitamin K and prothrombin complex concentrate for anti-coagulants like warfarin [40] or protamine sulfate for heparin [41]. Importantly, these drugs have relatively short-lived effects on the body, whereas the effect of CRISPR is permanent, making the availability of a kill-switch potentially even more vital.

Among the proposed applications of CRISPR is the development of gene drives to amplify a trait (for example malaria resistance in mosquitoes [15]) throughout a population or cause wild populations of organisms to crash entirely [42]. These methods, and others yet to be developed, constitute a form of environmental engineering that could affect ecosystems, human health, economies, and power structures on a global scale. The production of widely applicable CRISPR inhibitors to counteract instances of accidental or intentional misuse of gene drives, or the weaponization of CRISPR against human populations, may become an urgent global security priority.

A practical rationale for inhibiting CRISPR is also the prevention of off-target effects [43–45], defined as the unintended cleavage and mutation of sequences other than the target locus. In an extreme example of off-target effects, a recent study utilizing CRISPR in human embryos discovered that unrepaired cleavage products can persist through cell division resulting in allele-specific loss of entire chromosomes [46]. For off-target reduction, inhibitors might function by two methods. The first is prevention of significant off-target cleavage by timed inhibition. This method is built on the hypothesis that on-target cleavage, being more energetically favorable due to full guide-target complementarity, occurs rapidly while off-target activity is less favorable and accumulates primarily after the on-target locus has been cut and edited. For example, it has been shown that temporally limiting Cas9 and sgRNA persistence in cells raises the ratio of on-target to off-target editing [47–49]. Encoding a sgRNA that targets the gene for Cas9 itself has been demonstrated to cause a self-restriction of functional Cas9 expression, reducing off-target editing in human liver cells [50]. This method was further refined by the addition of an L7Ae:K-turn repression system to simultaneously attenuate Cas9 transcription and translation [47]. Similar results have also been achieved using timed delivery of the anti-CRISPR protein AcrIIA4 [51].

The second mechanism by which inhibitors can decrease off-target editing is by inhibiting excess enzyme. While this is similar to timed inhibition, it typically involves simultaneous delivery of the effector and inhibitor. For example, Aschenbrenner and coworkers describe what they call kinetic insulation against off-target activity when Cas9 is co-expressed or delivered as a fusion protein with a weakened anti-CRISPR (Acr) protein [44]. By carefully tuning the level of inhibition through Acr concentration or mutations that attenuated Acr efficiency in Acr-Cas9 fusions, the authors identified variants and conditions that maintained high on-target efficiency with low off-target activity.

Another related concept is “off-tissue” editing. In this case, it is not an incorrect genetic locus or target that is edited, but an on-target site in a tissue or organ where editing is not desired. Unrestricted CRISPR-mediated editing exposes diverse tissues and cell types, which may not be disease relevant, to potentially dangerous off-target mutations, including deletion of long genomic tracts or chromosomal rearrangements [52]. Off-tissue editing should thus be avoided if possible. While some methods such as tissue-specific expression of Cas9 and sgRNA [53–54] and modular LNP formulation for Cas9 ribonucleoprotein (RNP) delivery [55] have been described, the ability to inhibit Cas effector enzymes in non-target tissues would be a valuable alternative or supplement to other approaches. In fact, many of these findings may aid the development of tissue-specific inhibitor delivery or restricted CRISPR activity.

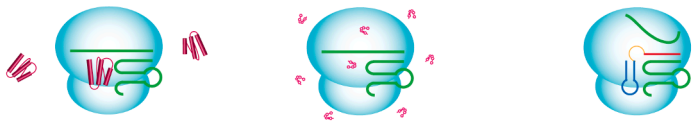
While various methods have been described to control gene editing activity, this review will focus on inhibitors that function without the need for Cas protein or CRISPR RNA engineering or regulation of expression vectors. Thus, the inhibitors of interest here are molecules that can largely act independently from enzyme engineering approaches and be added directly to an *in vitro* reaction, a cell, or potentially a living animal to block CRISPR-Cas endonuclease or gene targeting activity. Three current technologies potentially fit these criteria: Acr proteins, small molecule inhibitors of Cas enzymes, and small nucleic acid-base inhibitors (SNuBs) (Fig. 1). We discuss these technologies and their potential for human clinical use, with an emphasis on the potential benefits of CRISPR SNuBs.

3. Anti-CRISPR (Acr) proteins

Anti-CRISPR (Acr) proteins are encoded by phages to help evade bacterial and archaeal CRISPR systems. They are also found in certain bacteria and encoded by mobile genetic elements [56]. Acrs were first discovered as five genes encoded in phages of *Pseudomonas aeruginosa* [57]. They inhibited the bacterium’s type I-F CRISPR-Cas defense system, allowing them to infect *P. aeruginosa* cultures. Experimenting with translationally incompetent versions of the genes revealed that inhibition was translation-dependent and therefore likely to be protein-based. The same group shortly thereafter reported that some of these phages bore inhibitors of *P. aeruginosa* type I-E CRISPR systems [56]. Until 2017, all the Acr proteins identified targeted class 1 systems. However, Cas9 and Cas12 enzymes, which have been most heavily used for biotechnology, are derived from class 2 systems. The first reported examples of a class 2 Acr came in the form of three Acrs that inhibit the type II-C systems of *Neisseria meningitidis* and *Brackiella oedipodis* [58]. These type II-C inhibiting Acrs, AcrIIC1-3, were initially identified bioinformatically by their proximity to anti-CRISPR-associated (*aca*) genes. AcrIIC1-3 bind the *N. meningitidis* (Nme)Cas9-sgRNA complex and are able to inhibit its activity in human cells, albeit to varying degrees.

