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A Strategy for the Selective Imaging of Glycans Using Caged Metabolic Precursors

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Abstract: Glycans can be imaged by metabolic labeling with azidosugars followed by chemical reaction with imaging probes; however, tissue-specific labeling is difficult to achieve. Here we describe a strategy for the use of a caged metabolic precursor that is activated for cellular metabolism by enzymatic cleavage. An *N*-azidoacetylmannosamine derivative caged with a peptide substrate for the prostate-specific antigen (PSA) protease was converted to cell-surface azido sialic acids in a PSA-dependent manner. The approach has applications in tissue-selective imaging of glycans for clinical and basic research purposes.

A fundamental challenge in molecular imaging is the design of reagents that report on cell- or tissue-specific molecular processes. A popular approach to achieving such selectivity is the design of caged probes that are activated by enzymes produced by the target cells, a prototypical example being cancer-associated proteases.[1] Alternatively, probes can be conjugated to targeting elements that bind, noncovalently or covalently, to cell-surface markers.[2] We have been exploring this latter approach in the context of imaging cell-surface glycans. These biopolymers, collectively termed the glycome, change in structure and expression during development and cancer progression as well as many other physiological processes.[3] We previously reported that broad sectors of the glycome can be imaged in model organisms using a two-step procedure: (1) metabolic labeling of the glycans with azidosugar precursors and (2) chemical reaction of azide-labeled cell-surface glycans *in vivo* via Cu-free click chemistry with difluorinated cyclooctyne (DIFO) probes.[4]

The tissue selectivity of this approach is limited by the breadth of glycans targeted by a single metabolic precursor. For example, treatment of mice with peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz) leads to widespread labeling of glycans with *N*-azidoacetyl sialic acid (SiaNAz) in numerous tissues (e.g., liver, heart, kidney, and intestines) as well as serum glycoproteins.[5] The broad distribution of the azidosugar can undermine experiments that seek to probe glycomic changes in a single organ as a function of time or disease. One means to achieve tissue selectivity in glycan imaging is to restrict azidosugar metabolism to the cells of interest. Tissue-specific enzyme activities such as those mentioned above might be exploited to achieve this goal. Here, we describe a strategy for cell-selective glycan imaging using a caged metabolic precursor that is activated by a protease (Figure 1A).

We synthesized a variant of Ac_4ManNAz in which the 6-hydroxy group was conjugated through a self-immolating linker to a peptide substrate for the prostate-specific antigen (PSA) protease (**1**, Figure 1B). PSA is a serine protease that is secreted at low levels by normal prostatic glandular cells but is highly upregulated by prostate cancer

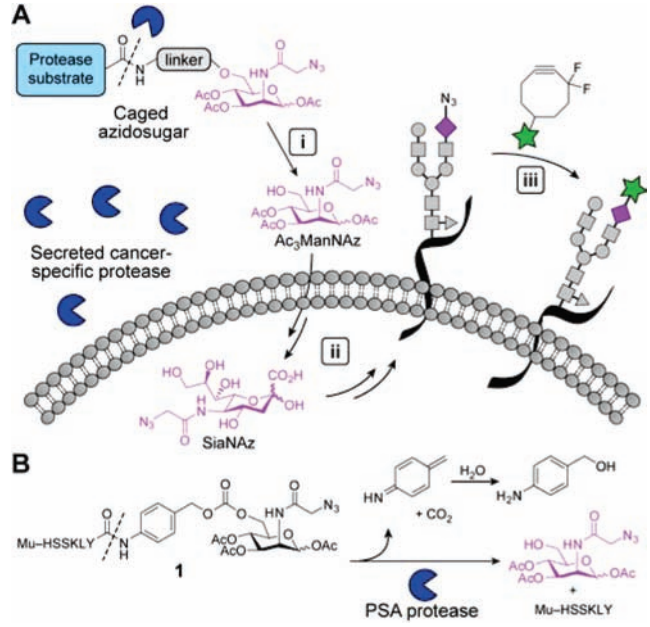

Figure 1. Strategy for tissue-specific release of Ac_3ManNAz via enzymatic activation. (A) (i) A nonmetabolizable caged azidosugar serves as a substrate for a secreted, cancer-specific protease, releasing Ac_3ManNAz. (ii) This azidosugar is then metabolized by the cell and incorporated into cell-surface glycans. (iii) The azide-labeled glycans are detected via Cu-free click chemistry using DIFO reagents. (B) Caged azidosugar used in this study (**1**). Cleavage of the indicated bond (dashed line) by the prostate-specific antigen (PSA) protease results in the release of a linker, the peptide, and Ac_3ManNAz.

