HHS Public Access

Author manuscript

Chembiochem. Author manuscript; available in PMC 2016 November 18.

Published in final edited form as:

Chembiochem. 2016 November 17; 17(22): 2149–2152. doi:10.1002/cbic.201600300.

Metabolic Incorporation of Azide Functionality into Cellular RNA

Sarah Nainar[a], **Samantha Beasley**[a], **Michael Fazio**[a], **Miles Kubota**[a], **Nan Dai**[b], **Ivan R. Corrêa Jr.**[b], and **Prof. Robert C. Spitale**[a,c]

Robert C. Spitale: rspitale@uci.edu

[a]Department of Pharmaceutical Sciences, University of California, 2403 Natural Sciences I, Irvine CA 92617 (USA)

[b]New England Biolabs, 240 County Road, Ipswich, MA 01938 (USA)

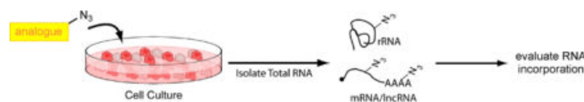
[c]Department of Chemistry, University of California, 2403 Natural Sciences I, Irvine CA 92617 (USA)

Abstract

Real-time tracking of RNA expression can provide insight into the mechanisms used to generate cellular diversity, as well as help determine the underlying causes of disease. Here we present the exploration of azide-modified nucleoside analogues and their ability to be metabolically incorporated into cellular RNA. We report robust incorporation of adenosine analogues bearing azide handles at both the 2′- and N6-positions; 5-methylazidouridine was not incorporated into cellular RNA. We further demonstrate selectivity of our adenosine analogues for transcription and polyadenylation. We predict that azidonucleosides will find widespread utility in examining RNA functions inside living cells, as well as in more complex systems such as tissues and living animals.

Graphical Abstract

Azide Functionality, Inc. We explored Functionality into Cellular RNA azidonucleoside incorporation into cellular RNA, revealing selectivity for azidoadenosine analogues and no incorporation of 5-azidouridine. This expansion of the bioorthogonal toolkit for RNA will further investigation into RNA expression and processing and provide a platform for analyzing the growing list of RNA functions beyond protein encoding.



Keywords

azides; imaging; modified nucleosides; RNA incorporation; transcription

---

Correspondence to: Robert C. Spitale, rspitale@uci.edu.

Supporting information for this article can be found under http://dx.doi.org/10.1002/cbic.201600300.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Recent analysis of complex transcriptomes has revealed that genomes are transcribed to generate diverse coding and non-coding RNAs that are critical for cell survival and identity.[1] The functions of both coding and noncoding RNAs continue to be elucidated.[2] As such, biochemical methods to track RNA transcription, posttranscriptional regulation, and RNA-based mechanisms that control their cellular function are in high demand.

Modified nucleoside analogues have been used to interrogate many facets of RNA biology. 4-Thiouridine (4SU) has been employed to track nascent transcription and monitor RNA decay.[3] However, recent evidence has suggested that the transient nature of disulfide bonds can bias RNA enrichment.[4] Extending beyond thiol-modified nucleosides introduces additional analytical properties, such as enrichment with stable covalent chemistry, imaging, and multiplex tracking. This can be accomplished through dosing of analogues containing diverse chemical functionalities. The analogue 5-ethynyluridine (5EU) has been used to track transcription and RNA localization by fluorescent imaging facilitated by copper-catalyzed azide–alkyne cycloaddition (CuAAC).[5] 2′-Azidonucleosides have proven useful for analysis of RNA produced in vitro by chemical synthesis.[6] N6-propargyl as well as C2- and C7-ethynyl adenosine have also been demonstrated to be useful probes for metabolic labeling of transcription and polyadenylation.[7] Despite this progress, a holistic description of the types of analogues that can be utilized to track RNA synthesis and processing inside living cells remains to be systematically interrogated.