Acr-encoding genes that inhibit type II-A systems, like the popular SpCas9, were discovered in phages of *Listeria monocytogenes*. Of the four genes identified, *acrIIA2* and *acrIIA4* encoded proteins that were able to inhibit target binding in SpCas9 despite the fact that LmoCas9 and SpCas9 share only 53% sequence identity, suggesting that AcrIIA2 and AcrIIA4 are broad-spectrum inhibitors of type II-A Cas9 orthologs [59]. This same study also demonstrated that AcrIIA2 and AcrIIA4 can inhibit SpCas9 in human cells. Using an approach focused on naturally occurring anti-CRISPR activity in phage strains to identify candidate Acrs, a fifth type II-A inhibitor was discovered, this time from phages of *Streptococcus thermophilus*. This protein, AcrIIA5, was also able to inhibit SpCas9 despite the fact that *S. thermophilus CRISPR1* (St1)Cas9 and SpCas9 share only 25% of their amino acid sequence identity [60]. AcrIIA5 was later found to broadly inhibit type II Cas9s, including every Cas9 homolog used in genome engineering. While inhibition was not observed in plaque assays for the type II-B *Francisella novicida* (Fn)Cas9 [61], AcrIIA5-dependent inhibition of FnCas9 was observed *in vitro* [62]. Recently, three new proteins, AcrIIA13-15, which specifically inhibit *Staphylococcus aureus* (Sa)Cas9 but not SpCas9 in human cells, were identified using a guilt-by-association method [63]. Because of SaCas9′s small size and relative ease of delivery, SaCas9 inhibitors are a valuable addition to the CRISPR-Cas9 toolset.

Acr inhibitors of another widely used Cas enzyme, the type V Cas12a, have also been identified. Two independent studies found self-targeting by Cas12a systems was inhibited by Acrs, called AcrVA1-5 [64]. Of these, AcrVA1 was found to broadly inhibit Cas12a homologs from *Moraxella bovoculi*, *Acidominococcus* sp., and *Lachnospiraceae bacterium ND2006* in human cells [65].



Technology (Example)	Acr Proteins (AcrIIA4)	Small Molecules (BRD0539)	CRISPR-SNuBs (Anti1_PAM-tracr)
Disrupts	Target binding	PAM binding	Target binding, RNP assembly
Delivery	Encoding; nucleofection	Carrier-free	Nucleofection; Carrier-free
K_d	~0.5-5 nM	~700 nM	< 5 nM
Specificity	Some broad specificity	SpCas9	SpCas9
Origin	Phage genomes	Compound screening	Rationally designed

Fig. 1. Comparison of anti-CRISPR technologies. Characteristics noted are based on published or implied properties and predicted delivery methods. K_d value for CRISPR SNuB [Anti1_PAM-tracr(FL), reported in Barkau et al., 2019] is unpublished.

3.1. Acr protein mechanisms

Acrs function by a variety of mechanisms, but interruption of target binding and protospacer adjacent motif (PAM) recognition is a recurring theme among Acrs whose mechanisms have been studied. Shortly after their initial discovery, the mechanisms of three of the originally described Acrs targeting the type I-F system of *P. aeruginosa* were characterized. They were found to each function distinctly. While one

