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An O-GlcNAcase-Specific Inhibitor and Substrate Engineered by the Extension of the *N*-Acetyl Moiety

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The control of post-translational modifications of nuclear and cytoplasmic proteins provides a means of influencing numerous cellular events and the potential for the management of various human diseases. A major post-translational cycle is the O-linked addition of *N*-acetylglucosamine (O-GlcNAc) by O-GlcNAc transferase (EC 2.4.1.94) (analogous to phosphate addition by the various kinases) and O-GlcNAc removal (analogous to phosphate removal by the various phosphatases) by a family of enzymes, including hexosaminidase A, hexosaminidase B (HEX A and HEX B, commonly referred to as the β-hexosaminidases), and O-GlcNAcase (EC 3.2.1.52).[1,2] The potent and selective manipulation of these post-translational events has, to date, received little attention relative to the vast interest in small molecule activators and inhibitors of kinases and phosphatases. The resulting shortage in useful biochemical tools is unfortunate given the importance of the apparent functional interplay between O-GlcNAc and O-phosphate.[3] The O-GlcNAc modification is emerging as an important factor in cellular regulation,[4] signal transduction,[5] protein structure,[6] and as one of the etiological determinants associated with insulin resistance and type II diabetes.[7]

The search for small molecule modulators of O-GlcNAc transferase and O-GlcNAcase has not been fully wanting. A recent report by the Walker laboratory has detailed the discovery of several O-GlcNAc transferase inhibitors.[8] In addition, there are a small number of known inhibitors of O-GlcNAcase, including *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) (**1**)[9] and a series of NAG-thiazolines[10] recently reported by Vocadlo and co-workers. The small number of well characterized modulators of these two important enzymes is largely due to the lack of high-throughput assays aimed at the discovery of novel small molecules with potent and specific activity at either O-GlcNAc transferase or O-GlcNAcase. The Walker laboratory has partially overcome this dilemma by developing a high-throughput donor displacement assay for O-GlcNAc transferase activity.[8] Further, we have recently reported a novel fluorogenic substrate (**3**) for the high-throughput characterization of O-GlcNAcase activity.[11]

Small molecules, such as PUGNAc, represent important advancements in the ability to dissect the roles of the O-GlcNAc modifications. The development of novel high-throughput methods will undoubtedly provide additional small molecular tools and pharmacological tools of higher quality. This is important as major obstacles exist within the use of current small molecule inhibitors to delineate the observed phenotypes associated with O-GlcNAcase down-regulation. Specifically, questions arise regarding the specificity of these small molecule inhibitors due to their comparable inhibition of O-GlcNAcase, HEX A, and HEX B. For instance, it has been well described that PUGNAc (**1**) alters O-GlcNAc modifications of proteins within the insulin signaling cascade and induces insulin resistance in fat cells.[12] However, one concern is that O-GlcNAcase inhibition cannot be held entirely responsible for this phenotype given that PUGNAc has the capacity to inhibit the related HEX A and HEX B. Furthermore, the inability to detect the activity of O-GlcNAcase apart from other endogenous hexosaminidases represents a key limitation to the use of the fluorogenic substrate (**3**).

The frequently ignored issue of selectivity of small molecule tools is becoming better understood. Many recent reports detail examples of phenotype disparity between small molecule inhibitors and the genetic knockouts of the same target.[13] With this in mind, we set out to re-engineer both PUGNAc (**1**) and the fluorogenic substrate **3** to generate improved specificity toward O-GlcNAcase.

The recent report by Vocadlo and co-workers describing the utility of NAG-thiazolines as potent inhibitors of O-GlcNAcase was based upon the realization that O-GlcNAcase utilizes a substrate-assisted mechanism of action.[10] These inhibitors contain a 2-alkyl-4,5-dihydrothiazole ring that mimics the biochemically relevant intermediate in such an enzymatic mechanism. Analysis of the NAG-thiazoline derivative with a 2-methyl-4,5-dihydrothiazole moiety showed that there was no observed selectivity between O-GlcNAcase and β-hexosaminidase inhibition. However, the extension of the alkyl moiety progressively increased the selectivity in favor of O-GlcNAcase inhibition up to the linear 4-carbon butyl chain. We hypothesized that the equivalent extension of the *N*-acetyl group of PUGNAc (**1**) to a novel pentanamide derivative (**2**) and expansion of the same moiety of the fluorogenic substrate (**3**) to the analogous pentanamide derivative (**4**) would provide a comparable enhancement in selectivity.

The synthesis of **2** was accomplished via the original pathway developed by Vasella and co-workers.[14] Purification by HPLC provided only the biochemically relevant Z oxime based upon NMR comparison of relevant protons to a series of Z PUGNAc derivatives. The synthesis of **4** was accomplished in accordance with our published method.[11] HPLC purification of **4** was performed prior to biochemical evaluation.

The analysis of **2** was accomplished using previously reported methods.[9,11] For the determination of the inhibitory selectivity of both PUGNAc (**1**) and **2** at O-GlcNAcase, HEX A, and HEX B, we utilized the nonselective fluorogenic substrate **3**. The level of inhibition was determined based upon the quantification of fluorescence measured in the absence and presence of both PUGNAc (**1**) and **2** (intensity of fluorescence was measured at $\lambda_{ex} = 485$ nm and at $\lambda_{em} = 535$ nm). The results are compiled in Figure 2. The analysis of **4** as a highly specific substrate for O-GlcNAcase activity was performed by the parallel treatment of O-GlcNAcase and HEX A with varying concentrations of **4** over a 45 min incubation period and the quantification of the resulting fluorescence. The results are compiled in Figure 3.

