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Comparative Analysis Reveals Furoyl *in Vivo* Selective Hydroxyl Acylation Analyzed by Primer Extension Reagents Form Stable Ribosyl Ester Adducts

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Abstract

RNA molecules depend on structural elements that are critical for cellular function. Chemical methods for probing RNA structure have emerged as a necessary component of characterizing RNA function. As such, understanding the limitations and idiosyncrasies of these methods is essential for their utility. Selective hydroxyl acylation has emerged as a common method for analyzing RNA structure. Ester products as a result of 2′-hydroxyl acylation can then be identified through reverse transcription or mutational enzyme profiling. The central aspect of selective hydroxyl acylation analyzed by primer extension (SHAPE) experiments is the fact that stable ester adducts are formed on the 2′-hydroxyl. Despite its importance, there has not been a direct comparison of SHAPE electrophiles for their ability to make stable RNA adducts. Herein, we conduct a systematic analysis of hydrolysis stability experiments to demonstrate that furoyl imidazole SHAPE reagents form stable ester adducts even at elevated temperatures. We also demonstrate that the acylation reaction with the furoyl acylimidaole SHAPE reagent can be controlled with dithiothreitol quenching, even in live cells. These results are important for our understanding of the biochemical details of the SHAPE experiment.

RNA molecules can perform a myriad of functions to control normal and disease physiology.[1] The ability of RNA to form into extensive secondary and tertiary structures is critical to its function inside cells.[2,3]

Several biochemical methods have been developed to measure RNA structure. Early attempts used RNase enzymes, which would recognize and cleave RNA single- and double-

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Author Contributions

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ASSOCIATED CONTENT

Supporting Information

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Experimental methods, synthetic schemes and spectra, for all compounds (PDF)

The authors declare no competing financial interest.

stranded motifs.[4] More recently, chemical probes have been utilized to analyze and interpret RNA structure, dynamics, and RNA interactions with *trans*-acting RNAs and proteins.[5,6] We can gain a deeper understanding of these reagents by studying the chemical properties of adducts formed with RNA.

Selective hydroxyl acylation analyzed by primer extension (SHAPE) is a structure probing technique that reveals the internucleotide flexibility in RNA by 2′-OH acylation. SHAPE reactions form ester adducts on the 2′-OH (Figure 1).[7] 2′-OH acylation is accomplished by incubation with acylation electrophiles such as anhydrides,[7] acyl cyanides,[8] and more recently acyl imidazole reagents.[9]

Understanding the properties of these electrophiles is necessary for the development of SHAPE probes. For example, alterations to the leaving group or the group attached after adduct formation can alter the rate of reactivity.[10] Additionally, recent evidence has demonstrated that the solubility and reactivity of SHAPE reagents are vital for their utility within living cells.[11]

Despite the large amount of work done on understanding what governs acylation adduct formation, almost nothing is currently known about the stability of SHAPE adducts. For example, the instability of SHAPE adducts may alter the reactivity profile for conventional structure probing. A more thorough analysis of such properties would be imperative for understanding the limitations of SHAPE. Recently developed SHAPE protocols merged with deep sequencing expose modified RNA to several heating and annealing steps for library generation; as such, the stability of SHAPE adducts under such conditions would be highly informative. Further exploration of the stability of SHAPE esters may assist in ongoing efforts to identify the best SHAPE reagent for in-cell applications.

In this work, we compare the ester stability of three SHAPE reagents: (1) *N*-methylisotoic anhydride (NMIA), (2) nicotinoyl acylimidazole (NAI), and (3) furoyl acylimidazole (FAI) (Figure 2A). We chose to focus on these three because NMIA has been suggested to form very stable esters because of the donation of an electron from the adjacent amide bond;[7] such stability makes NMIA a stable benchmark. NAI and FAI have markedly different SHAPE acylation kinetics because of the electron withdrawing or donating properties of their aromatic rings. Lastly, the NAI and FAI acyl imidazole scaffolds have been recently demonstrated to function in living cells and have been used for sequencing-based RNA structure measurements;[11–13] thus, their utility makes them important for analysis.

