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Chemical Modifications in RNA: Elucidating the Chemistry of dsRNA-Specific Adenosine Deaminases (ADARs)

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CONSPECTUS

The term RNA editing refers to any structural change in an RNA molecule (*e.g.* insertion, deletion, or base modification) that changes its coding properties and is not a result of splicing. An important class of enzymes involved in RNA editing is the ADAR family (adenosine deaminases acting on RNA), which facilitate the deamination of adenosine (A) to inosine (I) in double-stranded RNA (dsRNA). Inosines are decoded as guanosines (G) in most cellular processes; hence, A-to-I editing can be considered an A-to-G substitution. Among the RNA editing enzymes, ADARs are of particular interest because a large portion of RNA editing events are due to A-to-I editing by the two catalytically active human ADARs (ADAR1 and ADAR2). ADARs have diverse roles in RNA processing, gene expression regulation, and innate immunity; and mutations in the ADAR genes and dysregulated ADAR activity have been associated with cancer, autoimmune diseases, and neurological disorders. A-to-I editing is also currently being explored for correcting disease-causing mutations in the RNA, where therapeutic guide oligonucleotides complementary to the target transcript are used to form a dsRNA substrate and site-specifically direct ADAR editing. Knowledge of the mechanism of ADAR-catalyzed reaction and the origin of its substrate selectivity will allow understanding of ADAR's role in disease biology and expedite the process of developing ADAR-targeted therapeutics.

Chemically modified oligonucleotides provide a versatile platform for modulating the activity and interrogating the structure, function, and selectivity of nucleic acid binding or modifying proteins. In this account, we provide an overview of oligonucleotide modifications that have allowed us to gain deeper understanding of ADAR's molecular mechanisms, which we utilize in the rational design and optimization of ADAR activity modulators. First, we describe the use of the nucleoside analog 8-azanebularine (8-azaN) to generate high-affinity ADAR-RNA complexes for biochemical and biophysical studies with ADARs, with particular emphasis on X-ray crystallography. We then discuss key observations derived from the crystal structures of ADAR bound to 8-azaN-modified RNA duplexes and describe how these findings provided insight into ADAR editing optimization by introducing nucleoside modifications at various positions in synthetic guide strands. We also present the informed design of 8-azaN-modified RNA duplexes that selectively bind and inhibit ADAR1 but not the closely-related ADAR2 enzyme. Finally, we conclude with some open questions on ADAR structure and substrate recognition and share our current endeavors in the development of ADAR guide oligonucleotides and inhibitors.

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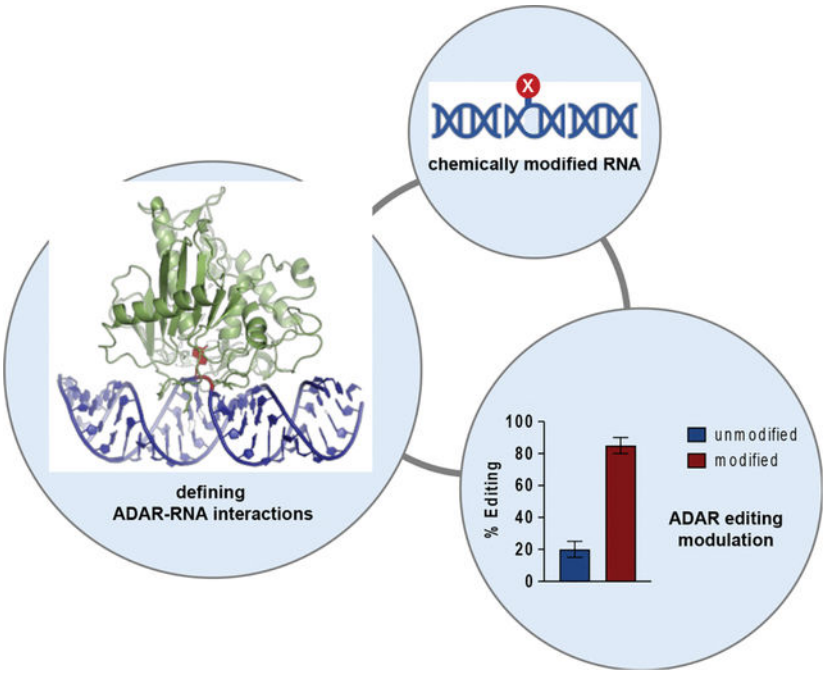
Graphical Abstract



1. Introduction

One of the most prevalent forms of RNA editing is the conversion of adenosine (A) to inosine (I) or A-to-I editing catalyzed by a group of duplex RNA-specific enzymes called ADARs (adenosine deaminases acting on RNA)[5]. ADARs facilitate the hydrolytic deamination of adenosine in double-stranded RNA (dsRNA) substrates, leading to the formation of the non-canonical nucleobase, inosine (Fig. 1a)[6]. Because of similar base pairing properties, inosine functions analogously to guanosine (G) in many cellular processes, including splicing, translation, and reverse transcription[7,8]. The proposed mechanism of adenosine deamination by ADARs is similar to other adenosine deaminases that act on monomers (ADA, AMP deaminase) or tRNAs (ADATs, TadA) (Fig. 1b)[9]. A Zn ion-bound water molecule in the active site is deprotonated by a catalytic glutamate residue, allowing for the nucleophilic attack of hydroxide at the C6 position of adenosine. Concurrent protonation of N1 results in the formation of a high energy tetrahedral intermediate, which, through a series of proton transfers and departure of ammonia, gets converted to inosine (Fig. 1b)[9].

Two catalytically active forms of ADARs, ADAR1 and ADAR2, facilitate the dsRNA-specific A-to-I editing in humans. These enzymes are comprised of a deaminase domain and dsRNA-binding domains (dsRBDs)[6], typical of dsRNA binding or modifying proteins (Fig. 1c)[10]. However, existing experimental data support that the deaminase domains of both enzymes also require a duplex RNA structure for substrate binding[11,12]. ADAR1 also contains an additional nucleic acid binding domain for Z-DNA/RNA binding (Zα and Zß) and exists primarily as the two isoforms, p110 and p150 (Fig. 1c)[6]. A nuclear localization

signal (NLS) near the dsRBD3 of ADAR1 and at the N-terminus of ADAR2 allows these proteins to localize in the nucleus. However, a nuclear export signal (NES) is also present adjacent to the Zα domain of ADAR1 p150, enabling this isoform to shuttle between the nucleus and cytoplasm[13].

The consequences of A-to-I editing by ADARs are context-dependent, and vary across different RNA molecules, tissues, and developmental stages. In general, A-to-I editing can result in amino acid substitutions as well as disruption or creation of splice sites. These recoding events contribute to the formation of novel protein isoforms (Fig. 1d)[5]. A-to-I editing can also lead to non-recoding events, which are collectively due to alterations in the RNA secondary structure. These structural changes can impact RNA stability and its interaction with other molecules, such as miRNAs and proteins involved in RNA degradation and immune responses (Fig. 1d)[5,14,15]. Mutations in the ADAR genes as well as aberrant ADAR-mediated A-to-I editing have been associated with various diseases, including neurological disorders and cancer[16,17], highlighting the functional significance of this tightly-regulated RNA modification process. Moreover, the growing list of ADAR's diverse roles in various cancer types has led to the increasing interest in the development of ADAR inhibitors as novel anti-cancer drugs[12,18,19].

