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## Fluorescent Adenosine Analog as a Substrate for an A-to-I RNA Editing Enzyme

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### Abstract

Adenosine to inosine RNA editing catalyzed by ADAR enzymes is common in humans and altered editing is associated with disease. Experiments using substrate RNAs with adenosine analogs at editing sites are useful for defining features of the ADAR reaction mechanism. Here we evaluated the reactivity of ADAR2 with RNA containing the emissive adenosine analog thieno[3,4-d]-6-aminopyrimidine (<sup>th</sup>A). This nucleoside was incorporated into a mimic of the glutamate receptor B (GluR B) mRNA R/G editing site. We found that <sup>th</sup>A is recognized by AMV reverse transcriptase as A, and is deaminated rapidly by human ADAR2 to give <sup>th</sup>I. Importantly, ADAR reaction progress can be monitored by following the deamination-induced change in fluorescence of the <sup>th</sup>A-modified RNA. The observed high <sup>th</sup>A reactivity adds to our understanding of the structural features that are necessary for an efficient hADAR2 reaction. In addition, the new fluorescent assay is expected to accelerate mechanistic studies of ADARs.

### Keywords

fluorescent nucleosides; RNA editing; ADAR; adenosine deaminase

Adenosine deaminases acting on RNA (ADARs) are enzymes that deaminate adenosine to form inosine in the context of double-helical RNA structure. Inosine base pairs with cytosine, and so is recognized by the translation machinery and polymerases as guanosine, often leading to codon changes in mRNA. This phenomenon is widespread in the human transcriptome with thousands of inosine sites identified.<sup>[1]</sup> In addition, misregulation of A to I RNA editing is associated with human disease,<sup>[2]</sup> such as Dyschromatosis Symmetrica Hereditaria (DSH)<sup>[3]</sup> and the autoimmune disease Aicardi-Goutieres Syndrome (AGS).<sup>[4]</sup> Hyper editing has been observed at certain sites in cancer cells, such as in the mRNA for AZIN1 (antizyme inhibitor 1),<sup>[5]</sup> leading to more aggressive tumor behavior during esophageal squamous cell carcinoma progression.

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SUPPORTING INFORMATION AVAILABLE: Methods for phosphoramidite synthesis, modified RNA purification and characterization, primer extension and fluorescence assays for ADAR are available online.

Studies of the ADAR proteins are hampered by the fact that kinetic experiments typically require time consuming methods such as (a) reverse transcription followed by either gel electrophoresis or PCR and DNA sequencing for each assay time point<sup>[6]</sup> or (b) <sup>32</sup>P labeling of the 5' phosphate of the reactive adenosine followed by laborious nuclease digestion and thin layer chromatography resolution of labeled nucleotides for each time point.<sup>[7]</sup> Monitoring ADAR-mediated editing by fluorescence changes of the reactive A would greatly simplify data acquisition, which in turn could facilitate mechanistic studies. However, there are no reports of adenosine analogs that are ADAR-reactive while also having useful fluorescent properties. We therefore developed methods to incorporate into ADAR substrates <sup>th</sup>A and <sup>th</sup>I, members of a new class of fluorescent nucleoside analogs derived from thieno[3,4-*d*]-pyrimidine as the heterocyclic nucleus.<sup>[8]</sup> These analogs exhibit desirable properties for use in biophysical experiments, such as isomorphism with natural bases, intact Watson-Crick hydrogen bonding faces, and intense visible emission.<sup>[8]</sup> Thieno[3,4-*d*]-6-aminopyrimidine (<sup>th</sup>A), the emissive analog for adenosine (Figure 1A), was recently shown to mimic adenosine in the reaction of adenosine deaminase (ADA), a metabolic enzyme that deaminates the free nucleoside.<sup>[9]</sup> <sup>th</sup>A is processed by ADA with a reduced  $V_{\max}$  (~ 3 fold) and increased  $K_M$  (~ 20 fold) compared to the natural substrate, likely because of the carbon-for-nitrogen substitution at the purine 7-position,<sup>[9]</sup> resulting in a loss of an important hydrogen bonding contact to an aspartic acid residue in the ADA active site.<sup>[10]</sup> Nevertheless, since <sup>th</sup>A and the corresponding inosine analog (<sup>th</sup>I, Figure 1A) exhibit different fluorescence properties, <sup>th</sup>A enabled a fluorescence-based, high throughput screen for inhibitors of ADA.<sup>[9]</sup> We anticipated that <sup>th</sup>A could be an even better substrate for human ADAR2 because the reaction rate for this enzyme is less dependent on the presence N7 in the edited purine than is ADA.<sup>[11]</sup>

Exploiting ADARs-mediated <sup>th</sup>A to <sup>th</sup>I fluorescence changes would be particularly useful given the complexities of measuring deamination rates for specific adenosines in RNA molecules.<sup>[7]</sup> However, the <sup>th</sup>A analog had not been previously incorporated into RNA and its reactivity in ADAR reactions was, therefore, unknown. The carbon-carbon bond linking the base to the sugar in <sup>th</sup>A and the sulfur atom corresponding to purine position 8 are structural features not previously tested in ADAR substrate analogs.

