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Metabolic inhibitors of *O*-GlcNAc transferase (OGT) that act *in vivo* implicate decreased *O*-GlcNAc levels in leptin-mediated nutrient sensing.[1]

Tai-Wei Liu,[†] Wesley F. Zandberg,[†] Tracey M. Gloster,[†] Lehua Deng, Kelsey D. Murray, Xiaoyang Shan and David J. Vocadlo*

Abstract: *O*-linked glycosylation of serine and threonine residues of nucleocytoplasmic proteins with N-acetylglucosamine (*O*-GlcNAc) residues is catalyzed by *O*-GlcNAc transferase (OGT). *O*-GlcNAc is conserved within mammals and is implicated in a wide range of physiological processes. Here we describe metabolic precursor inhibitors of OGT suitable for use both in cells and *in vivo* in mice. These 5-thiosugar analogues of N-acetylglucosamine are assimilated through a convergent metabolic pathway, most likely involving N-acetylglucosamine-6-phosphate de-N-acetylase (NAGA), to generate a common OGT inhibitor within cells. Of these inhibitors, we show that 2-deoxy-2-N-hexanamide-5-thio-D-glucopyranoside (5SGlcNHex) acts *in vivo* to induce dose- and time-dependent decreases in *O*-GlcNAc levels in various tissues. Decreased *O*-GlcNAc correlates, both *in vitro* within adipocytes and *in vivo* within mice, with lower levels of transcription factor Sp1 and the satiety-inducing hormone leptin – revealing a link between decreased *O*-GlcNAc levels and nutrient sensing in peripheral tissues of mammals.

β-*O*-linked *N*-acetylglucosamine (*O*-GlcNAc) is a dynamic modification of nucleocytoplasmic proteins that is present in all multicellular eukaryotes.[1] The attachment of GlcNAc (**1**, Figure 1) to hydroxyl groups of serine or threonine residues of hundreds of target proteins is catalyzed by the glycosyltransferase *O*-GlcNAc transferase (OGT), which uses uridine diphospho-*N*-acetylglucosamine (UDP-GlcNAc, **2**) as a donor sugar substrate. The glycosidase *O*-GlcNAcase (OGA) cleaves *O*-GlcNAc off from proteins.

O-GlcNAc is implicated in various diseases including cancer, neurodegeneration, cardiovascular disease, and obesity.[2] Notably, *O*-GlcNAc levels respond to nutrient availability both within cells and *in vivo* in animal models.[1b] Transgenic mice overexpressing OGT in fat or muscle tissue exhibit elevated serum leptin and insulin levels in addition to insulin resistance.[3] Further, deletion of the gene encoding OGT from neurons of the paraventricular nucleus (PVN) within the hypothalamus of mice results in uncontrolled eating.[2b]

Notably, OGA inhibitors that are active *in vivo* have helped gain insights into the roles of increased *O*-GlcNAc levels in mammals. Strikingly, OGA inhibitors sometimes yield different results from those made using genetic approaches to increase *O*-GlcNAc levels[4]; perhaps because OGT and OGA also have non-catalytic roles. Unfortunately, similar studies regarding the roles of decreased *O*-GlcNAc on mammalian physiology are lacking because there are no OGT inhibitors suitable for use *in vivo*. Given the emerging roles of OGT in nutrient sensing and other processes, inhibitors of OGT that can be used as research tools *in vivo* are of high interest.[5]

One approach to decreasing *O*-GlcNAc levels using small molecules has been to use broad-spectrum amidotransferase inhibitors such as 6-diazo-5-oxo-L-norleucine (DON), which promiscuously blocks all amidotransferases that biosynthesize many cellular metabolites including UDP-GlcNAc. High throughput screening has been pursued to deliver hits that can be improved upon.[5b, 5d] These leads, however, show modest cellular activity and limited solubility.[5b] They also exhibit off-target cellular toxicity.[5b] Another approach[5c] has been to generate a GlcNAc analogue, 2-acetamido-2-deoxy-5-thio-α-D-glucopyranose (5SGlcNAc, **3**), which in its per-*O*-acetylated form ($Ac_4$5SGlcNAc, **3**-OAc) can diffuse across the plasma membrane. Within cells, **3**-OAc is de-*O*-acetylated and assimilated via the GlcNAc salvage pathway (Figure 1) to generate UDP-5SGlcNAc (**4**), which is a competitive OGT inhibitor (K_i = 8 μM). $Ac_4$5SGlcNAc (**3**-OAc) lowers cellular *O*-GlcNAc levels (EC_{50} = 0.8 to 5 μM). This metabolic OGT inhibitor is not toxic but suffers from poor aqueous solubility. Indeed, while often used in cell studies,[6] solubilizing **3**-OAc requires high concentrations of DMSO, making it incompatible for dosing of mammals.