cells.[6] The enzyme has been widely targeted with caged drugs and imaging reagents in cell culture and in animal models.[6] The hexapeptide Mu-HSSKLY (Mu = morpholino ureidyl) was chosen as a PSA substrate based on reports of its high selectivity for this enzyme over other ubiquitous serine proteases.[6c,7] Upon cleavage of the peptide, the *p*-aminobenzyl alcohol (PABA) linker in **1** spontaneously fragments to release 1,3,4-tri-*O*-acetyl-*N*-azidoacetylmannosamine (Ac_3ManNAz), carbon dioxide, and an iminoquinone methide intermediate that is subsequently quenched by water (Figure 1B).[8] This process is known to occur rapidly and should therefore limit the diffusion of the released metabolic substrate from its target cell (for discussion see Figure S1 legend). Compound **1** was synthesized as a mixture of sugar anomers analogously to the route developed by Jones et al. (Scheme S1).[6c] Given the importance of hydroxy group acylation for the cellular uptake of Ac_4ManNAz,[9] we verified that Chinese hamster ovary (CHO) and prostate cancer (PC-3) cells incubated with Ac_3ManNAz could produce SiaNAz residues in their cell-surface glycans (Figure S1).

To confirm that **1** can serve as a substrate for PSA, the compound was incubated *in vitro* with active enzyme or, as negative controls, buffer only or heat-killed (HK) PSA. These enzymatic reactions were analyzed by reversed-phase HPLC and mass spectrometry. Incubation

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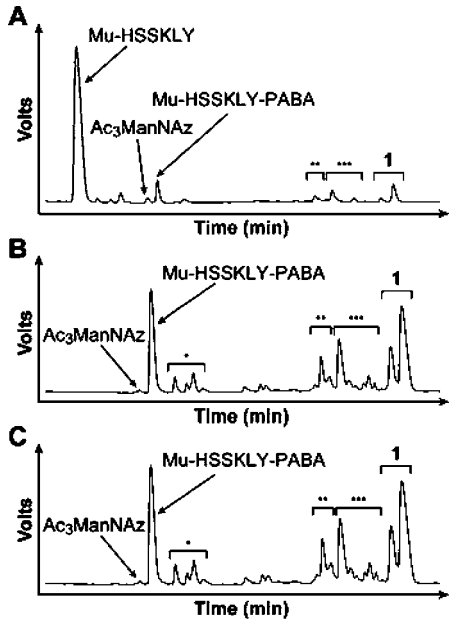

Figure 2. Compound **1** is a substrate for PSA *in vitro*. Shown are HPLC traces of *in vitro* 6 h enzymatic reactions of **1** (500 μM) in 50 mM Tris, 0.1 M NaCl, pH 7.8, with (A) active PSA (50 μg/mL), (B) buffer only, or (C) HK PSA (50 μg/mL). The identities of the various species based on mass spectrometry are indicated on the traces. *Monoacetylated Mu-HSSKLY-PABA, **Monodeacetylated **1**, and ***Isomers of **1**.

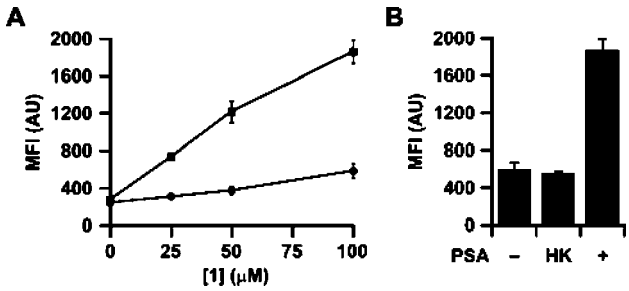

Figure 3. Cell-selective metabolic labeling of glycans using **1** and PSA. Flow cytometry analysis of CHO cells treated with (A) various concentrations of **1** (0−100 μM) and PSA (50 μg/mL, squares) or buffer only (circles) or (B) **1** (100 μM) and either buffer only (−), HK PSA (50 μg/mL, HK), or PSA (50 μg/mL, +). Cells were then labeled with DIFO−biotin (100 μM) and FITC-avidin. Error bars represent the standard deviation from the mean of three replicate samples. MFI = mean fluorescence intensity in arbitrary units (AU).

