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Substrate Analogues for an RNA-Editing Adenosine Deaminase: Mechanistic Investigation and Inhibitor Design

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Abstract: ADARs are *a*denosine *d*e*a*minases that act on *R*NA and are responsible for RNA-editing reactions that occur in eukaryotic mRNAs, including the mRNAs of glutamate and serotonin receptors. ADARs capable of editing biologically relevant RNA substrates have been identified. In addition, the consequence of the RNA-editing reaction on the function of the gene product is known in several cases. However, our understanding of the chemical mechanism of the ADAR-catalyzed adenosine deamination in RNA is lagging. By studying analogues of a naturally occurring substrate for ADAR2, we infer features of the enzyme's active site and reaction mechanism. 8-Aza substitution at adenosine in various RNA substrates accelerates the rate of deamination at these sites by ADAR2 (2.8−17-fold). The magnitude of this "aza effect" depends on the RNA structural context of the reacting nucleotide. N^6-Methyladenosine in RNA is a slow substrate for ADAR2 (rate is 2% that of adenosine), with no product observed with N^6-ethyladenosine, suggesting a limited size of the leaving group pocket. 2,6-Diaminopurine ribonucleoside in RNA is not a substrate for ADAR, in contrast to adenosine deaminase (ADA), which catalyzes a similar reaction on nucleosides. This and other results indicate that ADAR2 uses a base recognition strategy different from that of ADA. Consistent with the large 8-aza effect observed for the ADAR2 reaction, we find that 8-azanebularine, as the free nucleoside, inhibits the ADAR2 reaction ($IC_{50} = 15 \pm 3$ mM) with no inhibition observed with nebularine or coformycin.

Introduction

RNA editing is a term used to describe structural alteration, insertion, or deletion of nucleotides that changes the coding properties of an RNA.[1] A number of messenger RNA (mRNA) sequences are known to arise from RNA editing in a variety of organisms from *C. elegans* to humans.[2−5] An important consequence of RNA editing is that the sequences of certain proteins present at a given time in an organism are different from those predicted by the gene sequences. In several examples, properties of the protein products arising from translation of the edited and unedited RNAs have been studied.[3,6−9] These studies have shown that the proper function of the gene product requires efficient editing of its mRNA. The majority of research in RNA editing carried out to this point has focused on the identification of the enzymes and substrates involved and the consequence of the editing reaction on the function of the gene products. However, an understanding of the chemical mechanism for these processes is lagging. Thus, basic questions about RNA editing, such as why a certain nucleotide in an mRNA molecule is susceptible to editing while others are not, can often not be effectively answered at this time.

Editing reactions can be classified based on the type of reaction occurring on the RNA. Editing can occur via insertion or deletion of nucleotides and thus involves phosphodiesterases, RNA polymerases, and RNA ligases.[10,11] These are typically found in multicomponent ribonucleoprotein complexes responsible for the insertion/deletion editing. A second type of RNA-editing reaction is the deamination of the nucleotides adenosine and cytidine within an RNA strand.[12,13] Because hydrolytic deamination at C6 of adenosine and C4 of cytidine alters the hydrogen bonding properties at the Watson−Crick face of each base, the information encoded in the RNA sequence is changed by these reactions. RNA-editing activity via adenosine deamination in vitro requires only the RNA substrate and a single protein enzyme, making it a simple model system to explore mechanistic questions.[14−16]

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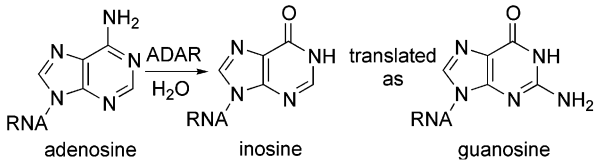

Figure 1. Because inosine in RNA is translated as guanosine, deamination of adenosine by the RNA-editing adenosine deaminases (ADARs) can change the coding properties of mRNA.

The deamination of adenosine (A) in RNA results in inosine (I) at that position (Figure 1). In one specific example, processing of the mRNA for the glutamate-gated ion channel B-subunit (GluR−B) involves editing reactions where genomically encoded adenosines are altered in the mRNA by deamination.[2,3] In this large RNA (> 3000 nt), there are only a few editing sites and only two that lead to codon changes. At one of these editing sites, an arginine codon is converted to a sequence that encodes glycine (the R/G site).[3] Receptors containing B-subunits with glycine at this position have been shown to recover from ligand desensitization at different rates than those with arginine at this position.[3] Specific deamination sites in other mRNA sequences have also been identified.[9]

ADARs are *a*denosine *dea*minases that act on duplex *R*NA substrates and are responsible for mRNA editing by adenosine deamination.[17] ADAR2 is an approximately 80 kDa protein that efficiently deaminates the R/G site adenosine in GluR−B pre-mRNA sequences in vitro.[16,18] This enzyme has two copies of a protein sequence (dsRBM) found in double-stranded RNA binding proteins such as PKR, the RNA-dependent protein kinase, and dicer, a ribonuclease involved in RNA interference.[19,20] Consistent with the presence of dsRBMs in ADAR2, duplex RNA secondary structure in the substrate is a requirement for the ADAR2-catalyzed reaction. Unfortunately, no high resolution structural data are currently available for ADARs or ADAR−RNA complexes.

ADARs are related to the nucleoside deaminases both by the reaction they catalyze (adenosine and AMP deaminases) and by their amino acid sequences (cytidine deaminases). It is believed that once ADAR recognizes a duplex RNA substrate, it flips the reactive nucleotide into an active site similar to that found in cytidine deaminases. Indeed, our laboratory has presented data that suggest both the RNA and protein undergo conformational changes during the editing reaction.[21,22] Then, in analogy to the mechanism of adenosine and cytidine deaminases, both of which are zinc enzymes, a metal-bound hydroxide ion attacks the purine ring to form a tetrahedral intermediate (Figure 2).[23] This intermediate collapses, ejecting ammonia and the inosine-containing product. Consistent with this mechanism, Bass and colleagues demonstrated that water is the source of oxygen in the inosine product.[24]

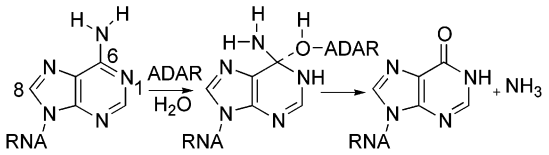

Figure 2. Reaction catalyzed by the RNA-editing adenosine deaminases proceeds through a proposed tetrahedral intermediate.

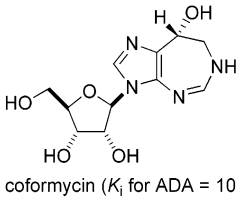

Figure 3. Structure of coformycin, an inhibitor of adenosine deaminase (ADA).

Nucleoside analogues that mimic a tetrahedral intermediate are inhibitors of nucleoside deaminases.[25,26] For instance, the natural product coformycin, which features a hydroxyl group bonded to a stable tetrahedral center at a site analogous to C6 of adenosine, is a picomolar inhibitor of adenosine deaminase (ADA) (Figure 3).[27] Interestingly, coformycin is not an inhibitor of the reaction of ADAR1.[24] Indeed, no mechanism-based inhibitors of the ADAR reaction have been reported to date.

In our ongoing work to define the mechanism of RNA-editing adenosine deaminases and the basis for editing selectivity, we have chosen to prepare and analyze variants of the substrate adenosine within the sequence and secondary structure context of a natural editing site.[21,22,28,29] These studies have allowed us to infer features of the ADAR2 active site and reaction mechanism. This information has also been useful in the pursuit of an active-site directed inhibitor of the ADAR reaction.

Results

8-Azaadenosine. It is apparent from analysis of the nucleoside chemistry literature that nitrogen for carbon substitution at the 8-position facilitates covalent hydration of the purine ring system.[30,31] In addition, 8-azaadenosine is converted to product by ADA faster than is adenosine.[32] Because formation of the covalent hydrate and hydrolytic deamination involve disruption of the π system, the π-electron deficiency conferred by the doubly bonded 8-nitrogen facilitates each. To determine if aza substitution at the 8-position of adenosine affected the ADAR2-catalyzed deamination of an editing substrate, we prepared a GluR−B model RNA with 8-azaadenosine at the R/G site (Figure 4; **X = 8-AA**).

