Discovery of a Low Toxicity O-GlcNAc Transferase (OGT) Inhibitor by Structure-based Virtual Screening of Natural Products

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O-GlcNAc transferase (OGT) plays an important role in regulating numerous cellular processes through reversible post-translational modification of nuclear and cytoplasmic proteins. However, the function of O-GlcNAcylation is still not well understood. Cell permeable OGT inhibitors are needed to manipulate O-GlcNAcylation levels and clarify the regulatory mechanism of this modification. Here, we report a specific natural-product OGT inhibitor (L01), which was identified from a structure-based virtual screening analysis. L01 inhibited O-GlcNAcylation both in vitro and in cells without significantly altering cell surface glycans. Molecular dynamics and site-directed mutagenesis indicated a new binding mechanism in which L01 could interact with Asn557 near the UDP binding pocket of OGT. This residue may contribute to the specificity of L01. Furthermore, as a specific OGT inhibitor, L01 produced low toxicity in cellular and zebrafish models. The identification of L01 validates structure-based virtual screening approaches for the discovery of OGT inhibitors. L01 can also serve as a chemical tool to further characterize O-GlcNAcylation functions or a new molecular core for structure-activity relationship studies to optimize the biochemical potencies.

O-GlcNAc transferase (OGT) mediates protein O-GlcNAcylation, a ubiquitous posttranslational modification characterized by the attachment of N-acetylglucosamine moieties from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) to serine or threonine residues of nuclear and cytoplasmic proteins in multicellular eukaryotes. Results from global proteomic experiments have shown that hundreds proteins involved in a wide range of cellular functions, are dynamically and reversibly modified with O-GlcNAc. O-GlcNAcylation has been proposed to modulate gene transcription, signal transduction, cellular stress response and protein stability. Altered protein O-GlcNAc profiles have been associated with the occurrence and development of numerous critical diseases, including diabetes, cardiovascular disease, cancer, Alzheimer’s disease and other neurodegenerative disorders. Aberrant OGT activity was reported to be a feature of several illnesses including cancer, and selective small-molecule OGT inhibitors would be useful as probes to investigate the primary biological functions of O-GlcNAc and could validate OGT as a therapeutic target. Therefore, OGT inhibitors that demonstrate selective, on-target inhibition and low toxicity in cells are required.

However, most of the reported compounds in the last few years have not been shown to inhibit OGT effectively or selectively. The uracil analogue Alloxan was the first reported OGT inhibitor but was questionable due to cellular toxicity and off-target effects. Ac5,5-S-GlcNAc and BADGP are mimics of the OGT donor substrate UDP-GlcNAc. These two compounds affected other glycosyltransferases by either direct or indirect inhibition, which induced abnormal cell surface glycan expression. Other substrate mimics were also proposed to inhibit OGT in vitro, but they were not cell-permeable and therefore were ineffective in cells. More recently, OGT inhibitors discovered by high-throughput screening showed some utility in cells. BZX is a covalent inhibitor identified by this strategy. However, it was shown to be potentially toxic and have off-target effects. Similarly, another cell-permeable inhibitor, OSMI-1, was recently designed and shown to minimally affect cell surface

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glycan synthesis. Nevertheless, it decreased cell viability by approximately 50% after treatment\(^1\). Therefore, future work is needed to develop cell-permeable, potent and low toxicity OGT inhibitors with novel scaffolds.

Natural products have long served as potentially rich source of novel bioactive scaffolds that display remarkable chemical diversity in structure and function\(^5\). An assessment of all FDA-approved new molecular entities reveals that natural products and their derivatives represent over one-third of all new molecular entities\(^6\). Moreover, modified natural products have shown special selectivity toward disease-related targets with cellular permeability\(^7\). Nevertheless, no natural-product OGT inhibitor has been reported. Structure-based virtual screening has emerged as an efficient strategy in natural product drug discovery, complementing conventional random screening\(^8\). By eliminating inactive non-binders in silico, the numbers of compounds to be tested in vitro can be dramatically reduced. As the crystal structure of human OGT in complex with the donor sugar substrate UDP-GlcNAc has been used to characterize enzyme-substrate interactions\(^9\), it is available for virtual screening for the rapid and efficient discovery of lead natural compounds against OGT.