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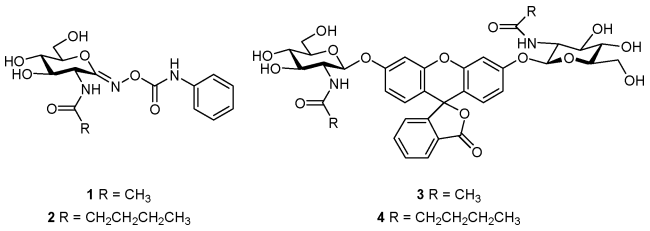

Figure 1. The structures of PUGNAc (**1**), pentanamide PUGNAc derivative (**2**), fluorogenic substrate (**3**), and pentanamide fluorogenic substrate (**4**).

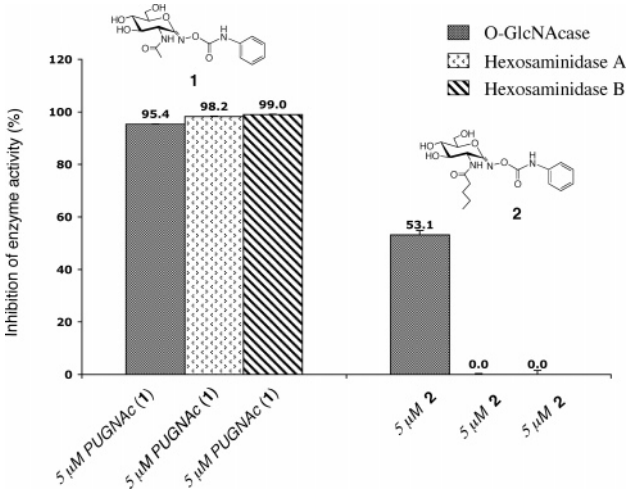

Figure 2. Analysis of inhibition of O-GlcNAcase, HEX A, and HEX B by PUGNAc (**1**) and pentanamide PUGNAc derivative (**2**).

PUGNAc was confirmed to potently inhibit all three enzymes, and it is apparent that the addition of the elongated butyl chain on the *N*-acyl moiety of **2** slightly decreases the inhibitory potency of **2** toward O-GlcNAcase. More compelling, however, is the total loss of inhibitory activity by **2** at both HEX A and HEX B. This high degree of selectivity was verified against HEX A up to 30 μM.[15] The extension of the *N*-acetyl moiety to a butyl chain was found to also confer selective O-GlcNAcase recognition of the fluorogenic substrate **4**. As per the schematic description in Figure 3, cleavage of one (or both) pentanamide sugar moiety of **4** by O-GlcNAcase will allow for ring opening of the fluorogenic substrate and result in a quantifiable fluorescence event. A strong fluorescent signal is observed with O-GlcNAcase glycosidic cleavage of **4**, whereas there is no apparent hydrolysis of **4** by HEX A up to and including exaggerated concentrations (400 μM).

These novel molecular tools represent a significant advance in appraising the role of O-GlcNAcase within cellular and whole organism functions. PUGNAc analogue **2** provides a powerful reagent for the delineation of the complex phenotype associated with the selective inhibition of O-GlcNAcase. The novel O-GlcNAcase-specific fluorogenic substrate **4** is a valuable new tool for the high-throughput analysis of O-GlcNAcase within cellular

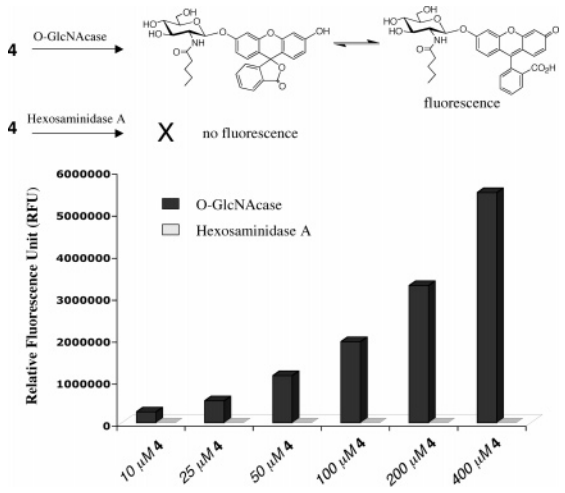

Figure 3. Schematic representation of fluorescence event following glycosidic bond cleavage, and analysis of relative fluorescence response by treatment of O-GlcNAcase and HEX A with pentanamide fluorogenic substrate (**4**).

assays and promises to be a novel imaging agent for the in vivo analysis of O-GlcNAcase function.

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Supporting Information Available: Experimental procedures and spectroscopic data for compounds **2** and **4** and synthetic intermediates, as well as procedural requirements for the expression and purification of recombinant O-GlcNAcase and enzyme experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) Analyses of **1** and **2** at O-GlcNAcase and HEX A were performed at each enzymes' optimal pH and additionally across a pH gradient to ensure the observed selectivity was not pH dependent (see Supporting Information)

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