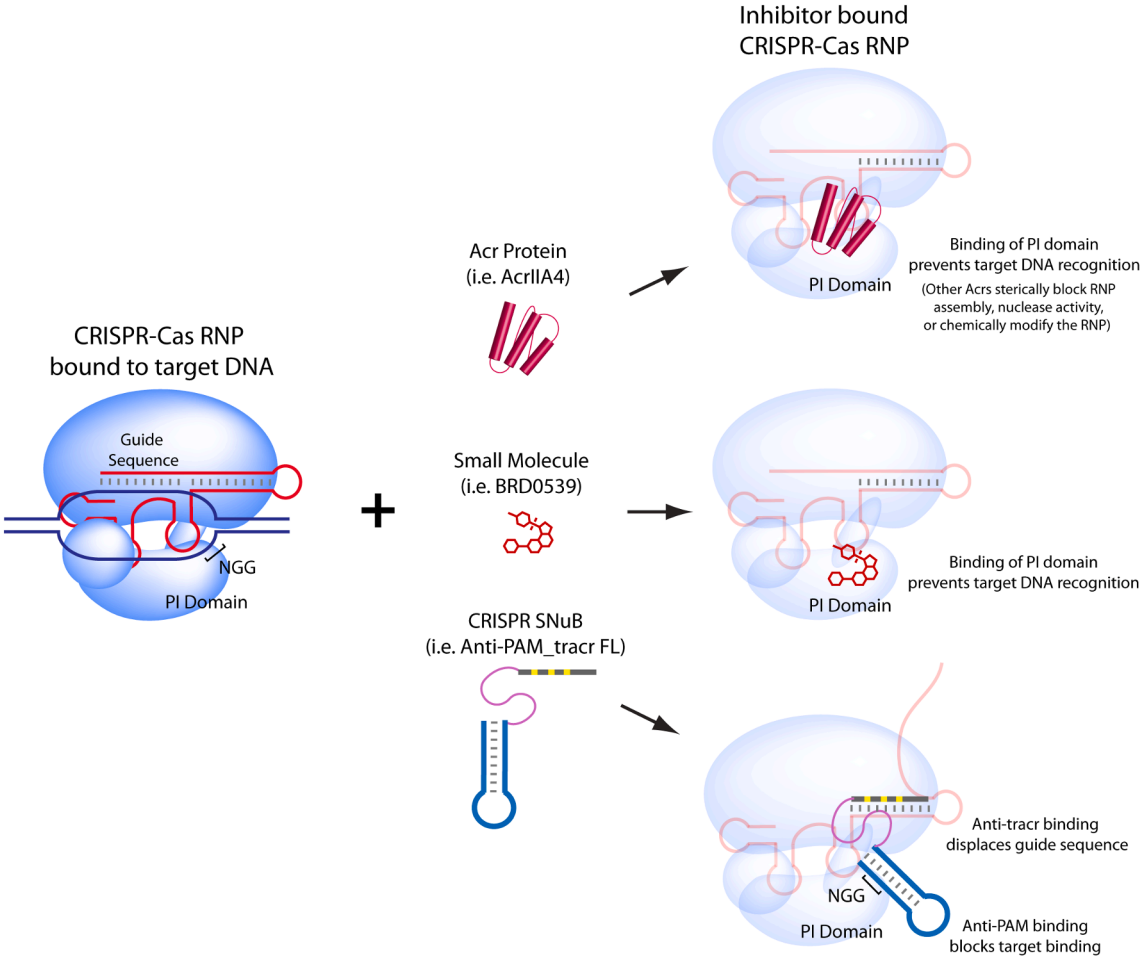


Fig. 2. Mechanism of Three Anti-CRISPR Technologies. An Acr protein, AcrIIA4, and small molecule, BRD0539, bind the PI domain of SpCas9, preventing PAM recognition and target binding. Different Acr proteins exhibit a diversity of mechanisms, but each usually has only one target in the Cas9 RNP. A CRISPR SNuB (Anti-PAM_tracr FL) exhibits a dual mechanism. The SNuB binds a sgRNA, obstructing proper assembly of the Cas9 RNP, and provides a DNA hairpin containing an NGG motif in close proximity to the PI domain as a substrate analog.

(AcrF3) prevented the assembly of the Cas3 helicase-nuclease protein to the DNA-bound Cas complex, the other two interrupted target DNA binding. One of these (AcrF1) bound the Csy1-Csy2 heterodimer, while the other (AcrF2) bound Csy3 [66]. Crystallography revealed that AcrF3 forms a homodimer which binds *P. aeruginosa* Cas3, trapping it in an inactive, ADP-bound form. AcrF3 also prevents DNA from entering into the helicase domain [67].

In the same publication which initially identified the type II-C inhibiting Acrs, AcrIIC3 was found to block DNA binding by catalytically "dead" Cas9 (dCas9) in human cells, indicating that its mechanism prevents target DNA binding rather than Cas9's catalytic activity [58]. Soon thereafter the Doudna group published mechanistic characterizations of AcrIIC1-3, reporting that AcrIIC1 functions by binding Cas9's HNH catalytic domain. The highly conserved nature of this domain allows AcrIIC1 to inhibit Cas9 orthologs from different species, including *Geobacillus stearothermophilus* (Geo)Cas9 and *Campylobacter jejuni* (Cj) Cas9. AcrIIC2 and AcrIIC3, however, were more specific. AcrIIC3 was shown to induce NmeCas9 dimerization and prevent DNA binding [68].

The mechanism of SpCas9 inhibitors AcrIIA2 and AcrIIA4 was first investigated by Dong and coworkers, who solved the crystal structure of AcrIIA4 bound to a Cas9-sgRNA complex. They found that these proteins bind Cas9 in a sgRNA-dependent manner. Intriguingly, rather than simply obstruct DNA binding, AcrIIA4 mimics PAM DNA, stopping Cas9 function at the earliest step of target recognition [69] (Fig. 2). AcrIIA4 was further found to only bind to a mature Cas9-sgRNA RNP complex before it engaged target DNA [51]. The mechanism of the broad-spectrum type II inhibitor AcrIIA5 was also reported to inhibit DNA binding and result in 3′ truncation of the sgRNA [61]. However, a conflicting study found that AcrIIA5 prevents target DNA cleavage by Cas9 but does not impact DNA binding, which would make it similar to AcrIIC1. It also exhibits differential and independent inhibition of the RuvC and HNH domains of Cas9, inhibiting RuvC to a much greater extent. The highly conserved nature of RuvC domains across Cas9s likely contributes to AcrIIA5′s ability to inhibit such diverse Cas9 homologs [62].

Although most Acrs function by nonenzymatic mechanisms, it has been found that the type V inhibitors AcrVA1 and AcrVA5 exhibit enzymatic inhibition of Cas12a. AcrVA1 induces 3′-end truncation, even reaching maximum activity at sub-stoichiometric levels, indicating that it is a multiple turnover mechanism [70]. AcrVA5, on the other hand, is an acetyltransferase. It acetylates MbCas12a at Lys653, which sterically prevents Cas12a from binding and recognizing its target PAM [71].

4. Small molecules

Despite the attractiveness of small molecules as CRISPR-Cas inhibitor drugs, relatively little work has been done on the discovery and characterization of small molecules as inhibitors. An early small molecule investigation screened a library of 189,606 compounds for their ability to inhibit either RuvC or HNH nuclease activity and found six compounds that exhibited greater than 30% inhibition of SpCas9 in their system [72]. Unfortunately, these molecules were found to be prohibitively toxic to cells at 10 μM and were thus not considered to be candidates for animal studies. The authors speculate that the high number of interactions, as well as interaction strength, between Cas9, sgRNA, and its target DNA make it difficult to target with small molecules. This is similar to known challenges faced with disrupting protein–protein interactions [73]. Although this initial small molecule screen was not successful in finding immediately useful compounds, it provided a potentially useful platform for quickly and efficiently screening other possible inhibitors.