The proposed reaction mechanism for ADARs involves formation of a hydrated intermediate that lacks the aromaticity of adenosine or inosine. Modifications that decrease the aromaticity of adenosine facilitate hydration and increase the reaction rate.<sup>[11b, 12]</sup> We have previously used quantum mechanical calculations to predict the propensity for hydration of various purine analogs relative to 9-methylpurine.<sup>[13]</sup> These generally correlate well to observed ADAR2-catalyzed deamination rates for the corresponding adenosine analogs in RNA, particularly when the substrates match the shape of adenosine.<sup>[11b]</sup> Using these same methods, we calculated the covalent hydration free energy for thieno[3,4-*d*]-pyrimidine to be 8.5 kcal/mol more favorable than for 9-methylpurine (Figure 2). Given our previous studies, this difference suggested <sup>th</sup>A would be an excellent ADAR2 substrate.<sup>[11b, 13]</sup>

The fluorescence and predicted ADAR reactivity of the thieno analogs led us to synthesize modified RNA, which required the preparation of the corresponding phosphoramidites

(Figure 3). For <sup>th</sup>A, regioselective protection of the 2'-hydroxyl group to give the corresponding 2'-O-TBDMS via the 3',5'-O-di-*tert*-butylsilyl-protected free nucleoside,<sup>[8]</sup> was followed by protection of the *N*<sup>6</sup> amine with the *N,N*-diisobutylformamidinium group (Supplementary Scheme 1). Tritylation of the 5'-hydroxyl group and phosphorylation at the 3'-hydroxyl group provided the <sup>th</sup>A phosphoramidite, suitable for automated RNA synthesis. The phosphoramidite of <sup>th</sup>I was synthesized in a similar manner (Supplementary Scheme 2). The free <sup>th</sup>I nucleoside synthesis was reported in our previous publication.<sup>[9]</sup> These phosphoramidites were used to incorporate the thieno analogs into a previously characterized mimic of the GluR B R/G editing site (Figure 1B) that is efficiently deaminated by human ADAR2 and various mutants of this enzyme.<sup>[7]</sup>

One method used for measuring ADAR activity consists of following the deamination reaction progress by a primer extension reaction.<sup>[14]</sup> We found both that <sup>th</sup>A is recognized by AMV reverse transcriptase like A, with only ddTTP incorporated at the complementary position (Figure 1C), and that <sup>th</sup>A is a substrate for ADAR2 (Table 1). Under single turnover conditions at saturating concentration of human ADAR2, we measured a  $k_{obs}$  for the <sup>th</sup>A deamination reaction approximately two-fold higher than that observed for the reaction with adenosine (Table 1). The rate of ADAR2 editing of adenosine in this substrate using this assay was comparable to that found using the AMP/IMP thin layer chromatography assay.<sup>[15]</sup> Thus, the <sup>th</sup>A analog is an excellent substrate for human ADAR2 consistent with the prediction based on calculated hydration free energies. The carbon-carbon bond linking the base analog to ribose and the thieno heterocycle are structural features that appear to be well tolerated by the ADAR2 active site.

We recorded fluorescence spectra for <sup>th</sup>A- and <sup>th</sup>I-modified GluR B R/G site RNAs either in the single stranded form or in a duplex (see Figure 1B for duplex structure) in the presence of ADAR assay buffer components (Figure 4).<sup>[7]</sup> Excitation at 380 nm of the <sup>th</sup>A-containing single strand showed strong emission with a maximum at 417 nm (Figure 4A). Interestingly, the <sup>th</sup>I analog emission is quenched to a greater degree in the context of the oligonucleotide than is the emission for the <sup>th</sup>A analog such that, in contrast to the case of the free nucleosides, emission of <sup>th</sup>I-containing RNA is substantially lower than for the <sup>th</sup>A-containing RNA (Figure 4A). While the precise origin of this difference is difficult to define, it is likely a combination of factors such as different ground state and excited state interactions, particularly with neighboring nucleobases, and differences in solvation. While fluorescence quenching is also observed upon duplex formation, the emission of <sup>th</sup>A and <sup>th</sup>I differs by approximately twofold and remains distinct (Figure 4B).