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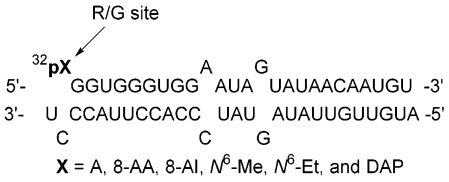
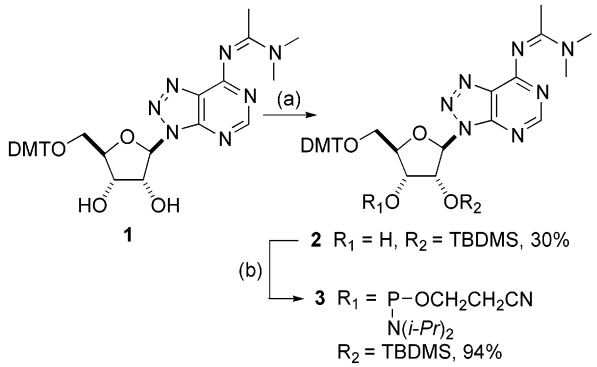


Figure 4. Synthetic oligoribonucleotide duplex substrate used for deamination assay with ADAR2, where **X** indicates nucleotide analogues with base modifications at the GluR−B R/G editing site as indicated.

Scheme 1. Synthesis of 8-azaadenosine Phosphoramidite



(a) TBDMSCl, TEA, $AgNO_3$, THF; (b) $ClP(OCH_2CH_2CN)(N(i\text{-}Pr)_2)$, DIEA, THF.

Automated RNA synthesis employing the phosphoramidite method was used to synthesize the substrate RNA. An 8-azaadenosine phosphoramidite was generated in two steps from the known *N*-(dimethylacetamidine)-5′-*O*-(4,4′-dimethoxytrityl)-8-azaadenosine (Scheme 1).[33] The 5′-*O*-DMT derivative **1** was converted to the 2′-*tert*-butyldimethylsilyl ether **2** followed by generation of the 3′-di-*iso*-propylaminocyanoethyl phosphoramidite **3**. For the initial deamination assays, 8-azaadenosine was positioned at the 5′ end of one strand such that it could be site specifically ^{32}P labeled (Figure 4).[28] Once the labeled single-stranded oligonucleotide was obtained, the necessary duplex was formed by hybridization to the complementary strand. A phosphoramidite of 8-azainosine was also prepared to allow for independent synthesis of the predicted ADAR2 product RNA (Scheme 2). This analogue was likewise positioned at the 5′ end of one strand such that it could be site specifically ^{32}P labeled (Figure 4; **X** = **8-AI**). 8-Azaadenosine nucleoside **4** was deaminated enzymatically using calf intestinal adenosine deaminase to generate 8-azainosine **5**. This nucleoside was converted as described above to the 5′-*O*-DMT, 2′-*O*-TBDMS derivative **6**, and finally to the 3′-di-*iso*-propylaminocyanoethyl phosphoramidite **7**.

To determine the effect **8-AA** substitution has on the reaction of ADAR2 with the GluR-B R/G site RNA, we carried out an analysis of the enzyme kinetics. Kinetic analyses with ADAR2 under conditions where [substrate] ≫ [enzyme] are complicated by slow reaction times for the RNAs shown in Figure 4, likely arising from substrate inhibition.[22] Furthermore, the time required to reach the steady state causes enzyme denaturation to be a concern. However, reactions carried out under single-turnover conditions at various concentrations of ADAR2 allow for an estimation of maximum rate constants (k_{max}) and binding affinities (K_d). Thus, rate constants were measured for the reaction of 1 nM GluR-B R/G site RNA bearing either **A** or **8-AA** at varying ADAR2 concentrations all in >10-fold excess of substrate and the data were fit to a preequilibrium model to extract the k_{max} and K_d values (Figure 5) (see Experimental). When **8-AA** was located at the editing site a $k_{max} = 0.43\ min^{-1}$ was observed, whereas $k_{max} = 0.07\ min^{-1}$ for the reaction with **A** at the editing site (Figure 5B, Table 1). Thus, 8-aza substitution at the editing site in this RNA increased k_{max} by over 6-fold. Importantly, the K_d values estimated from this analysis are similar (K_d = 18 nM for **8-AA**, K_d = 25 nM for **A**), indicating that the aza substitution has minimal effect on substrate binding affinity (Figure 5B, Table 1). The product of the reaction with **8-AA** containing RNA, after nuclease digestion to 5′-monophosphates, was shown to comigrate with 8-aza-

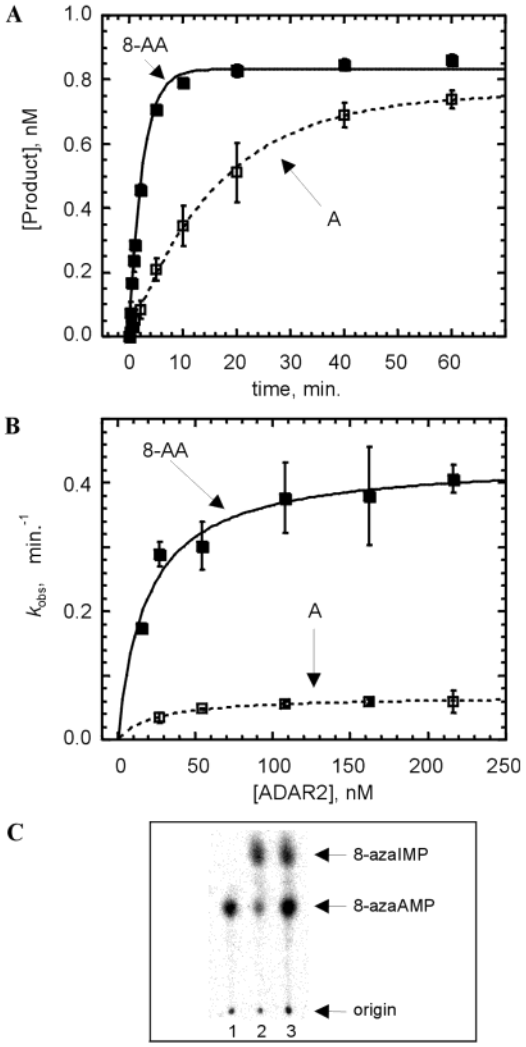


Figure 5. (**A**) Plot of product formation as a function of time when 1 nM substrate, with either **8-AA** (■) or **A** (□) occupying the R/G editing site, is allowed to react with 216 nM ADAR2. Data points reported are the average ± standard deviation for three independent experiments. (**B**) Plot of k_{obs} as a function of ADAR2 concentration. (■): **8-AA** at the R/G editing site. (□): **A** at the R/G editing site. Data points reported are the average ± standard deviation for three independent experiments at the relevant concentration. (**C**) Storage phosphor autoradiogram of TLC plate indicating comigration of independently synthesized 8-azainosine-containing oligomer after nuclease digestion into monophosphates. Lane 1, 0 time point from above; Lane 2, 60 min time point from above; Lane 3, co-spot of nuclease P1 digestion of a ^{32}P labeled 8-azainosine oligomer and the products of nuclease digestion of the 0 and 60 min reaction times from above.

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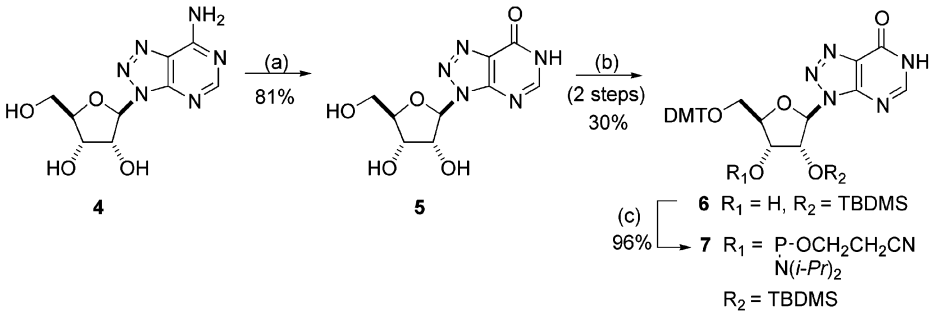
Scheme 2. Synthesis of 8-azainosine Phosphoramidites



(a) ADA, buffer pH 7.4; (b) (i) DMTCl, pyridine, $AgNO_3$, THF; (ii) TBDMSCl, TEA, $AgNO_3$, THF; (c) $ClP(OCH_2CH_2CN)(N(i\text{-}Pr)_2)$, DIEA, THF.

Table 1. Effect of 8-aza Substitution on ADAR2 Reaction Kinetics

edited nucleotide	$k_{max}(min^{-1})^a$	$K_D(nM)^a$
adenosine (**A**)	0.07	25
8-azaadenosine (**8-AA**)	0.43	18

[a] Determined from fit of $k_{obs} = (k_{max}[ADAR]/(K_D + [ADAR])$ under single turnover conditions (see the Experimental Section for details).