Although useful, installing alkyne-modified nucleosides into cellular RNA requires the use of CuAAC reactions, which produce copper-induced radicals that degrade RNA.[8] Such degradation can lead to deleterious effects on downstream analyses such as RNA sequencing.[9] As such, there is a critical need to expand the bioorthogonal toolkit for cellular RNA by endowing substrates with more versatile functionalities.

Azides are perhaps the most widely utilized among the long list of bioorthogonal functional groups used in cells. Azide-containing molecules can be probed through diverse chemical reactions, including both CuAAC[10] and copper-free strain-promoted azide–alkyne cycloadditions (SPAAC),[11] as well as Staudinger ligations.[12] Metabolic labeling with azide-functionalized sugars has been a gold standard for studying glycosylated proteins on the cell surface, and has revealed the importance of the glycocalyx in cancer and development.[12] Azide-modified unnatural amino acids have been used to track nascent protein synthesis and have revealed the intricacies of cell-type-specific translation.[13] These examples underscore just a few of the powerful techniques made possible by functionalizing endogenous biomolecules with azide handles.

Installing azide functionality into cellular RNA would set the stage for parallel investigations to greatly increase our understanding of RNA biology and function. Nevertheless, the metabolic incorporation of azide functionalities into cellular RNA has yet to be explored and reported.

Herein, we provide evidence that azidonucleosides can be metabolically incorporated into cellular RNA. We further demonstrated preference for adenosine analogues, whereas an azidouridine analogue was refractory to RNA incorporation. Our data also suggest that,

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

depending on the site of azide modification, the adenosine analogues could be selectively utilized for tracking either gene body transcription alone or gene body transcription and polyadenylation. By exploring the limitations and idiosyncrasies of different azidonucleosides, we can ascertain how they can be leveraged to expand the scope of bioorthogonal reactions for studying RNA biology within living cells.

We first incubated cells with chemically synthesized azidonucleoside analogues for 12 h (synthetic schemes in the Supporting Information) and then isolated the total RNA (Figure 1A, **1**–**4**). In order to detect the azide group, we appended a biotin-alkyne by CuAAC. We then performed streptavidin northern blotting to determine incorporation of azidonucleosides into cellular RNA (Figure 1B).

These results showed that azidonucleoside analogues **1**–**3** were robustly incorporated into cellular RNA, whereas metabolic labelling with **4** was not detected (Figure 1C). We examined the cytotoxicity of analogues **1**–**3** by using an MTT assay[13] and observed no significant difference in cell viability of control cells as compared to cells treated with **1–3** at 1 mm final concentration (Figure S1 in the Supporting Information). The data in Figure 1 suggest that the pathway for purine triphosphate biosynthesis might be quite flexible, as adenosine analogues **1–3** have modifications on the nucleobase and the ribose sugar. Uridine analogue **4**, which has a larger methylazido group at position 5, showed no detectable incorporation into RNA. Analogue **4** reacted smoothly with biotin-alkyne and propargyl alcohol by CuAAC to form adducts (Figure S2); furthermore, although analogues **1–3** were detected in cellular RNA by LC-MS, **4** was not (Figure S3 and Table S1). These results strongly suggest that 4 was not metabolically incorporated into cellular RNA. This result is in contrast to 5-ethynyluridine, which is routinely used for tracking RNA synthesis.[14]

The differences in relative signal prompted us to explore the kinetics of incorporation. We reasoned that the lack of incorporation might be due to slower kinetics or simply limited introduction into cellular RNA. We performed an incubation-time titration, which revealed that adenosine analogues **1** and **2** were slowly incorporated into RNA, with unobservable signal until 5 h. In contrast, **3** was robustly integrated into cellular RNA within 30 min (Figures 1C and S4). We also treated cells with increasing amounts of each analogue (Figure S5), and the data showed that **3** was the most robustly incorporated at a concentration as low as 10 μm. We posit that the differences in incorporation between the azidonucleosides might be a function of the pathways and various enzymes involved in ribonucleotide triphosphate synthesis (more discussion below). Overall, these results demonstrate that azidoadenosine analogues are amenable to metabolic incorporation into RNA and therefore present a new set of modified nucleosides that can be utilized for RNA labeling.

