Enzyme Inhibitors

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A Selective Inhibitor Gal-PUGNAc of Human Lysosomal β-Hexosaminidases Modulates Levels of the Ganglioside GM2 in Neuroblastoma Cells**

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Imbalances in the levels of many glycoconjugates have been implicated in human diseases. Many inhibitors of the large super family of glycoside hydrolases, which are the enzymes that break down these glycoconjugates, are known. However, few of these inhibitors have been developed and characterized as being able to selectively target the enzyme of interest over other functionally related glycoside hydrolases. Such highly selective inhibitors are critical tools to gain insights into the roles of specific glycoconjugates in disease etiology and normal cellular function.

In humans, there are three enzymes that cleave terminal β-linked *N*-acetylglucosamine residues from glycoconjugates. The two dimeric isozymes, hexosaminidase A and B (HEXA and HEXB),[1] share high sequence identity and are members of the family 20 glycoside hydrolases (GH20). HEXA and HEXB are localized in the lysosome, where they hydrolyze terminal *N*-acetylglucosamine and *N*-acetylgalactosamine residues of glycosphingolipids such as the ganglioside GM2 (Figure 1a), which contains a tetrasaccharide terminating at its non-reducing end with an *N*-acetylgalactosamine residue.[2] Inheritable mutations impairing either enzyme result in the accumulation of GM2 in the brain, causing the neurodegenerative disorders known as Tay-Sachs and Sandhoff diseases.[3] Deleterious effects arising from the accumulation of gangliosides are still being uncovered.[4] Interest in the biological roles played by gangliosides have increased as their roles in the modulation of cellular signaling have become apparent.[5,6]

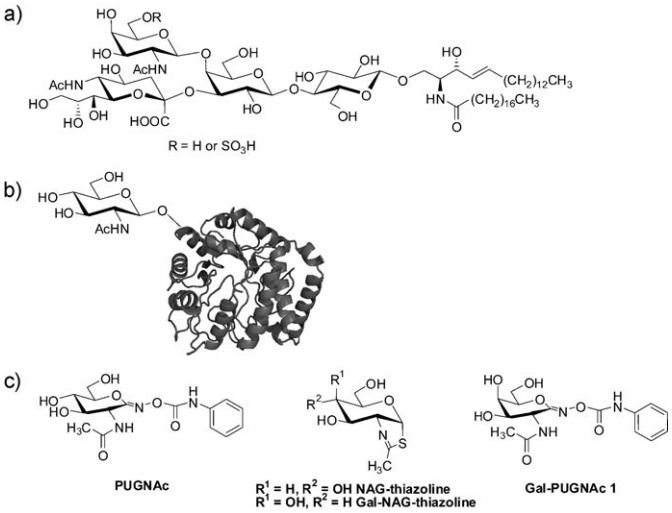

Figure 1. a) Structure of ganglioside GM2 and b) *O*-GlcNAc, and c) the compounds used herein.

The other human glycoside hydrolase that cleaves terminal β-linked *N*-acetylglucosamine residues is a family 84 enzyme termed *O*-GlcNAcase (OGA). This enzyme acts on post-translationally modified nucleocytoplasmic glycoproteins that bear 2-acetamido-2-deoxy-β-D-glucopyranoside residues linked to serine or threonine (*O*-GlcNAc, Figure 1b).[7,8] The consequences of impairing *O*-GlcNAcase in mammals are unknown; however, this enzyme has been implicated in Alzheimer disease and Type II Diabetes.[8]

Although *O*-GlcNAcase and the lysosomal β-hexosaminidases act on different substrates and are located in different cellular compartments, both of these enzymes use a catalytic mechanism involving nucleophilic participation of the 2-acetamido group of the substrate.[9–11] This commonality of the catalytic mechanism delayed the development of selective *O*-GlcNAcase inhibitors.[11] However, various inhibitors of *O*-GlcNAcase[12–15] are proving to be critical tools for evaluating the roles played by *O*-GlcNAc.[16] In contrast, no inhibitor has yet been described that inhibits β-hexosaminidases in a cellular setting to increase levels of GM2. Such a small molecule probe would however be a powerful tool for defining the roles played by gangliosides such as GM2 or for creating readily titratable models of certain disease states.