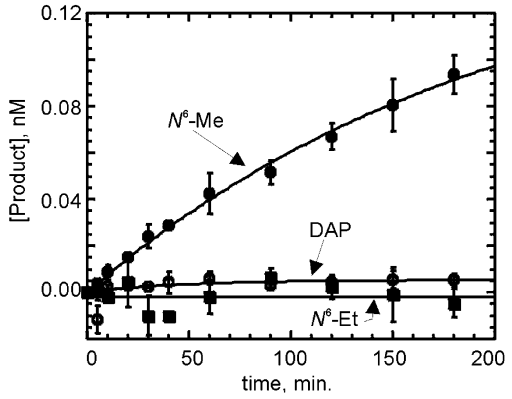


Figure 6. Plot of product formation as a function of time when 1 nM N^6-alkyl substituted or 2,6-diaminopurine substrate is allowed to react with 54 nM ADAR2. Legend: (●): **N^6-Me** at R/G site. (■): **N^6-Et** at the R/G editing site (○): **DAP** at the R/G editing site. Data points reported are the average ± standard deviation for three independent experiments.

inosine-5′-monophosphate, consistent with the expected deamination reaction at C6 (see Experimental) (Figure 5C).

N^6-Alkyladenosines and 2,6-Diaminopurine. We have previously shown that ADAR2 will process a base in RNA with a C6 substituent other than the primary amine of adenosine.[29] This prompted us to determine the extent to which the C6 substituent could deviate from that found in adenosine. Also, it was not previously known whether ADAR2 would accept a substrate with a substituent at C2 of the purine ring. 2,6-Diaminopurine in RNA at an ADAR2 editing site would allow us to address this question. To this end, we prepared RNA substrates containing N^6-methyladenosine (**N^6-Me**), N^6-ethyladenosine (**N^6-Et**) and 2,6-diaminopurine ribonucleoside (**DAP**). A phosphoramidite of 2,6-diaminopurine ribonucleoside is known and commercially available. However, synthesis of the N^6-alkyladenosine phosphoramidites was required. Both of these compounds were generated in four steps from 2′,3′,5′-tri-*O*-acetyl-6-bromopurine ribonucleoside (**8**), itself available in two steps from inosine (Scheme 3).[34] Compound **8** was converted to the N^6-substituted adenosine analogues by reaction with either methylamine or ethylamine in methanol at room temperature for 8h to obtain compounds **9** and **10**, respectively. For incorporation into RNA, **9** and **10** were protected at the 5′-hydroxyls as dimethoxytrityl ethers (**11** and **12**, respectively) and at the 2′-hydroxyls as *tert*-butyldimethylsilyl ethers (**13** and **14**, respectively). The resulting compounds were then converted to the 3′-di-*iso*-propylaminocyanoethyl phosphoramidites **15** and **16** in good yield under standard conditions.

The rates of deamination for the analogue-containing substrates (**N^6-Me**, **N^6-Et, DAP**) were measured under single turnover conditions (1 nM substrate, 54 nM enzyme) (Figure 6).[29] Under these conditions, ADAR2 converted **N^6-Me** to inosine with a $k_{obs} = 0.001 \pm 0.0001$ min^{-1} (Figure 6). The substrate is saturated with enzyme under these conditions since increasing [ADAR2] concentration from 54 nM to 216 nM had no apparent effect on the measured rate ($k_{obs} = 0.001 \pm 9 \times 10^{-5}$ min^{-1}). This rate was slower than that observed for adenosine but identical to that obtained for the 6-*O*-methyl derivative with a $k_{rel} = 0.02$ (k_{obs} for **N^6-Me**/k_{obs} for **A**).[29] Interestingly, no deamination reaction was observed with either the **N^6-Et** substrate or the **DAP** substrate under these conditions (Figure 6). Thus, ADAR2 appears to be sensitive to the size of the C6 substituent with the *N*-methyl derivative slowly converted to product, and no apparent reaction of the *N*-ethyl derivative. The fact that the *N*-methyl compound was processed at a rate identical to that of the *O*-methyl derivative indicates the ADAR2 reaction is not highly sensitive to the pK_a of the leaving group.

ADA has been shown to convert 2,6-diaminopurine ribonucleoside to guanosine with a rate only 4-fold slower than that of adenosine.[35] Indeed, this reaction has been used for the synthesis of certain guanosine analogues.[36] In contrast, 2,6-diaminopurine ribonucleoside in RNA is not a substrate for ADAR2 under the conditions tested here. Thus, the ADAR2 active site is unable to accommodate the purine substrate with an amino substituent at C2.

Magnitude of the "Aza Effect" is RNA Structure Dependent. The observation that 8-azaadenosine was deaminated considerably faster than adenosine in the model editing substrate shown in Figure 4 prompted us to investigate the generality of this "aza effect". We have shown that the rate of deamination of adenosine by ADAR2 is highly dependent on the structural context of the reacting base.[21] For instance, when the R/G site adenosine is flanked on both sides by duplex secondary structure, it is deaminated 20-fold faster than when it is at the 5′ end of a duplex as shown in Figure 4.[21] Also, an adenosine

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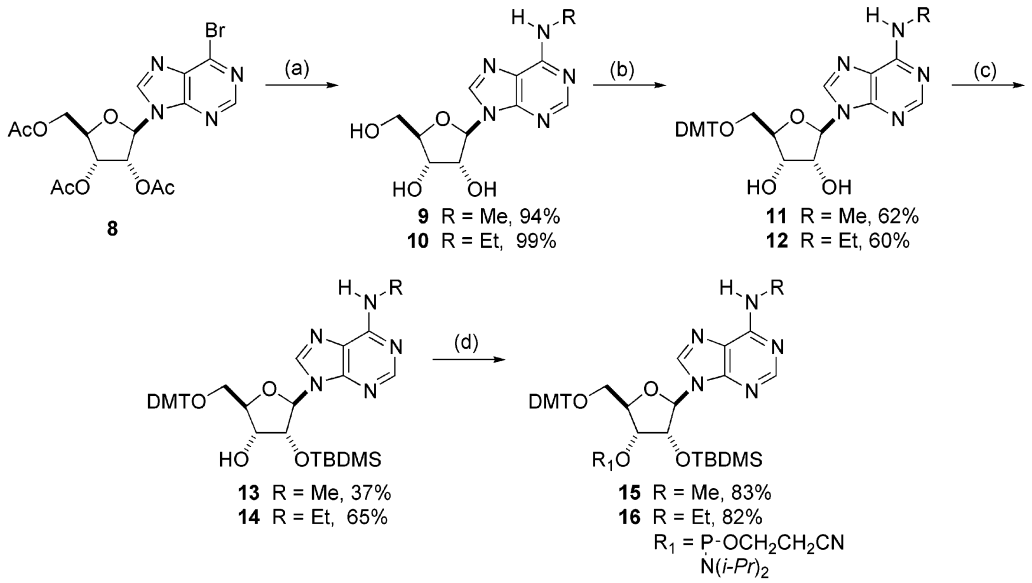
Scheme 3. Synthesis of N^6-alkyladenosine Phosphoramidites



(a) RNH_2, MeOH; (b) DMTCl, pyridine, $AgNO_3$, THF; (c) TBDMSCl, TEA, $AgNO_3$, THF; (d) $ClP(OCH_2CH_2CN)(N(i\text{-}Pr)_2)$, THF, DIEA.

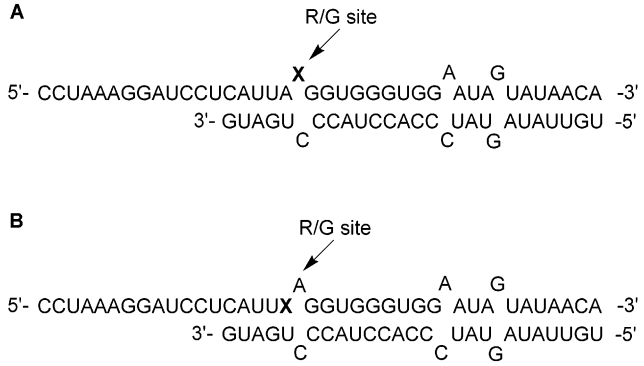


Figure 7. Duplex RNA substrates generated to measure the aza effect in different structural contexts. The **X** indicates the position of incorporation for either adenosine or 8-azaadenosine. Substrate **A** is a mimic of the GluR−B pre-mRNA which positions the R/G editing site internally within the substrate. Substrate **B** is likewise a mimic of the GluR−B pre-mRNA which positions the analogue used at a poorly edited site adjacent to the R/G editing site.

at a position within a duplex substrate not efficiently edited by ADAR2 is deaminated nearly 2 orders of magnitude more slowly than the R/G site A in the same RNA.[21] To measure the aza effect for adenosines in these different contexts, we prepared RNA substrates bearing 8-azaadenosine at an internal R/G site (Figure 7A) and also at an internal site that is poorly edited (Figure 7B). Site specific ^{32}P-labeling and splint ligations were used to generate these RNAs as previously described.[21] The longer duplex substrate binds ADAR2 with a slightly higher affinity than the substrate with the R/G adenosine positioned at the 5′ end.[21] For comparison, the rate of deamination of adenosine at these sites was also determined under the same conditions. When the internal R/G site was substituted with 8-azaadenosine, a 2.8-fold rate acceleration was observed (Table 2). However, at the adjacent adenosine, which is a poor ADAR2 substrate, 8-aza substitution caused a larger 17-fold rate acceleration. Thus, the magnitude of the observed aza effect is dependent on the structural context of the edited base with the larger effect observed at an adenosine that is deaminated poorly by the enzyme.

