Published in final edited form as:

ACS Chem Biol. 2015 June 19; 10(6): 1392–1397. doi:10.1021/acscchembio.5b00004.

A small molecule that inhibits OGT activity in cells

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Abstract

O-GlcNAc transferase (OGT) is an essential mammalian enzyme that regulates numerous cellular processes through the attachment of *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) residues to nuclear and cytoplasmic proteins. Its targets include kinases, phosphatases, transcription factors, histones, and many other intracellular proteins. The biology of *O*-GlcNAc modification is still not well understood and cell-permeable inhibitors of OGT are needed both as research tools and for validating OGT as a therapeutic target. Here we report a small molecule OGT inhibitor, OSMI-1, developed from a high-throughput screening hit. It is cell-permeable and inhibits protein *O*-GlcNAcylation in several mammalian cell lines without qualitatively altering cell surface *N*- or *O*-linked glycans. The development of this molecule validates high-throughput screening approaches for the discovery of glycosyltransferase inhibitors, and further optimization of this scaffold may lead to yet more potent OGT inhibitors useful for studying OGT in animal models.

O-GlcNAcylation, the attachment of *N*-acetylglucosamine (GlcNAc) to serine and threonine residues of nuclear and cytoplasmic proteins, is a ubiquitous post-translational modification of multicellular organisms¹. The *O*GlcNAc modification is attached by OGT and removed by a cellular glycosidase, OGA, in a process known as *O*-GlcNAc cycling (Figure 1)². Protein *O*-GlcNAc levels fluctuate in response to various cellular stimuli and are strongly correlated with glucose availability because OGT's substrate, uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), is one of the major products of glucose metabolism³⁻⁵. OGT's targets include virtually all classes of intracellular proteins, and *O*-GlcNAcylation has been shown to modify chromatin, modulate kinase signaling, regulate protein stability, alter proteasomal degradation, and affect gene expression⁶⁻⁹. Aberrant OGT activity is a feature of several cancers; a growing body of literature suggests that *O*-GlcNAcylation plays a role in reprogramming cellular metabolism to enable rapid growth¹⁰⁻¹⁴ and that inhibiting OGT may have a beneficial therapeutic effect for the treatment of some cancers^{15, 16}.

In order to validate OGT as a therapeutic target and gain a deeper understanding of its primary biological functions, small molecule OGT inhibitors that demonstrate selective, on-

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target inhibition in cells are required^{17, 18}. While various small molecules are reported to perturb *O*-GlcNAc in cells (Table S1; citations provided in SI references), including alloxan, a uracil mimic, and benzyl 2-acetamido-2-deoxy- α -D-galactopyranoside (BAGDP), a *N*-acetylgalactosamine (GalNAc) mimic, most of these compounds have not been shown to inhibit OGT selectively in cells. Indeed, many reports do not demonstrate OGT inhibition, but rather rely on cellular viability or other downstream readouts as a proxy. In the case of alloxan, it has even been shown that its ability to inhibit OGA surpasses its ability to inhibit OGT^{19, 20}, while BAGDP likely inhibits numerous carbohydrate processing enzymes²¹. Some substrate and bisubstrate mimics that inhibit OGT *in vitro* have been reported, but these inhibitors are not membrane permeable and hence are ineffective in cells²²⁻²⁵. However, in the case of one substrate mimetic inhibitor, UDP-5SGlcNAc, the lack of cell-permeability was overcome by feeding cells a metabolic precursor, Ac4-5SGlcNAc, which is converted to UDP-5SGlcNAc *in vivo*²⁵. Ac4-5SGlcNAc dramatically reduces global *O*-GlcNAcylation in cells, in part because the active form of the inhibitor, UDP-5SGlcNAc accumulates in cells²⁶. As an isostere of UDP-GlcNAc, UDP-5SGlcNAc may inhibit not only OGT, but also other UDP-GlcNAc-dependent enzymes²⁵. Moreover, UDP-5SGlcNAc is epimerized to UDP-5SGalNAc in cells and enzymes that use UDP-GalNAc may also be affected. While Ac4-5SGlcNAc is currently the best cellular inhibitor of OGT, these caveats must be considered when using it. Furthermore, prospects for overcoming off-target effects are limited for close substrate analogs, particularly if they require enzymatic processing in order to become active. Thus, there remains a pressing need for cell-permeable small molecule OGT inhibitors that are amenable to chemical modification.

