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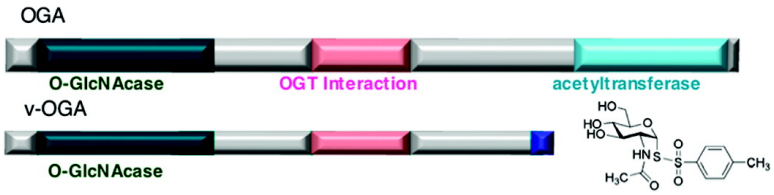

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Distinctive Inhibition of *O*-GlcNAcase Isoforms by an α-GlcNAc Thiolsulfonate

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Dynamic modification of cytoplasmic and nuclear proteins by O-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl (*O*-GlcNAc) residues is an important signaling mechanism that shares characteristics with phosphorylation.[1] "*O*-GlcNAc-ylation" functions in diverse cellular processes including nutrient sensing,[2] modifying protein target activity,[3] and controlling gene expression[4] and protein degradation.[5] Unusual patterns of *O*-GlcNAc-ylation have been linked to insulin resistance[6] and to several neurodegenerative diseases.[7] *O*-GlcNAcase (OGA) promotes *O*-GlcNAc removal and thus plays a key role in *O*-GlcNAc metabolism. Human OGA exists as two splice variants:[8] the *long isoform* (hlOGA) gene consists of 16 exons and encodes a protein that has a N-terminus domain homologous to hyaluronidase and a C-terminal region considered to be a histone acetyltransferase (HAT) domain;[9] the *short isoform* (hsOGA) is encoded by an alternatively spliced transcript consisting of only 10 exons and part of intron 10 that contains an alternative stop codon. Short OGA therefore lacks the C-terminal HAT domain and has 15 amino acids at its C-terminus different from those in long OGA. Interestingly, a single nucleotide polymorphism located in the intron 10 of the gene for OGA has been associated with type II diabetes in Mexican Americans.[10] We have recently demonstrated that the short OGA hydrolyzes the β-*O*-linked glycosidic bond of GlcNAc from glycoproteins *in vitro*.[11] A number of mechanistic studies of long OGA and crystallographic studies of homologous bacterial enzymes have demonstrated that the *O*-GlcNAcase active site resides in the N-terminal domain, and that catalysis requires aspartic acids at 174, 175, and 177 as key catalytic residues that enable a substrate-assisted mechanism.[12−14] Given that this domain is also found in short OGA, it is surprising that short OGA is comparatively resistant to previously described potent inhibitors of long OGA and lysosomal hexosaminidases including PUGNAc and NAG-thiazoline (see Supporting Information, S-1). We previously measured the kinetic parameters of both OGA enzymes by using the fluorogenic FDGlcNAc substrate **1**, and found that they behave differently as *O*-GlcNAcase catalysts.[11] We report here the differential inhibition characteristics of a new inhibitor, α-GlcNAc thiolsulfonate **2**, toward the OGA isoforms and the effects of *pseudo*-glycosylation of long OGA by **2** on the properties of the enzyme.

Previously described hexosaminidase inhibitors are much less potent against the short OGA isoform than the longer, HAT domain containing enzyme (see S-1). However, we have found that α-GlcNAc thiolsulfonate **2** (see Supporting Information for its synthesis) is a more effective inhibitor of short OGA than previous potent inhibitors of hexosaminidases (see S-2). Respective inhibition

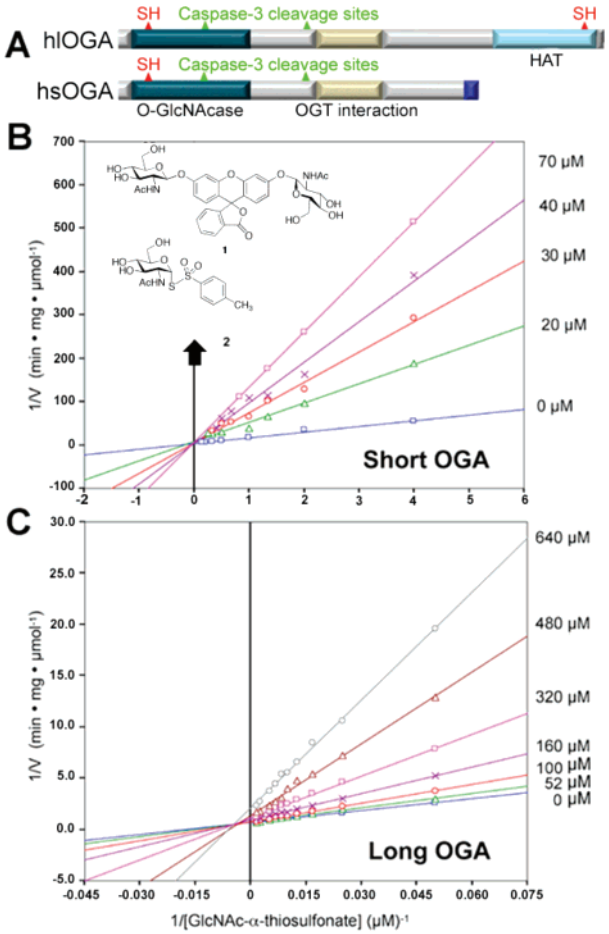

Figure 1. Schematic protein structures of long and short OGA (A) and chemical structures of FDGlcNAc (**1**) and GlcNAc α-thiosulfonate (**2**) and Lineweaver−Burk analyses of short (B) and long OGA (C) inhibition in the presence of increasing concentrations of **2**.

by **2** of long OGA and lysosomal hexosaminidase is about 4-fold and 9-fold less than that of short OGA, indicating some degree of selectivity. A Lineweaver−Burk plot of the initial velocity of FDGlcNAc hydrolysis by both OGA isoforms at varied concentrations of **2** reveals the mode of enzyme inhibition (Figure 1). The inhibitor, thiolsulfonate **2**, acts as a purely competitive inhibitor of short OGA, with $K_i = 10\ \mu M$, indicating that inhibitor (**2**) and FDGlcNAc substrate (**1**) are mutually exclusive in the active site of the enzyme. The presence of **2** changes both the apparent K_m and the apparent V_{max}, indicating a mixed-type inhibition for long OGA. Therefore, **2** can bind competitively with the substrate not only at the N-terminal active site but also at another site apart from the active site of OGA.