We started by isolating SHAPE electrophile−ATP adducts (Figure 2B) and tested their hydrolytic stability under a variety of conditions. Deacylation was monitored by gel shift. The main conditions on which we focused were 37, 52, and 95 °C. These temperatures are all utilized in conventional SHAPE structure probing by reverse transcription. Additionally, they also all represent conditions used for RNA sequencing library construction after SHAPE ester formation.[11,12,14] The recent extension of RNA structure probing to be merged with deep sequencing relies on extensive manipulation of RNA after isolation from chemical probing experiments. Once isolated, RNA is taken through many steps before reverse transcription and the production of a stable cDNA. For example, RNA fragmentation is

performed at 95 °C. Linker ligation has an annealing temperature of ~90 °C, and then linker ligation can be performed at elevated temperatures to prevent secondary structure interference. RNA annealing before reverse transcription (RT) is performed at 95 °C, and then RT is performed at 52 °C. As such, there are several annealing and enzymatic steps, each of which is performed at a high temperature.

As shown in Figure 2C (Figure S1), overnight incubation at 4 °C with NMIA and NAI in a neutral buffer revealed some ester hydrolysis. In contrast, FAI did not show appreciable ester hydrolysis. Incubation of all adducts at 37 °C showed that NMIA and FAI are quite stable (~5% hydrolysis). In contrast, NAI exhibits significant hydrolysis (10%) over time. Comparison at 52 °C (temperature of reverse transcription) yielded results similar to those at 37 °C, with all esters having an increased extent of hydrolysis (NAI ~ 20% after 4 min). Lastly, we compared ester stability at 95 °C, a temperature used repeatedly for annealing of RNA over several steps, including reverse transcription, primer binding, and also linker ligation. As shown in Figure 2C, within 1.5 min ~40% of the NAI ester adducts had been hydrolyzed. In contrast, NMIA products exhibited only ~15% hydrolysis, and FAI suffers only ~10% hydrolysis under the same condition. These results are critical for understanding the stability properties of SHAPE reagents and also demonstrate that NAI, a commonly used reagent for *in vitro* and *in vivo* SHAPE, is highly susceptible to hydrolysis under a wide variety of conditions. Furthermore, these results indicate that FAI forms the most stable ester adducts with RNA.

Further inspection of the formed ester product and comparison of the three SHAPE reagents tested herein provide some insight into why there may be differences in the hydrolysis (Figure S2). NMIA forms a stable ester adduct, and an adjacent amine can donate electron density to the carbonyl carbon through a resonance structure. In contrast, NAI has a highly electron-withdrawing substituent in the pyridine ring, which results in a less stable adduct. Similar to NMIA, the furan ring in FAI can donate a significant amount of electron density to the carbonyl carbon. The lone pair of electrons on the oxygen atom of furan is in conjugation with the π-bond of the carbonyl group. Considering the design of these reagents is therefore critical for understanding their reactivity and also stability once an ester is formed with a nucleotide.

The observation that a furoyl SHAPE reagent forms the most stable ester products implored us to revisit an experimental outline for its use in RNA structure probing. Because the half-life of FAI in solution was previously measured to be 73 min, the addition of a quencher would grant us complete control over structure probing. Thiol quenchers have been used for decades as part of RNA structure probing experiments[15] and, as such, should be smoothly implemented for SHAPE, as well.

The ability of NAI to have its reactivity quenched through thioesterification with dithiothreitol (DTT), a commonly used thiol quencher, has been shown previously.[15] As demonstrated in Figure 3A, FAI is also quenched with the addition of 1 molar equiv of DTT and thus prevents acylation of FAI with ATP. Preincubation of DTT before FAI addition results in no measurable acylation. Furthermore, addition of DTT gives more precise control

for acylation as we observed an increasing level of adduct formation over time [a similar trend was observed with NMIA and FAI (Figure S3)].