We previously developed high-throughput screening (HTS) approaches to identify small molecule inhibitor scaffolds for glycosyltransferases, including OGT²⁷⁻³⁰. Several OGT inhibitors of modest potency were identified in a polarization-based fluorescence displacement screen, and three such compounds were reported as *in vitro* inhibitors²⁷. Although we were unable to demonstrate robust inhibition of OGT in cells for these compounds³¹, they nonetheless came into use as OGT inhibitors for biological studies. Scattered reports of cellular efficacy led us to investigate one of these compounds more closely. The compound was shown to operate by an interesting covalent mechanism of action, but proved too reactive to use as a selective OGT inhibitor in cells^{10, 16, 32, 33}. Therefore, we reexamined the screening data to identify scaffolds amenable to optimization and a quinolinone-6-sulfonamide (Q6S) class of compounds appeared promising. Here we describe a cell-permeable OGT inhibitor identified from a biased library screen followed by analog synthesis. The inhibitor shows on-target OGT engagement, as judged by several readouts, but does not appear to alter *N*- or *O*-glycan structures substantially. This work validates the utility of a HTS approach for identifying scaffolds that can lead to probe molecules for studies of OGT's cellular roles.

Four previously undisclosed compounds from our HTS (Figure 2, top left)³¹ were found to contain either a Q6S or a 3,4-dihydro-quinoline-6-sulfonamide core (henceforth both will be referred to as Q6S; colored blue in Figure 2A). Related compounds with substitutions in the Q6S core were not hits in the screen. While the potency of the compounds was weak, the conserved core was deemed promising and so we assembled a library of 1,280 commercial

compounds bearing a Q6S moiety. Compounds were screened at five different concentrations in a fluorescence displacement assay (Figure 2A)²⁷. Using the known binding constant for the substrate analog, the dose-response data allowed us to estimate binding affinities (K_i values) for the hits. The top 40 hits from the primary screen, ranked by K_i , were tested in a secondary radiometric capture assay. The top four confirmed hits all bore a phenylglycine (colored in red, Figure 2A), the Q6S core, and an amide. Various analogs were prepared based on a modular assembly (as in Figure 2B), and OGT inhibition was assessed both *in vitro* and in cells. These efforts, which will be described in detail elsewhere, resulted in the identification of an analog, NCGC00344466, henceforth referred to as OSMI-1 (Figure 2B).

We tested the ability of OSMI-1 to inhibit full length human OGT (ncOGT) in a coupled enzyme assay that measures the UDP produced when GlcNAc is transferred from UDP-GlcNAc to a peptide acceptor. OSMI-1 inhibited ncOGT with an IC_{50} value of 2.7 μ M (Figure 3A). We obtained a similar IC_{50} value using a radiometric capture assay in which a well-characterized protein substrate, nucleoporin62 (Nup62), a heavily glycosylated component of the nuclear pore, is the acceptor substrate (Figure S1A)³⁴. In contrast, the IC_{50} values obtained for UDP-5SGlcNAc in these two assays were 78.8 and 11.1 μ M, respectively. The UDP-GlcNAc concentrations used in the two assays differed by about 6-fold, and given that UDP-5SGlcNAc is a competitive inhibitor with respect to UDPGlcNAc²⁵, this shift in IC_{50} value was expected (Figure S1 and Equation S1). Since the IC_{50} for OSMI-1 was largely insensitive to UDP-GlcNAc concentration (Figures S1C and S1D), we concluded that it did not act competitively with respect to the donor sugar substrate. Consistent with this, we observed that the V_{max} for glycosylation, under saturating acceptor and variable UDP-GlcNAc conditions, decreased with increasing OSMI-1 concentration (Figure 3B).

We next examined the ability of OSMI-1 to inhibit global *O*-GlcNAcylation in Chinese hamster ovary (CHO) cells. This cell line was used previously to evaluate Ac4-5SGlcNAc as an inhibitor²⁵. Cells were treated for 24 hours with varying concentrations of OSMI-1 ranging from 10-100 μ M and cell lysates were probed with the *O*-GlcNAc antibody RL2³⁵. OSMI-1 reduced global *O*GlcNAcylation (Figure 4A) in a dose-dependent manner (Figure S2), with the maximal effect being achieved at 50 μ M. Due to the limited aqueous solubility of OSMI-1, higher concentrations of OSMI-1 did not further reduce *O*-GlcNAc levels (Figure S3). When used at 50 μ M, Ac4-5SGlcNAc reduced global *O*GlcNAcylation to a greater extent than OSMI-1 even though it is a less potent inhibitor *in vitro*. UDP-5SGlcNAc reaches substantially higher intracellular concentrations than OSMI-1 because it cannot diffuse freely across the cell membrane; once formed from Ac4-5SGlcNAc, it accumulates intracellularly, allowing it to compete successfully with UDP-GlcNAc²⁵. Although OSMI-1 is cell permeable, it is relatively large and likely does not reach cellular concentrations comparable with the administered dose.