The current in vitro assays for ADAR deamination are not amenable to high throughput formats, and often necessitate the use of radiolabeled substrates. Since we have shown that RNAs containing <sup>th</sup>A or <sup>th</sup>I exhibit different fluorescence properties, we endeavored to develop a fluorescence assay for the ADAR reaction. The quenching of the fluorophores when in the context of an RNA duplex, not uncommon for fluorescent nucleosides, complicated direct measurement of fluorescence changes induced by ADAR (Figure 4B).<sup>[16]</sup> In addition, given the high enzyme/RNA ratios typical of in vitro ADAR reactions, real time fluorescence changes observed during an ADAR reaction are influenced by base flipping, a phenomenon known to alter the observed emission of the fluorescent base due to

unstacking.<sup>[15]</sup> To overcome these complications, we devised a protocol to stop the reaction at specified time points while rendering the <sup>th</sup>A/<sup>th</sup>I-containing oligonucleotide single stranded by adding an excess of a complementary unlabeled single stranded DNA and heating the sample (Figure 5A (top)). The progress of the reaction could then be monitored by measuring the fluorescence decrease at 417 nm (380 nm excitation) since <sup>th</sup>A in single stranded RNA is substantially more emissive under these conditions than is <sup>th</sup>I in single stranded RNA (see Figure 4A above). In addition, since ADAR has low affinity for single stranded RNA and the heat denatures and inactivates the enzyme, ADAR-induced base flipping would not affect the fluorescence of the sample. Using this protocol, we observed the expected decrease in fluorescence as a function of time for the reaction of the <sup>th</sup>A-modified GluR B R/G site duplex with an active N-terminal deletion mutant of human ADAR2 (hADAR2-RD)<sup>[17]</sup> (Figure 5A (bottom)). To confirm that the changes in fluorescence were indeed due to the deamination reaction, we carried out a parallel analysis of the reaction using the primer extension assay described above, finding good agreement between the two (Figure 5B).

We considered the use of time resolved fluorescence measurements to follow the ADAR reaction with the emissive RNA constructs in real time. Such an approach could have potentially distinguished between different helical or flipped out states of <sup>th</sup>A and <sup>th</sup>I in the ADAR–RNA complex. However, in addition to practical considerations, the excited state lifetimes of the two emissive nucleoside analogs are too close to one another to facilitate an unambiguous detection (see Figures S1–S3, Table S2). We therefore resorted to the use of indirect but assured fluorescence discrimination between substrate and product as described above.

Current estimates have the number of A to I editing sites in the human transcriptome at > 15,000 and our understanding of how the surrounding RNA sequence and structure controls editing efficiency is limited.<sup>[18]</sup> New assays that allow mechanistic data to be acquired more rapidly, particularly with short, synthetic RNA substrates, will accelerate efforts necessary to advance this field. The new assay described here is fast, does not require the generation of radiolabelled substrates with short half-lives and should be adaptable to a multi-well plate format.

In conclusion, we have evaluated the reactivity of the novel fluorescent adenosine analog <sup>th</sup>A with an RNA editing adenosine deaminase. This analog is an excellent substrate for human ADAR2 with a deamination rate comparable to the enzyme's natural substrate. <sup>th</sup>A-modified single stranded RNA is substantially more fluorescent than the same RNA modified with the deamination product, <sup>th</sup>I. This difference facilitated the development of a new ADAR assay based on monitoring the deamination-induced reduction in fluorescence of the <sup>th</sup>A/<sup>th</sup>I-containing RNA. This work will significantly accelerate mechanistic studies of ADARs and set the stage for high throughput screens for ADAR inhibitors.

## Supplementary Material

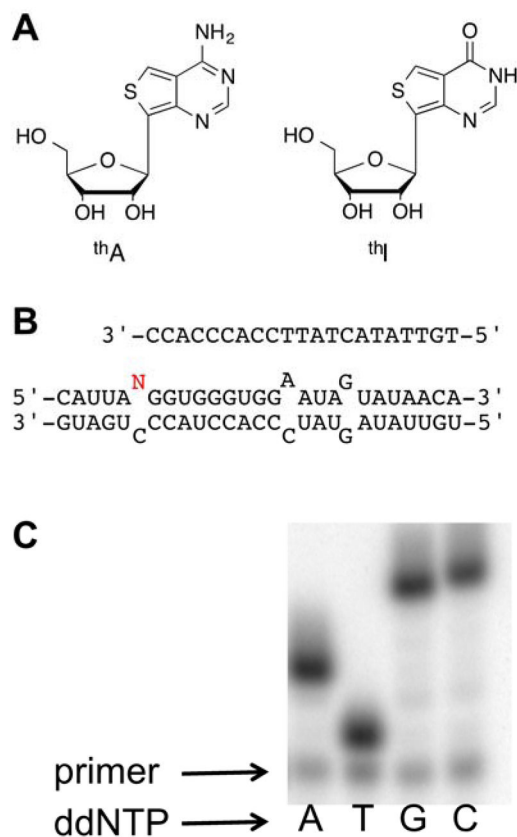
Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