Table 2. Observed and Relative Rate Constants for Deamination Assays Using 8-Azaadenosine in Varying Substrate Contexts

substrate	k_{obs} (min^{-1}) **A**	k_{obs} (min^{-1}) **8-AA**	k_{rel}[a]
A	0.88 ± 0.09	2.50 ± 0.29	2.8
B	0.0020 ± 0.0001	0.034 ± 0.002	17

[a] $k_{rel} = k_{obs}$ for **8-AA**/ k_{obs} for **A** at 25 nM RNA substrate, 165 nM ADAR2.

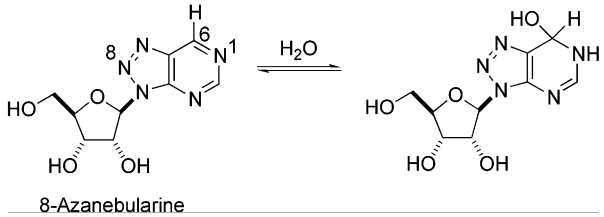


Figure 8. Hydration of the C6−N1 double bond in 8-azanebularine generates a good mimic of the tetrahedral intermediate formed during hydrolytic adenosine deamination.

8-Azanebularine Inhibits ADAR2. 8-Azanebularine is a derivative of 8-azaadenosine with hydrogen substituted for the C6 amino group (Figure 8). This compound is a potent inhibitor of adenosine deaminase with a $K_i = 40$ nM, a value 400-times lower than that for nebularine.[37] It has been suggested that this increase in potency is due to the greater propensity of the 8-aza analogue to undergo covalent hydration of the C6−N1 double bond (Figure 8).[30] Because the covalent hydrate is an excellent mimic of the tetrahedral intermediate formed during adenosine deamination, structural changes of the purine ring that facilitate hydration increase the potency of the inhibitor. 8-Aza substitution of adenosine caused an increase in the rate of the reaction for each of the ADAR substrates studied here. These results suggested that 8-azanebularine may also function as an ADAR inhibitor.

8-Azanebularine was prepared for these experiments using the sequence shown in Scheme 4. 2′-,3′-,5′-*O*-Triacetyl-8-azaadenosine (**17**) was synthesized as previously described.[33]

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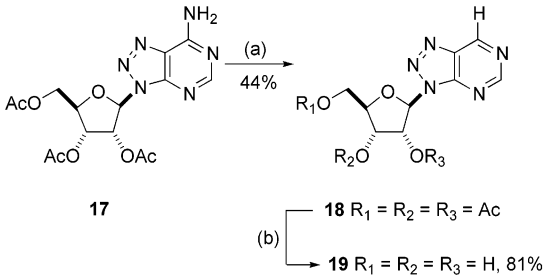
Scheme 4. Synthesis of 8-azanebularine



(a) *t*-BuONO, DMF, 65 °C; (b) NH_3/MeOH, 4 °C.

Reductive deamination at C6 of this compound was carried out using the Doyle protocol (*t*-BuONO in DMF),[38] allowing us to obtain the desired product **18** in 44% yield. Deacylation (NH_3/MeOH) afforded 8-azanebularine **19** in 81% yield.

Three nucleosides were tested for their ability to inhibit the RNA-editing reaction of ADAR2 (Figure 9A). As described above, coformycin is a potent transition state analogue inhibitor of ADA with an $K_i = 10$ pM, but does not inhibit ADAR1.[24,27] Nebularine and 8-azanebularine are also inhibitors of ADA with K_i's equal to 16 μM and 400 nM, respectively.[37] Interestingly, when these three compounds were titrated into an ADAR2 reaction, only 8-azanebularine inhibited the enzyme in the concentration ranges tested ($IC_{50} = 15 \pm 3$ mM) (Figure 9C).

Discussion

We describe here the reaction of the RNA-editing enzyme ADAR2 with RNA containing various adenosine analogues. This work extends our analysis of the sensitivity of the ADAR reaction to structural changes at the edited nucleotide.[28,29] The study of enzyme substrate analogues allows one to infer structural features of the active site and probe the reaction mechanism.[39−41] The results obtained also aid in the design of active-site directed inhibitors.[42] In the case of the ADAR reaction, the reactivity of adenosine analogues also allows us to gain insight into the mechanistic relationship between the RNA-editing enzyme and the well understood nucleoside modifying enzymes adenosine deaminase (ADA) and cytidine deaminase (CDA).[23]

ADA catalyzes a reaction similar to that of the RNA-editing ADARs on nucleoside substrates.[43] ADA uses a zinc-bound hydroxide to achieve hydrolytic deamination.[44] However, ADARs share little sequence similarity with ADAs.[15,16,45] Furthermore, ADARs are not inhibited by the potent ADA inhibitor coformycin, a mimic of the adenosine deamination transition state (Figure 3).[24] Interestingly, ADARs and a related family of tRNA-specific adenosine deaminases have conserved sequences similar to the consensus sequence that makes up the

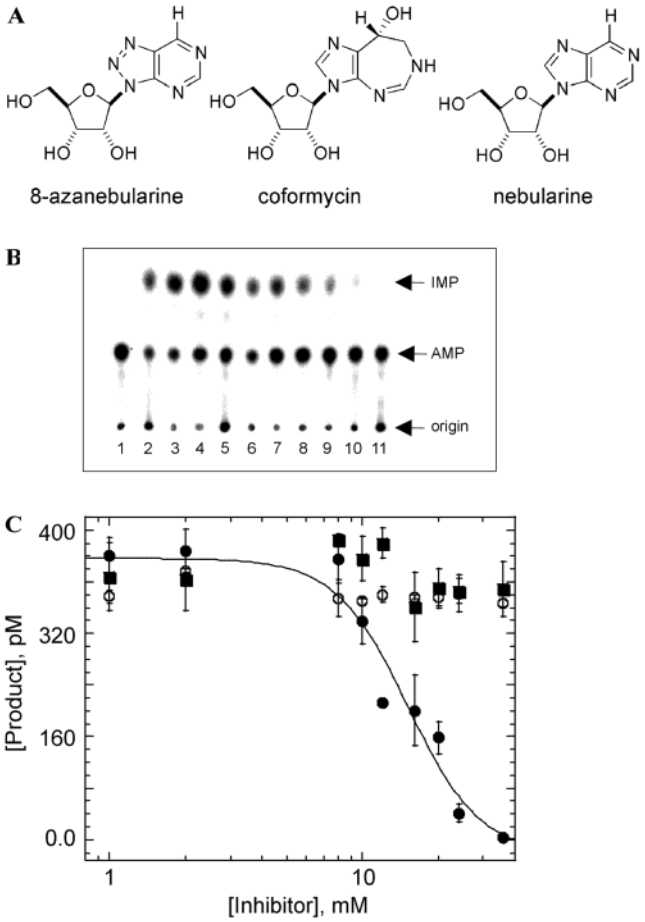


Figure 9. (**A**) Structures of nucleosides used in inhibition assays. (**B**) Storage phosphor autoradiogram of TLC plate used to separate products arising from the reaction of 1 nM substrate with adenosine occupying the R/G editing site and 54 nM ADAR2 in the presence of varying concentrations of 8-azanebularine after a 20 min reaction. Lane 1, Reaction standard; Lanes 2−11: 0, 1, 2, 8, 10, 12, 16, 20, 24, and 36 mM inhibitor added, respectively. (**C**) Plot of product formed as a function of inhibitor concentration. Legend for inhibitors: (●): 8-azanebularine (○): coformycin (■): nebularine. Data were fit to the equation: [Product] formed = {range/1 + ([Inhibitor]/IC_{50})^{slope})} + background using the least-squares method of KaleidaGraph. Data points reported are the average ± standard deviation for three independent experiments.