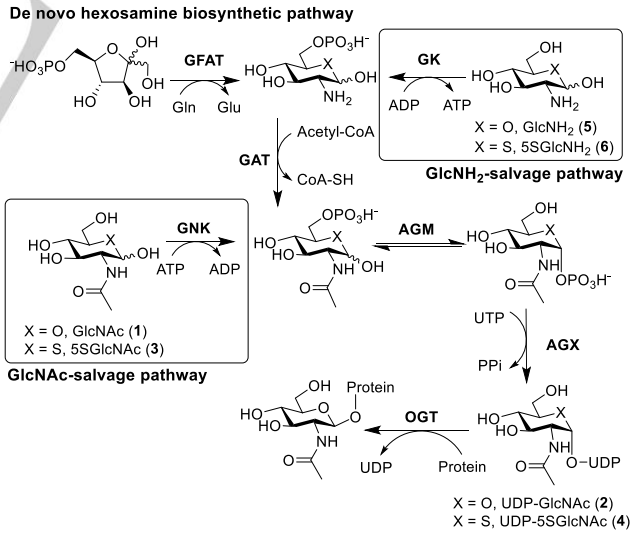

Figure 1. The hexosamine biosynthetic pathway (HBP) and the GlcNAc-salvage pathway yield UDP-GlcNAc (**2**). Glutamine fructose amidotransferase (GFAT) catalyzes the conversion of glucosamine (**5**) to glucosamine-6-phosphate ($GlcNH_2$-6PO_4; **5**-6PO_4). Acetylation of **5**-6PO_4 by glucosamine acetyltransferase (GAT) yields GlcNAc-6PO_4, an intermediate also produced by GlcNAc kinase (GNK) in the GlcNAc-salvage pathway. The sequential action of phosphoglucosamine mutase (AGM) and GlcNAc pyrophosphorylase (AGX) on GlcNAc-6PO_4 leads to the formation of UDP-GlcNAc (**2**). 5SGlcNAc (**3**) can be similarly salvaged and converted into UDP-5SGlcNAc (**4**).

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Accordingly, to explore the roles of decreased *O*-GlcNAc levels in organismal physiology, compounds that act *in vivo* are of high interest.

To generate a tool compound for inhibiting OGT *in vivo*, we synthesized a panel of water soluble analogues of 5SGlcNAc (**3**) possessing various *N*-acyl substituents (**7**–**17**, Figure 2a,b Scheme S1). We reasoned these hydrophobic N-acyl groups would confer a balance between hydrophilicity and lipophilicity, making them water soluble yet able to diffuse into cells. Furthermore, although the substrate specificity of *N*-acetylglucosamine-6-phosphate de-*N*-acetylase (NAGA) is not known,[7] we speculated that compounds **7–17**, once phosphorylated, might be substrates for this recently identified enzyme (Figure 2b). Action of NAGA on phosphorylated **7–17** would lead to formation of a common intermediate, 5-thio-glucosamine-6-phosphate (**5**-6PO_4), which can be assimilated by the hexosamine biosynthetic pathway (HBP). However, direct entry of **7**–**17** into the GlcNAc-salvage pathway cannot be ruled out since the specificity of NAGA is unknown and bulkier *N*-glycolyl (GlcNGc) and *N*-azidoacetyl (GlcNAz) containing analogues enter the HBP.[8]

To measure the potential of **7**–**17** and their per-*O*-acetylated congeners to block OGT activity in cells, we treated cells with these compounds. Analysis of *O*-GlcNAc levels of cell lysates showed only some deprotected analogues were effective in reducing *O*-GlcNAc levels (Figure 2c, Figure S1). Of these the promising 2-hexanamide derivative (5SGlcNHex, **12**) showed dose-dependent decreases in *O*-GlcNAc comparable to $Ac_4$5SGlcNAc (**3**-OAc) (Figure S2).