Based on these data, a structure-based high-throughput virtual screening was carried out. The ADME-Tox (absorption, distribution, metabolism, excretion and toxicity) prediction was applied to evaluate the properties of the small molecule candidates before screening, and twelve compounds of the top ranked 200 in silico were preliminarily tested for inhibition of OGT activity. L01 was further selected to undertake an exploratory study of its in vitro OGT inhibitory effects. Moreover, we demonstrated that L01 specifically inhibited O-GlcNAcylation in cells without significant acute toxicity \(\textit{in vivo}\). This potential lead compound identified from the current study can be useful in the design and development of a novel OGT inhibitor.

**Results**

**Identification of a novel OGT inhibitor from a virtual screen.** To date, although various classes of OGT inhibitors were reported\(^9\), virtual screening has not been used in OGT inhibitor discovery. In this study, structural-based virtual screening was conducted following the strategy shown in Fig. 1a. Sixteen X-ray structures of the human OGT binding to UDP or UDP-GlcNAc that represent various conformational changes of OGT were used as the receptor library for virtual screening. The search area for the screening was set near the UDP binding pocket of OGT\(^9\). A total of approximately 61,000 compounds from TCM Database@Taiwan, a chemical library of natural products and natural product-like molecules, were screened. ADME-Tox and other properties were predicted for all molecules by the FAF-Drugs server. Then, 4234 compounds were assessed by the virtual screening with the 16 structures in the OGT structures-ensemble library by Autodock Vina. The top 200 common compounds with high average Vina scores for each OGT structure were selected for further study.
After visual inspection considering the docking poses, structural diversity and novelty, 12 candidates (structures are shown in Supporting Information Table S1) from the 200 compounds were purchased. To evaluate their ability to inhibit OGT activity in vitro, these compounds were tested in a preliminary HPLC assay that detects the transfer of GlcNAc to a well-characterized OGT peptide substrate CKII (Fig. 1b). From these results, the biflavonoid derivative L01 (Fig. 1c and d) emerged as the top candidate.

L01 inhibits OGT in vitro. To confirm the ability of L01 to inhibit OGT, we assessed its inhibitory activity using a UDP-Glo glycosyltransferase assay, which measures the UDP production when GlcNAc is transferred from UDP-GlcNAc to a peptide acceptor (CKII)20. A dose-response experiment was performed to determine the potency of L01 in inhibiting the GlcNAc transfer activity of OGT. L01 effectively inhibited OGT with an IC50 of 21.8 μM (Fig. 2a). A similar IC50 value (20.2 μM) was obtained using the HPLC assay (Fig. S1a). OSMI-1 (50 μM) was recently reported to have a higher specificity against OGT than other extant compounds. In an in vitro assay, OSMI-1 had a 20-fold lower IC50 value compared to ST045849 (a commercially available OGT inhibitor). OSMI-1 has recently been used to study the role of O-GlcNAc in the replication of herpes simplex virus (HSV), suggesting the effective OGT inhibition of this compound20. In our experimental condition, the IC50 values of OSMI-1 in the UDP-Glo and HPLC assays were 3.5 and 6.2 μM, respectively (Fig. S1a and b). In addition, L01 was found to inactivate OGT in a time-dependent manner when we assessed OGT activity after preincubation with L01 for 30 min before addition of the substrate (Fig. 2b, OSMI-1 was used as a control). This abnormal time-dependent inhibition of OGT indicated that other inhibition mode may exist. The higher IC50 and longer working time of L01 compared with OSMI-1 also suggested that the affinity of L01 to OGT was weaker than OSMI-1. Additional experiments were performed to exclude potential nonspecific OGT inhibition of L01, such as through redox, aggregation, and irreversible inhibition (Fig. S1b and c). Then, we investigated the inhibition mode of L01. The shift in IC50 values for L01 did not correlate to low concentrations UDP-GlcNAc (Fig. 2c). The IC50 values of L01 increased in
a stepwise fashion with the increasing UDP-GlcNAc concentrations and IC$_{50}$ values correlated to UDP-GlcNAc when UDP-GlcNAc was above 25 $\mu$M, indicating that L01 may not completely act as a UDP mimic. Consistently, under saturating CKII peptide, variable UDP-GlcNAc and initial rate conditions, the V$_{\text{max}}$ for O-GlcNAcylation decreased with the increasing concentration of L01 (Fig. S2a).