More recently, Maji and colleagues introduced a high-throughput screen for the identification of small molecule inhibitors of SpCas9 [74]. This screening technique utilizes fluorescence polarization (FP) to measure binding of Cas9 to PAM-rich target DNA oligonucleotides as an initial readout for inhibitory potential. Importantly, this method yields inhibitors that impair PAM binding, a low affinity but essential step in Cas9-mediated DNA targeting and cleavage, rather than nuclease activity itself. An initial screen revealed hits from diversity-oriented synthesis (DOS) library compounds. The authors then carried out a second screen with 9,549 compounds calculated to optimally represent the structural diversity of the roughly 100,000 DOS compounds available. Further validation and characterization was performed *in vitro* and in cell-based EGFP disruption assays, eventually yielding compound BRD0539 as the best-performing molecule. R group variants of BRD0539 were systematically tested using cell-based EGFP disruption assays to further optimize the design. FP analysis of Cas9-gRNA binding revealed that BRD0539 does not interrupt RNP formation, meaning it specifically impedes target binding (Fig. 2). BRD0539 was further found to be reversible and specific to SpCas9, failing to inhibit *Francisella novicida* (Fn)Cas12a. Unfortunately, the estimated dissociation constant (K_d) of this inhibitor binding to SpCas9-gRNA complexes was 700 nM, far higher than those reported for Acr proteins, which are generally in the low nanomolar range [75–76]. Consequently, high concentrations of BRD0539 were necessary to inhibit SpCas9, with a 50% inhibitory concentration (IC_{50}) of around 15 μM in cell-based EGFP disruption assays.

Other small molecule-based approaches have been employed to control Cas9 activity. However, these have generally been limited to small molecule activators of Cas9 [77–78]. One notable exception is the small molecule-assisted shut-off (SMASh) method [79]. This technology consists of a fusion Cas9 protein containing a hepatitis C-derived protease (NS3) and degron (NS4A). This fusion causes Cas9 to be degraded by the proteasomal pathway in the presence of a protease inhibitor asunaprevir. In the absence of this inhibitor, the viral proteins excise themselves and are degraded alone, leaving Cas9 intact. While this method conceptually resembles small molecule inhibitors, it is distinct in its dependence on cellular processes, making it inviable for *in vitro* applications. The authors also observed a substantial delay in Cas9 loss ($t_{1/2}$ of 10.6 h) after asunaprevir addition in HEK293T cells transfected with Cas9-SMASh.

Small molecules remain to be fully explored as CRISPR inhibitors. Their cell permeability and likely low cost of manufacture makes them very attractive as drug candidates. Nonetheless, achieving efficacy at sufficiently low concentrations remains a technical hurdle. A cocktail of inhibitors that target different domains and interactions of the Cas RNP may help circumvent this problem by creating cumulative binding and selectivity at lower concentrations. Unlike proteins and nucleic acids, which are degraded in cells with fairly predictable byproducts, small molecules may have to be carefully studied for their potential off-target effects and metabolic breakdown products. In contrast to proteins and nucleic acids, which may offer more rational design rules, small molecules are unlikely to act as broad-spectrum inhibitors and will likely necessitate lengthy drug discovery pipelines for individual Cas enzymes.

5. Small nucleic acid-based inhibitors (SNuBs)

Inspired by the discovery of Acrs and previous work on chemically-modified CRISPR guide RNAs [80–81], small nucleic acids as potential inhibitors of Cas9 have been recently explored. The Cas9 RNP is a prime target for rational design of inhibitors that can mimic RNA and DNA binding, which are natural interactions for Cas enzymes. Nucleic acids can utilize multiple points of sequence-specific and sequence-nonspecific contact that can be exploited to disrupt RNP assembly or target binding. We previously found that oligonucleotides designed to have two key points of contact, Watson-Crick pairing to the guide RNA repeat region and binding to the PAM-interacting (PI) domain of Cas9, acted as strong inhibitors of SpCas9 [82]. These designs comprised a DNA hairpin containing an NGG sequence (anti-PAM), which mimics a PAM motif, that was tethered via a polyethylene glycol (PEG) linker to a 2′-*O*-methyl oligonucleotide for guide RNA base-pairing (anti-tracr) (Fig. 2). These two linked modules, anti-PAM and anti-tracr, function

synergistically to stably bind the Cas9-guide RNA complex and sterically block target binding. Initial designs produced an inhibitor with a K_d of ~ 25 nM while successive generations have since produced binding affinities at least an order of magnitude better, in the very low nanomolar range (Barkau and coworkers, unpublished results).

The presence of small nucleic acid-based inhibitors slowed Cas9 activity *in vitro* and in HEK293T cells in a dose-dependent manner, having a calculated half maximal effective concentration (EC_{50}) *in vitro* very near the concentration of the CRISPR RNA (crRNA) guide, with which it is designed to compete for binding. Modification of the 2′-*O*-methyl chemistry of the anti-tracr module to 2′-fluoro RNA (F) or 2′-fluoro with locked nucleic acid (LNA) bases at three positions (FL) increased inhibition *in vitro* and in cells, with the combination of F and LNA chemistries ("FL") emerging as the most favorable design [82]. These inhibitors small nucleic acid-based inhibitors, or SNuBs, of CRISPR are likely to benefit from additional structural and chemical optimizations. For example, phosphorothioate (PS) modification of the backbone may confer greater nuclease resistance and pharmacokinetic properties. Likewise, alternative pentose modifications (ribose, arabinose, etc.) could be used to modulate SNuB stability, binding affinity, and carrier-free uptake.