active-site of cytidine deaminases (CDAs).[46] CDA also uses a zinc-bound hydroxide to carry out deamination of its nucleoside substrate via attack at C4 of the pyrimidine and loss of ammonia.[23] Using the comparison to CDAs, amino acids identified as possible active site residues for the ADARs, including putative metal-binding ligands, have been altered by site-directed mutagenesis with a corresponding loss of editing activity.[47] Thus, it appears that ADARs have evolved from a CDA-like ancestor to carryout adenosine deamination within RNA and that the ADAR active site is more similar to that found in CDA than in ADA. Our results with nucleoside analogue substrates in RNA are consistent with this idea. Structural changes that change the nature of the leaving group or alter the purine's susceptibility to nucleophilic attack have similar effects on the ADAR and ADA reactions. This would be true if both used nucleophilic attack by a metal-bound hydroxide as the key catalytic step. However, structural modifications to the adenine

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(47) Lai, F.; Drakas, R.; Nishikura, K. *J. Biol. Chem.* **1995**, *270*, 17 098−17 105.

that change its recognition properties, such as the change in shape that occurs with 2-amino substitution or the removal of hydrogen bonding that occurs with 7-deaza substitution reveal significant differences in reactivity between ADAR2 and ADA.[29,48] Because it appears the active site of ADARs evolved from a cytidine deaminase and not an adenosine deaminase, base recognition in the ADAR active site could very well be different from that of ADA.

Substitution at C8 of the reactive adenosine with nitrogen accelerated the rate of the ADAR2 reaction for each of the substrates tested. 8-Aza substitution of adenosine decreases its pK_a from 3.5 to 2.2.[49,50] This structural change also facilitates hydration across the N1−C6 double bond.[30,31] Protonation at N1 is a necessary step in the formation of the tetrahedral intermediate in a hydrolytic adenosine deamination reaction. If this protonation were rate limiting, then a substitution that makes the purine less basic might be expected to slow the reaction. Because this substitution accelerates the ADAR reaction rate, it appears more likely that nucleophilic attack on the purine is rate limiting in these substrates. Importantly, 8-aza substitution of the adenosine also causes a rate acceleration for the ADA reaction (3-fold increase in k_{cat}).[32] There is, however, an alternative explanation for this aza effect that cannot be ruled out at this time for ADAR2. It is possible that the 8-nitrogen of 8-azaadenosine is involved in an interaction with a protein functional group in the active site that preferentially stabilizes the transition state. Further experiments will be required to determine the exact origin of the aza effect observed here for the ADAR reaction.

The magnitude of the aza effect was greater at sites that are edited inefficiently by the enzyme (Tables 1 and 2). It is possible that when the reactive adenosine is at the end of the duplex (Figure 4) or when the adenosine is in the wrong sequence context (Figure 7, Substrate B), interactions between the RNA and protein are not sufficient to correctly position the adenine base with respect to the metal-bound hydroxide in the active site. Increasing the intrinsic reactivity of the base has a greater effect at these sites compared to a good editing site, which may have evolved to maximize the rate of nucleophilic attack on adenosine. Future experiments designed to define how structural features of the RNA surrounding a good editing site control the rate of nucleophilic attack on the edited adenosine will lead to a better understanding of the basis for RNA editing selectivity.

As mentioned above, these substrate analogue studies are useful in the design of active-site directed inhibitors. Such a compound would be invaluable in structural studies in identifying key interactions in the ADAR active site. In addition, if this compound were cell permeable, it could be used in chemical genetic studies of ADAR function in whole cells.[51] It is important to note that coformycin, a transition state analogue inhibitor of ADA, inhibits neither ADAR1 nor ADAR2 at millimolar concentrations, whereas its K_i for ADA is 10 pM (Figure 3).[24,27] Given that ADA readily accepts DAP as a substrate and ADAR2 does not, we conclude that ADAR is more sensitive to the structure of the purine at the 2-position. Since coformycin has the larger seven-membered ring, it may not fit effectively in the ADAR active site. Thus, derivatives of coformycin will likely not be suitable for targeting the ADAR active site.

However, 8-azanebularine does inhibit the ADAR2 reaction (Figure 9C). The inhibition requires relatively high concentrations of the nucleoside ($IC_{50} = 15 \pm 3$ mM). This is likely due to the fact that much of ADAR2's substrate binding affinity comes from contacts made between the RNA and the RNA-binding domain which are not possible with this inhibitor. Indeed, we have reported that the isolated RNA-binding domain of ADAR2 binds an RNA duplex substrate with an affinity indistinguishable from that of the full-length enzyme bearing the deaminase domain.[22] The fact that nebularine shows no inhibition of ADAR2 in the concentration range tested is not surprising, given the aza effect we observed for ADAR2 substrates and the 400-fold more potent inhibition of ADA reported for 8-azanebularine compared with nebularine.[37] Thus, 8-azanebularine appears to be a good lead compound in the design of an active-site directed inhibitor of ADAR2. The challenge for future development of this compound will be to introduce structural features that increase potency and selectivity for ADARs over ADA. This may be possible, for instance, by phosphorylation of the 3′ and 5′ hydroxyls or introduction of phosphodiester mimics at these locations.

In summary, by studying analogues of the substrate for an RNA-editing adenosine deaminase, we have been able to infer features of the enzyme's active site and reaction mechanism. ADAR2 is able to displace groups other than ammonia from C6 of its substrate, but limits the size of the leaving group to something smaller than ethylamine. Unlike ADA, this enzyme cannot deaminate at C6 of 2,6-diaminopurine, suggesting a base recognition strategy different from that of ADA. The rate of the ADAR2 reaction is sensitive to the susceptibility of the substrate purine to nucleophilic attack and apparently insensitive to leaving group pK_a. Furthermore, a large effect on the rate of the reaction was observed for 8-aza substitution of the substrate adenosine. This suggested that 8-azapurines might be uniquely suited to bind the enzyme active site and led to the discovery of a nucleoside inhibitor of ADAR2 (8-azanebularine).

Experimental Section

General Synthetic Procedures. Glassware for all reactions was oven dried at 125 °C overnight and cooled in a desiccator prior to use. Reactions were carried out under an atmosphere of dry nitrogen when anhydrous conditions were necessary. All reagents were obtained from commercial sources and were used without further purification unless noted otherwise. Liquid reagents were introduced by oven-dried microsyringes. Tetrahydrofuran was distilled from sodium metal and benzophenone. Thin-layer chromatography (TLC) was performed with Merck silica gel 60 F254 precoated plates, eluting with the solvents indicated. Short and long wave visualization was performed with a Mineralight multiband ultraviolet lamp at 254 and 365 nm, respectively. Flash column chromatography was carried out using Mallinckrodt Baker silica gel 150 (60−200 mesh). ^{1}H, ^{13}C, and ^{31}P Nuclear Magnetic Resonance spectra of pure compounds were acquired at 300, 75, and 121 MHz, respectively. Chemical shifts are reported in parts per million in reference to the solvent peak. Chemical shifts for phosphorus NMR are reported in parts per million using 85% phosphoric acid as an external standard. The abbreviations s, d, dd, t, td, q, m, and brs stand for singlet, doublet, doublet of doublets, triplet, triplet of doublets, quartet, multiplet, and broad singlet, in that order. High-resolution mass

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spectra were obtained on a Finnigan Mat 95. *N*-(dimethylacetamidine)-5′-*O*-DMT-8-azaadenosine (**1**),[33] 8-azaadenosine (**4**),[33] 2′,3′,5′-tri-*O*-acetyl 6-bromonebularine (**8**),[34] and 2′,3′,5′-tri-*O*-acetyl-8-azaadenosine (**17**)[33] were synthesized according to literature procedures.

Procedure for the Preparation of 8-Azainosine (5). ADA (19.5 mg; Sigma, 2.2 units/mg protein, calf intestinal mucosa) was added to a solution of **4** (430 mg, 1.60 mmol) in aqueous sodium phosphate buffer (0.1 M, 39 mL; pH 7.4) and stirring was continued at room temperature overnight. The reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (10−20% MeOH/$CHCl_3$) to afford **5** (369.8 mg, 86%) as a white solid. Spectroscopic data agreed with reported values.[52]

General Procedure for the Preparation of N^6-Substituted Adenosine Analogues. The amine (10 equiv.) was added to a solution of the 6-bromonebularine triacetate (0.656−1.09 mmol) in MeOH (10 mL). The reaction mixture was stirred at room temperature for 8h. It was concentrated under reduced pressure and the solid was triturated with EtOAc to obtained the respective N^6-substituted product (vide infra).

