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Targeted Covalent Inhibition of O-GlcNAc Transferase in Cells†

Matthew Worth^{‡,a}, Chia-Wei Hu^{‡,b}, Hao Li^b, Dacheng Fan^b, Arielis Estevez^b, Dongsheng Zhu^b, Ao Wang^b, Jiaoyang Jiang^b

^aDepartment of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53705, USA

^bPharmaceutical Sciences Division, School of Pharmacy, University of Wisconsin–Madison, Madison, Wisconsin 53705, USA.

Abstract

O-GlcNAc transferase (OGT) glycosylates numerous proteins and is implicated in many diseases. To date, most OGT inhibitors lack either sufficient potency or characterized specificity in cells. We report the first targeted covalent inhibitor that predominantly reacts with OGT but does not affect other functionally similar enzymes. This study provides a new strategy to interrogate cellular OGT functions and to investigate other glycosyltransferases.

Graphical Abstract

The first targeted covalent inhibitor that reacts with O-GlcNAc transferase but not affects other functionally similar enzymes in cells

O-GlcNAcylation is a dynamic and reversible protein modification of serine and threonine residues with *N*-acetylglucosamine (O-GlcNAc) (Fig. 1a).¹ This monosaccharide modification is attached by O-GlcNAc transferase (OGT) using uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) as the sugar donor, and is removed by O-GlcNAcase (OGA).² OGT activity, which dynamically changes in response to the intracellular concentration of UDP-GlcNAc, modulates over 1,000 intracellular proteins in various biological processes,¹ including transcription,³ translation,⁴ and signal transduction.⁵ Notably, dysregulated O-GlcNAcylation and OGT have been found in cancer and many

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jiaoyang.jiang@wisc.edu.

[‡]These authors contributed equally to this work

Conflicts of interest

There are no conflicts of interest to declare.

other diseases.⁶ To dissect their cellular functions, a number of OGT inhibitors have been reported with a majority acting reversibly in the active site.^{7–13}

Despite this progress, applying OGT inhibitors in cells for studying O-GlcNAcylation remains challenging largely due to several factors: difficulty in directly validating the cellular targets of reversible OGT inhibitors, high cellular UDP-GlcNAc concentration competing with inhibitor binding, and rapid feedback response to reversible OGT inactivation.^{8,13} For example, one of the most promising OGT reversible inhibitors, OSMI-4, shows remarkable binding affinity to OGT *in vitro*, but its specificity in cells remains elusive.¹³ Furthermore, the recovery of O-GlcNAc level was observed following the prolonged treatment of OSMI class of compounds.¹³ UDP-5SGlcNAc is another elegantly designed inhibitor in which the endocyclic oxygen of GlcNAc is replaced with sulfur and thus reversibly inactivates OGT by dramatically impairing the glycosyl transfer step.⁸ Using its per-O-acetylated form Ac₄5SGlcNAc (Fig. 1b), this sugar analogue can be converted to UDP-5SGlcNAc in cells and efficiently inhibits cellular O-GlcNAcylation.⁸ However, with the cellular target(s) remaining uncharacterized, this compound may inhibit other related glycosyltransferases due to its structural similarity to UDP-GlcNAc.¹¹ BZX2 is currently the only reported covalent inhibitor of OGT,⁹ but its low reaction specificity makes it unsuitable for cellular use. The issues of existing inhibitors prompted us to explore the targeted covalent inhibition of OGT.

Targeted covalent inhibitors irreversibly react with a poorly conserved and non-catalytic nucleophilic residue, such as cysteine. Besides the advantages of traditional covalent inhibitors including simplified target validation, sustained target engagement, and overcoming the competition from high concentration of intracellular ligand, targeting a unique residue can improve specificity in living systems.¹⁴ This strategy has achieved success in several enzyme families including kinases,¹⁴ but has not yet been applied to inhibit glycosyltransferases. OGT contains a non-conserved, non-catalytic cysteine residue (C917) in proximity (3.6 Å) to the *N*-acetyl group of the active site bound UDP-GlcNAc (Fig. S1). We previously reported that this C917 can react with electrophile *in vitro*.¹⁵ Therefore, a GlcNAc analogue with suitable electrophile could potentially be exploited for targeted covalent inhibition of OGT in cells.