The size, chemistry, and mechanism of CRISPR SNuBs present some unique advantages over Acrs and small molecules for inhibiting Cas9. SNuBs are smaller than Acr proteins and advanced iterations of SNuB design may take advantage of chemical modifications that enhance carrier-free delivery and high resistance to nucleases, making them more deliverable than Acrs *in vivo*. Despite their small size, however, SNuBs can bind the Cas9 RNP with high affinity, possibly exceeding that of Acrs. Affinity and specificity would also be predicted to be superior to small molecules. While some Acrs exhibit broad spectrum activity [68], not every CRISPR nuclease can be inhibited by a single protein and some may not be inhibited by any naturally occurring Acr. Small molecules have yet to be found which inhibit multiple CRISPR enzymes and inhibiting enzymes other than SpCas9 will likely require *de novo* screens for new molecules. Though Cas9 orthologs vary in their structure, they all form RNPs with similar RNP interactions but different RNA sequences and PAM specificities. Thus, the rational design of SNuBs represents a platform that can likely be used to rapidly generate effective inhibitors against other Cas9 orthologs, such as SaCas9 and CjCas9, or even other CRISPR effector proteins like Cas12a.

6. Therapeutic nucleic acids

A key strength of CRISPR SNuBs lies in the translation of previous nucleic acid therapeutic successes to their development. Lessons learned from nucleic acid therapeutics can be applied to modulate their mechanistic properties, deliverability, tissue and organ distribution *in vivo*, nuclease resistance, and toxicity [83–86]. Several nucleic acid-based drugs, including antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs), have been approved by the United States Food and Drug Administration (FDA). The field surrounding them provides a wealth of experience that will guide the clinical development of SNuBs. Both ASOs and siRNAs are mechanistically or structurally similar to SNuBs.

Since their discovery, ASOs have played a vital role in the advancement of oligonucleotide-based therapies. The first ASO, a 13-nucleotide DNA oligomer with complementarity to the 35S RNA of Rous sarcoma virus, was found to inhibit viral infection when added to chicken embryonic fibroblast cultures [87]. The cause of this inhibition was determined to be the interruption of viral translation, likely through sequence-specific hybridization to viral RNA [88]. It was later discovered that ASOs inhibit RNA translation in cell-free reticulocyte lysate by an RNase H-dependent mechanism, suggesting that the RNA is digested while bound to the ASO [89]. Some ASOs, however, do not require RNase H in order to elicit hybridization-dependent inhibition, functioning instead by steric binding mechanisms like blocking translation, modulating splicing, or binding regulatory elements to control translation [90–93]. By these mechanisms, ASOs in principle can modulate the expression of nearly any gene, giving them enormous potential as therapeutics for genetic diseases.

RNA interference (RNAi) is a naturally occurring mechanism of gene regulation found in diverse eukaryotes. In canonical mammalian RNAi, microRNAs guide sequence-specific binding of Argonaute proteins to messenger RNA (mRNA) targets, which leads to repression of gene expression [94]. When perfect base-pairing occurs, which is induced by artificial siRNAs, target mRNAs are catalytically cleaved. siRNAs can be designed to target almost any transcript for degradation in human cells, making them an extremely useful tool in treating genetic diseases where a gene is overexpressed or perhaps aberrantly spliced [95].

The first FDA-approved oligonucleotide-based therapy was fomivirsen (commercially sold as Vitravene). Developed by ISIS (now Ionis) Pharmaceuticals and Novartis Ophthalmics, this 21-nucleotide fully phosphorothioated (PS) DNA oligo was designed to treat cytomegalovirus (CMV)-induced retinitis that is found in patients with compromised immune systems. It targets a CMV gene that encodes a protein, IE-2, which is necessary for replication [96]. For over a decade following the approval of fomivirsen by the FDA in 1998 and the European Medicines Agency (EMA) in 1999, no oligonucleotide drugs were approved for clinical therapeutic use. During the 2010s, however, nucleic acid therapeutics experienced a resurgence and the rate of FDA approval for oligonucleotide therapeutics has since accelerated [97]. This exciting trend is likely due to the increasingly shorter pre-clinical development times for oligonucleotides. Because nucleic acid therapeutics are straightforward to design using predictable rules of base pairing and secondary structure, they are readily synthesizable, and can be modulated in a variety of ways using well-characterized chemical strategies. Typically, it can take less than a year for a new oligonucleotide drug to enter clinical studies.

The rapid nature of oligonucleotide therapeutic development is exemplified by treatments designed for extremely rare genetic disorders, or so-called "N-of-1" treatments [98]. In one such case, a young girl was diagnosed with a fatal neurodegenerative disease caused by a completely novel mutation. It was found that this mutation caused missplicing of *MFSD8*, which led to premature translation termination. Milasen, a 22-nucleotide ASO, was designed to target the cryptic splice site where the abnormal splicing event occurs. This ASO utilized design elements of nusinersen, an FDA-approved ASO with a similar therapeutic mechanism. Within a year of the original diagnosis, milasen was given limited approval as an investigational drug to treat the patient [99]. Treatments such as these are testimony to the power of rational design and well-characterized chemistry that is shared by most nucleic acid-based drugs.

7. The challenge of delivery

One of the major challenges CRISPR-Cas inhibitors will face on their path to clinical application lies in efficient delivery. While the obvious application of inhibitors is to reduce off-target editing across the genome, they could also be used to suppress editing in non-target tissues and organs. The former would necessitate delivery of an inhibitor with the same target distribution as the CRISPR therapeutic itself, either at a later time point or simultaneously at a finely tuned ratio to the effector. The latter, however, would involve inhibitor delivery with an inverse distribution before or at the same time as CRISPR delivery. The distinct nature of each inhibitor technology discussed above—Acr proteins, small molecules, and nucleic acids—brings unique advantages and difficulties for their delivery. Potential avenues of delivery for each inhibitor type are shown in Fig. 3.

