

Metabolic Labeling of Sialic Acids in Living Animals with Alkynyl Sugars**

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Sialic acids, a family of monosaccharides widely distributed in higher eukaryotes and certain bacteria, are determinants of many functional glycans that play central roles in numerous physiological and pathological processes.^[1] For example, the sialic acid containing epitope Sia α 2–6Gal serves as the cellular receptor for human influenza A and B viruses during infection,^[2] and linear homopolymers of sialic acids, known as polysialic acid (PSA), modulate the formation of neuronal synapses in mammalian development.^[3] The expression of sialoglycoconjugates, such as sialyl Lewis^x, sialyl Tn (STn), and PSA, is also a common feature shared by numerous cancers.^[4] Interestingly, upregulation of these sialosides is strongly correlated with the transformed phenotype of many cancers.^[5,6] For example, STn, a mucin-associated disaccharide, is not normally found in healthy tissues but is expressed by malignant tumors, including those of the pancreas and breast.^[7,8] In addition, a strong correlation between the level of cell-surface sialic acids and metastatic potential has been observed in several different tumor types.^[9] Thus, as cancer cells generally display higher levels of sialic acid than their nonmalignant counterparts, sialylated glycoconjugates, collectively termed the “sialome”, constitute attractive targets in the search for novel cancer biomarkers.

A variety of methods have been reported for the enrichment and identification of sialylated glycoproteins from bodily fluids or cell lysates. For example, affinity chromatography using sialic acid specific lectins^[10–12] or selective periodate oxidation of sialic acids followed by hydrazide capture^[13] can provide glycoprotein samples that are enriched for sialylated species. These methods have been used for comparative analysis of the steady-state abundances of sialylated glycoproteins in serum from cancer patients and healthy subjects.^[1]

A complementary method that we have developed involves metabolic labeling of sialylated glycoproteins by treating cells or living animals with peracetylated analogues of *N*-acetylmannosamine (ManNAc) bearing chemical reporter groups such as the azide (i.e., peracetylated *N*-azidoacetylmannosamine, Ac₄ManNAz).^[14,15] Ac₄ManNAz is enzymatically deacetylated in the cytosol and then metabolically converted to the corresponding *N*-azidoacetyl sialic acid (SiaNAz), which is subsequently incorporated into sialoglycoconjugates.^[14,16] Once presented on the cell surface, the azide-labeled sialylated glycans can be visualized or captured for glycoproteomic analysis with a variety of reagents,^[17] including Staudinger ligation phosphines,^[14] terminal alkynes along with reagents for Cu^I-catalyzed azide–alkyne cycloaddition (CuAAC),^[18,19] or strained alkynes.^[20] Wong and co-workers reversed the polarity of the reagents, using an alkynyl ManNAc derivative for metabolic labeling of cultured cells and CuAAC-mediated reaction with an azide-functionalized probe for capture of sialylated glycoproteins.^[21,22]

The metabolic/chemical labeling method holds several advantages over previous approaches to sialylated glycoprotein analysis. First, metabolic labeling selects for those glycoproteins that are biosynthesized at high levels, irrespective of their steady-state abundance. Thus, metabolic labeling may reveal novel sialylated biomarkers that are turned over rapidly and therefore missed by steady-state labeling methods. Second, metabolic labeling can be performed in live animals,^[15] permitting the selective tagging of sialylated glycoconjugates within their native tissue environments. However, the efficiency of sialic acid labeling using Ac₄ManNAz is fairly low in vivo. Mouse heart tissue glycoproteins incorporate SiaNAz at approximately 3% of total sialic acid, and the azidosugar is undetectable in some organs that are known to possess sialylated glycoconjugates.^[15]

The efficiency of sialic acid biosynthesis is very sensitive to the *N*-acyl structure of nonnatural ManNAc analogues.^[23,24] Analogues with long or branched *N*-acyl chains are poor substrates for the biosynthetic enzymes, while those contain-

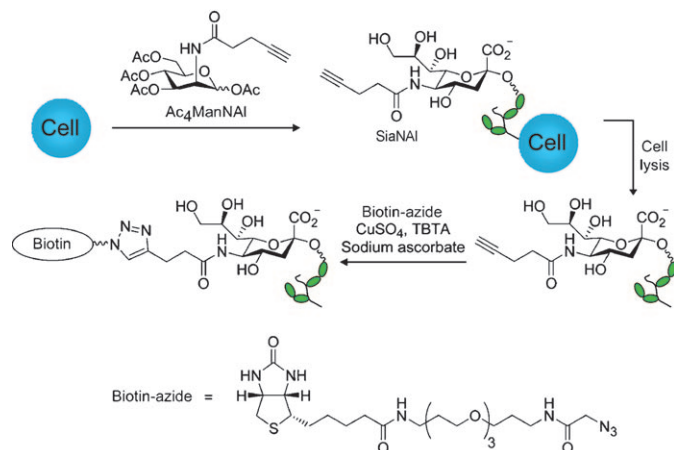
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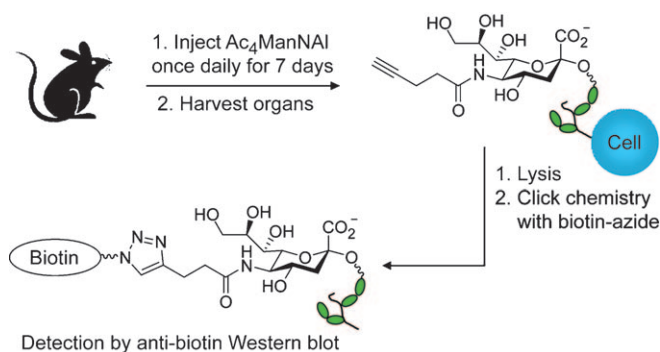
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ing short, linear side chains are better tolerated.^[24] Thus, we were curious how the alkynyl ManNAc analogue reported by Wong and co-workers^[22] would fare in live animal metabolic labeling studies compared to ManNAz. Toward this goal, we synthesized peracetylated *N*-(4-pentynoyl)mannosamine (Ac_4ManNAI , Scheme 1) and confirmed its metabolic con-