We next tested whether these compounds were directly activated as UDP-linked analogues or if they were metabolized by NAGA. We therefore analyzed the pool of nucleotide sugars from cells treated with compounds **7**, **8**, and **12** by capillary electrophoresis (CE) (Figure 3). Electropherograms for nucleotide sugars from cells treated with **7**, **8**, and **12** revealed two new peaks that had CE mobilities matching those of the chemoenzymatically-prepared standards UDP-5SGlcNAc (**4**) and its epimer UDP-5SGalNAc (Figure 3a). Extracts from cells treated with per-*O*-acetylated 5SGlcNH_2 (**6**-OAc, Figure S3) also yielded the same two unnatural nucleotide sugars in cells. These data suggest that these 5SGlcNR analogues are processed within cells to the common intermediate 2-amino-2-deoxy-5-thio-glucopyranose 6-phosphate (**6**-PO_4) and then assimilated by the HBP to form UDP-5SGlcNAc (**4**).

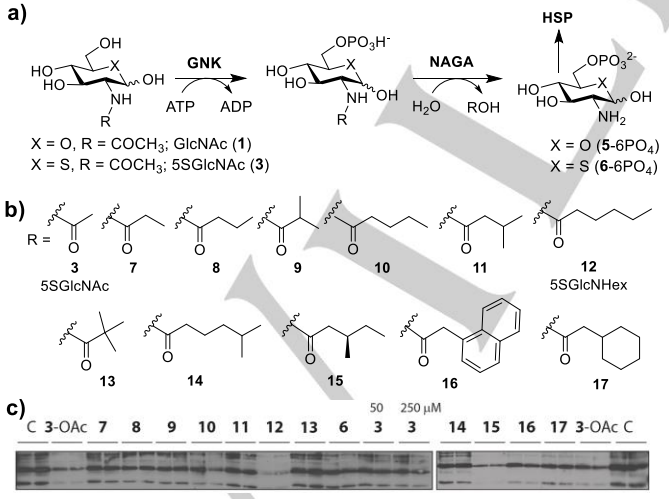

Figure 2. Synthetic analogues of 5SGlcNAc (**3**) are metabolized within cells, leading to decreased *O*-GlcNAc levels in cells. **a)** Possible metabolism of 5SGlcNR analogues (**7** – **17**) by NAGA prior to their entry into the HBP. **b)** Synthetic analogues (**7** – **17**) bearing various *N*-acyl R groups. **c**) Effects of analogues of *O*-GlcNAc levels of HEK293 cells. C = vehicle (PBS alone).