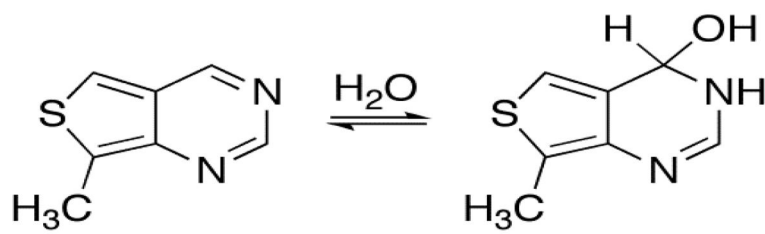
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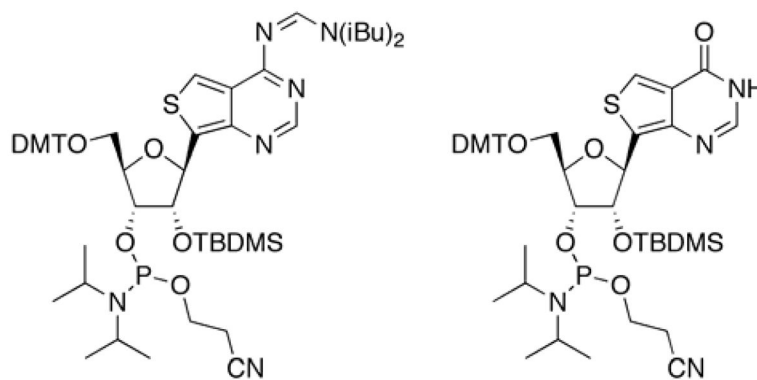
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**Figure 1.**

(A) Structures of emissive RNA nucleosides <sup>th</sup>A and <sup>th</sup>I. (B) Top: labeled primer used in the primer extension assay, Bottom: RNA duplex with editing site shown in red. (C) Only dideoxyT base pairs with <sup>th</sup>A, indicating that it is recognized by AMV RT as A.

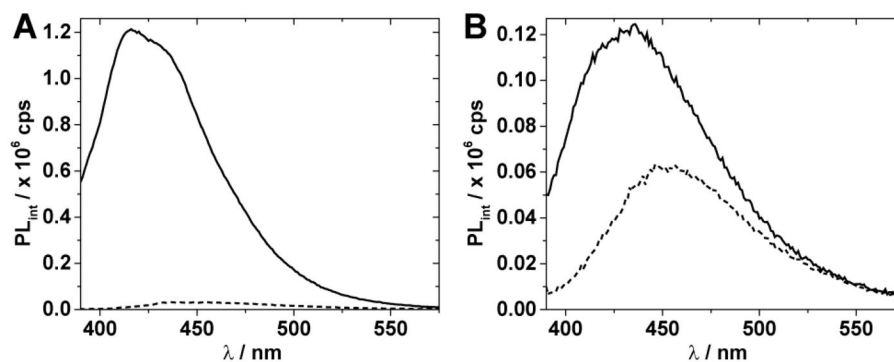


**Figure 2.** Thieno[3,4-*d*]-pyrimidine covalent hydration. The calculated hydration free energy compared to that of 9-methylpurine predicts high ADAR reactivity.<sup>[13]</sup>