Toward this goal, we first docked UDP-GlcNAc analogues with various *N*-acyl electrophiles into the OGT active site structure (PDB: 4GZ5).¹⁶ We found that analogues with a more distant reactive center (three to four bonds from the carbonyl group) and relatively small size were promising candidates. Based on this, we chose four electrophiles of moderate reactivity (**ES1-ES4**, Fig. 1b) to selectively react with the proximal thiol in OGT.^{17–19} To prevent glycosyl transfer (or UDP hydrolysis) before covalent reaction, an endocyclic sulfur as in Ac₄5SGlcNAc was incorporated into our design. Given the fact that various acetylated GlcNAc analogues can be metabolically converted to the corresponding UDP-GlcNAc analogues in cells,^{8,20,21} we synthesized **ES1-ES4** through a series of reactions starting from D-glucosamine (Fig. S2).

To determine whether ES compounds can exhibit sustained OGT inactivation for cellular applications, we first evaluated the effects of **ES1-ES4** on O-GlcNAcylation in cells using a

pre-treatment and post-incubation strategy. Human breast cancer MCF7 cells, which possess abundant O-GlcNAcylation, were treated with different ES compounds overnight to allow acetylated GlcNAc analogues to be metabolically converted to the active nucleotide-sugar forms. Following that, the compounds were washed out and the cells were incubated in inhibitor-free media for another several hours. For comparison, two previously reported OGT inhibitors, Ac₄5SGlcNAc (reversible inhibitor) and BZX2 (covalent inhibitor), were also tested under the same conditions. As evaluated by Western blot, **ES1-ES3** reduced global O-GlcNAcylation substantially and showed greater inhibitory effect than BZX2 (Fig. 1c). Notably, **ES1** displayed stronger O-GlcNAc inhibition than other tested ES compounds and one of the best reported reversible OGT inhibitors, Ac₄5SGlcNAc. Further evaluation of **ES1** at different doses in MCF7 cells showed more effective O-GlcNAc reduction than Ac₄5SGlcNAc at concentrations as low as 0.5 μ M (Fig. 2a). While **ES1** might induce modest toxicity after treatment for more than 24 h, the cytotoxicity of **ES1** was negligible (< 10%) at the concentrations needed to reduce O-GlcNAc level during the pre-treatment and post incubation (Fig. S3). We observed that the effective doses for O-GlcNAc reduction could vary between different cell lines. However, even when higher concentrations were applied in another cell line (COS-7), **ES1** achieved more complete O-GlcNAc inhibition than Ac₄5SGlcNAc (Fig. S4), possibly due to the different mechanisms of inhibition. To further investigate whether **ES1** irreversibly reduces O-GlcNAcylation in cells, glucosamine was added to the MCF7 cells during the post-incubation stage to rapidly increase cellular UDP-GlcNAc level after inhibitor treatment. The augmented sugar donor concentration is expected to outcompete reversible inhibitors from the OGT active site and restore the O-GlcNAcylation in cells. In contrast to the Ac₄5SGlcNAc-treated cells, **ES1** was resistant to the recovery effect from glucosamine (Fig. 2b), supporting its irreversible mechanism of inhibition. Interestingly, an even more dramatic difference between these two compounds was observed in COS-7 cells (Fig. S5). These findings demonstrate that **ES1** can efficiently achieve sustained O-GlcNAc inhibition in cells.

We next synthesized the respective UDP-sugar analogue **UDP-ES1** to validate the irreversible inhibition mechanism of **ES1** *in vitro* (Fig. S6). Because UDP-sugar analogue synthesis is challenging and typically low yielding, we employed a one-pot chemoenzymatic reaction to efficiently generate the key intermediate **13** in 92% yield using the following three enzymes: NahK (*N*-acetylhexosamine 1-kinase), AGX1 (UDP-*N*-acetylhexosamine pyrophosphorylase), and PPA (pyrophosphatase) (Fig. S6).^{22,23} With the intermediate **13** in hand, the final product **UDP-ES1** was conveniently synthesized in two additional steps. We first examined whether **UDP-ES1** irreversibly inhibits OGT *in vitro*. In a radiometric assay, OGT was preincubated with **UDP-ES1** for different amounts of time followed by reaction with its protein substrate NUP62 (ref. 24) and UDP-³H-GlcNAc. We found that **UDP-ES1** showed clear time-dependent OGT inactivation with an inhibition efficacy ($k_{\text{inact}}/K_{\text{I}}$) of 0.06 $\mu\text{M}^{-1} \text{min}^{-1}$ (Fig. 3a). Next, to detect the potential covalent modification of **UDP-ES1** and evaluate the reaction specificity, purified OGT protein was incubated with excess **UDP-ES1** and the reaction product was analyzed using intact protein MS. Notably, only one unit of inhibitor modification (+647 Da) was detected on OGT, and its C917S mutation abolished the modification (Fig. 3b). Further LC-MS/MS analysis of tryptic peptides derived from the full-length OGT treated with **UDP-ES1** identified C917 as the exclusive modification site