A key benchmark for introducing modified molecules into cells is their ability to be used as reporter molecules for imaging experiments. We tested whether incorporation of each analogue could be tracked by cellular imaging using fluorophores. Cells were grown in the presence of **1**, **2**, or **3** and were subsequently fixed and treated with alkyne–rhodamine by CuAAC (Figure 2). Imaging showed clear staining of the nucleolus for all three analogues, indicating robust incorporation into ribosomal RNA, which accounts for the majority of cellular RNA. Each analogue also showed varying amounts of staining in the cytoplasm,

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

likely the result of trafficking, and this was most pronounced for analogue **3**. Consistent with our observations by northern blot, we were also able to observe RNA incorporation for analogue **3** at concentrations as low as 10 μm (Figure S6). Treatment with hydroxyurea showed no difference in imaging signal, thus strongly suggesting that the signal was derived from RNA and not DNA (Figure S7). RNase digestion of fixed cells also resulted in loss of signal, thus further demonstrating that the signal resulted from RNA labeling (Figure S8).

Azides present a unique opportunity to be probed by less damaging copper-free chemistry. To test our azidonucleosides inside living cells, we incubated cells with analogues **1**–**3** and dibenzylcyclooctyne (DIBO) biotin for SPAAC reactions.[11] From northern blotting, we concluded that bioorthogonal reactions with azide-modified RNA could be performed in living cells (Figure S9). Furthermore, SPAAC imaging experiments demonstrated that azide-labeled RNA could be robustly imaged with copper-free cycloadditions (Figure S10). Such observations demonstrate that nascent RNAs could be tracked in situ while eliminating the cytotoxic effects of copper-induced radical formation. Overall, our data support the theory that azide-modified adenosine analogues are useful for labeling RNA by CuAAC as well as SPAAC.

The differing rates of incorporation observed in Figure 1 suggest that there might be selectivity for incorporation into specific types of cellular RNA. It has previously been demonstrated that triphosphate analogues bearing a wide variety of modifications are well tolerated by a variety of polymerases.[14] Poly(A) polymerase selectivity has been investigated in vitro,[7c,8b] but has yet to be fully explored inside living cells. Adenosine analogues with alkyne groups at the N6, C2, and C7 positions have been demonstrated to be acceptable substrates.[7] However, larger azide modifications at the 2′- and N6 positions have not been investigated.

To gain insight into the possibility of substrate selectivity by poly(A) polymerase, we analyzed the crystal structure of the mammalian enzyme bound to adenosine triphosphate (PDB ID: 1F5A).[15] Amino acid residues tightly surround the N6 position of adenosine. By contrast, the 2′-position is relatively open to solvent. We therefore speculated that the different sites of azide modification might impart selectivity for either transcription by polymerases and/or polyadenylation by poly(A) polymerase.

To further explore polymerase selectivity, we challenged the incorporation of all three analogues with actinomycin D (ActD, an inhibitor of RNA polymerases I and II) and/or cordycepin (Cdy, an inhibitor of RNA poly(A) polymerase) at concentrations known to inhibit both processes.[7a,b] Analogues **1** and **2**, which contain N6 alkyl chains, showed strong inhibition by ActD treatment, but showed a limited decrease in signal when treated with Cdy (Figure 3B, Figure S11). In contrast, compound **3** signal decreased but was not completely abolished when challenged with either Cdy and ActD individually or together. These data suggest that analogues **1** and **2** were predominantly incorporated during transcription, whereas analogue 3 was robustly incorporated into RNA by both nascent RNA synthesis and polyadenylation (Figure 3B). We anticipate that such differences could be exploited to track nascent RNA synthesis and poly(A) elongation differentially, solely

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

through analogue selection. This will provide unique tracking capabilities for RNA synthesis inside living cells.