We next probed the inhibition of O-GlcNAc levels in vitro on a purified protein acceptor nucleoporin 62 (Nup62, Rattus norvegicus), which is a heavily glycosylated component of the nuclear pore. Immunoblot analysis using an anti-O-GlcNAc antibody (CTD110.6) showed that O-GlcNAc modification of recombinant Nup62 by purified OGT in a cell free reaction system was decreased along with a shift in molecular weight by L01 treatment (Fig. 2d, OSMI-1 was used as a control). Similar results were obtained using a commercially available enzymatic method (O-GlcNAc Enzymatic Labeling System) that involves chemoselective ligation of biotin to terminal GlcNAc residues (Fig. 2e). We also performed a parallel experiment with another glycosyltransferase, ppGalNAc-T2, to evaluate the specificity of L01. This compound did not strongly inhibit ppGalNAcT2 (IC$_{50}$ > 500 $\mu$M, Fig. S3a). Moreover, L01 did not affect the activity of other cellular enzymes (glucose oxidase, lactic dehydrogenase and thioredoxin reductase, Fig. S3b), suggesting that L01 cannot interact with these proteins and affect their activity in a non-specific mode. These results suggest that L01 effectively blocks the glycosylation by OGT in vitro and it is therefore a potential OGT inhibitor.

L01 putatively interacts with residues in the UDP-binding pocket of OGT. To address the structural mechanism of the strong inhibitory activities of the newly identified natural-product inhibitor with OGT, the binding mode of the L01-OGT complex was studied by molecular dynamics (MD) simulation and free energy calculation methods (Fig. S4 and Table S2). All MD simulations adopted the OGT structure 3PE3. MD results showed that the binding site of L01 in OGT was similar to that of UDP (Fig. S5a). The B and C rings of L01 were deep inside the UDP binding cavity, which mapped onto the phosphate groups of UDP forming hydrogen bonds with Lys842, His920 and Thr922. The D ring of L01 was predicted to be located at the entrance of the UDP binding pocket, where the uridine group of UDP was located (Fig. 3a). Compared with the binding free energy of UDP ($-554.772$ kJ/mol), L01 ($-100.334$ kJ/mol, Table S2) did not show full inhibition of OGT activity, indicating that the IC$_{50}$ of L01 was reasonably high.

Interestingly, another hydrogen bond between Asn557 and hydroxyl groups in the D ring made a strong contribution to the interaction between L01 and OGT. This bond has not been previously described in the OGT-UDP simulations or crystal structures. Therefore, we estimated the impact of an Asn557 to Ala mutation (N557A) in silico and in vitro. As expected, the binding residues of L01 were substantially different between wild type OGT (OGT$^{WT}$) and N557A OGT (OGT$^{N557A}$) (Fig. 3b). Moreover, this mutation also resulted in a significant
decrease in the binding energies of L01 (\(\text{OGT}^{\text{WT}} = -100.334\,\text{kJ/mol}, \text{OGT}^{\text{N557A}} = -60.005\,\text{kJ/mol}\)) but not UDP (Table S2). Site-directed mutagenesis of this residue followed by an in vitro glycosylation assay showed similar results, indicating that L01 interacts with Asn557 near the UDP-GlcNAc-binding pocket of OGT (Fig. 3c). Additional kinetic assay showed that L01 was insensitive to UDP-GlcNAc concentration in OGT N557A (Fig. S2b). These dates indicated that N557A mutation inhibited the binding of L01 to OGT and might affect the competition of this compound with UDP-GlcNAc. Consistent with previous reports, mutation of Lys898 (K898A), which is involved in UDP binding, results in the complete inactivation of the OGT activity.