We next reaffirmed that FAI is capable of measuring RNA structure in the same way as NAI, the acylation reagent used for many *in vitro* and *in vivo* RNA structure studies (Figure 3B).

Lastly, we wanted to demonstrate that FAI SHAPE reactivity can be quenched inside living cells. Either cells were preincubated with DTT, followed by the addition of FAI to the medium, or FAI was added and the mixture incubated for several minutes, followed by DTT. Figure 3C unambiguously shows that DTT is capable of quenching the FAI acylation reaction with RNA.

We also assessed ester stability in a mimic of cellular conditions. In such a case, the concentration of the acylation electrophile is much higher than the RNA concentration. We incubated ATP with each of the SHAPE electrophiles and then added high concentrations of DTT to the reaction mixtures. After long incubations, the reactions were resolved by denaturing electrophoresis, revealing that a large portion of the ATP remained acylated. This result suggests that the SHAPE reaction has stable acylations under conditions in which the electrophiles are in great excess (Figure S4). The important difference here, in comparison to the results in Figure 1, is that once the acylated adduct is isolated it can become hydrolyzed (similar to when the RNA is isolated). Overall, these data demonstrate that FAI acylation reactivity can be controlled with DTT quenching, and it should thus make for a robust protocol for utilizing FAI for stable 2′-OH ester adducts.

Herein, we have performed the first evaluation of SHAPE probe stability after the acylation reaction. Such evaluations are rare, as most of the focus is placed on the initial reaction in structure probing. However, it is vitally important to fully understand how structure probing reagents react with RNA and in what ways the final product can be altered by RNA manipulation after the reaction. The observation that furoyl SHAPE reagents form stable adducts after acylation suggests this scaffold may be better suited for structure probing analysis of acylation products, and that FAI adducts can survive many of the steps necessary for more complex procedures of RNA handling and processing, such as sequencing library construction. As such, we have performed a more comprehensive analysis of its use and how to control FAI SHAPE reactions with quenching. We anticipate that the furoyl scaffold will now be more widely used and will serve as a design starting point for additional SHAPE reagents and even those used in transcriptome-wide analysis of RNA structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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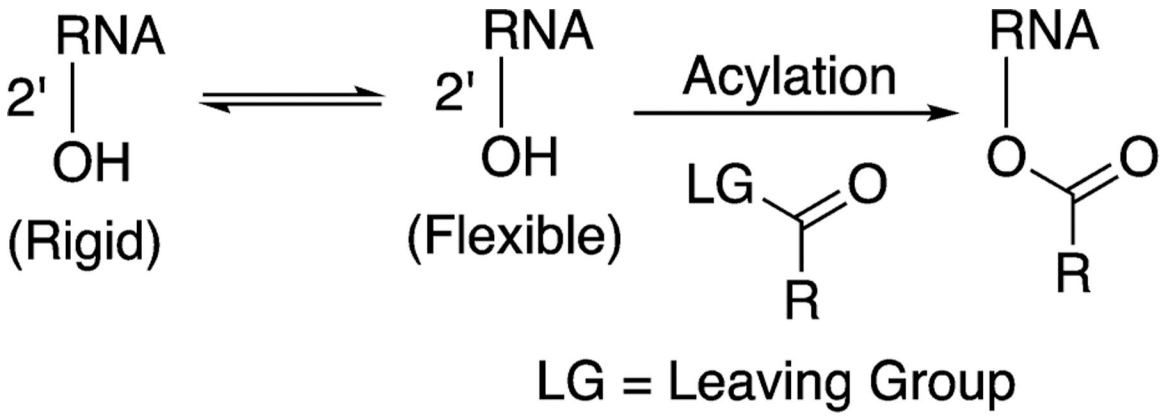

Figure 1.
SHAPE reaction.