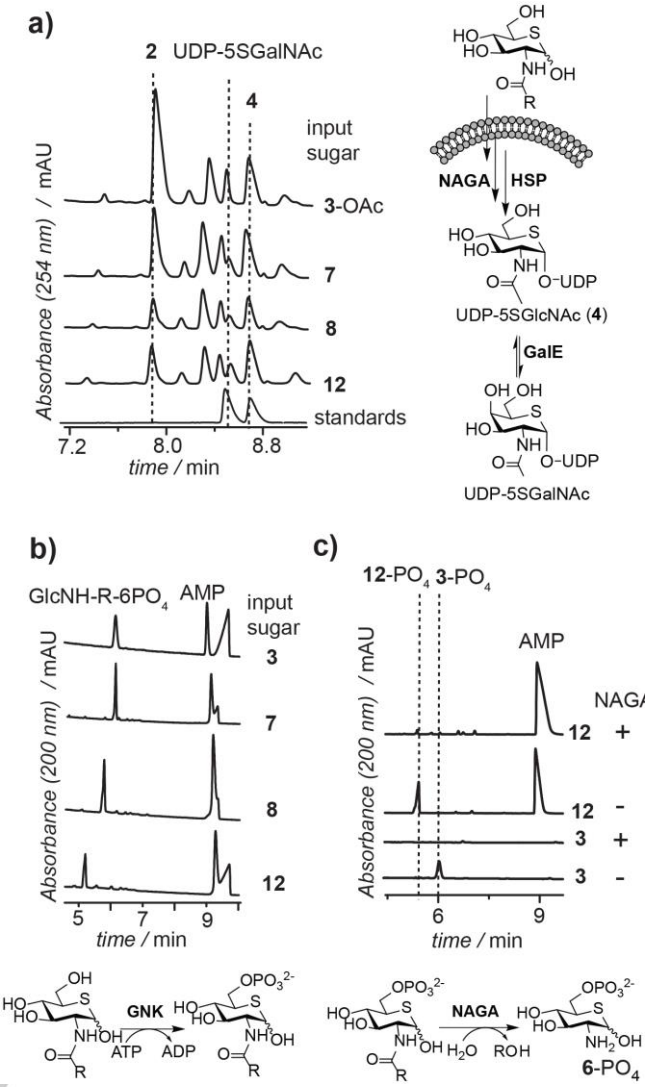

Figure 3. 5SGlcNR analogues are sequentially processed in cells by GlcNAc kinase (GNK) and, likely, *N*-acetylglucosamine-6-phosphate-de-N-acetylase (NAGA) to generate 5SGlcNH_2-6PO_4 (**6**-PO_4). a) Nucleotide sugar analysis of cells treated with selected inhibitors indicated that all the 5SGlcNR analogues tested (**3**-OAc, **7**, **8**, and **12**) were converted within cells into two new nucleotide sugars, indistinguishable from synthetically prepared UDP-5SGlcNAc (**4**) and UDP-5SGalNAc. b) 5SGlcNR analogues were phosphorylated by GNK *in vitro*. c) 5SGlcNR-6-phosphates were not substrates for AGM or AGX but were readily hydrolyzed *in vitro* by NAGA to produce 5SGlcNH2-6PO_4 (**6**-PO_4).

To clarify the metabolic processing of compounds **7**, **8**, and **12**, we used recombinant enzymes of the mammalian HBP (Figure 1). **7**, **8**, and **12** were all phosphorylated by GNK (Figure 3b), yet none of the phosphorylated products were substrates for AGM except for **7**. However, the nucleotide sugar produced *in*

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vitro from **7** by the combined HBP enzymes had a different mobility than that of nucleotide sugar UDP-5SGlcNAc (**4**) that we detected in cells treated with compound **7** (Figure S4). We accordingly tested whether NAGA converts the corresponding 5-thiosugar-6-PO_4 derivatives of **7**, **8**, and **12** into 5SGlcNH_2-6-PO_4 (**6**-6PO_4). We confirmed this scenario by digestion of **12**-PO_4 with recombinant human NAGA. Electropherograms obtained by CE analysis of the processing of **12**-PO_4 by NAGA showed it was converted to 5SGlcNH_2-6-PO_4 (**6**-PO_4) (Figure 3c). These data suggest that 5SGlcNR analogues **7**-**17**, including **7**, **8**, and the most potent derivative 5SGlcNHex (**12**), are phosphorylated within cells by GNK and then deacylated by NAGA, which we found has remarkable substrate tolerance. The resulting common intermediate, 5SGlcNH_2-6-PO_4 (**6**-PO_4), is then assimilated via the HBP to form UDP-5SGlcNAc (**4**), which leads to metabolic inhibition of OGT and decreased *O*-GlcNAc levels in tissues.