7.1. Delivery of Anti-CRISPR proteins

Acr proteins may be the most challenging type of inhibitor to deliver

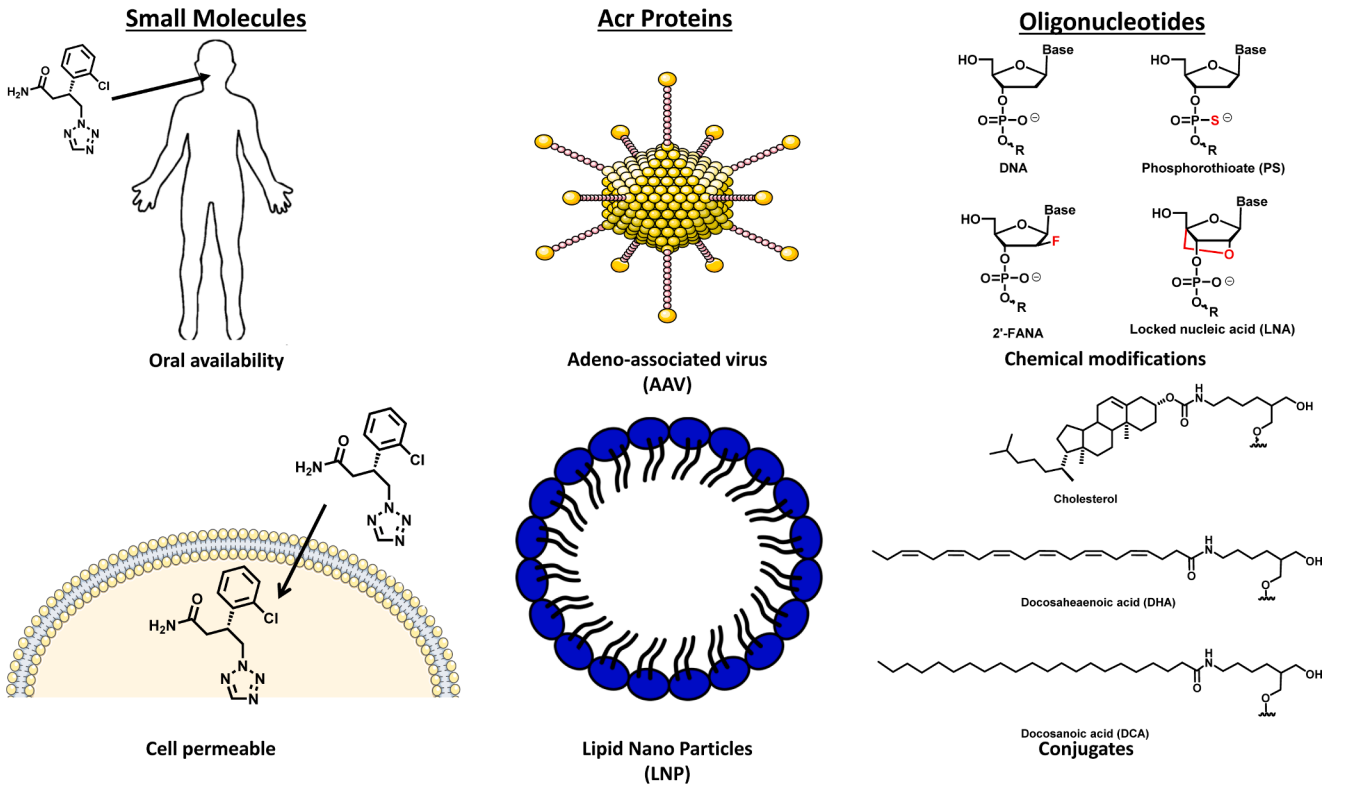


Fig. 3. Methods of Anti-CRISPR Delivery. Small molecules can be taken orally and are cell permeable. Acr proteins must be encoded in an AAV vector or packaged in lipid nanoparticles (LNPs) and delivered by injection. Oligonucleotides (SNuBs) can be injected directly or via LNP and exhibit uptake and distribution patterns modulated by chemical modifications and conjugates.

directly in a clinical setting. Though small by protein standards, Acrs would instead most likely rely on delivery via adeno-associated virus (AAV) vectors or as mRNAs packaged into LNPs, the same methods by which most CRISPR-based therapeutics are expected to be delivered in patients [100]. Packaging into an AAV vector has long been a challenge for CRISPR effectors alone, as the sheer size of most Cas enzymes, such as SpCas9, presents packaging issues in AAVs. This has been overcome by using dual AAVs carrying either Cas9 and its sgRNA encoded separately or a split Cas9 encoded on two separate AAVs [101] and by using smaller orthologs of Cas9, such as SaCas9 [102]. The addition of Acrs to these systems would further decrease the space that can be used for additional sgRNAs, fusion proteins, or regulatory elements. Alternatively, Acrs could be delivered as stand-alone AAVs. Another consideration for AAV-based or LNP-based delivery of Acrs is their dependence on the cellular processes of transcription and translation, which can be impacted by a number of cellular factors, such as stress or cell type. This reliance makes it more difficult to accurately predict the timing and degree of inhibition that can be achieved by Acrs. It also means that inhibition, possibly being far removed from the point of administration, might require hours to take effect.

One significant benefit of using AAVs to encode inhibitors is that AAVs themselves can exhibit tissue specificity, making targeted delivery of both CRISPR and Acrs for the purpose of increasing tissue specificity of editing potentially straightforward. For example, different AAV serotypes have been found to be selectively transduced most efficiently to specific tissues in the muscle, lung, and liver [103]. Co-delivery by the same method as CRISPR might also ensure that similar timing of Acr and Cas enzyme expression can be achieved if desired, regardless of factors that might globally affect transcription and translation. Acrs have been routinely tested in human cell lines but they are also the only inhibitor technology that has been tested in a living animal. Acrs were recently used to inhibit Cas9 in a miRNA-repressible manner to strengthen tissue specificity of editing in mice [104].

