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***N*-Propargyloxycarbamate monosaccharides as metabolic chemical reporters of carbohydrate salvage pathways and protein glycosylation†,‡**

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Abstract

Metabolic chemical reporters of glycosylation allow for the visualization and identification of a variety of glycoconjugates by taking advantage of the promiscuity of carbohydrate metabolism. Here we describe the synthesis and characterization of metabolic chemical reporters bearing an *N*-propargyloxycarbamate (Poc) group that creates discrimination between glycosylation pathways.

An ever expanding repertoire of bioorthogonal reactions has enabled the specific labeling of reporter molecules in a range of biological contexts.^{1–3} In many applications, this two-step detection strategy relies upon the metabolic delivery and subsequent enzymatic installation of a chemical reporter. Emblematic of this technology, metabolic chemical reporters of glycosylation have been in use for over a decade, and contain the founding member of azide-containing reporters an analogue of *N*-acetyl-mannosamine (ManNAc) termed *N*-azidoacetyl-mannosamine (ManNAz), which was developed for the visualization of sialic acid-containing carbohydrates upon reaction with an immuno-tag using the Staudinger ligation.⁴ Inspired by this result, a variety of other metabolic chemical reporters have been developed to target sialic acid modification,^{5–7} mucin O-linked glycosylation,^{8,9} fucosylation,^{7,10} and intracellular O-GlcNAc modification (O-GlcNAcylation).^{11,12} These chemical reporters take advantage of carbohydrate scavenging pathways that convert them into the corresponding nucleotide sugar donors for utilization by glycosyltransferases. Until recently, these metabolic chemical reporters were thought to function largely in a one-input one-output paradigm, where treatment with one chemical reporter would read-out on one type of glycoconjugate (*e.g.* ManNAz treatment results in sialic acid labeling). However, cells are armed with metabolic pathways that can enzymatically interconvert monosaccharides and uridine diphosphate (UDP) sugar donors.¹³ For example, *N*-acetyl-glucosamine (GlcNAc) can be reversibly converted to both *N*-acetyl-galactosamine (GalNAc)¹⁴ and *N*-acetyl-manno-samine (ManNAc).¹⁵ Therefore, it was not entirely surprising when multiple reports demonstrated that the azide-containing chemical reporter, *N*-azidoacetyl-galactosamine (GalNAz), can be transformed to *N*-azidoacetyl-glucosamine

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(GlcNAz), resulting in the labeling of a combination of mucin O-linked, O-GlcNAcylation, and some N-linked glycans.^{12,16,17} While these different types of glycosylation can be separated using biochemical methods, a more ideal metabolic chemical reporter would specifically read-out on only one type of glycosylation. Because each of the carbohydrate scavenging pathways and glycosyltransferases is likely to display unique tolerance to chemically-modified monosaccharides, it should be possible to create chemical reporters that discriminate between glycosylation pathways. In support of this, we previously reported on alkyne-containing chemical reporters, *N*-butynyl-glucosamine (GlcNAIk) and *N*-butynyl-galactosamine (GalNAIk), which are not interconverted, enabling the more selective visualization and identification of O-GlcNAc modified proteins using the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC).¹²

Herein, we continue to examine the chemical tolerance of mammalian glycosylation pathways through the synthesis and characterization of the *N*-propargyloxycarbamate (Poc) containing chemical reporters GlcPoc,¹⁸ GalPoc, and ManPoc¹⁹ (Fig. 1A). Interestingly, we find that each chemical reporter displays unique labelling efficiencies and that qualitatively GlcPoc and GalPoc are incorporated into the same proteins while ManPoc labels a different pattern. Finally, we show that all three chemical reporters allow for the selective enrichment of a known O-GlcNAc modified protein NEDD4, suggesting that they all can be metabolically converted to enter the O-GlcNAcylation pathway.