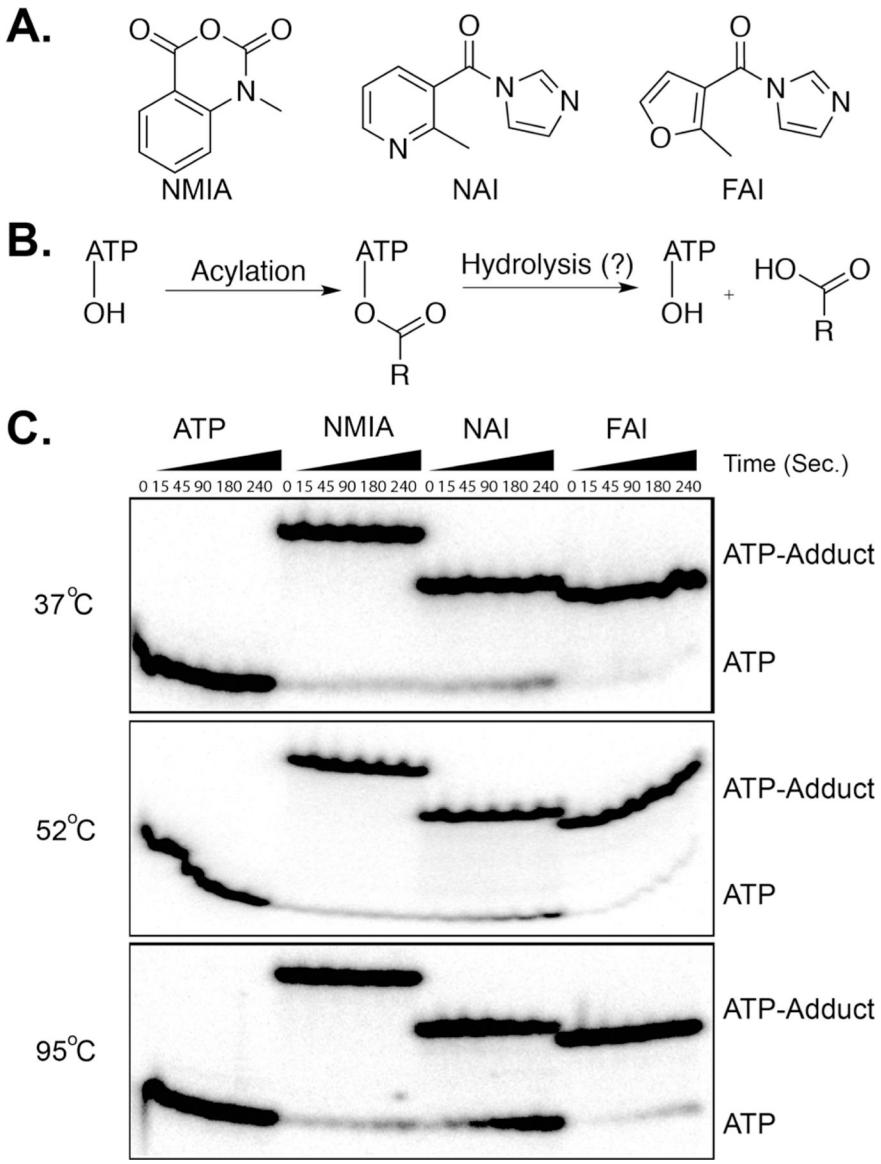

Figure 2.

SHAPE electrophile profiles for ester stability. (A) Chemical structures of SHAPE electrophiles used in this study. (B) Schematic of the ATP acylation and stability assay represented in Figure 3. (C) Hydrolysis time courses for all conditions tested in this study.