**L01 is an efficient, specific OGT inhibitor in cells.** Given that L01 could block OGT glycosylation activity in a cell-free system, we subsequently investigated the ability of L01 to antagonize OGT activity in living cells. After 4 h glucose starvation, COS7 cells were incubated with L01 ranging from 10 to 150 \(\mu\text{M}\) for 6 h. L01 attenuated global O-GlcNAcylation in a dose-dependent manner as evaluated by immunoblotting using CTD110.6 (Fig. 4a). L01 was less effective in inhibiting cellular O-GlcNAcylation than OSMI-1 at 10 \(\mu\text{M}\). The maximal effect of L01 was observed at 100 \(\mu\text{M}\). By contrast, due to the limited aqueous solubility, concentrations higher than 50 \(\mu\text{M}\) of OSMI-1 did not decrease O-GlcNAc levels further. Although L01 showed weaker inhibition of OGT than OSMI-1 in vitro, it was comparable with OSMI-1 in reducing of global O-GlcNAc levels in living cells at 50 \(\mu\text{M}\). We also compared L01 with other established OGT inhibitors such as BADGP and ST045849. At the same concentration (50 \(\mu\text{M}\)), L01 showed stronger OGT inhibition than the other two compounds in HeLa and K562.
cells (Fig. S6). In immunocytochemistry analysis, L01 treatment uniformly lowered O-GlcNAcylation within COS7 cells (Fig. 4b). This result was subsequently confirmed using click chemistry involving metabolic labeling of the O-GlcNAcylation protein with tetraacetylated N-azidoacetylglucosamine (Fig. 4c). We also observed that COS7 cells treated with 50 μM L01 showed a time-dependent decrease in global O-GlcNAcylation (Fig. 4d). However, in a parallel test, OSMI-1 showed a more rapid onset of glycosylation reduction than L01. A significant reduction of O-GlcNAcylation was observed for L01 only at 6 h, whereas OSMI-1 showed a similar effect within 2 h. In addition, L01 also reduced global O-GlcNAcylation with similar effect as OSMI-1 at 50 μM in several other mammalian cell lines (293, Hela, K562, Fig. S6).

Having established that L01 reduces global O-GlcNAcylation in cells, we next investigated O-GlcNAc levels on specific cellular markers after treatment with this compound by immunoprecipitating these proteins from cell lysates (Fig. 4e). As expected, Nup62 from untreated cells showed O-GlcNAc modification by using CTD110.6, whereas little O-GlcNAc modification of Nup62 was found in the cells treated with L01. Immunoprecipitated Nup62 was also probed with an O-GlcNAc Enzymatic Labeling System, and similar results were obtained by detection with streptavidin. Furthermore, L01 caused Nup62 to shift to a lower molecular weight, consistent with a loss of the O-GlcNAc residues. OGA is also O-GlcNAcylated and OGA levels of are regulated by this modification. In this study, L01 reduced cellular OGA levels without affecting cellular OGT levels. From Fig. 4e, we can also observe that L01 was as effective as OSMI-1 in suppressing O-GlcNAcylation of specific targets in living cells. Therefore, these results demonstrate that L01 inhibits OGT activity in cells.