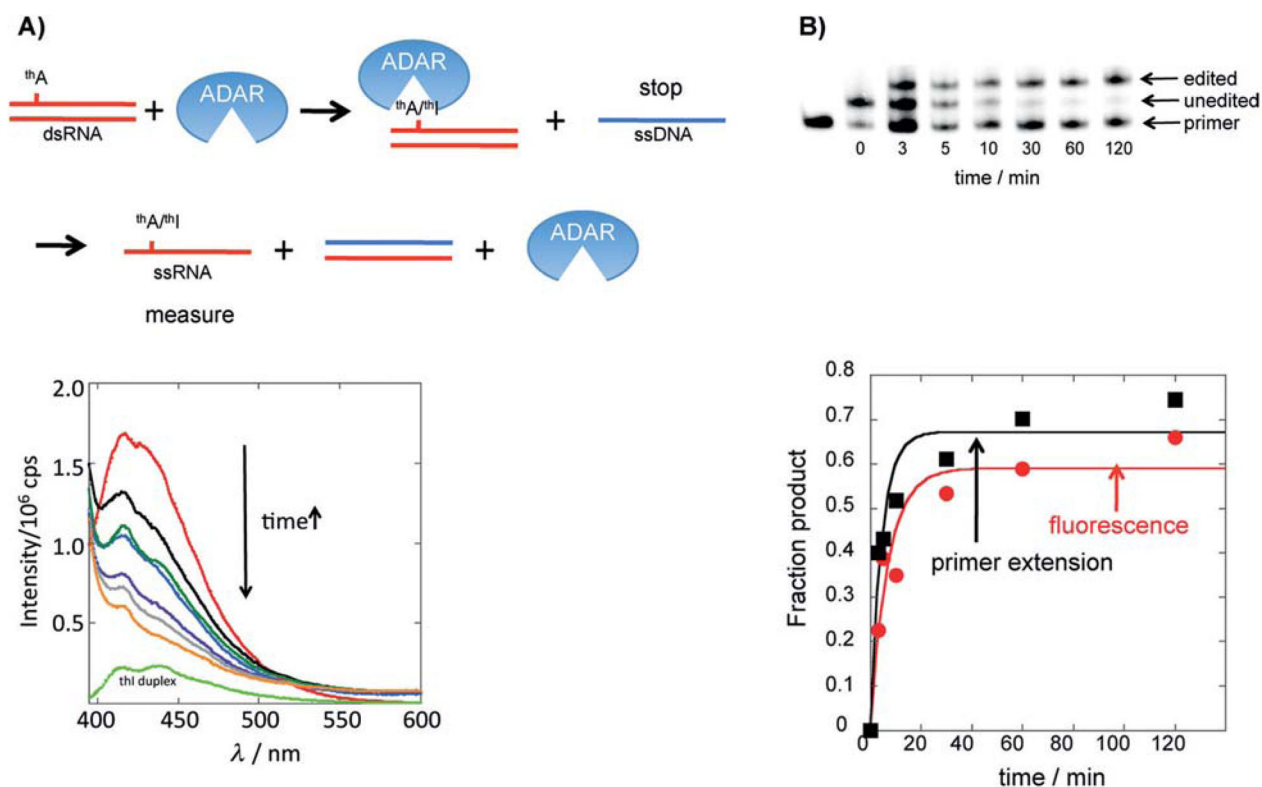


**Figure 3.** Structures of phosphoramidites for <sup>th</sup>A (left) and <sup>th</sup>I (right) used to prepare analog-containing RNA.





**Figure 4.** Emission spectra of 1  $\mu$ M GluR B R/G site single stranded RNA (**A**) and duplex RNA (**B**) with incorporated <sup>th</sup>A (solid line), and <sup>th</sup>I (dashed line) in ADAR2 buffer at 30 °C containing DTT (0.5 mM), yeast tRNA (1  $\mu$ g/ $\mu$ L), and RNasin (0.16 U/ $\mu$ L). Spectra were recorded on a Horiba Fluoromax-3 after excitation at 380 nm using slit-widths of 5 nm. Spectra are corrected for the blank fluorescence signal measured using the same instrument settings.



**Figure 5.**

Fluorescence-based assay for an ADAR reaction. A) Top: The procedure used to stop the reaction such that thA/thI fluorescence can be measured in ssRNA form. Bottom: Plot of change in fluorescence as a function of time for the reaction of 1 mM thA-modified GluR B R/G site substrate and 1 mM hADAR2-RD. Red: 0 min, black: 3 min, blue: 5 min, dark green, 10 min, purple: 30 min, gray: 60 min, orange: 120 min, light green: thI-containing duplex. B) Top: Image of gel used to resolve products for primer extension editing assay for reaction evaluated by changes in fluorescence in (A). Bottom: Fraction product vs time for primer extension assay (black squares) and fluorescence assay (red circles) ( $k_{obs}=0.14\ 0.01\ \text{min}^{-1}$  (fluorescence);  $k_{obs}=0.15\ 0.07\ \text{min}^{-1}$  (primer extension)).

**Table 1**Rate of deamination of A and <sup>th</sup>A by ADAR2 in the primer extension assay

substrate (N)	$k_{\text{obs}}, \text{min}^{-1}$	$k_{\text{rel}}$
A	$0.87 \pm 0.42$	1
<sup>th</sup> A	$1.83 \pm 0.66$	2.1

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