7.2. Delivery of small molecules

Perhaps the simplest type of CRISPR inhibitor to deliver *in vivo* would be small molecules. Due to their high likelihood for cell permeability, most small molecule inhibitors could in theory be administered orally in a manner similar to conventional drugs. Small molecules generally have rapid uptake kinetics as well, perhaps taking effect on the order of minutes [105]. Unlike proteins or nucleic acids, which are broken down and metabolized by well-known mechanisms, small molecules exhibit diverse pharmacokinetic properties and metabolic outcomes. In addition to toxicity and efficacy, any small molecule designed to inhibit CRISPR systems in a clinical setting must be thoroughly characterized in terms of its absorption, distribution, metabolism, and excretion (ADME). Most enzymatic inhibitors used in medicine are small molecules. However, considering the potential necessity of dedicated discovery pipelines for each Cas enzyme and the complexity of small molecule medicinal chemistry and pharmacology, small molecules that inhibit CRISPR-based therapeutics may remain a longer-term prospect for the clinic.

7.3. Delivery of CRISPR-SNuBs

The challenge of delivery is distinct for nucleic acids as opposed to both Acr proteins and small molecules. To be successful in clinical applications, SNuBs will need to overcome barriers such as degradation by serum and cellular nucleases, renal clearance, and cellular uptake. Fortunately, this challenge is a major topic of study in nucleic acid-based therapeutics and a number of approaches have been developed to address these issues. Much is also known about effective methods for delivery, major barriers to delivery inside the human body, and how oligonucleotides can be chemically modified to overcome these.

When considering modifications to enhance delivery, it is important to remember that CRISPR SNuBs are essentially composed of two

modules. These are the anti-PAM, a native DNA hairpin, and the anti-tracr, made of RNA-like nucleotides that maintain an A-form structure [82]. Several chemical modifications are known to improve cellular uptake of oligonucleotides. Due to their foreign nature, these modifications often also bestow some degree of nuclease resistance to nucleic acids, as they are not readily recognized by physiological nucleases. Modification can be performed to the nucleic acid backbone, the ribose sugar, or the base itself. One backbone modification, phosphorothioate (PS), is the most common modification throughout all classes of therapeutic oligonucleotides. PS confers both nuclease resistance [106] and improved cellular uptake [107] in exchange for a small reduction in binding affinity [108]. Importantly, PS modification allows DNA to largely retain its native structural properties [109]. These features are desirable but PS modification has also been shown to cause toxicity through non-specific binding of proteins [110]. Therefore, the use of PS in SNuB designs should be limited to enhance activity and serum stability but reduce toxicity.

DNA-like modifications, such as 2′-deoxy-2′-fluoroarabino nucleic acid (FANA) could be incorporated into the anti-PAM module to maintain B-form structure while increasing serum stability [111] and promoting cellular uptake [112–113]. RNA-like modifications, such as 2′-*O*-methyl, 2′-fluoro, and LNA can promote stability for the anti-tracr module [114–116]. Like FANA, LNA is also conducive to gymnotic delivery, wherein the oligo is taken up by the cell without the need for a transfection reagent [117–118]. Other modifications such as phosphorodiamidate morpholino oligomers (PMO) and peptide nucleic acids (PNA) might also provide nuclease resistance and high binding affinity [119] and may be suitable in the anti-tracr module.

Another important avenue for improving uptake and distribution of nucleic acid-based inhibitors is conjugation. A number of conjugates are known to facilitate cellular uptake of nucleic acids [120]. One of the first conjugates found to be useful for this purpose were lipids such as cholesterol. Cholesterol was found to induce distribution of oligonucleotides to diverse tissues [121–122]. It achieves this by associating with lipoproteins, transmembrane proteins, and lipoprotein receptors [123]. There is a growing diversity of lipid conjugates, such as docosanoic acid and docosahexaenoic acid (DHA), which have been utilized to enhance extrahepatic distribution of oligonucleotides [120,124]. Another conjugate, N-acetyl galactosamine (GalNAc), can be used to direct oligonucleotides to hepatocytes via endocytosis. GalNAc binds with high affinity to the asialoglycoprotein receptor 1 (ASGR1) found in high abundance in the liver, making it very effective at facilitating organ-specific cellular uptake [125–126]. Conjugation of oligonucleotides to antibodies or other scaffold proteins [127] can promote uptake by a number of different cell types and may even be useful in targeting specific types of cancers [128–132]. Other conjugates, such as short peptides [133], aptamers [134], and cell-penetrating peptides [135], can enable the delivery of oligonucleotides to diverse tissues and cell types.

While it is possible that SNuBs could be delivered as heavily modified oligonucleotides, in some cases it may also be desirable to use carriers such as LNPs. Short-lived lipid-nucleic acid complexes can be formed simply by mixing nucleic acids (polyanions) with lipid molecules but more complex LNP structures are required to evade reticuloendothelial clearance. These typically comprise a lipid bilayer with a heterogeneous lipid composition, including fusogenic lipids to assist endosomal escape, cholesterol, and pegylated lipids [136–137]. One notable drawback of LNPs is that their size limits their target potential. LNPs, being ~100 nm particles, can only exit circulation at areas with sufficiently large fenestrations in the linings of blood vessels, such as liver, spleen, lymphoid organs, and bone marrow [138].