Concerning the target promiscuity, lectins were used to analyze cell glycans following the treatment of cells with L01. Ten biotinylated lectins, Concanavalin A (ConA), Dolichos biflorus Agglutinin (DBA), Ulex europaeus Agglutinin (UEA), Jacalin (JAC), Soybean Agglutinin (SBA), Vicia villosa Lectin (VVL), Lotus Lectin (LL), Peanut Agglutinin (PNA), Ricinus Communis Agglutinin (RCA), and succinylated Wheat Germ Agglutinin (sWGA), which recognize different features of N- and O-glycans, were employed to probe the glycan composition. COS7 cells were treated with 50 μM L01 or OSMI-1 for 24 h. For a positive control, the levels of succinylated WGA (sWGA), which recognizes N-acetylglucosamine, were reduced to similar levels by either L01 or OSMI-1 (Fig. 4f). No visible changes in bands detected by ConA, DBA (Fig. 4e), UEA, VVL, LL, PNA, RCA or SBA (Fig. S7) were observed in COS7 cells treated with both compounds. However, for JAC, L01 but not OSMI-1 treatment caused slight changes in the glycans from COS7 cells (Fig. 4f), indicating that L01 may block mucin-type O-glycan synthesis. Nevertheless, we confirmed that L01 is an efficient, specific OGT inhibitor in cells.

L01 is a low toxicity OGT inhibitor. Most reported OGT inhibitors possess off-target toxicity. To determine whether there were non-specific effects of L01, we first tested the effects of up to 100 μM L01 on COS7 and Hela cells. Minimal dose-dependent effects on cell viability were observed after L01 treatment for 24 h as shown by CCK8 analysis (Fig. 5a). We hypothesized that L01 may reduce the cell proliferation by inhibiting O-GlcNAcylation of proliferation related proteins. By contrast, 50 μM OSMI-1 decreased the cell viability by more than 50%, indicating more side effects. To further evaluate L01’s specificity, three mononuclear cells isolated from primary normal peripheral blood mononuclear cells (PBMCs) were studied (Fig. 5b). Primary cells were treated with an increasing concentration of L01 or OSMI-1, and Annexin V was measured by flow cytometry after 24 h. L01 was relatively nontoxic towards normal PBMCs, whereas OSMI-1 showed a dose-dependent induction of cell death.

As a convenient and predictive animal model, the zebrafish model organism is increasingly used for assessing drug toxicity. Numerous studies have confirmed that mammalian and zebrafish toxicity profiles are strikingly similar, and zebrafish can usually serve as an intermediate step between cell-based evaluation and conventional animal testing. In this study, the zebrafish model was used to investigate L01 acute toxicity in vivo (Fig. 5c). The well-known toxic compound staurosporine (Stau) was used as a benchmark. Concentrations of L01 from 0.0054 mg/mL (10 μM) to 0.054 mg/mL (100 μM) were investigated, and no zebrafish death was observed even at the highest dose at 12 and 24 h, while the LC50 of OSMI-1 was 0.031 mg/mL (56 μM, 12 h) and 0.025 mg/mL (45 μM, 24 h). From these data, we demonstrated that L01 has low toxicity in vitro and in vivo.

Discussion

Natural products offer a rich source of bioactive structural motifs for identifying novel low molecular weight lead structures that are active against a wide range of assay targets. However, there have been no reports of natural product-like OGT inhibitors. In this study, we described a cell-permeable and relatively low toxicity small molecule OGT inhibitor, L01, which was identified through structure-based virtual screening from natural products. L01 is an amentoflavone derivative and was reported as the major anti-inflammatory compound in the seed of Semecarpus anacardium. Amentoflavone also possesses several bioactivities, such as antitumor and antifungal effects. Nevertheless, the mechanism by which amentoflavone exhibits its antitumor activity is not completely clear. To the best of our knowledge, this class of compounds has not been reported as OGT inhibitors in the literature. O-GlcNAcylation is one of the most common posttranslational modifications and modulates the function of a wide-range of proteins, regulating various biological processes, such as gene expression, metabolism, signal transduction, cellular stress response and protein stability. Given the complexity of OGT’s biochemical activities, inhibition of OGT by L01 would interfere with other cellular processes beyond O-GlcNAcylation. This may partially explain the anticancer mechanism of L01’s analogue amentoflavone.