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Despite a number of inhibitors having been generated for these three enzymes (HEXA, HEXB, and *O*-GlcNAcase), the number of synthetic steps and overall yield often limit their use in biological studies, as large quantities of these inhibitors are often required. Furthermore, most of these inhibitors concomitantly inhibit both family 20 (GH 20) and family 84 (GH 84) enzymes and are thus less useful for studying the role of one particular enzyme in a biological setting as they may elicit a complex phenotype. Accordingly, there is a need for potent inhibitors that are selective for lysosomal β-hexosaminidases over *O*-GlcNAcase, and are able to elevate levels of ganglioside GM2 in cells.

One inhibitor of all three enzymes is *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-*N*-phenylcarbamate PUGNAc.[17] Despite its benefits, one limitation of PUGNAc is its indiscriminate inhibition of both family 20 lysosomal β-hexosaminidases[11,18] and family 84 *O*-GlcNAcase.[11,19] Given that HEXA and HEXB can process both *gluco*- and *galacto*-configured hexosaminides,[1] while *O*-GlcNAcase is selective for *gluco*-configured substrates,[19] a *galacto*-configured C-4 epimer of PUGNAc appeared to be a simple route to ensure selectivity by exploiting these differences in substrate specificities. We therefore synthesized Galactose-PUGNAc **1** (Gal-PUGNAc), and investigated its activity toward human β-hexosaminidase in enzyme assays and in cultured human neuronal cells.

Starting from galactosamine hydrochloride, Gal-PUGNAc **1** was prepared in eight steps, with an overall yield of nearly 20% (see Supporting Information), using a route modified from that previously described for synthesis of PUGNAc.[12] Gal-PUGNAc **1** was found to be a potent and competitive inhibitor of HEXA and HEXB, with an inhibition constant (K_i) of 18 ± 1 nM against HEXB (Figure 2) and 51 ± 3 nM against HEXA (see Supporting Information). Gal-PUGNAc **1** is therefore comparable to the most potent inhibitors of family 20 β-hexosaminidases that have K_i values in the low nanomolar range (see Supporting Information).[11,20,21]

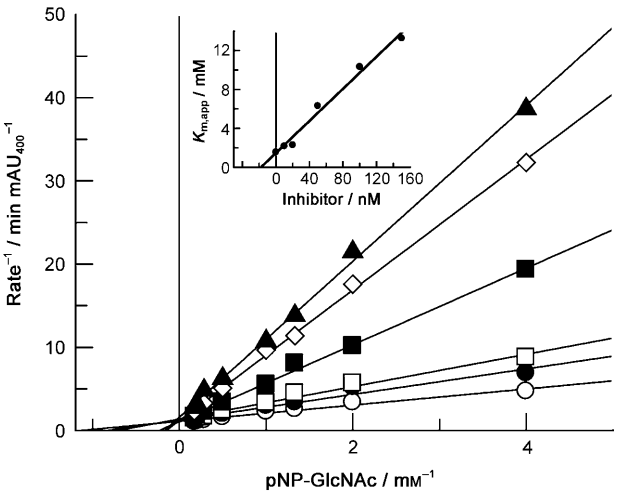

Figure 2. Inhibition of human HEXB catalyzed hydrolysis of pNP-GlcNAc by Gal-PUGNAc **1** shows a pattern of competitive inhibition. The concentrations of Gal-PUGNAc **1** (μM) used were 0.15 (▲), 0.10 (◇), 0.050 (■), 0.020 (□), 0.010 (●), and 0.00 units(○). Inset: graphical analysis of K_i from plotting K_M apparent against the concentration of Gal-PUGNAc **1**. $K_{m,app}$ is the apparent K_m value obtained at each inhibitor concentration.