An emerging therapeutic modality called site-directed RNA editing harnesses the natural process of A-to-I editing by ADARs to correct disease-causing mutations at the transcript level[20,21]. In this approach, an antisense or guide oligonucleotide complementary to the target transcript is introduced to form the required duplex substrate, recruiting ADAR to the desired edit site (Fig. 2a). ADAR performs the corrective A-to-I edit, resulting in a transcript that would now translate a functional protein (Fig. 2a). Current directed RNA editing methods either employ endogenous human ADARs or overexpress engineered ADAR proteins with improved efficiency and target specificity[22,23]. To achieve better editing outcomes for therapeutic RNA editing using endogenous ADARs, chemically modified guide oligonucleotides (~30–40 nt long) are presently utilized[3,22]. An example of a chemically modified guide that has been previously used by our lab is shown in Fig. 2b[3] and a detailed discussion of the chemical modifications that specifically enhance ADAR catalysis is presented in section 3. However, other backbone (phosphorothioate (PS) and phosphoryl DMI amidate (PN) linkages) and sugar (2'-deoxy, 2'-*O*-methyl (2'-OMe), 2'-fluoro (2'-F), locked nucleic acid (LNA)) modifications are also being employed to improve metabolic stability, target binding kinetics and specificity of guide oligonucleotides (Fig. 2b)[3,22,24].

Paramount to the success of utilizing ADARs for directed editing as well as the development of therapeutic interventions for ADAR-associated diseases is a deeper understanding of ADAR structure, substrate interaction, and selectivity. By fine-tuning oligonucleotide properties, chemically modified oligonucleotides offer a flexible framework for activity manipulation and investigation of the structure, function, and specificity of nucleic acid binding or modifying proteins[25,26]. In this account, we outline our lab's recent breakthroughs on elucidating the nature of the ADAR-RNA interface and optimization of ADAR editing modulators enabled by the use of chemical modifications in RNA.

2. Illuminating the ADAR-RNA interface using 8-azanebularine-modified RNA duplexes

The nucleoside analog 8-azanebularine (8-azaN) was critical in our lab's earlier efforts to develop ADAR editing substrate mimics which could then be used to probe the ADAR A-to-I editing reaction[9,27]. Upon hydration by ADAR, 8-azaN in RNA is converted to a tetrahedral covalent hydrate that is a structural analog of the predicted ADAR reaction intermediate (Fig. 3a, Fig. 1a–b) and the proposed hydrolytic deamination transition state[9]. Aza substitution at C8 of nebularine increases susceptibility of hydration across N1-C6[27], while the absence of a good leaving group at C6 allows for the mechanistic trapping of the covalent hydrate[9]. Therefore, 8-azaN-modified RNA duplexes facilitate the formation of active site-directed and high affinity ADAR-RNA complexes suitable for biochemical and biophysical investigations with ADARs[9,11,12].

The important early crystal structure of the ADAR2 deaminase domain (ADAR2-D) revealed the presence of an inositol hexakisphosphate (IP6) molecule buried within the enzyme core and important features of the active site, including the catalytic Zn ion described earlier in section 1[28]. However, a major breakthrough in our study of ADAR-RNA interactions came with the first set of high resolution crystal structures of ADAR2-D bound to 8-azaN-containing RNA substrates[1]. In the next sections, we describe key findings from these crystal structures and from a more recent crystal structure of a larger ADAR2 fragment, ADAR2 deaminase domain + dsRBD2 (ADAR2-R2D), in complex with an 8-azaN-modified RNA duplex[2].

2.1. Base-flipping loop and orphan position.

The mechanism of ADAR deamination clearly requires the edited adenosine to be flipped-out of the RNA duplex into the enzyme active site. Indeed, substituting the edited adenosine by 8-azaN in the duplex substrates used for crystallography studies allowed for the mechanistic trapping of the flipped-out base in the ADAR2 active site (Fig. 3b)[1]. Like several other enzymes that invoke this base-flipping mechanism[29,30], ADAR2 stabilizes the flipped-out conformation by utilizing a base-flipping loop. This loop, consisting of amino acid residues 486–489, intercalates into the space vacated by the flipped-out base and forms hydrogen bonding interactions with the orphaned base via residue E488 (Fig. 3b). Flanking E488 are two glycine residues, G487 and G489, which allow for the loop's conformational flexibility required for helix penetration. Interestingly, ADAR's observed preference for editing adenosines across a cytidine (C) or a uridine (U)[31] can be explained by a possible clash between E488 and a purine at the orphan position.

2.2. Other ADAR-induced structural changes in the RNA.

From the crystal structures, the intercalating loop is observed to approach from the minor groove side at the edited site. To accommodate this approach, the RNA duplex undergoes other conformational changes resulting in a structure with considerable deviations from the regular A-form helix adopted by dsRNAs (Fig. 3c). In particular, the −1 base (position across the nucleotide directly 5' to edited site) appears to be unstacked, pointing towards the helical axis in the orphan base direction. Accompanying this is a change in the −1 ribose

pucker from the usual C3'-*endo* in A-form RNA helices to C2'-*endo* typical of B-form DNA helices. Overall, these conformational variations at the −1 position cause the formation of a kink in the unedited strand and a widening of the major groove opposite the editing site (Fig. 3c). The observations just described can also rationalize the inability of ADARs to modify deoxyadenosines in duplex DNA. The minor groove in a B-form helix is narrower and would not permit helix penetration and occupancy of the edited base position by ADAR's base-flipping loop[1]. Indeed, we have shown that ADARs can deaminate 2'-deoxyadenosines when present on the DNA strand of DNA/RNA hybrid duplexes consistent with the idea that ADARs require the A-form duplex conformation for reactivity[32].

2.3. Nearest neighbor preferences.

The observed contacts between ADAR2-D and the base pairs adjacent to the editing site provided a rationale for the known nearest neighbor preference of ADARs. In general, ADARs favor editing adenosines flanked by a 5'-U or A and a 3'-G[33,34]. Based on the crystal structures, we predicted that the 2-amino group of a 5'-G (or −1 G in the case of a 5'-C) in the minor groove would clash with the protein at G489 (Fig. 3d). However, a U or A should not experience this clash, explaining why these nucleotides are favored at the 5' position (Fig. 3d). Indeed, deamination of an RNA substrate with the 5'-U:A pair replaced with a U:2AP (2-aminopurine) pair resulted in an five-fold reduction in deamination rate[1], validating the negative effect of a 2-amino group in the minor groove at this site.