In an 8-hour time course study of CHO cells treated at 50 μ M with either OSMI-1 or Ac4-5SGlcNAc, OSMI-1 showed a more rapid onset of *O*-GlcNAc reduction. A substantial reduction of global *O*-GlcNAcylation was observed for OSMI-1 within two hours, whereas Ac4-5SGlcNAc showed an effect only at four hours (Figure S3). We also examined several

additional mammalian cell lines and found that OSMI-1 treatment reduced global *O*-GlcNAcylation in all of them (Figures S4 and S5). Having established that OSMI-1 reduces global *O*GlcNAcylation in cells, we investigated the effects of OSMI-1 on specific cellular markers of OGT inhibition. Nup62 bears at least ten *O*-GlcNAc moieties that contribute over 2.5 kDa to the protein mass, and we found that treating cells with OSMI-1 caused Nup62 to shift to a lower molecular weight, consistent with loss of the *O*-GlcNAc residues (Figure 4B)³⁶. It is also known that levels of OGA, the glycosidase that removes *O*-GlcNAc residues from proteins, decreases when cellular *O*-GlcNAcylation is blocked^{25, 37}. We found that OSMI-1, like Ac4-5SGlcNAc, reduced cellular OGA without affecting cellular OGT levels (Figure 4B). Hence, we conclude that OSMI-1 inhibits OGT activity in cells.

A concern with any inhibitor is target promiscuity, and targets of the same enzyme class are a particular concern. Although it is possible to profile kinase inhibitors against panels of kinases, glycosyltransferase panels for inhibitor profiling have not been developed. An accepted strategy to evaluate the selectivity of glycosyltransferase inhibitors uses lectins to probe cell surface glycans following treatment of cells with compound. Commercially available biotinylated lectins that recognize different features of *N*- and *O*-glycans are available for this purpose, and while their binding epitopes are not fully understood, they are useful for assessing whether a given treatment substantially alters glycan composition (Figure 4C). We used nine different biotinylated lectins (ConA, LCA, Jacalin, Pha-E, ECL, Pha-L, GSL-I, PNA or DBA) to probe the glycan composition of CHO cells treated with 50 [proportional]M OSMI-1 or Ac4-5SGlcNAc for 24 hours. For both compounds, we observed minimal changes in bands detected by ConA, PHA-L, ECL, GSL-I, PNA, or DBA, indicating that neither OSMI-1 nor Ac4-5SGlcNAc treatment grossly perturbed the carbohydrate structures recognized by these lectins (Figure 4D; Figure S6)²⁵. For Jacalin, PHA-E and LCA, however, we observed dramatic changes in the glycans from cells treated with Ac4-5SGlcNAc, but not from cells treated with OSMI-1 (Figure 4D). Jacalin detects the GalNAc-peptide portion of mucin-type *O*-glycans, and Ac4-5SGlcNAc treatment resulted in decreased masses for several prominent bands (Figure 4D)³⁸, suggesting that UDP-5SGlcNAc may block some glycosyltransferase(s) involved in mucin-type *O*-glycan synthesis. Ac4-5SGlcNAc treatment also resulted in greatly diminished signal intensity for the lectins PHA-E (Figure S6) and LCA (Figure 4D), suggesting that the inhibitor broadly affects several other types of cell surface glycans³⁹. Given the close resemblance of UDP-5SGlcNAc to substrates used by cellular glycosyltransferases, off-target effects for this inhibitor are perhaps not unexpected, particularly given its high intracellular concentrations⁴⁰.

OGT is essential for development and remains essential in many cell types in both adult organisms and in tissue culture⁴¹, but OGT inhibition by Ac4-5SGlcNAc was reported to have no effect on cell viability^{25, 42, 43}. We tested the effects of 50 μ M OSMI-1 on CHO cells and found that viability decreased by about 50% after 24 hours (Figure S7). In order to evaluate whether this effect resulted from inhibition of OGT or some other target, we prepared a structurally related compound, PG34 (Figure S7A), which bears a phenylalanine in place of the 2-methoxyphenylglycine. PG34 demonstrated poor *in vitro* inhibitory activity against OGT and did not reduce global *O*-GlcNAcylation in cells (Figure S7). However, it

affected cell viability similarly to OSMI-1. Hence, it is possible that OSMI-1 shares a target other than OGT with PG34. Further work is needed to resolve this question, but in the meantime, PG34 may serve to distinguish OGT-dependent phenotypes from effects on other cellular targets.