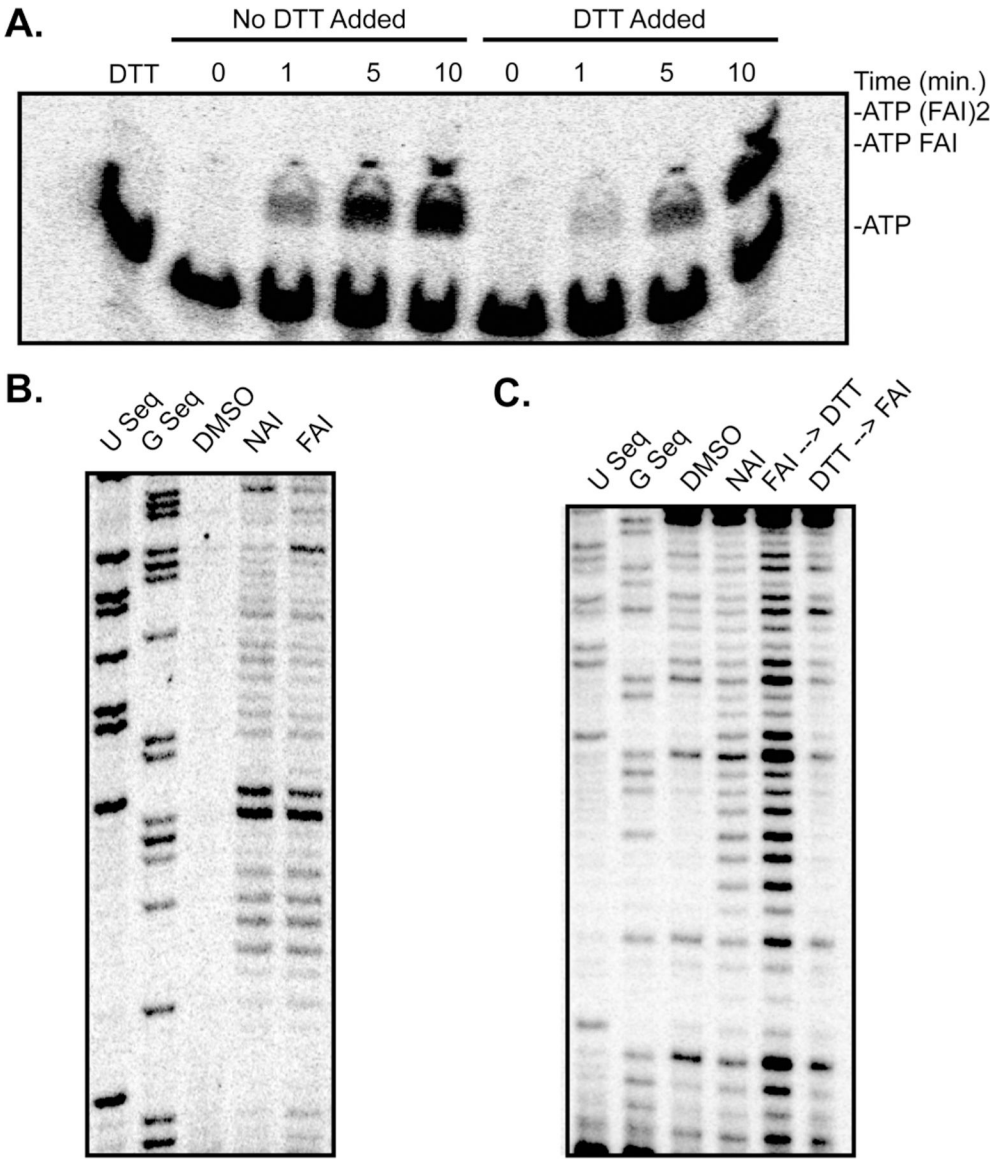

Figure 3.

Establishing robust SHAPE conditions for FAI. (A) DTT quenches FAI SHAPE reactivity. (B) NAI and FAI have very similar acylation profiles *in vitro*. (C) DTT can quench FAI activity in living cells.