The preference for a G at the 3' position arises from the hydrogen bonding contact between the 2-amino group of G and the backbone carbonyl group of S486 (Fig. 3d). Of the four canonical nucleobases in RNA, only G presents an H-bond donor in the minor groove, hence the predilection for this base at the 3' position. Indeed, deamination of an RNA substrate with the 3'-G replaced with I (no 2-amino group) or N^2-methylguanosine (N^2MeG, blocked access to 2-amino group) resulted in a 50% decrease in rate to no observed editing, respectively[1]. These results clearly indicate the importance of a stabilizing hydrogen bonding interaction at this position.

2.4. The 5' binding loop and other RNA contacts.

Aside from the interactions near the edited site, two other regions in the RNA's 5' and 3' direction relative to the flipped-out base show sites for ADAR2-D engagement. At 7–8 bp in the 3' direction, a lysine residue (K594) makes a hydrogen bonding contact with the phosphodiester backbone of the unedited strand (Fig. 4a). Importantly, mutating K594 to alanine (K594A) appreciably reduced ADAR2-D's editing activity, suggesting the significance of this contact for substrate recognition and editing[1]. A lysine residue is also conserved at this position in ADAR1 (K1120) and is predicted to make a similar hydrogen bonding contact in the same location of the RNA duplex based on a Rosetta homology model of the ADAR1 deaminase domain (ADAR1-D, Fig. 4a)[12,35]. Indeed, experiments using known ADAR1 and ADAR2 substrates with shortened duplex 3' to the edit site (≤ 6 bp) displayed a substantial loss in binding or editing activity for both enzymes[12,36]. These observations imply that both ADARs require an RNA duplex with at least 8 bp in the 3' direction relative to the edited base for efficient editing.

Another region of protein-RNA contact is observed ~ 10 bp in the 5' direction from the edited base facilitated by a stretch of amino acid residues (454 to 477) comprising ADAR2's 5' binding loop (Fig. 4b). Interestingly, the deaminase domains of ADAR1 and ADAR2 have a relatively high sequence homology except in their 5' binding loops, providing one possible origin of the difference in substrate selectivity of the two enzymes. Indeed, experiments using a chimeric protein with ADAR2's 5' binding loop grafted onto ADAR1-D exhibited ADAR2-like substrate selectivity[37]. Furthermore, a Rosetta homology model of ADAR1-D shows 5' binding loop contacts that are closer to the editing site (~ 5 bp, Fig. 4b)[35], suggesting that ADAR1-D requires less duplex 5' to the edit site compared to ADAR2-D. These observations are remarkably consistent with the minimum substrate length reported for ADAR1 (14 bp with 5 bp 5' and 8 bp 3' to edit site)[12,37].

2.5. ADAR dimerization.

The crystal structures of ADAR2-D in complex with 8-azaN-bearing substrates provided highly valuable insights into the deaminase domain's function on RNA binding and editing. However, questions on the role of the dsRBDs on ADAR activity still remained. For instance, the deaminase domain of ADAR2 alone has been shown to be sufficient in recognizing and deaminating adenosines in certain substrates (*e.g.* GLI1 mRNA)[1,38], but requires the presence of dsRBDs in editing others (*e.g.* 5-HT_{2C} pre-mRNA, D-site)[34]. Furthermore, multiple studies have provided evidence of ADAR dimerization, its possible importance in ADAR activity, and the likely role of dsRBDs in dimer formation[39–41]. All these accounts further emphasized the need for uncovering the detailed function of dsRBDs on RNA recognition and editing.

Our lab then sought to crystallize a fragment of ADAR2 bearing the deaminase domain and dsRBD2 (ADAR2-R2D) bound to an 8-azaN-modified RNA duplex (Fig. 5a)[2]. From the resulting X-ray crystal structure, we found that the RNA-bound protein forms an asymmetric homodimer (Fig. 5a–b), with the deaminase domain of one monomer (monomer A) interacting with the RNA in the same fashion observed in ADAR2-D-RNA crystal structures. On the other hand, the deaminase domain of the other monomer (monomer B) is not directly involved in RNA binding. Instead, its dsRBD2 contacts the RNA at a site found in the 3' direction relative to the flipped-out base (Fig. 5a), consistent with results from protein-RNA footprinting experiment with ADAR2-R2D[2]. While dsRBD2 B is shown to interact with the RNA, there is no clear electron density to model the location of dsRBD2 A.

As stated earlier, deaminase B does not directly contact the RNA. However, it interacts with the RNA-bound deaminase A via an extensive network of protein-protein interactions centered around an α-helical dimerization interface (dimerization helix; Fig. 5a and 5c). The dimerization helix (residues 501–509) is presented by deaminase A to a binding site in deaminase B, which consists of similar residues involved in RNA duplex binding around the catalytic pocket of deaminase A. Interestingly, three amino acid residues in the dimerization helix (T501, W502, and D503) are highly conserved in ADARs across different species. Mutating these residues to alanine led to reduced ADAR2-R2D dimer formation and inhibition of deamination of most substrates studied, including 5-HT_{2C} at D-site[2].

The D503A mutant, in particular, exhibited the most detrimental effect on dimerization and editing, consistent with observed key protein-protein interactions facilitated by D503 between monomers A and B (Fig. 5c). Importantly, overexpression of ADAR1 p110 with the corresponding aspartic acid residue mutation (D1023A) also displayed decreased editing of most endogenous substrates studied in HEK293T cells[2].

Altogether, the reported crystal structure of the ADAR2-R2D homodimer revealed both protein-RNA and protein-protein interactions but did not exactly disclose the involvement of dsRBD2 binding in dimer formation. However, gel shift experiments using duplex substrates with or lacking sufficient base pairs 3' to edit site for dsRBD2 contact clearly showed dependence of ADAR2-R2D dimer formation on dsRBD2-RNA interaction in this region[2]. These results, coupled with the structural and biochemical data presented above, validate the essential role played by dsRBDs in dimerization, substrate recognition, and editing. Specifically, dimerization enables the simultaneous binding of a deaminase domain and a dsRBD on the same RNA molecule. For substrates with target adenosines situated in an ideal position in the duplex (mostly base-paired) and in the preferred orphan base and nearest neighbor context (*e.g.* GLI1), dimerization or additional binding of dsRBDs might not be necessary for efficient editing. However, the opposite is true for substrates characterized by mismatches, bulges, and loops, such as the 5-HT_{2C} RNA. For these substrates, the deaminase domain is inadequate for effective binding and deamination, hence dimerization is essential for supplementary RNA contact via the dsRBDs.

3. Oligonucleotide modifications for RNA editing modulation

A key component of the directed RNA editing toolkit is the enzyme that will carry out the deamination at the therapeutically relevant site. The use of endogenous human ADARs offers significant advantages over ectopically expressed engineered proteins, including comparatively lower levels of off-target editing and reduced likelihood of immune stimulation[20,42]. However, directed editing with native ADARs often suffers from inefficient editing of desired target sites, stimulating efforts to optimize guide oligonucleotides being used in this approach[3,4,22,43].