In summary, we have described a cell-permeable small molecule OGT inhibitor that was identified through a combination of high-throughput screening and follow-up chemistry. Through the use of a biased library and follow-up medicinal chemistry we were able to dramatically improve on the potency of the initial screening hits (nearly 100x), and we identified a compound that inhibits OGT in cells⁴⁴. We demonstrated that this compound has on-target activity in cells based on its ability to reduce global *O*-GlcNAcylation, inhibit *OGlcNAcylation* of cellular Nup62, and reduce OGA levels. We suggest that validation of other small molecule OGT inhibitors include evaluation of Nup62 glycosylation, which is a convenient biomarker because the protein is ubiquitously expressed and so highly glycosylated that inhibition of *OGlcNAcylation* results in a detectable mass shift. Although OSMI-1 is not as effective at reducing global *O*-GlcNAcylation at 24 hours as the same concentration of Ac4-5SGlcNAc, it has a more rapid onset of action owing to its ability to enter cells in an active state, and it does not appear to drastically alter other cellular glycans. OSMI-1 may thus be useful in conjunction with other inhibitors and methods for OGT inhibition/depletion to probe OGT inhibition phenotypes in cells. Furthermore, because OSMI-1 is not a substrate analog, it may be possible to optimize it further to increase potency. In order to accomplish this, it will be important to understand its mode of action better. In particular, it was unexpected that inhibition is not overcome by high UDP-GlcNAc concentrations because the compound series was discovered in a UDP-GlcNAc displacement screen and several closely related inhibitors have IC₅₀ values that do depend linearly on UDP-GlcNAc concentration. Understanding how OSMI-1 inhibits OGT may lead to more potent compounds for investigating OGT as a therapeutic target.

Materials and Methods

Chemistry

Methods for the synthesis of OSMI-1 and PG34, compound characterization and spectra are described in detail in the supporting information. Protein biochemistry: Methods for the production and purification of GST-Nup62 and ncOGT can be found in the supporting information. In vitro and in vivo inhibition: Methods used for evaluating the in vitro inhibition of OGT, as well as the methods for measuring the degree of OGT inhibition in cells and the effect of OSMI-1 on cellular viability are described in the supporting information. Methods for evaluating changes to cell-surface glycan structures are also detailed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by the Division of Preclinical Innovation, National Center for Advancing Translational Sciences, the Molecular Libraries Initiative of the National Institutes of Health Roadmap for Medical Research, grant #U54CA143930 and the National Institutes of Health (GM094263 to S.W. and GM100539 to R.F.O.M.). M.O. was supported by training grant 5 T32 GM095450-04. J.J. was supported by NSERC PGS-M and D3 fellowships. We thank D. Vocadlo for generously providing UDP-5SGlcNAc, Ac4-5SGlcNAc and thoughtful comments on the manuscript. We also thank J. Gildersleeve for helpful advice on lectin specificity.

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44. In general, compounds were found to be about an order of magnitude more potent against sOGT than against ncOGT.

Figure 1.
OGT adds *O*-GlcNAc to proteins and OGA removes this modification.

Figure 2.

Identification and optimization of OSMI-1. A) Several Q6S (moiety colored in blue)-containing hits were identified in a high-throughput screen²⁷ and their IC_{50} values against sOGT are shown⁴⁴. A 1,280-member library of commercially available Q6S-containing molecules was subsequently screened using an FP displacement assay, and selected hits are shown. Q6S derivatives bearing a phenylglycine residue (moiety colored in red) were some of the best hits in a secondary radiometric capture assay. This scaffold was optimized through medicinal chemistry to OSMI-1. B) The synthetic route to OSMI-1.

Figure 3. OSMI-1 inhibits OGT *in vitro*. A) OSMI-1 inhibits OGT activity in a dose-dependent manner. B) When using fixed saturating concentrations of GSTNup62 (protein acceptor), the V_{max} changes as a function of OSMI-1 concentration, suggesting that it is not competitive with respect to UDP-GlcNAc (See also Figure S1).

Figure 4.

OSMI-1 inhibits OGT *in vivo* and does not grossly perturb cell-surface glycan structures. A) Lysates from CHO cells, untreated or treated with either OSMI-1 or Ac4-5SGlcNAc, at 50 μ M, were immunoblotted for global O-GlcNAc. A full RL2 blot is shown in Figure S5B. B) Markers of OGT inhibition include a mass shift of Nup62 and a decrease in OGA levels while OGT levels remain unchanged. C) Lectins ConA, LCA and jacalin (JAC) can recognize extracellular glycan structures, which should not be affected by a specific inhibitor of OGT. D) Lysates from cells, untreated or treated, at 50 μ M, with either OSMI-1 or Ac4-5SGlcNAc, were probed with lectins ConA (left), LCA (middle) and JAC (right).