To test the ability of Poc-modified monosaccharides to transit carbohydrate salvage pathways and serve as substrates for glycosyltransferases, the hydrochloride salts of glucosamine, galactosamine, and mannosamine were reacted with propargylchloroformate. The remaining free hydroxyl groups were subsequently acetylated to give Ac₄GlcPoc, Ac₄GalPoc, and Ac₄ManPoc (Fig. 1A). NIH3T3 cells were metabolically labeled at 200 μ M for 16 hours. The cells were washed, lysed, and the soluble protein fraction was reacted with a previously reported azide-containing rhodamine fluorescent dye (Az-Rho) using CuAAC.⁹ In-gel fluorescent scanning showed labelling of a variety of proteins with all three chemical reporters (Fig. 1B). ManPoc labels cells at the highest level followed by GlcPoc and, finally, GalPoc. Interestingly, GlcPoc and GalPoc qualitatively label the same subset of proteins, while ManPoc enables visualization of a non-overlapping population, suggesting that the Poc group is tolerated to different degrees by all the monosaccharide salvage pathways but discriminated against at the level of the metabolic interconversion and/or glycosyltransferases. To test the concentration dependence of the chemical reporters, NIH3T3 cells were treated with various concentrations of each molecule for 16 hours, followed by lysis, CuAAC with Az-Rho, and analysis by in-gel fluorescent scanning (Fig. S1A, ESI[†]). All three reporters labeled proteins at concentrations as low as 50 μ M.

A key feature of all metabolic chemical reporters is their ability to label a newly-generated protein fraction in a pulse experiment; however, this requires that they are efficiently incorporated on short time-scales. To qualitatively determine the rate of chemical reporter labeling, NIH3T3 cells were treated with Ac₄GlcPoc (150 μ M), Ac₄GalPoc (200 μ M), or

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Ac₄ManPoc (150 μ M) for different amounts of time. Fluorescent modification using CuAAC and in-gel fluorescence demonstrated incorporation of all three chemical reporters in as little as 4 h (Fig. S1B, ESI†), consistent with the labeling times of other reporters including GlcNAz and GlcNAk. Additionally, metabolic chemical reporters can be used to determine the stability of a modification or protein in a pulse-chase format by measuring the decrease in fluorescence signal over time. Accordingly, NIH3T3 cells were treated with Ac₄GlcPoc (150 μ M), Ac₄GalPoc (200 μ M), or Ac₄ManPoc (150 μ M) for 8, 16, and 16 hours, respectively. At these times, the cells were washed with PBS and media containing the corresponding unmodified sugar (Ac₄GlcNAc, Ac₄GalNAc, or Ac₄ManNAc) at 150 μ M was added. Cells were treated for 0–72 hours before analysis by in-gel fluorescence as above (Fig. S1C, ESI†). Interestingly, GlcPoc and GalPoc displayed similar signal decay rates, again suggesting that they label the same protein substrates. In contrast, loss of ManPoc signal was somewhat slower, supporting its incorporation into a different type of glycosylation.

We further analyzed the efficiency of the Poc metabolic chemical reporters using flow-cytometry. Accordingly, NIH3T3 cells were treated with each chemical reporter at 150 μ M concentration for 8 hours. At this time, the cells were fixed with paraformaldehyde (PFA) and permeabilized with Triton X-100 before CuAAC in the presence of Az-Rho. Consistent with our fluorescence scanning, ManPoc showed the highest level of signal, followed by GlcPoc and finally GalPoc (Fig. 2). Again this demonstrates that the Poc group is differentially tolerated by the carbohydrate salvage pathways and/or glycosyltransferases.