The crystal structures of ADAR2 bound to 8-azaN-modified RNA duplexes shed light on multiple facets of the ADAR editing mechanism, substrate recognition, and selectivity. In the next sections, we discuss how these findings, coupled with relevant biochemical and biophysical experimental data, inform the rational design of efficient and site-specific chemically modified guide oligonucleotides for directed editing applications, including at disfavored 5'-G<u>A</u> sites. We focus our discussion on two sites for optimization in the guide strand: the orphan and the −1 positions. Finally, we also describe our efforts in the development of selective inhibitors of the A-to-I editing reaction based on the apparent differences in the 5' binding loops of the two active human ADARs[1,12,37].

3.1. Nucleoside analogs at the orphan position.

In section 2.1, we described how the E488 residue in the intercalating loop participates in the stabilization of the flipped-out base conformation by hydrogen bonding with the nucleotide at the orphan position (Fig. 3b)[1]. An important requirement in this interaction

is the protonation of the E488's carboxylate group to facilitate H-bond donation to N3 of the orphan C (Fig. 6a). However, this protonation is pH-dependent and a substantial fraction of E488 species may exist in the deprotonated state at physiologically relevant pH. Interestingly, a previously reported mutation of E488 to glutamine (E488Q) resulted in enhanced base-flipping and deamination activities[44], which is believed to arise from the pH-independent H-bond donation from Q488's carboxyamide group to orphan C's N3 (Fig. 6b)[1]. Indeed, crystal structures of RNA-bound ADAR2-D E488Q reveal that Q488 makes the same hydrogen bonding contacts with the orphan C as does E488[1]. The base-flipping ability and catalytic activity of wild-type ADAR2 have also been shown to respond to changes in pH but this pH dependence is much less pronounced for the hyperactive E488Q[45].

These findings led us to test orphan base analogs that could facilitate a pH-independent hydrogen bond formation with the E488 residue, allowing the wild-type protein to elicit the same hyperactive effect as the E488Q mutant[3]. To do this, we tested analogs that present an H-bond donor instead of H-bond acceptor at the N3 position or that display a *donor-donor-acceptor* instead of *donor-acceptor-acceptor* face found in C (Fig. 6c). Of the three analogs studied, 6-amino-5-nitro-3-(1'-β-D-2'-deoxyribofuranosyl)-2(1H)-pyridone (referred to as 2'-deoxy Benner's base Z or dZ, Fig. 6c–d) displayed the most enhancement in wild-type ADAR2's base-flipping and deamination activities when placed at the orphan position of guide RNAs (gRNAs). These results are in comparison with gRNAs containing a dC at the orphan site (2'-deoxyribonucleotide counterparts were used due to phosphoramidite availability). The rate enhancement was not as large for 2'-deoxypseudoisocytidine (dpiC) because the hydrogen at N3 could possibly migrate through tautomerization. Conversely, the significant rate decrease observed for 8-oxo-2'-deoxyadenosine (8-oxodA) may be due to 8-oxodA adopting an *anti* instead of the required *syn* conformation to orient its *donor-donor-acceptor* face to E488. Notably, orphan dZ-modified gRNAs exhibited up to 3-fold increase in editing rates or yields across multiple disease-relevant transcripts *in vitro*, in human cells, and in mouse primary liver fibroblasts. Furthermore, using a gRNA bearing an orphan dZ in combination with wild-type ADAR1 p110 also resulted in increased editing rate *in vitro*[3], which agrees with the similar hyperactive effect of the corresponding E1008Q mutation in ADAR1[46]. Interestingly, a recent paper by Monian *et al.* suggests that an 8-oxodA modification at the guide orphan position can support directed A-to-I editing by endogenous ADAR1[22]. Collectively, these results demonstrate the effective use of endogenous ADARs with a chemically modified guide oligonucleotide that mimics the enhanced base-flipping contact in the hyperactive mutant. This strategy also allows improvement in editing activity of the wild-type protein without the higher levels of off-target editing associated with the hyperactive protein[42,47,48].

The putative base-flipping contact between wild-type ADAR2 and orphan dZ-containing RNA duplex (Fig. 6d) was also investigated by X-ray crystallography[3]. As hypothesized, E488 is shown to interact with dZ in the crystal structure via the expected hydrogen bonding contact between E488 side chain and N3 of dZ (Fig. 6e). Interestingly, another hydrogen bonding interaction is seen between the E488's carboxylate group and the amino group at the 4-position of dZ (Fig. 6e), which likely contributes to improved stabilization of the base-flipped conformation in comparison with dC. Also, density functional theory (DFT)

calculations suggest that the *donor-donor-acceptor* face of dZ has a more positive partial charge than the *donor-acceptor-acceptor* face of dC complementing the negatively changed carboxylate of E488[3].

3.2. Nucleoside analogs at the −1 position.

The unusual conformation assumed by the −1 nucleotide in our ADAR2-RNA crystal structures (Fig. 3c) inspired us to test adenosine analogs at this position in the guide strand for directed editing at 5'-UA sites[43]. The −1 A analogs studied either contain modifications in the sugar, in the nucleobase, or a combination of both. In general, we found that modifications that enable the irregular conformation at the −1 position (*e.g.* C2'-*endo* sugar pucker and base unstacking)[1] improved both ADAR2 and ADAR1 p110 deamination rates or yields for most substrates tested *in vitro* and in human cells. Specifically, we observed that −1 dA gRNAs gave better editing rates compared to −1 A gRNAs, given that 2'-deoxynucleotides preferentially adopts the C2'-*endo* sugar pucker[49]. However, an LNA modification (Fig. 7a) in the −1 sugar displayed very little to no editing, presumably because it can not adopt the C2'-*endo* pucker[50]. 2'-Deoxynebularine (dN, Fig. 7a), on the other hand, consistently increased editing rates when placed at the −1 site in gRNAs. We hypothesize that the lack of amino group at the 6-position of dN could potentially reduce steric clash upon unstacking of the −1 base. Interestingly, we also observed that a gRNA containing both dN −1 to a target site and LNA −1 to a bystander site can eliminate bystander editing while maintaining enhanced target editing. Finally, the beneficial effect of dN modification at the −1 position of the guide was shown to be enhanced when combined with a dZ modification at the orphan position. However, this additive effect appears to be ADAR-dependent as it was only observed in deamination experiments with ADAR2.