Previous efforts to use $Ac_4$5SGlcNAc (**3**-OAc) *in vivo* in mammals failed because of its poor aqueous solubility. We therefore evaluated if these new water-soluble metabolic OGT inhibitors could be used *in vivo*. Accordingly, we dosed mice by intraperitoneal (IP) delivery with our most cell active compound **12**. A concentration-dependent decrease in spleen *O*-GlcNAc levels was observed upon treatment with **12**, with apparent effects at even 3 mg kg^{-1} (Figure S5a). Compound **12** (300 mg kg^{-1}) decreased spleen *O*-GlcNAc levels over time with maximal inhibition by 8 h that was maintained for at least 48 h (Figure S5b). **12** reduced *O*-GlcNAc levels in the kidneys, lungs, fat, pancreas, heart, spleen and muscle tissue but not in the blood or brain (Figure S5c). Mice injected with high doses of **12** (300 mg kg^{-1}), became lethargic, which is a known consequence of low leptin levels.[9] A second injection of 300 mg kg^{-1} on the second day caused mice to be moribund, and we discontinued treatment. We therefore dosed mice IP with a lower dose of **12** (50 mg kg^{-1}, n=3) and found reduced *O*-GlcNAc levels in various tissues 16 h after dosing but no effect in brain, liver, pancreas, and kidney (Figure 4a,b and Figure S6a-c). *O*-GlcNAc levels returned to baseline levels after 16 h (Figure 4a). Metabolic inhibitor **12** therefore acts *in vivo* to reversibly lower *O*-GlcNAcylation in various tissue types. Notably, others have shown $Ac_4$5SGlcNAc (**3**-OAc) appears able to inhibit other glycosyltransferases in cell lines.[5b] We find, however, by lectin blot assessment that **12** induced no apparent changes in other forms of protein glycosylation in all tissues tested (Figure S7), even at a dose of 300 mg kg^{-1} after 48 h. Notably, these observations are consistent with a recent report using parent compound $Ac_4$5SGlcNAc (**3**-OAc) in cells.[10]

Curiously, we observed that mice treated with either low or high doses (50-300 mg kg^{-1}) of **12** exhibited engorged stomachs that were full of rodent chow after 16 h (Figure S8). Because lethargy and excessive food consumption (hyperphagia) are seen in mice deficient in leptin signaling,[11] we measured the serum leptin concentration of animals treated with 50 mg kg^{-1} **12** (Figure 4b). Their leptin levels dropped transiently to a minimum at 16 h (Figure 4b), consistent with the engorged stomachs[9,12]. Leptin levels were also lower in mice treated with a high dose of **12** (300 mg kg^{-1}) (Figure S9). We therefore hypothesized that OGT inhibition might impair leptin production (Figure S10).

To test this hypothesis we used 3T3-L1 adipocytes and found these cells secreted less leptin when treated with either **12** (Figure 4d) or $Ac_4$5SGlcNAc (**3**-OAc) (Figure S11) without affecting