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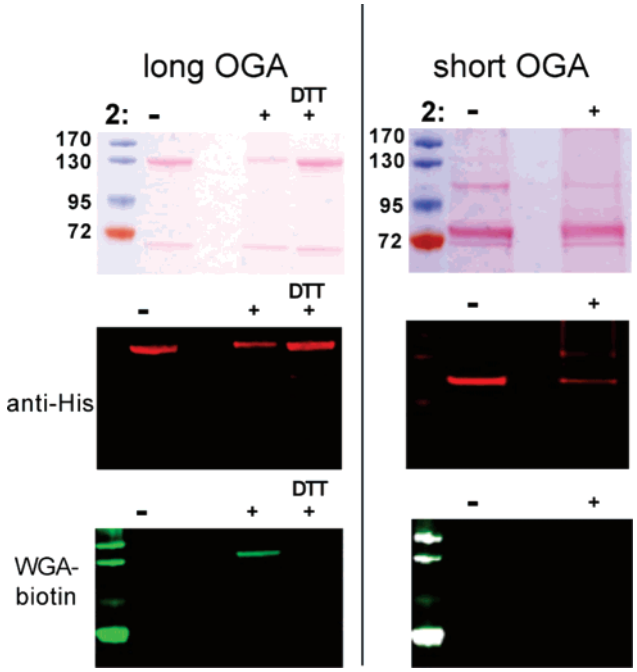

Figure 2. Western blot analyses of long OGA (left) and short OGA (right) treated with (+) and without (−) **2**. Blot was stained by Ponceu S. solution (upper) and probed with anti-His (middle panels) and WGA-biotin/Streptavidin (lower panels). DTT treatment of the modified long OGA reversed its modification (DTT+).

The calculated specific inhibition constant (K_i) and catalytic inhibition constant (K_i') of **2** with respect to long OGA are 35 and 280 μM, respectively. Thus, the mixed inhibition of long OGA results from a combination of competitive inhibition with a K_i similar to that observed for the short isoform and a second nonspecific component. The distinctive kinetic behavior toward *O*-GlcNAcase catalysis of the two OGA isoforms implies that the local environment of the enzyme active site differs. This difference likely results from the presence of the C-terminal HAT domain in long OGA and its absence in short OGA.

When long OGA was incubated with a saturating solution of **2** at 37 °C, a modification of the enzyme occurred: Western blot analysis using the *O*-GlcNAc-specific CTD110.6 antibody (data not shown) and wheat germ agglutinin lectin (WGA) confirmed this to be a covalent attachment of GlcNAc (Figure 2, left panel). Comparable modification did not occur in short OGA (Figure 2, right panel). Thiolsulfonate modification has been reported to occur at cysteine residues through the formation of disulfide linkages.[15,16] Glycosyl thiolsulfonates have been used by Davis and co-workers as reagents for protein pseudo-glycosylation at cysteine.[15,16] The chemical feasibility of disulfide bond formation with **2** and cysteine residues was modeled by conjugating **2** with an N,C-protected cysteine (S-3). We also find that treatment of the modified long OGA with dithiothreitol reverses the modification (Figure 2, left panel), suggesting the presence of a reducible α-GlcNAc−protein disulfide bond. Long OGA is known to have two free cysteines (positions 166 and 878[17]) that are possible modification sites. Indeed, alkylation of free sulfhydryls in cysteine residues of long OGA with *N*-ethylmaleimide completely blocks modification by **2** (S-4). On the basis of the enzyme inhibition studies, the presumed GlcNAc disulfide modification by **2** likely occurs in the C-terminal HAT domain. This region features a free cysteine at 878 that is absent in short OGA.

Strikingly, modification of OGA with thiol-linked GlcNAc abolishes almost all of its enzymatic activity (S-5A). We also note that the amount of effective enzyme decreases as the time of preincubation of long OGA with **2** increases (S-6). Furthermore, thiol-GlcNAc-modified long OGA is completely insensitive to cleavage by caspase 3. Long OGA is known to be a substrate for caspase 3, and its cleavage sites are speculated to reside in the N-terminal and middle domains.[18] Complete caspase 3 resistance of modified long OGA suggests a dramatic change in the conformation or steric properties of the latter, rendering the cleavage sites inaccessible (S-5B). It is presently unclear whether the modification of long OGA with S-GlcNAc occurs while the inhibitor is bound to the active site or occurs independent of the active site binding event; however, our results suggest that a change in the conformation of enzyme upon remote modification drastically alters the active site architecture, resulting in a complete blockage of substrate access. The covalent attachment of an α-*S*-GlcNAc unit to long OGA accounts for the irreversible and time-dependent irreversible inhibition of catalysis.

How might the α-linked GlcNAc thiolsulfonate **2** competitively inhibit an enzyme that specifically processes β-linked substrates? One hypothesis is that **2** assumes the $^1S_3$ twist boat conformation of the bound substrate. In this conformation, the sulfonyl oxygens might be in position to act as H-bond acceptors[19] from the general acid residue and/or other donor residues in the active site (see S-7 for illustration).

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Supporting Information Available: Preparation of **2** and details of the enzyme inhibition assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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