So far, the −1 nucleoside modifications described were aimed towards editing adenosines in the preferred nearest neighbor context[1,33,34]. The inherent sequence bias of ADARs, especially against 5'-GA sites (see section 2.3), clearly limits the scope of utilizing these enzymes for therapeutic directed RNA editing applications. Previously, fusion proteins bearing ADAR deaminase domains revealed that editing at 5'-GA sites can be improved by pairing the 5'-G with a −1 G or A[51]. Indeed, we observed that both 5'-G:G and G:A pairs led to faster *in vitro* deamination rates of both full-length ADAR2 and ADAR1 p110 in comparison to G:U and G:C[4]. We imagined that a purine:purine mismatch near the edit site could destabilize the duplex and facilitate the base-flipping required for the editing reaction. However, since purine:purine combinations have also been shown to exist as stable H-bonded pairs in RNA[52], we also considered that the 5'-G:G and G:A pairs could activate ADAR by assuming a hydrogen bonded structure that avoids the detrimental steric clash in the minor groove.

We turned to X-ray crystallography to test our hypotheses by introducing a G:G pair adjacent to an editing site in an ADAR2-RNA complex that previously crystallized[2]. Interestingly, in the new structure, we found that the 5'-G:G pair adopts a G_{syn}:G_{anti} conformation that is H-bonded between the Hoogsteen face of 5'-G_{syn} and the Watson-Crick face of −1 G_{anti} (Fig. 7b)[4]. The *syn* conformation assumed by 5'-G places its 2-amino group in the major groove, hence eliminating the minor groove clash and enabling the deamination

reaction (Fig. 7b). In addition, a slight shift of the base-flipping loop accommodates the 2-amino group of −1 G_{anti} in the minor groove, compared to previous ADAR2-RNA crystal structures with a 5'-U:A nearest neighbor pair[1–3].

The increase in editing rates observed for 5'-G:A pair compared to G:U and G:C pairs can also be rationalized by the formation of a G_{syn}:AH^{+}_{anti} interaction (Fig. 7c). To facilitate this conformation, N1 of −1 A_{anti} should be protonated to effectively donate a hydrogen bond to N7 of 5'-G_{syn}. The importance of this N1 protonation is demonstrated by the significantly improved editing rates observed with 3-deaza-2'-deoxyadenosine (3-deaza-dA) when placed at the −1 position of a gRNA targeting a 5'-G<u>A</u> site[4]. The substantially higher pK_a for N1 protonation in 3-deazaadenosine[53] suggests that this site is more likely protonated at physiologically relevant pH compared to A (Fig. 7c). It is important to note that a −1 dA gRNA also led to ~2.5-fold faster ADAR2 editing rates compared to a −1 A gRNA as expected based on the preferred sugar pucker conformation at this site. However, a −1 3-deaza-dA remarkably showed ~8.5-fold higher rate of ADAR2 deamination compared to a −1 A gRNA, highlighting the beneficial effect of the increased N1H pK_a in the base modification. Importantly, 3-deaza-dA gave the greatest enhancement in both ADAR2 and ADAR1 p110 deamination rates among other purine analogs tested, inspiring us to solve the crystal structure of ADAR2-R2D bound to an RNA duplex containing a 5'-G:3-deaza-dA pair[4]. Indeed, the crystal structure confirmed the predicted G_{syn}:3-deaza-dA_{anti} pairing interaction, which is further stabilized by a hydrogen bond between the 2-amino group of 5'-G_{syn} and its 5'-phosphodiester oxygen (Fig. 7c).

3.3. Selective inhibition of ADAR1.

The growing evidence linking ADAR1 to cancer progression has triggered the search for potent and targeted inhibitors of this enzyme[12,18,19]. Unfortunately, the lack of high resolution structures for ADAR1 has hampered these research endeavors. Our more detailed understanding of ADAR2 can be attributed to the successful utilization of chemically modified substrates, with the particular use of 8-azaN[1,2,11]. We then sought to determine if we could similarly employ 8-azaN-modified RNA duplexes for probing ADAR1-RNA interactions. Indeed, from gel-shift and *in vitro* deamination experiments, we found that short RNA duplexes (≤ 16 bp) with 8-azaN replacing adenosine at the editing site can form high affinity complexes with ADAR1[12]. Structure-activity relationship studies with these modified substrates defined a 14 bp (with 5 bp 5' and 8 bp 3' to edit site) minimum length requirement for ADAR1 deaminase recognition. Importantly, we showed that these 8-azaN-containing duplexes can be used as substrate decoys for competitive inhibition of both ADAR1 p110 and p150 but not of ADAR2 (Fig. 8)[12]. ADAR1-selective inhibition is achieved because of the difference in the 5' binding loops of the two ADARs (see section 2.4), with ADAR2 requiring a longer duplex (~ 10 bp 5' to edit site) for effective substrate recognition[1,37].

4. Concluding remarks

The use of chemically modified oligonucleotides has opened up several avenues to augment our understanding of ADARs. We expect that this platform will continue to guide us in

solving some of our prevailing questions on ADAR structure, substrate selectivity, and recognition. For instance, what is the role of ADAR2's dsRBD1? Solving the structure of the full-length protein bound to RNA should provide insight into the proposed function of dsRBD1[41] and its interplay with the deaminase domain and dsRBD2. A high resolution structure of ADAR1 will also help accelerate the development of targeted inhibitors and the rational design of gRNAs specific to this more ubiquitous member of the human ADAR family[22,54]. Apart from the orphan and −1 positions, other protein-RNA contacts revealed from our crystal structures can serve as possible sites for modification for RNA editing modulation. Non-protein binding sites are also promising locations for incorporation of chemical moieties that will improve metabolic stability and delivery without compromising potency. The combined effect of these modifications must be established across multiple substrates and sequence contexts. Furthermore, inhibition of dimerization presents an alternative mechanism for ADAR editing inhibition, and identification of molecules that can block ADAR dimerization could lead to a new class of potential therapeutics for cancer and other ADAR-associated disorders.