Even with its current limitations, in silico virtual screening offers a practical pipeline to discover new potential drug-like compounds for pharmaceutical research. Many novel scaffolds have been successfully discovered using structure-based computational analysis. Based on high-resolution X-ray 3D crystal structures of OGT, structure-based virtual screening was employed. The workflow of our screening centered on chemical structure standardization and preparation, followed by careful evaluation of the sugar donor binding site of OGT in the receptor library. The multiple protein structures helped to realize the receptor flexible partially due to relatively...
independent and flexible side chains. We narrowed our interests from a total of 61,000 compounds to 200 top candidate compounds. Among these, 12 were tested for inhibition of OGT activity in HPLC based preliminary experiments. Of the candidate compounds tested, L01 showed remarkable inhibition of OGT activity. Based on the docking and molecular dynamics analysis, we anticipate that L01 may form hydrogen bonds with several important residues in the UDP binding cavity of OGT to insert deeply into the cleft. Among these residues, Asn557 forms an important interaction with L01, which was not found in UDP (Fig. S5a). In virtual mutation simulation, L01 tended to interact with Phe837 and Phe868 in OGT N557A by hydrophobic and van der Waals interactions, but weakly interacted with the other part of the UDP binding cleft. However, this mutation did not significantly change the binding energy of UDP, suggesting the specificity of N557 in L01 binding. As L01 has larger topological polar surface area and higher rigidity than UDP, ring D of L01 occupied the entrance of the UDP binding pocket between N557 and A897, which was difficult for UDP to reach. Further site-directed mutagenesis to target the potentially important OGT amino acid residue Asn557 followed by an in vitro glycosylation assay showed that N557A influences the binding of L01 but not UDP. These data suggest that L01 can bind to the UDP-GlcNAc-binding pocket, and Asn557 contributes to L01’s inhibitory activity. We hypothesize that N557 is a characteristic residue that differentiates the UDP binding pocket of OGT from others and might contribute to the specificity of L01. Thus, N557 might be a specific binding site for OGT inhibitor screening. Multiple methods were used to verify the OGT inhibitory activity of L01 in vitro. Similar IC_{50} values (21.8 μM and 20.2 μM) of L01 were measured by UDP-Glo glycosyltransferase and HPLC assays, respectively. Although the binding sites of L01 were similar to that of UDP, the binding free energy of L01 was much higher than UDP. Thus, it is reasonable that the IC_{50} of L01 is high. Notably, by using O-GlcNAc antibody CTD110.6, we demonstrated that this compound could inhibit O-GlcNAcylation of purified Nup62 by OGT in a cell-free system. To eliminate the nonspecific immunostaining of CTD110.6, O-GlcNAc Enzymatic Labeling System was employed. This methodology is highly specific, because the GalT (Y289L) in this system recognizes terminal GlcNAcs with high affinity. Using this approach, inhibition of purified Nup62’s O-GlcNAcylation by L01 was detected. IC_{50} values did not completely depend on UDP-GlcNAc concentration, indicating that L01 did not fully act as a mimic of UDP-GlcNAc. This was consistent with its weak affinity to OGT. We noticed that the IC_{50} values increased in a stepwise fashion with the increasing UDP-GlcNAc concentration. L01 was presumed to occupy the UDP-binding pocket of OGT and compete with UDP. However, our data suggested that L01 does not completely act as predicted. We supposed that other binding sites near UDP might exist and influence the binding mode of L01. These assumed binding sites may affect the binding of UDP-GlcNAc directly. Further investigation of the binding mode...
and structure-activity relationship analyses are needed to understand its precise mechanism of OGT inhibitory activity. Additionally, on-target activity of L01 was then detected in cells based on its ability to reduce cellular global O-GlcNAcylation; this compound inhibited O-GlcNAcylation of cellular Nup62, and reduced OGA protein levels. The reduction of global O-GlcNAcylation in several additional mammalian cell lines was also found by L01 treatment.