***N*^{6}-Methyladenosine (9).** White solid (94%). Spectroscopic data agreed with reported values.[53]

***N*^{6}-Ethyladenosine (10)**. White solid (99%). Spectroscopic data agreed with reported values.[54]

General Procedure for the Preparation of 5′-*O*-(4,4′-dimethoxytrityl)-N^6-Substituted Adenosine Analogues and 8-Azainosine. To a solution of the ribonucleoside (0.615−1.68 mmol) in freshly distilled THF (15 mL) was added sequentially anhydrous pyridine (6.0 equiv.), 4,4′-dimethoxytrityl chloride (1.1 equiv), and $AgNO_3$ (1.1 equiv). The reaction mixture was stirred at room temperature overnight. The mixture was diluted with EtOAc (25 mL), filtered, and washed with 5% aqueous $NaHCO_3$ (1 × 40 mL) and brine (1 × 30 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The crude products were purified by flash column chromatography (DCM/MeOH/TEA 98:1:1), except for the 8-azainosine derivative which was coevaporated with toluene to remove pyridine and used without purification.

5′-*O*-(4,4′-Dimethoxytrityl)-N^6-methyladenosine (11). Light orange foam (62%). ^{1}H NMR (CD_2Cl_2, 300 MHz): δ (ppm) 8.29 (br s, 1H), 8.00 (s, 1H), 7.31−7.27 (m, 2H), 7.21−7.16 (m, 5H), 6.73 (dd, J = 9, 3.9 Hz, 4H), 6.03 (br s, 1H), 5.95 (d, J = 6 Hz, 1H), 4.77 (t, J = 5.1 Hz, 1H), 4.39−4.35 (m, 2H), 3.75 (s, 3H), 3.74 (s, 3H), 3.41 (dd, J = 10.5, 3.6 Hz, 1H), 3.23 (dd, J = 10.5, 3.6 Hz, 1H), 3.16 (br s, 3H). ^{13}C NMR (CD_2Cl_2, 75 MHz): δ (ppm) 159.2, 153.1, 145.1, 138.5, 136.2, 136.0, 130.5, 130.4, 128.5, 128.3, 127.3, 113.6, 91.3, 87.0, 86.7, 76.5, 73.3, 64.2, 55.7, 54.8, 53.3, 30.4. HRFABMS: calcd for $C_{32}H_{34}N_5O_6$ $(M + H)^+$ 584.2509, obsd 584.2525.

5′-*O*-(4,4′-Dimethoxytrityl)-N^6-Ethyladenosine (12). Light orange foam (60%). ^{1}H NMR (CD_2Cl_2, 300 MHz): δ (ppm) 8.22 (br s, 1H), 8.05 (s, 1H), 7.39 (app. d, J = 6.9 Hz, 2H), 7.30−7.17 (m, 7H), 6.78 (dd, J = 8.7, 2.1 Hz, 4H), 6.45 (br s, 1H), 6.09 (d, J = 5.1 Hz, 1H), 4.82, (t, J = 5.1 Hz, 1H), 4.48 (t, J = 4.8 Hz, 1H), 4.38 (q, J = 3.3 Hz, 1H), 3.74 (s, 6H), 3.62 (br s, 2H), 3.45 (dd, J = 10.2, 3.0 Hz, 1H), 3.34 (dd, J = 10.5, 3.9 Hz, 1H), 1.27 (t, J = 7.5 Hz, 3H). ^{13}C NMR (CD_2Cl_2, 75 MHz): δ (ppm) 159.0, 155.2, 153.2, 145.2, 138.6, 136.1, 136.1, 130.5, 128.5, 128.3, 127.3, 120.2, 113.5, 90.2, 86.9, 85.6, 75.9, 72.3, 64.1, 55.6, 36.0, 15.1. HRFABMS: calcd for $C_{33}H_{36}N_5O_6$ $(M + H)^+$ 598.2666, obsd 598.2647.

General Procedure for the Preparation of 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)-N^6-Substituted Adenosine Analogues, 8-Azaadenosine, and 8-Azainosine. The 5′-*O*-DMT derivative (0.377−0.742 mmol) was dissolved in freshly distilled THF (15 mL) and to this was added triethylamine (1.9 equiv), followed by the addition of TBDMSCl (1.1 equiv). After 5 min, $AgNO_3$ (1.1 equiv) was added to the solution. After 8 h of stirring at room temperature, the reaction mixture was diluted with EtOAc (25 mL), filtered, and washed with 5% aqueous $NaHCO_3$ (1 × 30 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The crude products were purified by flash column chromatography on silica gel using appropriate solvent systems (listed under individual compound headings, vide infra).

***N*-(Dimethylacetamidine)-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*t*-butyldimethyl-silyl)-8-azaadenosine (2).** Chromatography, EtOAc/hexanes 4:1. White foam (30%). ^{1}H NMR (CD_2Cl_2, 300 MHz): δ (ppm) 8.57 (s, 1H), 7.47 (dd, J = 8.1, 1.8 Hz, 2H), 7.35 (dd, J = 6.3, 2.7 Hz, 4H), 7.27−7.19 (m, 3H), 6.79 (d, J = 7.5 Hz, 4H), 6.40 (d, J = 4.5 Hz, 1H), 5.36 (t, J = 5.1 Hz, 1H), 4.48 (q, J = 4.5 Hz, 1H), 4.27 (q, J = 4.8 Hz, 1H), 3.78 (s, 6H), 3.43 (dd, J = 10.5, 3.6 Hz, 1H), 3.30 (s, 3H), 3.26 (dd, J = 10.5, 5.1 Hz, 1H), 3.21 (s, 3H), 2.76 (d, J = 4.8 Hz, 1H), 2.30 (s, 3H), 0.87 (s, 9H), 0.05 (s, 3H), −0.11 (s, 3H). ^{13}C NMR (CD_2Cl_2, 75 MHz): δ (ppm) 164.7, 161.0, 159.1, 157.5, 151.0, 145.6, 136.6, 136.4, 130.6, 128.7, 128.2, 127.1, 113.5, 89.9, 86.7, 85.1, 75.1, 72.4, 64.4, 55.7, 39.3, 38.9, 25.9, 18.4, 18.1, −4.7, −4.8. HRFABMS: calcd for $C_{40}H_{52}N_7O_6Si$ $(M + H)^+$ 754.3748, obsd 754.3708.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)-8-azainosine (6). Chromatography, EtOAc/hexanes 5:1. White foam (30%). ^{1}H NMR (CD_2Cl_2, 300 MHz): δ (ppm) 8.34 (s, 1H), 7.47 (d, J = 6.9 Hz, 2H), 7.35 (d, J = 7.8 Hz, 4H), 7.29−7.21 (m, 3H), 6.81 (d, J = 8.4 Hz, 4H), 6.37 (d, J = 4.5 Hz, 1H), 5.23 (t, J = 4.8 Hz, 1H), 4.47 (t, J = 4.5 Hz, 1H), 4.31 (q, J = 3.3 Hz, 1H), 3.45 (dd, J = 10.5, 3.3 Hz, 1H), 3.31 (dd, J = 10.5, 5.4 Hz, 1H), 2.71 (br s, 1H), 3.77 (s, 6H), 0.89 (s, 9H), 0.07 (s, 3H), −0.06 (s, 3H). ^{13}C NMR (CD_2Cl_2, 75 MHz): δ (ppm) 159.1 157.7, 150.2, 149.4, 145.5, 136.5, 136.3, 130.6, 128.7, 128.3, 127.3, 113.6, 90.2, 86.8, 85.5, 75.6, 72.4, 64.2, 55.7, 25.9, 18.4, −4.7, −4.8. HRFABMS: calcd for $C_{36}H_{43}N_5O_7Si$ M^+ 685.2932, obsd 685.2949.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)-N^6-methyladenosine (13). Chromatography, 20−40% EtOAc/hexanes. White foam (37%). ^{1}H NMR (CD_2Cl_2, 300 MHz): δ (ppm) 8.28 (br s, 1H), 7.98 (s, 1H), 7.48 (d, J = 7.2 Hz, 2H), 7.34 (d, J = 8.7 Hz, 4H), 7.31−7.20 (m, 3H), 6.83 (d, J = 9 Hz, 4H), 6.40 (br s, 1H), 6.01 (d, J = 5.1 Hz, 1H), 5.04 (t, J = 5.1 Hz, 1H), 4.37 (br s, 1H), 4.24 (q, J = 3.9 Hz, 1H), 3.77 (s, 6H), 3.50 (dd, J = 10.5, 4.2 Hz, 1H), 3.15 (br s, 3H), 2.83 (br s, 1H), 0.86 (s, 9H), 0.01 (s, 3H), −0.10 (s, 3H). ^{13}C NMR (CD_2Cl_2, 75 MHz): δ (ppm) 159.2, 156.0, 153.8, 145.4, 139.1, 136.3, 130.6, 130.6, 128.7, 128.4, 127.4, 120.8, 113.7, 89.0, 87.0, 84.5, 75.9, 72.0, 64.0, 55.7, 30.2, 25.9, 18.4, −4.7, −4.9. HRFABMS: calcd for $C_{38}H_{46}N_5O_6Si$ $(M - H)^+$ 696.3217, obsd 696.3197.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*t*-butyldimethylsilyl)-N^6-ethyladenosine (14). Chromatography, 30% EtOAc/hexanes. White foam (40%). Equilibration of the 3′-*O*-TBDMS derivative in 0.1% TEA/MeOH for 8h, followed by purification (chromatography, 30% EtOAc/hexanes) afforded 25% of the product. Total yield: 65%. ^{1}H NMR (CD_2Cl_2, 300 MHz): δ (ppm) 8.25 (br s, 1H), 7.95 (s, 1H), 7.49 (dd, J = 8.1, 1.5 Hz, 2H), 7.37 (d, J = 8.7 Hz, 4H), 7.32−7.23 (m, 3H), 6.83 (d, J = 8.7 Hz, 4H), 6.07 (br s, 1H), 6.00 (d, J = 5.1 Hz, 1H), 5.05 (t, J = 5.1 Hz, 1H), 4.38 (br s, 1H), 4.24 (q, J = 3.6 Hz, 1H), 3.78 (s, 6H), 3.66 (br s, 2H), 3.49 (dd, J = 10.5, 3.3 Hz, 1H), 3.39 (dd, J = 10.5, 4.5 Hz, 1H), 2.87 (br s, 1H), 1.29 (t, J = 6 Hz, 3H), 0.86 (s, 9H), 0.01 (s, 3H), −0.10 (s, 3H). ^{13}C NMR (CD_2Cl_2, 75 MHz): δ (ppm) 159.2, 155.4, 153.7, 145.4, 139.1, 136.3, 130.6, 130.6, 128.6, 128.4, 127.4, 120.7, 113.6, 89.0, 84.5, 75.8, 72.0, 64.1, 55.7, 36.0, 25.9, 18.3, 15.3, −4.7, −4.9. HRFABMS: calcd for $C_{39}H_{50}N_5O_6Si$ $(M + H)^+$ 712.3530, obsd 712.3536.

