Published in final edited form as:

J Am Chem Soc. 2018 October 24; 140(42): 13542–13545. doi:10.1021/jacs.8b07328.

Structure-based evolution of low nanomolar O-GlcNAc transferase inhibitors

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Abstract

Reversible glycosylation of nuclear and cytoplasmic proteins is an important regulatory mechanism across metazoans. One enzyme, O-linked N-acetylglucosamine transferase (OGT), is responsible for all nucleocytoplasmic glycosylation and there is a well-known need for potent, cell-permeable inhibitors to interrogate OGT function. Here we report the structure-based evolution of OGT inhibitors culminating in compounds with low nanomolar inhibitory potency and on-target cellular activity. In addition to disclosing useful OGT inhibitors, the structures we report provide insight into how to inhibit glycosyltransferases, a family of enzymes that has been notoriously refractory to inhibitor development.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Materials and Methods, SI figures and tables, NMR spectra (PDF); coordinates and structure factors for complexes of OGT with **1a**, **ent-1a**, **2a**, **3a**, and **4a** (PDB codes: 6MA1, 6MA2, 6MA3, 6MA4, 6MA5); proteomics data (XLSX).

The authors declare no competing financial interest.

O-GlcNAc transferase (OGT) is an essential mammalian enzyme that modifies myriad nuclear and cytoplasmic proteins with O-linked N-acetylglucosamine (OGlcNAc), affecting their stability, localization, activity, and interactions with other proteins.¹ Evidence points to a crucial role for O-GlcNAc in metabolic homeostasis and elevated O-GlcNAc levels have been linked to metabolic adaptations associated with several disease phenotypes, including the abnormal proliferative capacity of cancer cells.² To better understand OGT function, small molecule OGT inhibitors are required. OGT inhibitors with some cellular activity have been reported, but most are substrate analogs that offer limited opportunities for modifications to improve potency or selectivity.³

The active site of OGT is particularly challenging to inhibit. The nucleotide-sugar substrate, UDP-GlcNAc, lies in an extended conformation underneath the peptide substrate; filling the active site requires molecules that can mimic this stacked substrate geometry (Figure S1).⁴ Complicating matters, OGT's active site is hydrophilic and accommodates many peptide sequences, with substrate selection being determined not by specific contacts to OGT side chains, but by binding of proteins to the tetratricopeptide repeat (TPR) domain.⁵ At a loss for how to design inhibitors for OGT's large, hydrophilic, and promiscuous active site, we previously carried out a high-throughput screen that led to a weakly active compound containing a quinolinone-6-sulfonamide (Q6S).^{3b,6} Here we report structures of OGT complexed with several cell-permeable Q6S-based inhibitors, including two having low nanomolar K_d s. To our knowledge, these are the first structures of a nucleotide-sugar glycosyltransferase complexed with biologically active inhibitors that are not substrate mimics.

We made a series of compounds containing the Q6S scaffold and eventually obtained a crystal structure of OGT bound to compound **1a** (Figure 1A, B). This structure inspired three related agents, **2a**, **3a**, and **4a**, that were also crystallized with OGT (Figure 1A). The structures of these complexes revealed that the Q6S moiety is a faithful uridine mimic (Figure 1C). Like uracil, the quinolinone ring stacks directly over the imidazole of His⁹⁰¹; the nitrogen and adjacent carbonyl of the hetero-cycle make the same contacts to Arg⁹⁰⁴ and Ala⁸⁹⁶ as N3 and O4 of uracil. In addition, a sulfonamide oxygen hydrogen bonds with Lys⁸⁹⁸, mimicking contacts made by the ribose hydroxyls. The remarkable overlap between the quinolinone and uridine suggests this motif may serve as a privileged fragment for designing inhibitors against other glycosyltransferases (Figure S2).