We evaluated the selectivity of Gal-PUGNAc **1** and found that at a concentration 1 mM, Gal-PUGNAc **1** showed less than 1% inhibition of *O*-GlcNAcase, which is consistent with the inability of *O*-GlcNAcase to process *galactose*-configured saccharides.[19] The K_i value of Gal-PUGNAc **1** against *O*-GlcNAcase must therefore be greater than 10 mM, making it the most selective inhibitor for lysosomal β-hexosaminidases over *O*-GlcNAcase (over 500000-fold; see Supporting Information). α-*N*-Acetylgalactosaminidases are functionally related to β-hexosaminidases in that they also process terminal *N*-acetylgalactosamine residues, albeit only those having an α-glycosidic linkage. Therefore, to further investigate the selectivity of Gal-PUGNAc **1** we evaluated its potency towards the *G. gallus* GH 27 α-*N*-acetylgalactosaminidase, which shares 50% identity with the human homologue (NAGA) including completely conserved active site residues[22] and determined a K_i value of 140 ± 10 μM. Gal-PUGNAc **1** is therefore 2800-fold more selective for β-hexosaminidase over the GH 27 α-*N*-acetylgalactosaminidase. Gal-PUGNAc **1** also binds over 40 times more tightly than another *galactose*-configured inhibitor of lysosomal β-hexosaminidases, Gal-NAG-thiazoline (Figure 1c), which was reported to have a K_i of 820 nM against HEXA.[23] Gal-NAG-thiazoline, however, has not been tested against *O*-GlcNAcase nor used in cell culture. We therefore prepared this compound and found that, like Gal-PUGNAc **1**, it is also a poor inhibitor of *O*-GlcNAcase.

Having demonstrated the selectivity of Gal-PUGNAc **1** and Gal-NAG-thiazoline for the lysosomal β-hexosaminidases in vitro, we evaluated these inhibitors in cultured SK-N-SH cells. These cells are a well-characterized human neuroblastoma cell line that has appreciable levels of GM2.[24] Cells were treated with a panel of inhibitors of HEXA, HEXB, and *O*-GlcNAcase; this panel was comprised of Gal-PUGNAc **1**, PUGNAc,[12,17,25] NAG-thiazoline,[9,11] Gal-NAG-thiazoline,[23] and NButGT.[11] After nine days in the presence of inhibitors, glycosphingolipids were extracted from the cells and ganglioside levels were assessed by high-performance thin-layer chromatography using resorcinol staining. Levels of GM2 were significantly elevated (30–40%) in cells treated with Gal-PUGNAc **1**, PUGNAc, NAG-thiazoline, and Gal-NAG-thiazoline (Figure 3). Notably, NButGT, the *O*-GlcNAcase specific inhibitor,[11] did not elevate cellular GM2 levels (Figure 3d). This result is consistent with NButGT being a poor inhibitor of HEXB ($K_i = 340$ μM)[11] and highlights the selectivity of this inhibitor towards *O*-GlcNAcase.

To further investigate the value and specificity of these inhibitors we examined their effect on cellular *O*-GlcNAc levels. We treated SK-N-SH cells overnight with the panel of inhibitors used above and analyzed cell lysates for levels of *O*-GlcNAc by Western blot. As expected, known *O*-GlcNAcase inhibitors used previously in cells, including PUGNAc, NAG-thiazoline, and NButGT, all significantly increased cellular *O*-GlcNAc levels. In contrast, neither Gal-PUGNAc **1** nor Gal-NAG-thiazoline increased cellular *O*-GlcNAc levels