We demonstrated that azide-modified nucleoside analogues can be metabolically incorporated into cellular RNA. We also observed that upon incorporation, our analogues were responsive to bioorthogonal ligations mediated by CuAAC as well as SPAAC, which is amenable to live-cell analysis. Overall, our data support the use of this novel set of azidonucleosides for labeling and monitoring nascent RNA. These analogues are sure to find widespread utility in the RNA community and beyond. Modified nucleosides have thus far been critical toward understanding the rate of RNA synthesis and turnover,[3] characterizing the RNA-binding proteome,[16] and even tracking viral infection.[17] As such, our azide-modified nucleosides are poised to expand the arsenal of methods used to analyze RNA in cells as well as in complex environments such as tissues and living animals.

Expanding the bioorthogonal toolkit for RNA requires further investigation of the limitations and selectivity for modified nucleosides. Our data have demonstrated that there is selectivity in both upstream biosynthesis and downstream synthesis of mature polyadenylated transcripts. The observation that 5-methylazidouridine (**4**) was not incorporated into cellular RNA compelled us to cautiously posit that this difference might arise from the first phosphorylation step in pyrimidine triphosphate synthesis. Indeed, it has been demonstrated with other cytidine analogues that the first phosphorylation step can be rate limiting.[18] A comparison analysis of the adenosine kinase and uracil/cytidine kinase crystal structures revealed that adenosine with modifications at the 2′ and N6 positions should indeed be an amenable substrate. (Figure S12). In contrast, the 5-position of uridine forms a tight van der Waals interaction with the enzyme and is relatively compact. This structural difference might account for the observed selectivity, which presents an exciting opportunity for enzyme engineering to introduce additional modifications into cellular RNA. Such investigations are currently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Spitale Laboratory for critical reading of the manuscript. S.N. is supported as a Vertex Fellow. M.D.K. is an Amgen Fellow. RNA research in the Spitale Lab is supported by startup funds through the University of California, Irvine, a National Institutes of Health (NIH) Director's New Innovator Award (1DP2M119164), and additional NIH funding (1R01MH109588).

References

1. Morris KV, Mattick JS. Nat Rev Genet. 2014; 15:423–437. [PubMed: 24776770]
2. Quinn JJ, Chang HY. Nat Rev Genet. 2016; 17:47–62. [PubMed: 26666209]
3. Rabani M, Levin JZ, Fan L, Adiconis X, Raychowdhury R, Garber M, Gnirke A, Nusbaum C, Hacohen N, Friedman N, Amit I, Regev A. Nat Biotechnol. 2011; 29:436–442. [PubMed: 21516085]
4. Duffy EE, Rutenberg-Schoenberg M, Stark CD, Kitchen RR, Gerstein MB, Simon MD. Mol Cell. 2015; 59:858–866. [PubMed: 26340425]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