The structures showed that the Q6S compounds have a U-shaped architecture that helps explain their ability to inhibit OGT. The S-N bond veers up from the plane of the quinolinone ring and the backbone of the molecule folds back over it, positioning the substituents on the disubstituted amide directly over the quinolinone (Figure 1D). Density functional theory calculations show that the conformer observed in the crystal structures is also the most stable conformer (Figure S3). The inhibitor's U-shape allows it to fully occupy a space that accommodates the uridine and the segment of peptide that lies over it. Indeed, the thiophene substituent on the disubstituted amide penetrates so deeply into the active site that Gln⁸³⁹, Leu⁸⁶⁶, and Phe⁸⁶⁸ must rotate to make room (Figure S4).

We also obtained a structure of OGT bound to the S-enantiomer of **1a** (*ent*-**1a**), which binds more weakly to OGT than the R-enantiomer (Table S1). The Q6S element in *ent*-**1a** binds exactly as in **1a**, confirming the importance of this fragment in binding (Figure S5). The switch in chirality of the substituted phenylglycine means that the substituents on the disubstituted amide project away from, rather than into, the deeper recesses of the active site. The weaker interactions of the amide substituents with active site residues undoubtedly drive the lower affinity of the S-configured compounds.

Analysis of the OGT complex with **1a** suggested two strategies to modify these inhibitors to make additional contacts. One strategy exploited the observation that the *ortho*-methoxy group points toward an unoccupied region of the active site where the diphosphate of UDP-GlcNAc would bind (Figure S6). Previous structural studies of the OGT:UDP-GlcNAc complex showed that one of the anomeric phosphate oxygens forms hydrogen bonds to the backbone amides at the N-terminus of a proximal helix^{4b}, while another contacts the side chain amine of catalytically essential Lys⁸⁴².^{4a, 4b, 4d} The binding pose of **1a** suggested that it would be possible to mimic these interactions by attaching a carboxylate to the phenyl ring via a sufficiently long linker to bridge the distance to the phosphate binding site. We prepared compounds **2a** and **3a** with linkers containing three and four methylenes and solved structures of OGT bound to both inhibitors. Both compounds make additional contacts to OGT in the expected region of the active site (Figure 1E, S7A, B). Strikingly, the pendant carboxylate of compound **3a** overlaps almost perfectly with the UDP-GlcNAc phosphate and makes the same interactions with the proximal helix (Figure S7C). While the carboxylate on **2a** is not within H-bonding distance of the N-terminal amides, one of its oxygens interacts with the side chain of Thr⁹²¹ while the other is oriented towards Lys⁸⁴², which is 3.7 Å away (Figure 1E).

The second strategy focused on including a small substituent on the quinolinone *ortho* to the sulfonamide because analysis of the crystal structures suggested it would be possible to achieve a tighter fit in the uridine pocket. We prepared chlorine derivative **4a** and the crystal structure of the complex with OGT confirmed the expected binding pose (Figure 1F).

To measure the dissociation constants for each of the inhibitors and their enantiomers, we used microscale thermophoresis (MST) after confirming that the method produced K_{d} s for UDP and UDP-GlcNAc similar to those obtained by surface plasmon resonance (Table S1, Figure S8).^{4d} Compounds **2a** and **4a** bound OGT with K_{d} s of ~5 and ~8 nM, respectively, while the K_{d} s of **1a** and **3a** were an order of magnitude or more higher (Figure 2A). The *S*-

enantiomers tested bound at least tenfold more weakly to OGT than the corresponding *R*-enantiomers. The tighter binding of **2a** compared with **3a** is likely explained by a stronger interaction of the negatively-charged carboxylate of **2a** with the positively charged Lys⁸⁴² amine combined with a smaller loss in conformational entropy due to immobilization of the shorter linker. The tighter binding of **4a** compared with **1a** is attributed to its snugger fit in the binding pocket.