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Biographies

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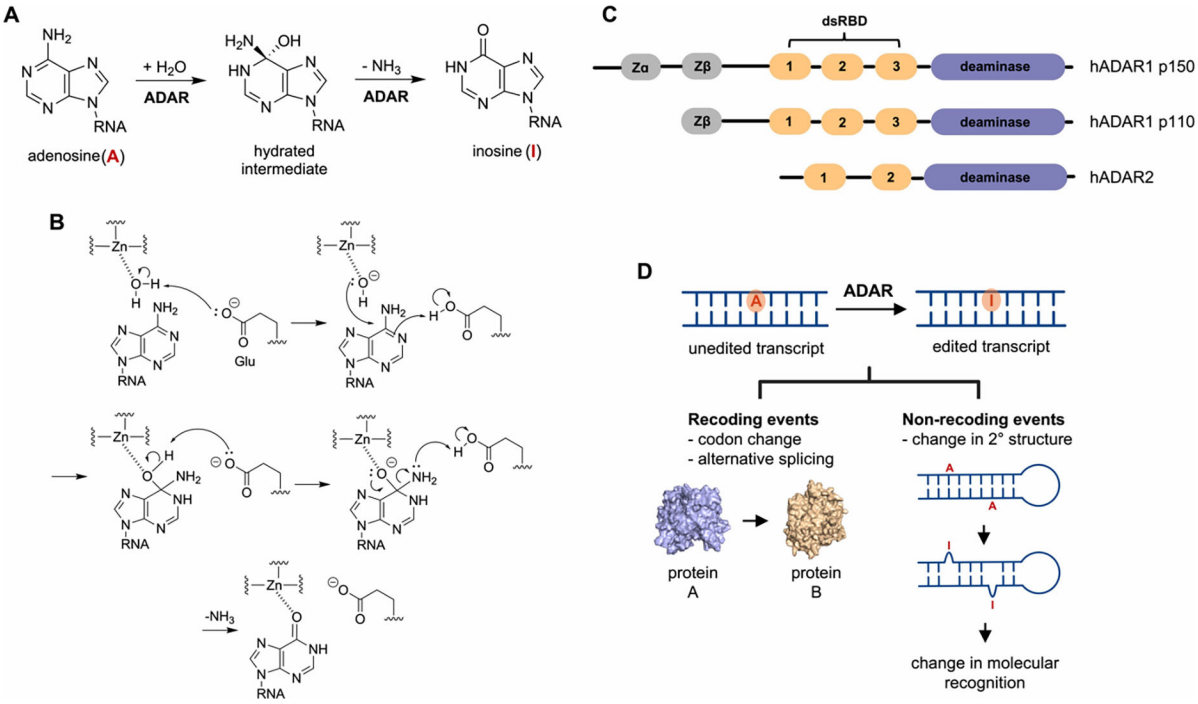

Figure 1.

(**A**) ADAR catalyzes the hydrolytic deamination of adenosine to inosine in dsRNA via a tetrahedral reaction intermediate. (**B**) Proposed mechanism of catalysis by ADARs. (**C**) Protein domain maps of human ADAR1 and ADAR2. (**D**) General consequences of A-to-I editing by ADARs.

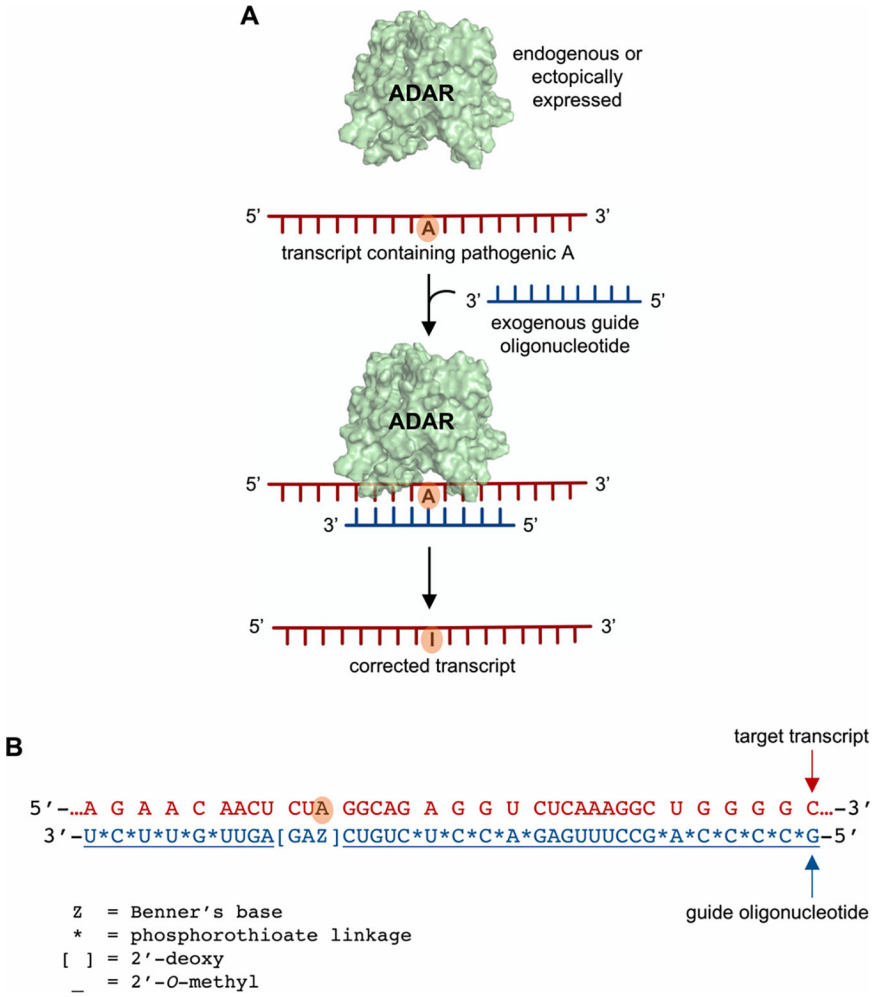

Figure 2.

(**A**) ADARs for site-directed RNA editing applications. (**B**) An example of a chemically modified guide design used for therapeutic RNA editing with endogenous ADARs.