5. Jao CY, Salic A. Proc Natl Acad Sci USA. 2008; 105:15779–15784. [PubMed: 18840688]
6. a) Fauster K, Hartl M, Santner T, Aigner M, Kreutz C, Bister K, Ennifar E, Micura R. ACS Chem Biol. 2012; 7:581–589. [PubMed: 22273279] b) Santner T, Hartl M, Bister K, Micura R. Bioconjugate Chem. 2014; 25:188–195.
7. a) Grammel M, Hang H, Conrad NK. ChemBioChem. 2012; 13:1112–1115. [PubMed: 22513998] b) Curanovic D, Cohen M, Singh I, Slagle CE, Leslie CS, Jaffrey SR. Nat Chem Biol. 2013; 9:671–673. [PubMed: 23995769] c) Zheng Y, Beal PA. Bioorg Med Chem Lett. 2016; 26:1799–1802. [PubMed: 26927424]
8. a) Paredes E, Das SR. ChemBioChem. 2011; 12:125–131. [PubMed: 21132831] b) Winz ML, Samanta A, Benzinger D, Jaschke A. Nucleic Acids Res. 2012; 40:e78. [PubMed: 22344697]
9. a) Feng H, Zhang X, Zhang C. Nat Commun. 2015; 6:7816. [PubMed: 26234653] b) Gallego Romero I, Pai AA, Tung J, Gilad Y. BMC Biol. 2014; 12:42. [PubMed: 24885439]
10. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. Angew Chem Int Ed. 2002; 41:2596–2599.Angew Chem. 2002; 114:2708–2711.
11. Agard NJ, Prescher JA, Bertozzi CR. J Am Chem Soc. 2004; 126:15046–15047. [PubMed: 15547999]
12. Saxon E, Bertozzi CR. Science. 2000; 287:2007–2010. [PubMed: 10720325]
13. Dieterich DC, Link AJ, Graumann J, Tirrell DA, Schuman EM. Proc Natl Acad Sci USA. 2006; 103:9482–9487. [PubMed: 16769897]
14. a) Sun H, Jiang S, Caton-Williams J, Liu H, Huang Z. RNA. 2013; 19:1309–1314. [PubMed: 23887148] b) Samanta A, Krause A, Jaschke A. Chem Commun. 2014; 50:1313–1316.c) Rao H, Tanpure AA, Sawant AA, Srivatsan SG. Nat Protoc. 2012; 7:1097–1112. [PubMed: 22576108]
15. Martin G, Keller W, Doublie S. EMBO J. 2000; 19:4193–4203. [PubMed: 10944102]
16. Castello A, Horos R, Strein C, Fischer B, Eichelbaum K, Steinmetz LM, Krijgsveld J, Hentze MW. Nat Protoc. 2013; 8:491–500. [PubMed: 23411631]
17. Neef AB, Pernot L, Schreier VN, Scapozza L, Luedtke NW. Angew Chem Int Ed. 2015; 54:7911–7914.Angew Chem. 2015; 127:8022–8025.
18. Golitsina NL, Danehy FT Jr, Fellows R, Cretton-Scott E, Standring DN. Antiviral Res. 2010; 85:470–481. [PubMed: 19883694]

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

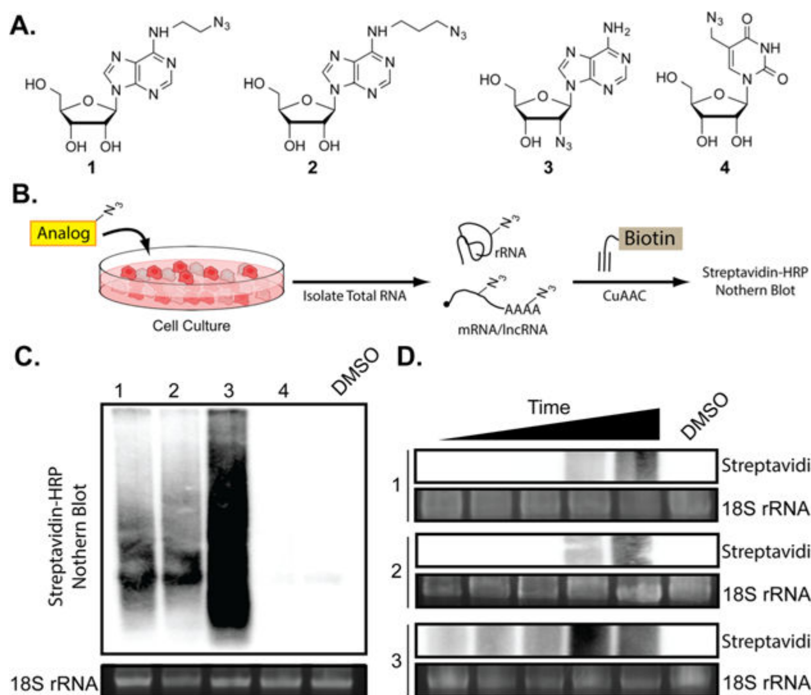


Figure 1.