of **1** with PSA resulted in the release of Mu-HSSKLY as the major peptide product, along with Ac_3ManNAz (Figure 2A). Minor products were also observed. These included Mu-HSSKLY-PABA, presumably produced by nonenzymatic carbonate hydrolysis, as well as monodeacetylated **1** and products formed by migration of acetyl groups within **1**. Control reactions lacking PSA (Figure 2B) or with HK PSA (Figure 2C) produced only nonenzymatic hydrolysis and acetyl migration products.

We next tested the performance of **1** as a caged substrate for metabolic glycan labeling. CHO cells were incubated with **1** at various concentrations (0−100 μM) in the presence of PSA, no enzyme, or HK PSA for 12 h at 37 °C. The cells were then washed and labeled with a DIFO−biotin conjugate,[4b] incubated with fluorescein isothiocyanate-labeled avidin (FITC-avidin), and analyzed by flow cytometry. We observed labeling that was both PSA- and substrate concentration-dependent, suggesting that the signal is due to enzymatic activation of **1** (Figure 3A). In a separate experiment, we demonstrated that the labeling intensity correlates with PSA concentration (Figure S2). Additionally, we verified that treatment of PC-3 cells with **1** resulted

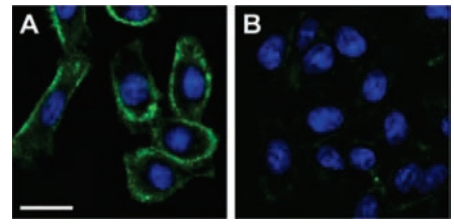

Figure 4. Selective imaging of cells using **1** in the presence of PSA. Fluorescence microscopy analysis of CHO cells treated with **1** (100 μM) and (A) PSA (50 μg/mL) or (B) HK PSA (50 μg/mL), followed by DIFO−biotin (100 μM) and a quantum dot 605−streptavidin conjugate. Green = Texas Red channel; Blue = DAPI channel. Scale bar = 20 μm.

in PSA-dependent metabolic labeling (Figure S3). In the absence of PSA or with HK PSA, both CHO and PC-3 cells exhibited modest background labeling that likely reflects low levels of Ac_3ManNAz produced by nonenzymatic carbonate hydrolysis (Figures 3B and S3). Importantly, we verified that **1** did not cause any cytotoxicity by incubating CHO cells labeled as above with phycoerythrin-conjugated annexin V, a marker of apoptosis (Figure S4).

Finally, we tested **1** as an enzyme-activatable metabolic substrate for glycan imaging. CHO cells were incubated with **1** in the presence of PSA or HK PSA for 12 h at 37 °C. The cells were then washed and labeled with DIFO−biotin, followed by quantum-dot-conjugated streptavidin. We observed substantial cell-surface labeling of cells treated with **1** and PSA (Figure 4A) and minimal fluorescence on cells treated with **1** and HK PSA (Figure 4B).

In conclusion, we have developed a strategy for targeted metabolism of azidosugars using an enzymatically activated substrate. While we chose PSA to demonstrate proof-of-concept, it should be noted that the concentrations of PSA employed in our studies are physiologically relevant; i.e., they are similar to the levels of PSA secreted by both prostate cancer xenografts in mice and prostate tumor tissue obtained from human patients.[10] In addition, many cancers, including prostate cancer, are known to express elevated levels of sialic acid compared to surrounding tissue.[11] Thus, clinical imaging applications may be worth pursuing. More generally, however, the approach has promise for use in tissue-specific glycan imaging, a major future direction.

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Supporting Information Available: Synthetic procedures and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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