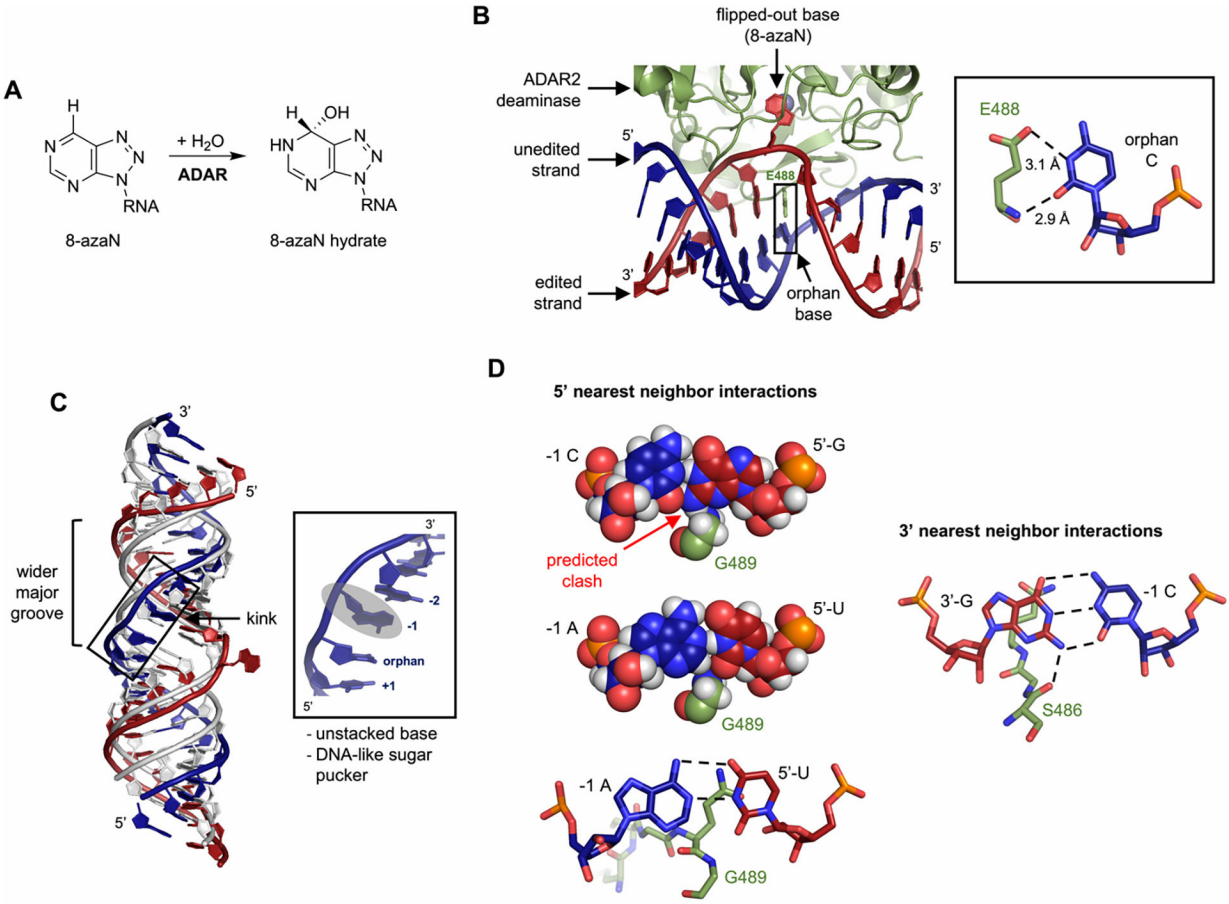

Figure 3.

The ADAR2-RNA interface as illuminated by crystal structures of ADAR2-D bound to 8-azaN-containing RNA duplexes. (**A**) ADAR reacts with 8-azaN in RNA to form a hydrate that mimics the hydrated intermediate of ADAR's adenosine deamination (see Fig. 1a). (**B**) Crystal structure of human ADAR2-D (green) in complex with an 8-azaN-containing RNA duplex showing the flipped-out base in the edited strand (red) and intercalating loop bearing E488 (PDB 5hp3). E488 hydrogen bonds with the orphan base in the unedited strand (blue). (**C**) Crystal structure of the RNA duplex from an ADAR2-D-RNA co-crystal structure (PDB 5hp2) overlayed with an ideal RNA duplex (silver). The unstacked base and DNA-like sugar pucker at the −1 position are highlighted in grey in the inset. (**D**) Predicted steric clash between the G489 backbone and 2-amino group of a 5'-G (top left). A 5'-U avoids this steric clash as observed in crystal structures (middle and bottom left, PDB 5ed2). The backbone carbonyl of residue S486 hydrogen bonds with the 2-amino group of a 3'-G (right, PDB 5ed2).

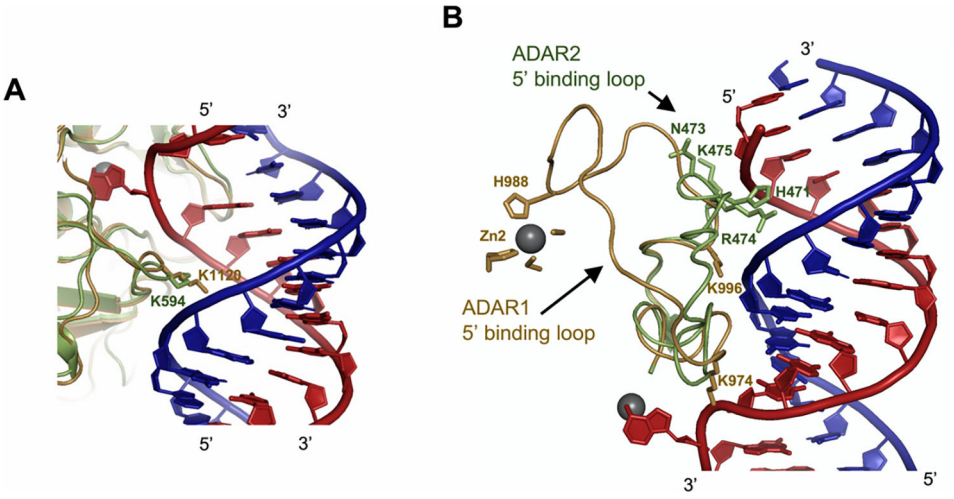

Figure 4.

Overlay of the deaminase domains of ADAR2 (green, PDB 5ed2) and ADAR1 (gold, from a Rosetta homology model). (**A**) RNA interaction facilitated by a conserved lysine residue 3' to the edit site. (**B**) The 5' binding loops of ADAR2 and ADAR1. The second Zn metal (Zn2, grey sphere) binding site for ADAR1 is also shown.

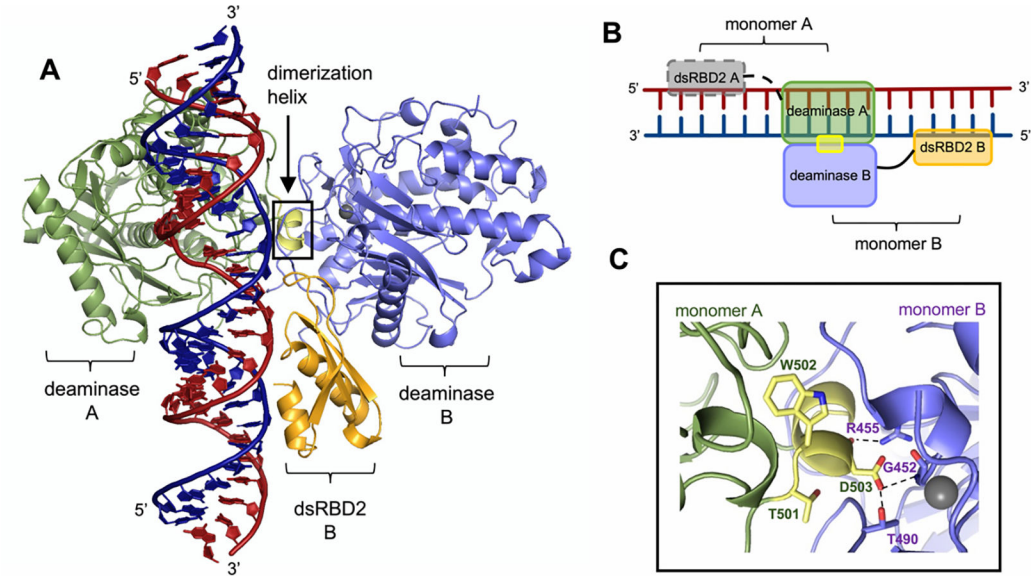

Figure 5.

(**A**) Crystal structure of ADAR2-R2D bound to an 8-azaN-containing RNA duplex (PDB 6vff) and (**B**) cartoon representation of the asymmetric homodimer (RNA binding contacts not drawn to scale). Broken lines represent regions that were not resolved in the crystal structure. (**C**) Inset shows the α-helical dimerization interface (yellow) in the deaminase domain of monomer A and relevant hydrogen bonding contacts between the deaminase domains of monomers A and B facilitated by D503 in the dimerization helix.