Screening of azidonucleosides. A) Structures of azidonucleosides used in this study. B) Schematic of incubation and RNA processing protocols. C) Northern blot after 12 h. incubation with 1–4 at 1 mm. D) Time titration analysis after 1 mm incubation with 1–3.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

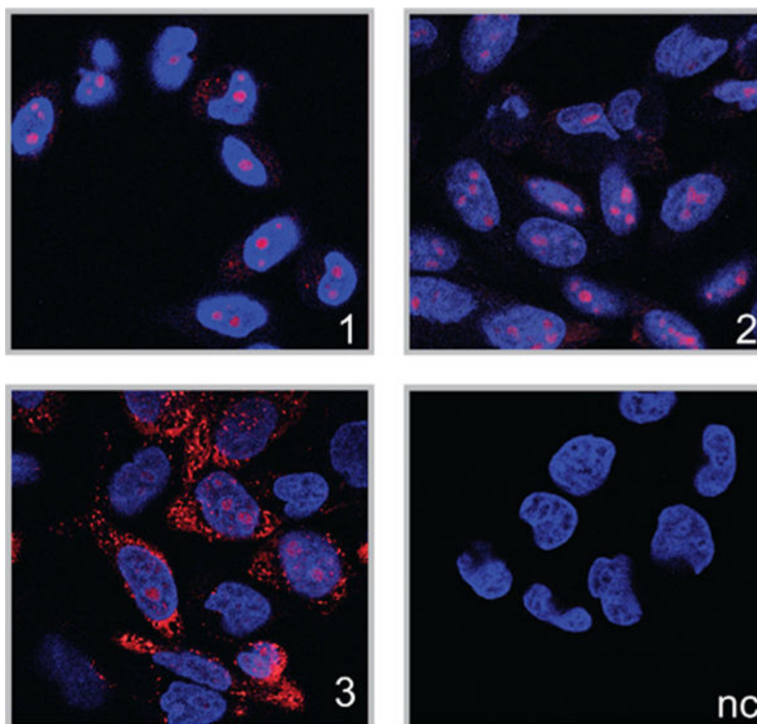


Figure 2.
Incorporation of adenosine analogues into cellular RNA, interrogated by CuAAC fluorescent imaging. Numbering is the same as in Figure 1. nc= negative control, no analogue.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

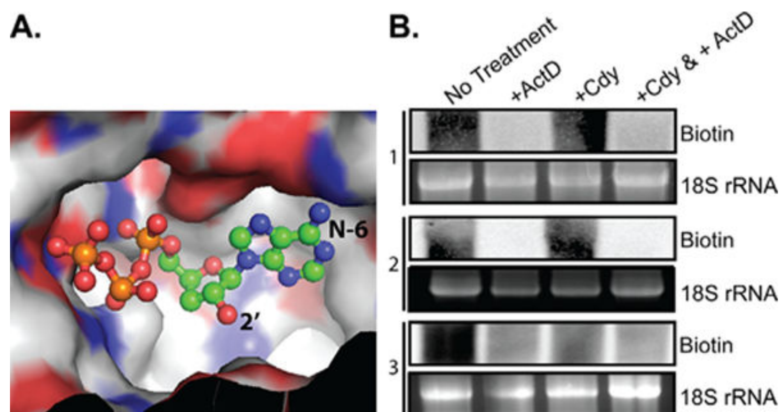


Figure 3.

Nucleoside analogues are differentially incorporated into cellular RNA. A) PDB model of mammalian poly(A) polymerase. B) Northern blot analysis of polymerase inhibition and analogue incorporation. ActD= Actinomycin, Cdy= Cordecypin.