Fluorescent Nucleosides

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A Fluorescent Adenosine Analogue 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 analogues at editing sites are useful for defining features of the ADAR reaction mechanism. The reactivity of ADAR2 was evaluated with RNA containing the emissive adenosine analogue thieno[3,4-d]-6-aminopyrimidine (thA). This nucleoside was incorporated into a mimic of the glutamate receptor B (GluR B) mRNA R/G editing site. We found that thA is recognized by AMV reverse transcriptase as A, and is deaminated rapidly by human ADAR2 to give thI. Importantly, ADAR reaction progress can be monitored by following the deamination-induced change in fluorescence of the thA-modified RNA. The observed high thA reactivity adds to our understanding of the structural features that are necessary for an efficient hADAR2 reaction. Furthermore, the new fluorescent assay is expected to accelerate mechanistic studies of ADARs.*

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.[1] Furthermore, misregulation of A to I RNA editing is associated with human disease,[2] such as dyschromatosis symmetrica hereditaria (DSH)[3] and the autoimmune disease Aicardi–Goutieres syndrome (AGS).[4] Hyper editing has been observed at certain sites in cancer cells, such as in the mRNA for AZIN1 (antizyme inhibitor 1),[5] leading to more aggressive tumor behavior during esophageal squamous cell carcinoma progression.

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[6] or b) ^{32}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.[7] 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 analogues that are ADAR-reactive while also having useful fluorescent properties. We therefore developed methods to incorporate thA and thI into ADAR substrates,-members of a new class of fluorescent nucleoside analogues derived from thieno[3,4-*d*]-pyrimidine as the heterocyclic nucleus.[8] These analogues exhibit desirable properties for use in biophysical experiments, such as isomorphicity with natural bases, intact Watson–Crick hydrogen bonding faces, and intense visible emission.[8] Thieno[3,4-*d*]-6-aminopyrimidine (thA), the emissive analogue for adenosine (Figure 1 A), was recently shown to mimic adenosine in the reaction of adenosine deaminase (ADA), a metabolic enzyme that deaminates the free nucleoside.[9] thA is processed by ADA with a reduced V_{max} (ca. 3 fold) and increased K_M (ca. 20 fold) compared to the natural substrate, which is likely because of the carbon-for-nitrogen substitution at the purine 7-position,[9] resulting in a loss of an important hydrogen bonding contact to an aspartic acid residue in the ADA active site.[10] Nevertheless, since thA and the corresponding inosine analogue (thI, Figure 1 A) exhibit different fluorescence properties, thA enabled a fluorescence-based, high-throughput screen for inhibitors of ADA.[9] We anticipated that thA could be an even better substrate for human ADAR2 because the reaction rate for this enzyme is less dependent on the presence of N7 in the edited purine than is ADA.[11]

Exploiting ADARs-mediated thA to thI fluorescence changes would be particularly useful given the complexities of measuring deamination rates for specific adenosines in RNA molecules.[7] However, the thA analogue 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 thA and the

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Supporting information for this article (including methods for phosphoramidite synthesis, modified RNA purification and characterization, primer extension, and fluorescence assays for ADAR) is available on the WWW under http://dx.doi.org/10.1002/anie.201502070.

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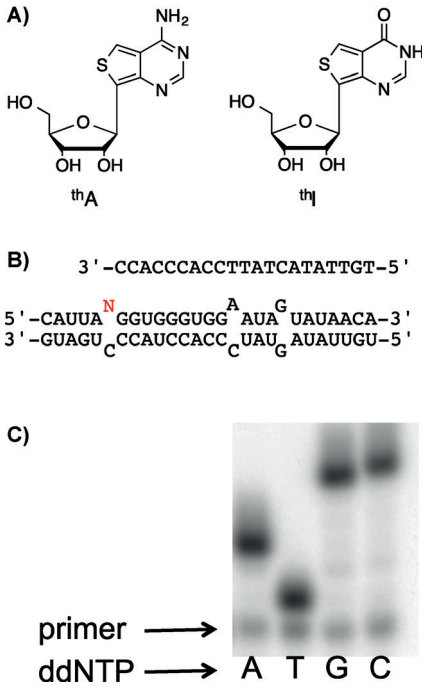

Figure 1. A) Structures of emissive RNA nucleosides thA and thI. B) Top: labeled primer used in the primer extension assay; Bottom: RNA duplex with editing site shown in red. C) Only dideoxyT base pairs with thA, indicating that it is recognized by AMV RT as A.

sulfur atom corresponding to purine position 8 are structural features not previously tested in ADAR substrate analogues.