Scheme 2. Experimental overview for probing Ac_4ManNAI metabolism in vivo. Wild-type B6D2F1/J mice were injected with Ac_4ManNAI or vehicle intraperitoneally once daily for seven days. On the eighth day, the organs were collected and homogenized, and organ lysates were probed using CuAAC chemistry for the presence of alkyne-bearing glycoproteins by reaction with biotin-azide, followed by Western blot analysis using an HRP-conjugated anti-biotin antibody.

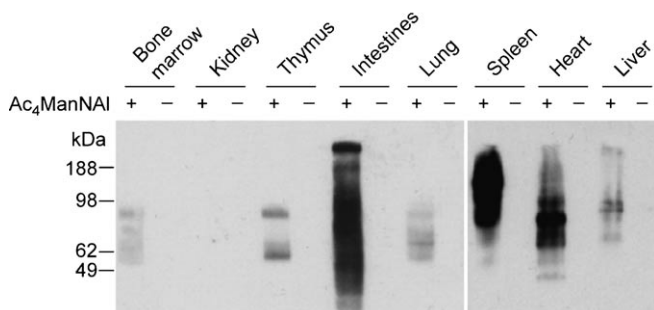


Figure 2. Western blot analysis of tissue lysates from B6D2F1/J mice administered Ac_4ManNAI (+) or vehicle (-). Mice were injected with Ac_4ManNAI (300 mg kg^{-1}) or vehicle once daily for seven days. On the eighth day, the organs were harvested and homogenized. The lysates were then reacted with biotin-azide ($100 \mu\text{M}$) in the presence CuSO_4 (1 mM), sodium ascorbate (1 mM), and TBTA ($100 \mu\text{M}$) for 1 h at room temperature and analyzed by Western blot using an HRP-conjugated anti-biotin antibody. Shown are representative data from three replicate experiments. Total protein loading was confirmed by Coomassie Blue staining of a duplicate protein gel (data not shown).

stronger labeling in organ lysates than Ac_4ManNAz treatment (Figure 3). Based on quantification by densitometry, we estimate that the labeling using ManNAI is at least 25% greater than that using ManNAz (see the Supporting Information). However, estimating metabolic incorporation based on these data is difficult because CuAAC displays different reaction kinetics depending on whether the limiting reagent is the alkyne or the azide.^[26] Given that the reaction kinetics are two to three times faster in the latter case, we believe our estimate based on densitometry to be a lower limit.

In summary, we have demonstrated that Ac_4ManNAI can metabolically label sialic acids in cultured cells and mice with greater efficiency than Ac_4ManNAz . The alkynyl sugar may therefore be useful in the discovery of sialylated cancer biomarkers using murine cancer models. Moreover, these results underscore the sensitivity of sialic acid biosynthetic enzymes to subtle differences in the *N*-acyl structures of the two ManNAc analogues. Accordingly, further structural

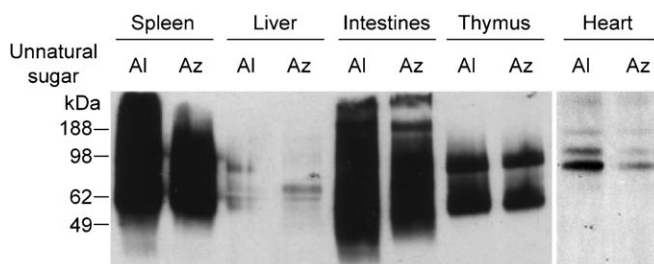


Figure 3. Ac_4ManNAI is converted into the corresponding sialic acid more efficiently than Ac_4ManNAz in mouse organs. A panel of organ lysates from mice treated with Ac_4ManNAI (Al) or Ac_4ManNAz (Az) (300 mg kg^{-1}) once daily for seven days were reacted with $100 \mu\text{M}$ biotin-azide or biotin-alkyne,^[20] respectively, in the presence CuSO_4 (1 mM), sodium ascorbate (1 mM), and TBTA ($100 \mu\text{M}$) for 1 h at room temperature and analyzed by Western blot using an HRP-conjugated anti-biotin antibody. Shown are representative data from three replicate experiments. Total protein loading was confirmed by Coomassie Blue staining of a duplicate protein gel (data not shown).

modulation of alkynyl and azido ManNAc analogues is worth pursuing in order to further increase metabolic labeling efficiency in vivo.

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