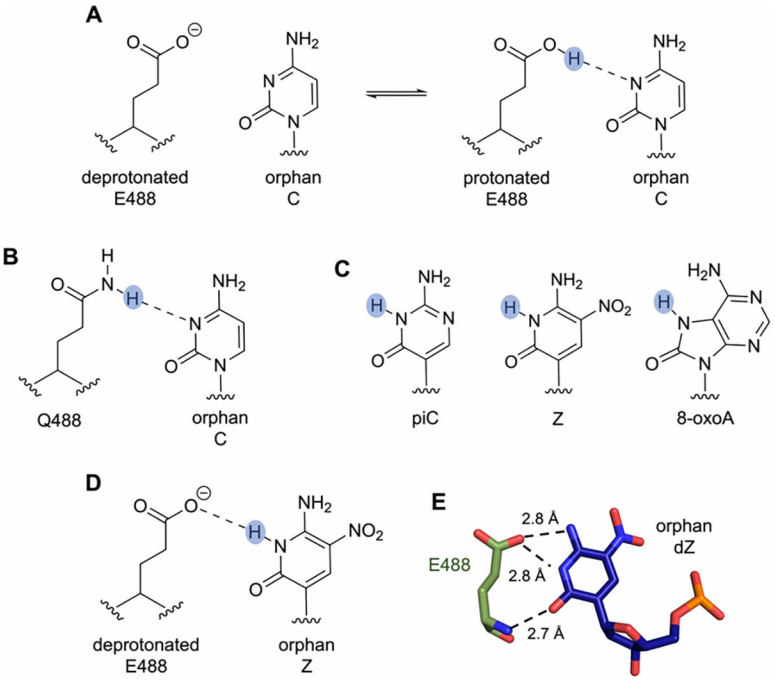

Figure 6.

Chemical modification at the orphan position. (**A**) Wild-type ADAR2 relies on the protonation of residue E488 for hydrogen bonding with N3 of the orphan C. (**B**) Protonation-independent hydrogen bonding between ADAR2 E488Q and orphan C. (**C**) Chemical structures of orphan base analogs tested. (**D**) A Benner's base Z at the orphan position provides a protonation-independent hydrogen bond with wild-type ADAR2. (**E**) Crystal structure showing the hydrogen bonding interaction between E488 and orphan dZ (PDB 7kfn).

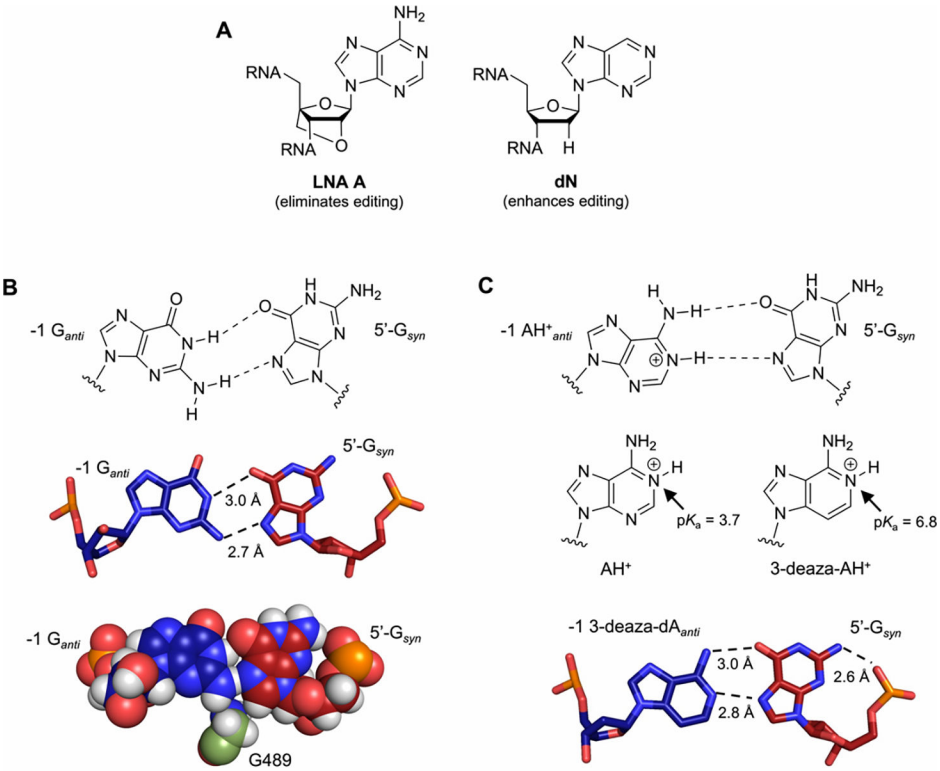

Figure 7.

Chemical modifications at the −1 position. (**A**) Nucleoside analogs at the −1 position that modulate editing at 5’-U<u>A</u> sites. (**B**) Chemical structures (top) and crystal structure (middle, PDB 8e0f) showing hydrogen bond formation between a 5’-G*syn*:G*anti* pair. The 5’-G adopts a *syn* conformation to avoid steric clash with residue G489 (bottom). (**C**) Predicted base-pairing interaction in a 5’-G*syn*:AH$^+$*anti* pair (top). The pK_a of 3-deaza-AH$^+$ N1H is higher than that of AH$^+$ (middle). Crystal structure showing hydrogen bond formation between a 5’-G*syn*:3-deaza-dA*anti* pair (bottom, PDB 8e4x).

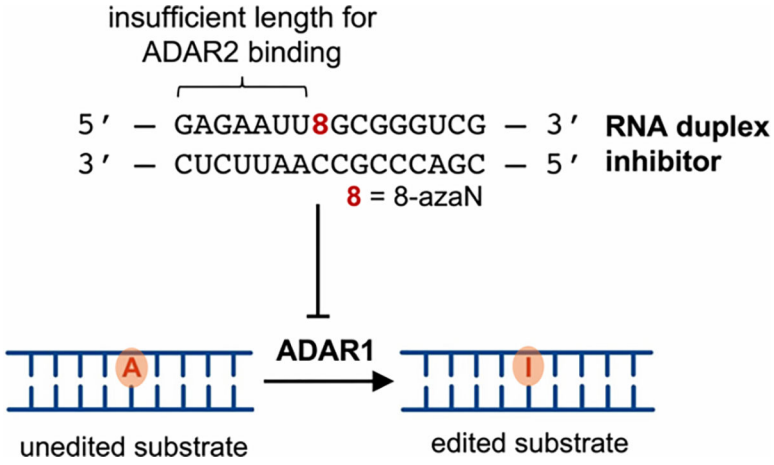

Figure 8.
ADAR1-selective inhibition using a 16 bp 8-azaN-modified RNA duplex.