General Procedure for the Preparation of 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphino]-2′-*O*-(*t*-butyldimethylsilyl)-N^6-substituted Adenosine Analogues, 8-Azaadenosine, and 8-Azainosine. The phosphoramidite formation was performed according to standard procedure on the 5′-*O*-DMT-2′-*O*-

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TBDMS protected derivatives (0.115−0.169 mmol).[55] The crude products were purified by flash column chromatography on silica gel using appropriate solvent systems (listed under individual compound headings, vide infra).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphino]-2′-*O*-(*t*-butyldimethylsilyl)-8-azaadenosine (3). Chromatography, EtOAc/hexanes 3:1. White foam (94%). ^{31}P NMR (121 MHz, CH_2Cl_2, 85% H_3PO_4 as external standard): δ (ppm) 152.5, 150.8. HRFABMS: calcd for $C_{49}H_{69}N_9O_7PSi$ $(M + H)^+$ 954.4827, obsd 954.4824.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphino]-2′-*O*-(*t*-butyldimethylsilyl)-8-azainosine (7). Chromatography, EtOAc/hexanes/TEA 4:0.9:0.1. White foam (96%). ^{31}P NMR (121 MHz, CH_2Cl_2, 85% H_3PO_4 as external standard): δ (ppm) 152.7, 151.2. HRFABMS: calcd for $C_{45}H_{61}N_7O_8SiP$ M^+ 886.4089, obsd 886.3995.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopro-pylamino)phosphino]-2′-*O*-(*t*-butyldimethylsilyl)-*N*^{6}-methyladenosine (15). Chromatography, EtOAc/hexanes/TEA 3:0.9:0.1. White foam (90%). ^{31}P NMR (121 MHz, CH_2Cl_2, 85% H_3PO_4 as external standard): δ (ppm) 152.3, 150.7. HRFABMS: calcd for $C_{47}H_{65}N_7O_7PSi$ $(M + H)^+$ 898.4452, obsd 898.4463.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphino]-2′-*O*-(*t*-butyldimethylsilyl)-*N*^{6}-ethyladenosine (16). Chromatography, EtOAc/hexanes/TEA 3:0.9:0.1. White foam (82%). ^{31}P NMR (121 MHz, CH_2Cl_2, 85% H_3PO_4 as external standard): δ (ppm) 152.2, 150.5. HRFABMS: calcd for $C_{48}H_{67}N_7O_7PSi$ $(M + H)^+$ 912.4609, obsd 912.4577.

Procedure for the Preparation of 8-Azanebularine. 2′,3′,5′-Tri-*O*-acetyl-8-azanebularine (18). Freshly distilled *t*-BuONO (517.8 mg, 5.02 mmol) was added to a degassed hot solution (65 °C) of 8-azaadenosine triacetate **(17)** (990 mg, 2.51 mmol) in anhydrous DMF (15 mL) and stirred at this temperature for 1.5h. The reaction mixture was diluted with EtOAc/hexanes (50 mL, 7:3), successively washed with water (3 × 20 mL) and brine (1 × 25 mL), dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The syrup obtained was purified by flash column chromatography (1% MeOH/$CHCl_3$) to afford **18** (420.5 mg, 44%) as a light yellow syrup. Spectroscopic data agreed with reported values.[56]

8-Azanebularine (19). A solution of 8-azanebularine triacetate (**18**) (690 mg, 1.82 mmol) in methanolic ammonia (8 mL) was stored at 4 °C overnight. The mixture was concentrated under reduced pressure and purified by flash column chromatography (preadsorbed on silica gel, 10% MeOH/$CHCl_3$) to afford **19** (370.6 mg, 81%) as a light yellow soft solid. Spectroscopic data agreed with reported values.[56]

General Biochemical Procedures. Distilled, deionized water was used for all aqueous reactions and dilutions. Biochemical reagents were obtained from Sigma/Aldrich unless otherwise noted. Common enzymes were purchased from Stratagene (La Jolla, CA), Boehringer-Mannheim (Indianapolis, IN), Promega (Madison, WI), or New England Biolabs (Beverly, MA). 5′-Dimethoxytrityl-2′-*tert*-butyldimethylsilyl protected adenosine, guanosine, cytidine, and uridine phosphoramidites were purchased from Glen Research (Sterling, VA). Additionally, 5′-dimethoxytrityl-2′-*O*-triisopropylsilyloxymethyl-2,6-diaminopurine phosphoramidite was purchased from Glen Research. Coformycin nucleoside was a generous gift from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Phosphoramidites **3**, **7**, **15**, and **16** were synthesized as described above. [γ-^{32}P]ATP (6000 Ci/mmol) was obtained from Perkin-Elmer Life Sciences. Storage phosphor autoradiography was carried out using imaging plates obtained from Eastman Kodak Co. A Molecular Dynamics STORM 840 was used to obtain data from the phosphorimaging plates. Liquid scintillation counting was carried out with a Beckman LS 6500 scintillation counter using Bio-Safe II cocktail from Research Products International, Corp. Full-length human ADAR2 was overexpressed in *Saccharomyces cerevisiae* using the plasmid pScE[hA2a-His6] containing the human ADAR2 gene under the transcriptional control of a galactose-inducible GAL1 promoter. This expression system, as well as the purification protocol employed, was developed by Herbert L. Ley III and Prof. Brenda Bass, Department of Biochemistry, University of Utah.[57]

Synthesis and Purification of RNAs. Oligoribonucleotides were chemically synthesized on a Perkin-Elmer/ABI 392 DNA/RNA synthesizer using β-cyanoethyl phosphoramidites on a 1.0 μmol scale with coupling times extended to 20 min for more efficient coupling. In addition to the extended coupling time, a double coupling procedure was also used for the incorporation of phosphoramidites **3**, **7**, **15**, and **16** into their respective oligoribonucleotides. Deprotection of synthetic oligoribonucleotides was carried out in 1.5 mL NH_3-saturated methanol for 24 h at room temperature followed by 600 μL of 0.1 M tetrabutylammonium fluoride in THF for 48h at room temperature. Deprotected oligonucleotides were purified by polyacrylamide gel electrophoresis (19%), visualized by UV shadowing, and extracted from the gel via the crush and soak method with 0.5 M NH_4OAc, 0.1% SDS, and 0.1 mM EDTA. Extinction coefficients for these RNAs were calculated as the sum of the extinction coefficients of the component nucleotides utilizing the adenosine value for the analogues studied.[58] RNA concentrations were determined by UV/vis measurements at 260 nm using a Beckman DU 7400 spectrophotometer.