over a dozen other cysteine residues in OGT (Fig. 3c). Moreover, we determined a high resolution (2.55 Å) crystal structure of OGT complexed with **UDP-ES1** (Fig. 3d, Table S1). Continuous electron density observed from the sugar to the sulfur of C917 provided unambiguous evidence for the covalent inhibition mechanism of **UDP-ES1**. These results showed that **UDP-ES1** specifically reacts with C917 to inactivate OGT *in vitro*.

We next sought to characterize the cellular specificity of **ES1**. As an initial evaluation, the effect of **ES1** on glycan biosynthesis was examined by lectins ConA and HPA, which can recognize N-linked and O-linked glycans, respectively.²⁵ Using the lectin blot, no significant change was detected on the glycan expression in MCF7 cells following **ES1** treatment for 24 h at concentrations that induced clear O-GlcNAc reduction (Fig. S7). Encouraged by this result, we thus synthesized **6AzES1** (Fig. S8), which incorporates an azide handle to the 6-position on **ES1**, for direct detection of its labeled proteins through click chemistry with fluorophore alkyne and in-gel fluorescence scanning (Fig. 4a). Considering the potential challenge of using in-gel fluorescence to detect relatively low-abundant OGT in the cellular proteome,²⁶ we first employed cells expressing exogenous OGT to evaluate the reactivity profile of **6AzES1** and compared it with the control cells expressing Venus yellow fluorescent protein (YFP) (Fig. 4b). Notably, after 24 h treatment of **6AzES1**, OGT-expressing cells demonstrated a strong fluorescent band at a molecular weight that is consistent with the OGT protein size (~115 kDa, Fig. 4b). The OGT identity of this band was supported by Western blot (Fig. S9). Interestingly, **6AzES1** labeling of OGT was increased in a dose-dependent manner and no **6AzES1** modification was observed on the highly expressed YFP, which possesses cysteine residues including one exposed to solvent.²⁷ To further assess the prolonged engagement of **ES1** in OGT, we tested the cellular OGT labeling using the pre-treatment and post-incubation approach as mentioned above. Consistent with the 24 h treatment results in Fig. 4b, OGT remained the strongest labeled band even eight hours after inhibitor removal (Fig. 4c). Importantly, a major band of the same size, despite the lower intensity, was observed in the nuclear extracts from non-transfected cells following similar **6AzES1** treatment, click chemistry and detection (Fig. S10a). We further validated that endogenous OGT was targeted by **6AzES1** and enriched in the purified **6AzES1**-labeled protein sample (Fig. S10b). Additionally, we applied a high concentration of glucosamine to the cells before and during **6AzES1** treatment. As expected, the OGT labeling was competed out by augmented UDP-GlcNAc from glucosamine (Fig. S10c). These data support the on-target cellular engagement of **6AzES1**.

To further evaluate the inhibition specificity of **ES1** in cells, we examined its effect on a functionally similar glycosyltransferase, epidermal growth factor (EGF) domain-specific OGT (EOGT).²⁸ Despite utilizing the same sugar donor UDP-GlcNAc, EOGT acts differently from OGT by specifically O-GlcNAcylating a distinct group of secreted and membrane proteins at EGF repeats, such as NOTCH1.²⁸ To test whether **ES1** inhibits EOGT-dependent O-GlcNAcylation, we expressed the verified EOGT substrate NOTCH1 EGF repeats (EGF1–36) in cells, conducted inhibitor treatment, and inspected the O-GlcNAc level of EGF1–36 using immunoprecipitation and Western blot (Fig. 5a). As a control, similar experiments were performed on NUP62 expressing cells (Fig. 5b). Since EOGT and OGT share low sequence similarity (12%) and the C917 is unique in OGT, we

speculated that **ES1** would not affect EOGT activity. Remarkably, while the O-GlcNAcylation of NUP62 was greatly reduced by **ES1**, the O-GlcNAc level of EGF showed no substantial change upon inhibitor treatment (Fig. 5). These findings strongly support that **ES1** inhibits OGT in cells with minimal impact on other related glycosyltransferases.