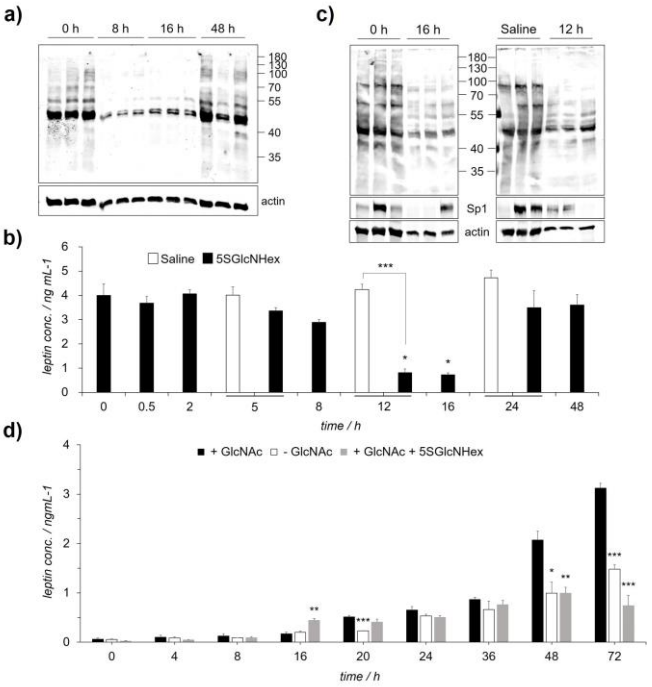

Figure 4. Dosing of mice with 50 mg kg^{-1} 5SGlcNHex (**12**) reduced *O*-GlcNAc levels and impaired the secretion of the hormone leptin. **a)** Skeletal muscle from mice dosed with 50 mg kg^{-1} of **12** showed a transient reduction in *O*-GlcNAc levels by immunoblot analysis (CTD110.6). **b)** Leptin levels as measured by ELISA decreased in mice dosed with **12** to a minimum at 16 h. **c)** Dosing with **12** decreased *O*-GlcNAc and Sp1 levels to a minimum at 16 h in fat pad tissue. **d)** Compound **12** lowered GlcNAc-induced leptin secretion from 3T3-L1 adipocytes. Results are given as the means ± SEM of three independent samples (n = 3). Each independent sample was tested in duplicate; symbols denote statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001 compared to control. Student's t-test).

glucose uptake. Notably, transcription factors C/EBP-α, -β, and Sp1 all regulate leptin production. Compound **12** lowered levels of these known *O*-GlcNAcylated proteins in adipocytes (Figure S9) and in mice (Figure S6d). These collective data are consistent with OGT acting as a nutrient sensor in a process coupling HBP flux to leptin levels (Figure S10).

We envision that future studies to detail dosing regimens with **12** will be important to explore the effects of decreased *O*-GlcNAc on varied physiological processes. Experiments will also be needed to detail the mechanistic links between reduced *O*-GlcNAc levels due to *in vivo* OGT inhibition, impaired leptin secretion, and apparent hyperphagia. However, these data provide the first direct correlation between decreased *O*-GlcNAc levels and impaired leptin production *in vivo*. Our findings are in accord with studies showing that reduced *O*-GlcNAc lowers levels of Sp1 in cells[6f,13] and OGT having a pivitol role in fat tissues[14]. Notably, these data also suggest that regulation of leptin by *O*-GlcNAc is bidirectional *in vivo*, since overexpression of OGT[3], knock out of OGA,[15], and metabolic upregulation of UDP-GlcNAc levels[16] all increase leptin expression.

In summary, we describe convenient new tools to inhibit OGT in cells and *in vivo*. Strikingly, inhibition of OGT with 5SGlcNHex (**12**) provides support for the hypothesis that reduced *O*-GlcNAc levels signal impaired nutrient supply in mammals. We expect these observations will stimulate activity in the creation of

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additional metabolic OGT inhibtiors, as well as metabolic inhibitors of other glycosyltransferases for use *in vivo*. Moreover, we envision that this chemical strategy of metabolic OGT inhibition will serve as a valuable complement to using genetic methods in various rodent models to accelerate studies to understand the *in vivo* roles of *O*-GlcNAc in mammals.

Experimental Section: Please see supporting information for full experimental procedures. All experiments carried out on animals were approved by the university animal care committee of SFU.

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Keywords: glycoprotein • inhibitors • thiosugar • nucleotide-sugars • leptin

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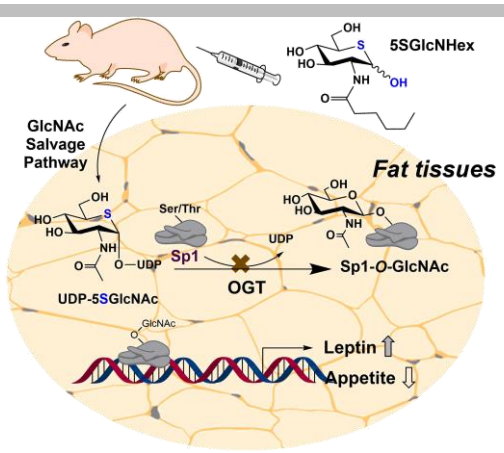
New metabolic inhibitors of OGT enable chemical control of *O*-GlcNAc *in vivo* in mice and link decreased *O*-GlcNAc modification in fat with leptin-mediated nutrient sensing in vivo.



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Page No. – Page No.

Metabolic inhibitors of O-GlcNAc transferase (OGT) that act in vivo implicate decreased O-GlcNAc levels in leptin-mediated nutrient sensing.

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