To assess the cellular activity of these inhibitors, we prepared the corresponding ester derivatives (compounds **1b-4b**) to enhance cell penetration and examined their ability to inhibit global O-GlcNAcylation in HCT116, HEK293T, PC3, and LNCaP cells (Figure 2BE, S9–S11). Intracellular esterases cleave esters rapidly, and the corresponding carboxylic acid forms of these compounds are likely the active species in cells. Compounds **2b** and **4b** had the best activity, with **4b** reducing O-GlcNAc levels almost completely by 5 μ M (Figure 2B, S11). At short treatment times (<8h), **1b** was also a decent inhibitor (Figure 2C, S9A); however, O-GlcNAc levels began to recover at longer treatment times with this compound (Figure S9B–D, S10A, B). We attribute the more sustained cellular effects of **2b** and **4b** compared with **1b** to the greater affinity of their deesterification products (**2a** and **4a**) for OGT. The enantiomers tested did not substantially affect protein O-GlcNAc levels (Figure 2D, S9E).

In addition to glycosylating Ser/Thr residues of proteins, OGT catalyzes cleavage of the cell-cycle regulator HCF-1 by glycosylating a glutamate in the HCF-1 cleavage sequence.^{4c, 7} An OGT inhibitor would be expected to block cleavage. Indeed, we observed a decrease in HCF-1 cleavage products and the appearance of uncleaved HCF-1 in cells treated with **1b**, **2b** or **4b** (Figure 2E, S12). Because OGT knockdown is known to decrease cell proliferation,⁸ we also monitored the effects of **1b**, **2b**, and **4b** on cell growth in culture over 96h. Although there was no evidence of apoptosis, we observed reduced growth of cells over time (Figure S13), consistent with the knockdown results.

We performed quantitative proteomics to assess how HEK293T cells responded to inhibitor treatment (Figure S14). A time course performed with 20 μ M **1b** showed reciprocal changes in the abundance of OGT and OGA, with a particularly large increase in OGT (Figure 3A, S15); this may explain the recovery in O-GlcNAc levels with this compound. At 24h where O-GlcNAc levels fully recovered, we observed that few other proteins changed in abundance with **1b**, suggesting minimal off-target activity. Compensatory changes in OGT and OGA abundance have been observed previously when levels or activity of these proteins are perturbed.^{3a, 3b, 8–9} Cells contain a large nuclear pool of partially spliced OGT transcript, and one mechanism for the rapid increase in OGT levels is increased cotranscriptional splicing of a detained intron to form productive mRNA.^{9–10} Indeed, we found that 2h treatment with 10 μ M **1b**, **2b** or **4b** increased detained intron splicing (Figure 3B). That OGT transcript splicing is responsive to OGT inhibition indicates a feedback mechanism linking splicing with enzymatic activity, and highlights the importance of maintaining adequate cellular O-GlcNAc.

We also performed quantitative proteomics with **4b**, which causes prolonged inhibition of O-GlcNAc over 24h. 86 proteins, most showing increased abundance, changed significantly

(Figure 3C, S16). These proteins included SQSTM1 (sequestosome-1), a protein involved in autophagy that was shown to increase upon conditional deletion of OGT in the liver,¹¹ as well as additional proteins involved in autophagy. Proteins involved in other processes previously linked to OGT,¹² including transcription and ER stress (Figure 3D), also changed. We also observed increased abundance of key enzymes involved in the biosynthesis of cholesterol, including 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGCR), which catalyzes the rate-limiting step in cholesterol synthesis, squalene synthase (FDFT1), which catalyzes the first committed step of cholesterol synthesis, and squalene epoxidase (SQLE). This finding suggests a connection between OGT activity and sterol homeostasis that merits further investigation.