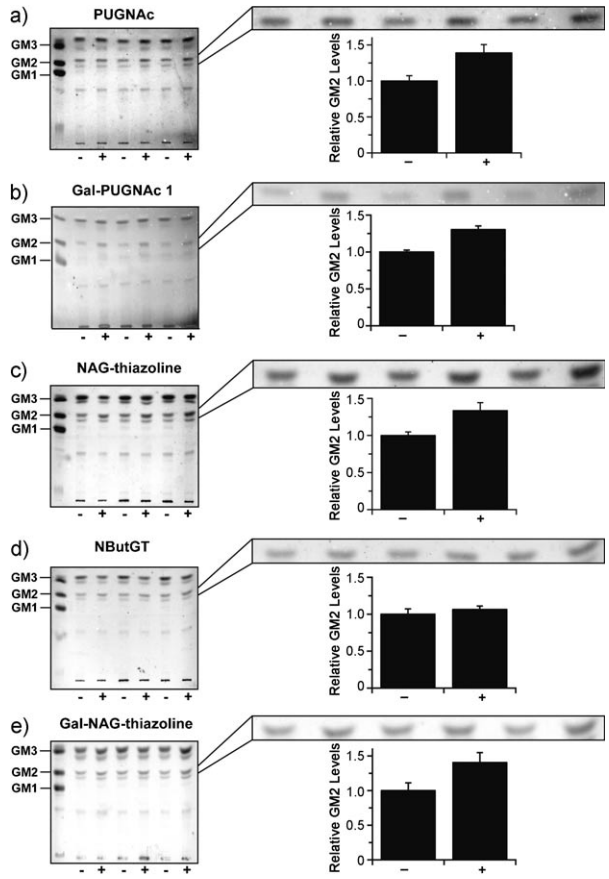

Figure 3. Small-molecule inhibitors of HEXA and HEXB increase levels of GM2 in SK-N-SH cells. HPTLC analysis of lipid extracts of cells treated with 200 μM of each compound for nine days (left panels). In each experiment, control cells were grown parallel to treated cells. Magnification of the band representing GM2 is shown on the right panels along with densitometric analyses of the intensities of the GM2 bands.

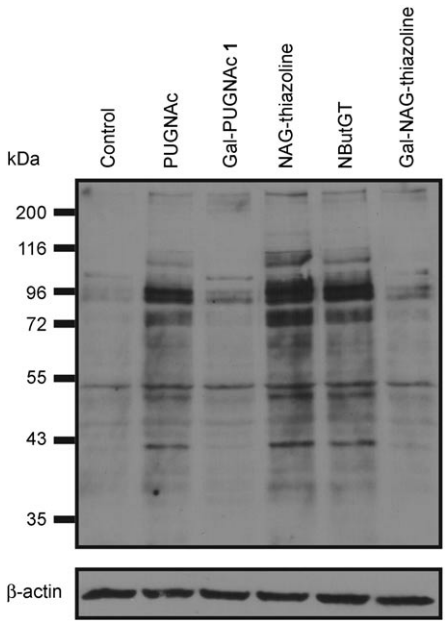

Figure 4. Gal-PUGNAc **1** and Gal-NAG-thiazoline do not increase levels *O*-GlcNAc-modified proteins. SK-N-SH cells were treated overnight with 50 μM of each of the compounds and analyzed for levels of *O*-GlcNAc-modified proteins by Western blot (upper panel). Western blot of cellular β-actin levels demonstrates equal loading for each lane (lower panel).

(Figure 4) highlighting their selectivity for HEXA and HEXB.

One important observation made here for cell biologists using non-selective *O*-GlcNAcase inhibitors to uncover the biological roles of *O*-GlcNAc is that PUGNAc and NAG-thiazoline perturb both *O*-GlcNAc and ganglioside levels. A further cautionary note is that PUGNAc has also been recently shown to inhibit an α-*N*-acetylglucosamindase bacterial homologue from GH 89 that has high active site similarity with human NAGLU.[26] Therefore, results obtained using PUGNAc in cells should be interpreted with these off-target effects in mind, as levels of multiple glycoconjugates will be perturbed.

In summary, Gal-PUGNAc **1** is a readily accessed and selective inhibitor of the human lysosomal β-hexosaminidases. This selectivity is manifested in the ability of Gal-PUGNAc **1** to increase GM2 ganglioside levels in cultured cells, but to not affect *O*-GlcNAc levels. We find that Gal-NAG-thiazoline affords similar control over GM2 levels and expect other β-hexosaminidase inhibitors that permeate into cells, including those that are non-carbohydrate-based,[27] to act similarly. Owing to the greater stability and 40-fold higher potency of Gal-PUGNAc **1** over Gal-NAG-thiazoline, Gal-PUGNAc **1** offers advantages for studying lysosomal β-hexosaminidase inhibition and ganglioside GM2 in a cellular context without generating a complex chemical phenotype stemming from concomitant inhibition of *O*-GlcNAcase. We expect these compounds will prove to be valuable tools for researchers interested in the roles of GM2 in cells and in vivo.

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