Finally, to examine the metabolic interconversion of the Poc-bearing chemical reporters we examined their ability to read out on O-GlcNAc modifications. Accordingly, NIH3T3 cells were treated with Ac₄GlcPoc (150 μ M), Ac₄GalPoc (200 μ M), or Ac₄ManPoc (150 μ M) for 8 h. The corresponding cell-lysates were then reacted under CuAAC conditions with a known azide-containing biotin affinity probe (azido-azo-biotin).¹² Labeled proteins were enriched with streptavidin beads, washed, and eluted with sodium dithionite. Western blot analysis of these enriched proteomes was then performed using an antibody against a known O-GlcNAc substrate NEDD4 (Fig. 3).¹² Interestingly, NEDD4 was enriched by all three chemical reporters, with GlcPoc and ManPoc displaying approximately the same level of enrichment followed by GalPoc, demonstrating that both ManPoc and GalPoc can be metabolically converted to GlcPoc and enter the O-GlcNAc modification pathway.

The continually expanding range of metabolic chemical reporters of glycosylation has enabled the visualization and identification of many types of glycans and underlying protein substrates. We and others have demonstrated that many chemical reporters can be metabolically transformed and therefore label multiple glycosylation pathways. However, given the complete chemical control of these small molecules, it might be possible to structurally bias their metabolic fates to generate more specific reporters. To further test this possibility, we have developed the N-propargyloxycarbonyl (Poc) bearing metabolic chemical reporters GlcPoc, GalPoc, and ManPoc. Comparison of these reporters revealed that they are all incorporated into large numbers of cellular proteins, which can be readily visualized using both in-gel fluorescence scanning and flow cytometry. The pattern of GlcPoc and GalPoc labeled proteins are approximately identical, suggesting that they are

incorporated into the same type of glycosylation. In contrast to the azide-bearing chemical reporter GalNAz when compared to GlcNAz,^{16,20} GalPoc is utilized at a much lower efficiency than GlcPoc, consistent with our previous analysis of the structurally similar alkyne-containing derivative GalNAlk.¹² However, based on the enrichment of NEDD4, GalPoc can be interconverted to GlcPoc, which does not occur with GalNAlk and other chemical reporters with larger substituents at the N-acyl position.^{12,21} This demonstrates that even small alterations to the structure of these chemical reporters can influence their metabolism and final destination. Likewise, ManPoc can be converted to GlcPoc and subsequently participate in O-GlcNAc modification of NEDD4. Interestingly, the level of NEDD4 enrichment is similar in both GlcPoc and ManPoc treated cells. Together with the much higher global levels of ManPoc incorporation and its distinct labeling pattern, we believe that only a minority of ManPoc enters the O-GlcNAcylation pathway, while the majority is transformed to sialic acid, supporting previous *in vitro* experiments.¹⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

N-Propargylcarbamate containing metabolic chemical reporters are incorporated onto proteins. (A) Metabolic chemical reporters Ac₄GlcPoc: *N*-propargylcarbamate-1,3,4,6-tetra-*O*-acetyl-glucosamine, Ac₄GalPoc: *N*-propargylcarbamate-1,3,4,6-tetra-*O*-acetyl-galactosamine, Ac₄ManPoc: *N*-propargylcarbamate-1,3,4,6-tetra-*O*-acetyl-mannosamine. (B) NIH3T3 cells were treated with each chemical reporter (200 μM) for 16 hours before reaction with azido-rhodamine under CuAAC conditions and analysis by in-gel fluorescence. Coomassie blue shows protein loading.

Fig. 2. Flow cytometry analysis of metabolic chemical reporter incorporation. NIH3T3 cells were treated with each chemical reporter (150 μ M) for 16 hours. Cells were then fixed with 3.7% PFA and permeabilized with 0.1% Triton X-100 before reaction with azido-rhodamine under CuAAC conditions and analysis by flow cytometry. Error bars represent S.E.M. of three experiments.

Fig. 3.

Incorporation of metabolic chemical reporters into the O-GlcNAcylation pathway. NIH3T3 cells were treated with each chemical reporter (150 μ M) for 16 hours. Labelled proteins were reacted with azido-azo-biotin using CuAAC before streptavidin enrichment and analysis of O-GlcNAc modification by anti-NEDD4 Western blotting.