In summary, we rationally designed and synthesized targeted covalent inhibitors that modify a unique cysteine of OGT. **ES1** provides sustained reduction of O-GlcNAcylation with OGT being directly validated as the predominant target in cells. This represents, to our knowledge, the first rationally designed targeted covalent inhibitor of any glycosyltransferase, which provides a new strategy to interrogate OGT biological functions and other glycosyltransferases.

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Supplementary Material

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Design and application of ES compounds for targeted covalent inhibition of OGT in cells. (a) O-GlcNAcylation is controlled by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). (b) Chemical structures of **ES1-ES4** and the reported reversible inhibitor Ac₄SSGlcNAc. (c) Western blot using RL2 antibody shows the O-GlcNAc inhibition in MCF7 cells. Cells were pre-treated with each of ES compounds, Ac₄SSGlcNAc, or BZX2 at 12.5 μM for 13 h followed by post-incubation in inhibitor-free medium for 8 h. The relative O-GlcNAc level (%) was normalized to the respective actin and presented as mean ± S.D. from three independent experiments.

Figure 2.

ES1 irreversibly inhibits O-GlcNAcylation in MCF7 cells. Western blot using RL2 antibody shows the O-GlcNAc level of MCF7 cells pre-treated with DMSO, **ES1**, or Ac₄5SGlcNAc at indicated concentration (13 h) followed by post-incubation in inhibitor-free medium (8 h). Cells were either left non-supplemented (a) or supplemented with 15 mM glucosamine (b) five hours before harvest.

Figure 3.

UDP-ES1 specifically reacts with C917 residue to inactivate OGT *in vitro*. (a) The k_{inact}/K_i of **UDP-ES1** with purified OGT. OGT was pre-incubated with **UDP-ES1** for varying amounts of time and then diluted 50-fold before reacting with NUP62 and UDP-³H-GlcNAc. Data represent the average values $\pm$ S.D. from triplicate experiments. (b) Deconvoluted intact protein MS spectra of **UDP-ES1** reacted with wild-type (WT) OGT_{4.5} (an N-terminal truncated OGT)¹⁶ or its C917S mutant at indicated concentration ratio. (c) LC-MS/MS spectrum maps the modification site of **UDP-ES1** on C917 residue (highlighted in red) of full-length OGT. *c*, carbamidomethylation. (d) X-ray crystal structure of OGT:UDP-ES1:CKII complex. Highlighted electron density shows the covalent reaction of **UDP-ES1** with OGT C917 side chain.

Figure 4.

6AzES1 predominantly targets OGT in cells. (a) Strategy to detect the cellular reactivity profile of **6AzES1** using fluorophore alkyne and fluorescence scanning. Total protein loading amount was obtained by Coomassie Blue staining of the same gel. (b) and (c) In-gel fluorescence results of OGT- and YFP-expressing cells. In (b), cells were treated with different concentrations of **6AzES1** for 24 h. In (c), cells were pre-treated with **6AzES1** at indicated concentration for 13 h before 8 h of post-incubation in inhibitor-free medium.

Figure 5.

ES1 showed minimal effect on cellular EOGT. HEK293 cells were transfected either with EGF1-36-*c-myc* or NUP62-*c-myc* followed by 17 h of **ES1** treatment. The O-GlcNAc level of EGF1-36 (a) and NUP62 (b) from *c-myc* immunoprecipitation was detected by Western blot using CTD110.6 and RL2 antibodies, respectively. (c) The relative O-GlcNAc changes of EGF1-36 and NUP62 after **ES1** treatment. Data represent the mean $\pm$ S.D. from three independent experiments. **, $p < 0.01$ (Student *t*-test). G-EGF, O-GlcNAcylated EGF. G-NUP62, O-GlcNAcylated NUP62.