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.[11b,12] We have previously used quantum mechanical calculations to predict the propensity for hydration of various purine analogues relative to 9-methylpurine.[13] These generally correlate well to observed ADAR2-catalyzed deamination rates for the corresponding adenosine analogues in RNA, particularly when the substrates match the shape of adenosine.[11b] Using these same methods, we calculated the covalent hydration free energy for thieno[3,4-*d*]-pyrimidine to be 8.5 kcal mol^{-1} more favorable than for 9-methylpurine (Figure 2). Given our previous studies, this difference suggested thA would be an excellent ADAR2 substrate.[11b,13]

The fluorescence and predicted ADAR reactivity of the thieno analogues led us to synthesize modified RNA, which required the preparation of the corresponding phosphoramidites (Figure 3). For thA, regioselective protection of the 2′-hydroxy group to give the corresponding 2′-O-TBDMS via

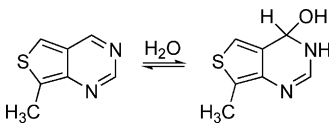

Figure 2. Thieno[3,4-*d*]-pyrimidine covalent hydration. The calculated hydration free energy compared to that of 9-methylpurine predicts high ADAR reactivity.[13]

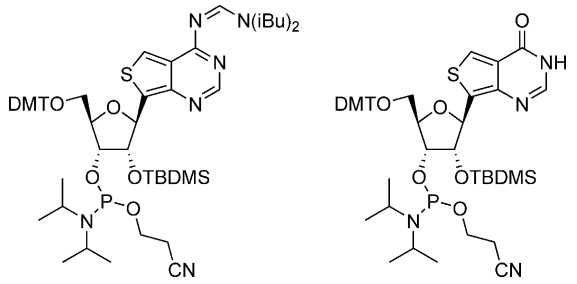

Figure 3. Structures of phosphoramidites for thA (left) and thI (right) used to prepare analogue-containing RNA.

the 3′,5′-O-di-*tert*-butylsilyl-protected free nucleoside[8] was followed by protection of the N^6 amine with the *N*,*N*-diisobutylformamidine group (Supporting Information, Scheme S1). Tritylation of the 5′-hydroxy group and phosphitylation at the 3′-hydroxy group provided the thA phosphoramidite, which is suitable for automated RNA synthesis. The phosphoramidite of thI was synthesized in a similar manner (Supporting Information, Scheme S2). The free thI nucleoside synthesis was reported previously.[9] These phosphoramidites were used to incorporate the thieno analogues into a previously characterized mimic of the GluR B R/G editing site (Figure 1 B) that is efficiently deaminated by human ADAR2 and various mutants of this enzyme.[7]

One method used for measuring ADAR activity consists of following the deamination reaction progress by a primer extension reaction.[14] We found both that thA is recognized by AMV reverse transcriptase like A, with only ddTTP incorporated at the complementary position (Figure 1 C), and that thA is a substrate for ADAR2 (Table 1). Under single turnover

Table 1: Rate of deamination of A and thA by ADAR2 in the primer extension assay.

substrate (N)	k_{obs} [min^{-1}]	k_{rel}
A	0.87 ± 0.42	1
thA	1.83 ± 0.66	2.1

conditions at saturating concentration of human ADAR2, we measured a k_{obs} for the thA 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.[15] Thus, the thA analogue is an excellent substrate for human ADAR2 consistent with the prediction based on calculated hydration free energies. The carbon–carbon bond linking the base analogue 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 thA- and thI-modified GluR B R/G site RNAs either in the single-stranded form or in a duplex (see Figure 1 B for duplex structure) in the presence of ADAR assay buffer components (Figure 4).[7] Excitation at 380 nm of the thA-containing single strands showed strong emission with a maximum at 417 nm (Fig-

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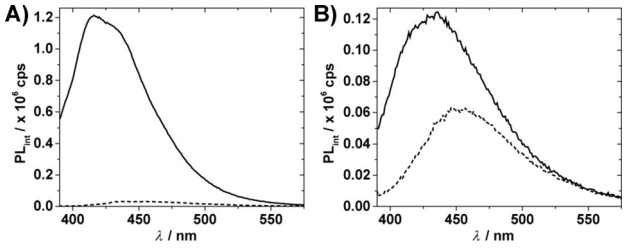

Figure 4. Emission spectra of 1 μM GluR B R/G site single-stranded RNA (A) and duplex RNA (B) with incorporated thA (——) and thI (-----) in ADAR2 buffer at 30 °C containing DTT (0.5 mM), yeast tRNA (1 μg μL^{-1}), and RNasin (0.16 U/μL). Spectra were recorded on a Horiba Fluoromax-3 after excitation at 380 nm using slit-widths of 5 nm. Spectra are corrected for the blank fluorescence signal measured using the same instrument settings.