In conclusion, we have described the structure-based evolution of small molecule OGT inhibitors and have reported three useful cell-permeable compounds, henceforth known as OSMI-2 (**1b**), OSMI-3 (**2b**), and OSMI-4 (**4b**). The active forms of OSMI-3 and OSMI-4 (**2a** and **4a**) have low nanomolar binding affinities, a milestone in glycosyltransferase inhibitor development. Indeed, OSMI-4 is the best OGT inhibitor reported to date, with a ~3 μ M EC₅₀ in cells (Figure S11F), making it especially attractive for probing OGT's complex biology. Addressing a longstanding need in the field, the inhibitor:OGT complexes we describe may also provide a framework to guide inhibitor development for other glycosyltransferases.

Supplementary Material

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ACKNOWLEDGMENT

This work was supported by GM094263 (S.W.) and GM124838 (M.B.L.). S.E.S.M. was supported by an NIH Kirschstein NRSA (GM117704), Z.T. by a Singapore A*STAR NSS fellowship, and J.J. by NSERC of Canada PGS-M and D3 fellowships. This work used NE-CAT beamlines (GM103403), a Pilatus detector (RR029205), and an Eiger detector (OD021527) at the APS (DE-AC02-06CH11357). This work used MicroScale Thermophoresis instrumentation in the Center for Macromolecular Interactions at Harvard Medical School, and we thank Kelly Arnett for training and assistance. We thank Yuhong Fang at the NIH National Center for Advancing Translational Sciences for chiral separations.

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Figure 1.

Structures of OGT:inhibitor complexes allowed structure-based improvements. (A) Structures of reported R-series inhibitors. (B) Overview of the OGT:**1a** structure (gray) showing **1a** (cyan) bound in the active site. All crystals were obtained using a TPR-binding peptide derived from HCF-1 (pink) to improve resolution.^{4c} (C) Overlay of **1a** (cyan) and UDP-GlcNAc (orange, PDB:4N3C) showing that the Q6S moiety mimics uridine. Dashed lines indicate inferred hydrogen bonds from **1a** to OGT (green sticks). (D) The U-shaped conformation of **1a** enables the amide substituents to fill the space above the quinolinone. (E) Overlay of **1a** (cyan), **2a** (light purple), and **3a** (dark blue). Dashed lines indicate hydrogen bond contacts to Thr⁹²¹ from **2a** (red) and **3a** (blue), and to backbone amides from **3a**. Pink and beige sticks show the side chains in the **2a** and **3a** complexes, respectively. See figures S4, S6, and S7 for additional views. (F) Space-filling views of **1a** (cyan) and **4a** (yellow) with the **1a** hydrogen and **4a** chlorine shown in white and green, respectively.

Figure 2.

Inhibitors bind OGT with nanomolar affinity and block OGT activity in cells. (A) MST binding curves for **2a**, **3a**, and **4a**, with dissociation constants of selected inhibitors listed (inset). Error bars represent S.E.M. of at least three replicates. See Table S1 for all K_d s and Figure S8 for other MST curves. (B) O-GlcNAc (RL-2) blot of HEK293T cell lysates after treatment with **4b** for 24h. (C) O-GlcNAc blot of HCT116 cells after treatment for 4h with **1b** and **2b**. (D) O-GlcNAc blot of HEK293T cell lysates after treatment for 24h with **1b**, **2b**, and their enantiomers. (E) Treating HEK293T cells with 20 μM of **2b** or **4b** for 48h blocked HCF-1 cleavage. Asterisk (*): uncleaved HCF-1; arrows: cleavage products.

Figure 3.

OGT inhibition results in increased abundance of OGT and changes in multiple proteins involved in ER stress and sterol metabolism. (A) Volcano plot of proteomic data after treating HEK293T cells with 20 μM **1b** for 24h. (B) Bar graph summarizing quantitative PCR results using primers to OGT's detained intron (intron 4) and spliced exons (exon 4 and 5) after 2h treatment of HEK293T cells with 10 μM **1b**, **2b** and **4b**. Error bars represent s.d. (n=3). (C) Volcano plot of proteomic data after treating HEK293T cells with 20 μM **4b** for 24h. (D) Heatmap of proteins involved in indicated processes after treatment with **4b**.