8. Metabolism and safety concerns of Anti-CRISPRs

An important aspect of any CRISPR inhibitor proposed for use in human patients is the eventual metabolic fate and potential for unintended effects on the human body. While detailed studies on this subject have yet to be done, the three types of anti-CRISPRs discussed in this review could be expected to experience different fates inside the human body due to their contrasting molecular compositions.

8.1. Acr protein metabolism

Peptide chains are typically very stable in solution, taking perhaps years to hydrolyze nonenzymatically [139]. While there are a number of pathways that degrade proteins in the human body, anti-CRISPR protein metabolism should be relatively predictable if they are assumed to be transcribed or translated, and likewise degraded, inside of cells. Intracellular proteolysis is carried out mostly by the autophagy-lysosomal pathway or by the ubiquitin-proteasomal pathway. Proteins are non-selectively taken up into autophagic vacuoles within the cell. These bodies can then fuse with lysosomes containing proteases that degrade captured proteins [140]. By contrast, the ubiquitin-proteasomal pathway is more selective. Briefly, proteins are tagged for degradation by covalent linkage of ubiquitin polypeptides to the target protein (ubiquitination). Ubiquitinated proteins are then degraded within the proteasome. Amino acids released by proteolysis can then be used by cellular processes, including the synthesis of new proteins [141]. In fact, fusion of Acrs to the cell cycle regulated protein Cdt1 has been used to demonstrate cell cycle dependent inhibition of Cas9 via the ubiquitin-proteasomal pathway [142]. One possible drawback to the use of Acr proteins is that their half-life is dependent on factors might affect cellular metabolism, such as cell type, stress, and cell health.

8.2. Metabolism of small molecule inhibitors

Somewhat less predictable are the metabolic outcomes of small molecule inhibitors. While only a few have been identified to date, anti-CRISPR small molecules could ultimately assume a wide diversity of structures containing any number of various chemical groups, each of which greatly influences the metabolism of the molecule. It is worth noting that BRD0539 exhibited high stability in human plasma (50% serum for 5 h). Beyond this, low cytotoxicity was observed [74]. However, this cannot be extrapolated to reflect toxicity to organ systems or whole animals [143].

8.3. Metabolism of nucleic acid inhibitors

The metabolic fate of nucleic acids inside of cells, like that of proteins, is relatively well understood. As naturally occurring biological molecules, unmodified DNA and RNA oligonucleotides are readily degraded via nucleases that occur ubiquitously in human tissues. Upon degradation, the resulting nucleotide or nucleoside monomers can be recycled for nucleic acid synthesis or further broken down and excreted as waste products such as uric acid. As mentioned above, several chemical modifications can be used to alter the properties and pharmacokinetics of nucleic acid drugs [144]. CRISPR SNuBs will have to be heavily modified for use inside human patients if delivered directly or as LNPs. The way these modifications affect metabolism is therefore an important consideration in the development of SNuBs or other nucleic acids for inhibition of gene editing in a therapeutic context. Non sequence-specific toxicities associated with ASOs *in vivo* are generally mild, treatable, and dose-dependent. These include thrombocytopenia, hyperglycemia, and hypotension associated with PS modification. Acute toxicity related to PS ASOs has also been observed in the form of transient complement cascade activation or inhibition of the clotting cascade.

Another potential concern with oligonucleotide drugs is the presence of unmethylated CpG sites, which stimulate an immune response [145], but this is easily addressed by chemical modifications [146]. Beyond sequence, every unique chemical modification employed can potentially introduce its own metabolic breakdown pathway, and therefore

potential toxicity profile, in human patients. It has been found that 2′ modifications such as 2′-*O*-methyl and 2′-O-methoxyethyl (MOE) induce similar side effects as PS oligos, but to a lesser degree. LNA has been found to exhibit few side effects at low doses clinically, but have been shown to be hepatotoxic at higher doses in mice, possibly in a sequence and length-specific manner [147]. At normal *in vivo* dosage levels, 2′-fluoro monomers were found not to significantly accumulate in rats given revusiran over a two-year period. Additionally, the byproducts of 2′-fluoro modified oligo did not appear to be competent polymerase substrates or chain terminators.

As mentioned previously in this review, several oligonucleotide drugs containing various chemical modifications have been approved by regulatory agencies for use in human patients. The demonstrated safety of chemical modification schemes for therapeutic oligonucleotides illuminates a clear path to clinically applicable anti-CRISPR nucleic acids with little to no toxicity.

9. Conclusion

As CRISPR becomes a more common component of human therapeutics, methods for inhibiting Cas enzymes will become ever more important. In the future, inhibitors may be used to suppress CRISPR in non-target tissues during gene therapy, added preemptively to minimize off-target activity, or even administered in emergency situations where CRISPR has been misapplied or caused adverse events. Indeed, it is easier to envision a future for CRISPR-based therapeutics with anti-CRISPR technologies at our disposal.

The next logical step toward the clinic for anti-CRISPR technologies is optimization and testing in human tissue culture and animal models of gene editing. Fortunately, the accelerating rate of success for human nucleic acid therapeutics is paving a clear path to the clinic for nucleic acid-based anti-CRISPR molecules. Chemistry and improved manufacturing and quality control methodologies, pharmacology, and delivery of nucleic acid drugs are all reaching a threshold of maturity. For these reasons, as well as the intrinsic power of using nucleic acids as inhibitors of nucleic acid-binding proteins, CRISPR SNuBs represent a promising platform for developing a clinically successful anti-CRISPR drug.

CRediT authorship contribution statement

C.L.B. conceived, wrote, and edited the manuscript and prepared figures. D.O. and S.B.E. assisted in writing, literature assessment, and figure preparation. M.J.D assisted in writing and editing the manuscript. K.T.G. conceived and assisted in writing and editing of the manuscript.

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