ure 4A). Interestingly, the thI analogue emission is quenched to a greater degree in the context of the oligonucleotide than is the emission for the thA analogue such that, in contrast to the case of the free nucleosides, emission of thI-containing RNA is substantially lower than for the thA-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 thA and thI differs by a factor of about two 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 thA or thI 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, which is not uncommon for fluorescent nucleosides, complicated direct measurement of fluorescence changes induced by ADAR (Figure 4B).[16] Furthermore, 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.[15] To overcome these complications, we devised a procedure to stop the reaction at specified time points while rendering the thA/thI-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 thA in single-stranded RNA is substantially more emissive under these conditions than is thI in single-stranded RNA (Figure 4A, top). Furthermore, 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 procedure, we observed the expected decrease in fluorescence as a function of time for the reaction of the thA-modified GluR B R/G site duplex with an active N-terminal deletion mutant of human ADAR2 (hADAR2-RD)[17] (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 thA and thI in the ADAR-RNA complex. However, along with practical considerations, the excited-state lifetimes of the two emissive nucleoside analogues are too close to one another to facilitate an unambig-

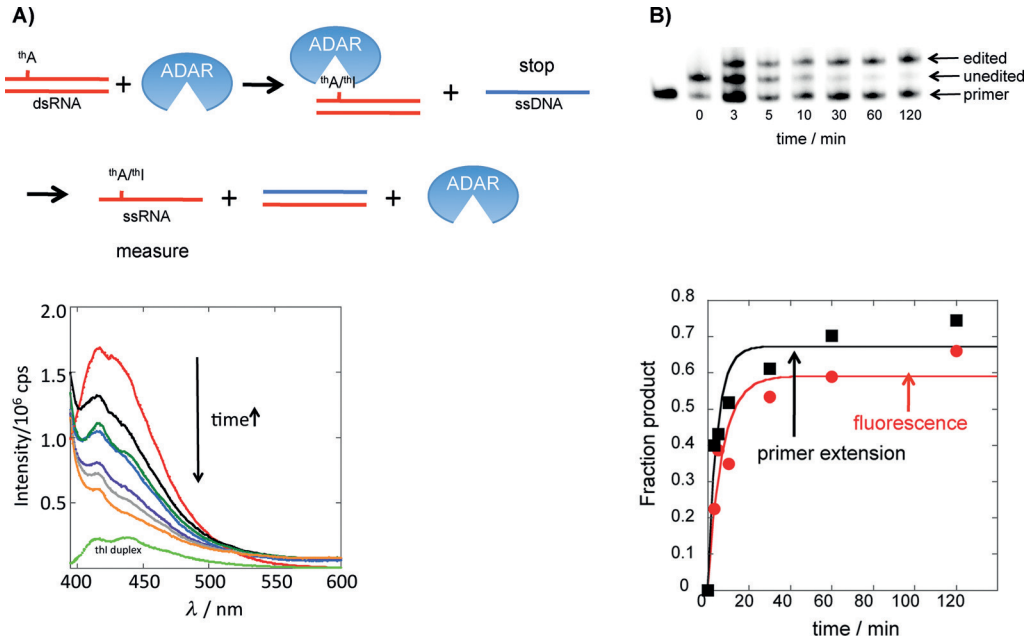

Figure 5. Fluorescence-based assay for an ADAR reaction. A) Top: The procedure used to stop the reaction such that thA/thI fluorescence can be measured in ssRNA form. Bottom: Plot of change in fluorescence as a function of time for the reaction of 1 μM thA-modified GluR B R/G site substrate and 1 μM 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) ($k_{obs} = 0.14 \pm 0.01$ min^{-1} (fluorescence); $k_{obs} = 0.15 \pm 0.07$ min^{-1} (primer extension).

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uous detection (Supporting Information, 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 more than 15000, and our understanding of how the surrounding RNA sequence and structure controls editing efficiency is limited.[18] 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 analogue thA with an RNA editing adenosine deaminase. This analogue is an excellent substrate for human ADAR2 with a deamination rate comparable to the natural substrate of the enzyme. thA-modified single-stranded RNA is substantially more fluorescent than the same RNA modified with the deamination product, thI. This difference facilitated the development of a new ADAR assay based on monitoring the deamination-induced reduction in fluorescence of the thA/thI-containing RNA. This work will significantly accelerate mechanistic studies of ADARs and set the stage for high throughput screens for ADAR inhibitors.

Keywords: ADAR enzymes · adenosine deaminase · fluorescent nucleosides · RNA editing

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