Preparation of RNA for Mass Spectrometry. An oligoribonucleotide of the sequence 5′-CAUUA**X**GGUGGG-3′, where **X** = **N^6-Me**, **N^6-Et**, **8-AA**, or **8-AI** was deprotected and desilylated as described above. The resulting 12 nucleotide oligomer (**N^6-Me** 12-mer, **N^6-Et** 12-mer, **8-AA** 12-mer, or **8-AI** 12-mer) was then purified by polyacrylamide gel electrophoresis (19%) in the absence of sodium or potassium ions. The appropriate gel slices were visualized by UV shadowing and extracted from the gel via the crush and soak method into distilled, deionized water at room temperature for 24 h. The solution was passed through a Centrex filter (0.2 μm cellulose acetate membrane) to remove the gel particles. The resulting solution was transferred to a 1000 MWCO dialysis tubing (total volume ~4 mL). This solution was dialyzed against 3 L of 10 mM NH_4OAc (pH 7.0) three times at 4 °C with exchange into fresh solution after 4 h. The 10 mM NH_4OAc was replaced with deionized H_2O and again dialyzed overnight with three changes taking place every 4 h. The resulting solution was lyophilized to dryness. The RNA pellet was resuspended in 1 mM NH_4OAc (pH 7.0) to a concentration of 80 μM and an equivalent volume of 2-propanol was added (final concentration = 40 μM). The samples were analyzed by matrix assisted laser desorption ionization-time-of-flight spectrometry (MALDI-TOF) at the University of Utah Mass Spectrometry Facility. The mass spectrum were obtained on a PerSeptive Biosystems voyager-DE SDR MALDI-TOF spectrometer. MALDI-TOF analysis: **N^6-Me** 12-mer, calcd 3890.0 obsd 3893.5, **N^6-Et** 12-mer, calcd 3906.0, obsd 3909.4, **8-AA** 12-mer, calcd 3888.0 obsd 3886.5, and **8-AI** 12-mer, calcd (M + Na) 3911.0 obsd 3913.0. The standard error related to the MALDI-TOF analysis is ±0.1%.

Preparation of Duplex Substrates. For the formation of labeled duplex RNA, a given oligonucleotide was labeled at the 5′ end using [γ-^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase. The labeled strand was first purified on a 19% denaturing polyacrylamide gel, visualized by storage phosphor autoradiography, excised, and extracted before it was hybridized to the unlabeled complement strand in TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) with 50 mM NaCl. The mixture was heated at 95 °C for 5 min and allowed to slow cool to room temperature. The duplex was purified on a 16% nondenaturing

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polyacrylamide gel. The appropriate band was visualized by storage phosphor autoradiography, excised, and extracted into TE buffer overnight at room temperature. Polyacrylamide particles were removed using a Centrex filter, as described above. The RNA duplex was ethanol-precipitated, redissolved in deionized water, and stored at −20 °C. The concentration of the duplex was determined using scintillation counting and the specific activity of the labeled strand.

Preparation of 8-Azainosine-5′-monophosphate. For the preparation of 8-azainosine to be used as a 5′-monophosphate standard, a 26-mer RNA strand with 8-azainosine as the 5′-nucleotide prepared with phosphoramidite **7** was end-labeled using [γ-^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase. The labeled strand was purified on a 19% denaturing polyacrylamide gel, visualized by storage phosphor autoradiography, excised, and extracted in TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) with 50 mM NaCl. After ethanol-precipitation, the labeled strand was digested with nuclease P1 to generate the 5′-monophosphates which were co-spotted onto thin-layer chromatography (TLC) plates in addition to the products of the nuclease digestion of the 8-azaadenosine oligonucleotide, as previously described.[12]

Construction of Internally Labeled RNA Substrates. The synthesis of substrates containing nucleotides 5′ to the R/G editing site was based on splint ligations reported previously by this laboratory.[21] In short, a 22 nt RNA oligonucleotide (30 pmol) (5′-**X**GGUGGGUGGAAUAG-UAUAACA-3′, where **X** = adenosine or 8-azaadenosine) was 5′ end-labeled as described above. To the resulting labeled strand, a 10 μM solution of DNA splint (5′-CTATTCCACCCACCTTAATGAGGATC-CTTTAGG-3′), a 20 μM solution of the 18-mer RNA extension strand (5′-CCUAAAGGAUCCUCAUUA-3′), RNasin (Promega), T4 DNA ligase 10× buffer (New England Biolabs), ATP, and T4 DNA ligase (45 Weiss units; NEB) were added. The ligation procedure was carried out using a Perkin-Elmer GeneAmp PCR System 2400 as described previously.[21] The resulting products were purified to nucleotide resolution by PAGE. To the labeled strand was added a known amount (>70-fold excess) of unlabeled strand of the same sequence. The strands were hybridized, and the resulting duplex was gel-purified as described above. For the generation of substrate **B** in Figure 7 with adenosine or 8-azaadenosine adjacent to the R/G site labeled, the oligonucleotide 5′-**X**AGGUGGGUGGAAUAGUAUAACA-3′ was 5′ end-labeled and ligated to 5′-CCUAAAGGAUCCUCAUU-3′ using the same DNA splint described above.

Deamination Assay. The deamination assays were carried out as previously described.[28] Briefly, each deamination reaction contained ADAR2 at various concentrations, 1 nM labeled RNA duplex, and assay buffer containing 15 mM Tris-HCl, pH 7.5, 6% glycerol, 0.5 mM dithiothreitol (DTT), 60 mM KCl, 1.5 mM EDTA, 0.003% NP-40, 160 U/mL RNasin, and 10 μg/mL yeast tRNA^{Phe}. Additionally, at each time point, an aliquot was removed and the reaction quenched by the addition of 0.5% SDS at 95 °C, followed by digestion with nuclease P1, and resolution of the resulting 5′-monophosphates by TLC as previously described.[12] For the internally labeled substrates, the amount of RNA duplex was increased to 25 nM, while the ADAR2 concentration was fixed at 165 nM. The higher RNA concentration favors duplex formation over alternative structures formed by the 40 nt single stranded oligonucleotide. The higher enzyme concentration is used to maintain a large excess of ADAR2. The data were analyzed by performing volume integrations of the regions corresponding to starting material, product, and the background sites using the ImageQuant software. The data were fit to the equation: $[P]_t = \sigma[1 - e^{(-k_{obs}\bullet t)}]$, where $[P]_t$ is the concentration of product at time t, σ is the fitted reaction end point, and k_{obs} is the fitted rate constant using KaleidaGraph. Each experiment was carried out in triplicate and plotted values are averages ± standard deviation. For enzyme saturation experiments, k_{obs} was measured as a function of [ADAR2] varying from 15 to 216 nM with the RNA substrate concentration fixed at 1 nM. The values of K_d and k_{max} were obtained from the fit to the equation $k_{obs} = k_{max}[ADAR2]/(K_d + [ADAR2])$ based on the kinetic scheme shown below, where E is ADAR2, S is substrate RNA, and P is product RNA

$$E + S \overset{K_d}{\rightleftharpoons} ES \xrightarrow{k_{max}} EP$$

Inhibition Assay. Each inhibition reaction was carried out by combining 54 nM ADAR2 with varying concentrations of the desired inhibitor and preincubating at 30 °C for 30 min. This period of preincubation was followed by addition of 1 nM ^{32}P-labeled RNA which was prepared as described above. In addition to the RNA, the reaction mixture contained 15 mM Tris-HCl, pH 7.5, 6% glycerol, 0.5 mM DTT, 60 mM KCl, 1.5 mM EDTA, 0.003% NP-40, 160 units/mL RNasin, and 1.0 μg/mL yeast tRNA^{Phe}. Reaction mixtures containing the RNA duplex were incubated at 30 °C for 20 min. The reaction was then quenched by the addition of 0.5% SDS at 95 °C, followed by digestion with nuclease P1 and resolution of the resulting 5′-monophosphates by TLC as previously described.[12] The data were obtained as detailed above and fit to the equation: [Product] formed = {range/(1 + ([Inhibitor]/IC_{50})$^{sl\circ pe}$)} + background. Each experiment was carried out in triplicate and plotted values are averages of the normalized data ± standard deviation.

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