Taken together, these data suggest that L01 acts as an inhibitor of OGT activity by binding, at least in part, to the OGT substrate binding pocket. As described in the introduction, the potential non-specific effects of the developed OGT inhibitors caused their off-target toxicity. Target promiscuity of these inhibitors may contribute to their toxicity. Thus, targets of the same enzyme class are a particular concern in developing an on-target OGT inhibitor. ppGalNAcT2 is a member of the UDP-GalNac polypeptide N-acetylgalactosaminyl-transferase (ppGalNac-T) family of enzymes which transfers GalNac to serine or threonine residues of proteins. This glycosyltransferase regulates the initial key steps of O-glycosylation41. L01 could not inactivate ppGalNAcT2, suggesting L01’s selectivity and specificity for OGT. Probing with various lectins to detect the change of cell surface glycans following treatment of cells with the compound is another accepted strategy to evaluate the selectivity of glycosyltransferase inhibitors. Minimal interference with cell surface glycosylation was observed, indicating this compound did not substantially alter glycans composition.

To further assess L01’s on-target activity, the toxicity of this compound was tested in vitro and in vivo. First, in contrast to OSMI-1, L01 had a slight effect on cell viability in a variety of mammalian cell lines, including tumor and normal cell lines. It was reported that 250 μM amentoflavone induced apoptosis in cancer cells26. We speculate that high concentrations of amentoflavone may enhance other side effects to induce cell death in cancer cells. Additionally, genetically ablating OGT in some cells led to their death32, suggesting that OGT plays a critical role in cellular physiology. However, the OGT functions that are essential for cell survival are not well understood. Based on different cell backgrounds, reduction of O-GlcNAc-modified proteins may lead to proliferation inhibition or modest cell death levels. Second, mononuclear cells isolated from primary normal PBMCs were used, and similar results were obtained. Finally, the zebrafish model was employed to investigate the acute toxicity of L01. In contrast to OSMI-1, L01 presented low acute toxicity in the zebrafish model. From these data, we demonstrated that L01 has on-target OGT inhibitory activity with low toxicity in vitro and in vivo.

In conclusion, we utilized structure-based virtual screening to discover a cell-permeable natural product inhibitor of OGT. L01 specifically inhibited OGT activity in a cell-free system and reduced global O-GlcNAcylation in living cells, with potencies comparable to the well-known OGT inhibitor OSMI-1. Additionally, compared with other OGT inhibitors, L01 showed low toxicity in vitro and in vivo, indicating its on-target inhibition of OGT. Our molecular modeling analysis suggests that L01 might form multiple hydrogens bonds with OGT in the same positions as the sugar donor UDP. Notably, L01 could bind to N557, which is near the UDP-binding pocket of OGT and might contribute to the specificity of L01. This study also validates the use of structure-based molecular docking to discover novel inhibitors of OGT. We envisage that L01 may be employed as a useful scaffold for the development of more potent OGT inhibitors.

**Materials and Methods**

**Cells and reagents.** COS7, MCF10A, HeLa and K562 cells were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 containing 10% fetal bovine serum at 37 °C with 5% CO₂. Primary normal hematopoietic cells were obtained from three healthy volunteers donating their peripheral blood mononuclear cells (PBMCs). The authors confirm that all methods were carried out in accordance with written consent under approval of the Dalian University of Technology Review Board, that all experimental protocols were approved by the Medical Ethics Committee of School of Life Science & Medicine and that informed consent was obtained from all subjects. Mononuclear cells from peripheral blood samples were isolated by Ficoll-Hypaque sedimentation (Sigma Chemical Co., St. Louis, MO, USA). Cells were either used directly or cryopreserved in liquid nitrogen.

L01 and other compounds were purchased from BioBioPha Co., Ltd. (Kunming, China). UDP-GlcNAc, OSMI-1 and BADGP were purchased from Sigma-Aldrich (MO, USA). ST045849 was purchased from TimTec (DE, USA). All compounds were solubilized in dimethyl sulfoxide (DMSO) to a storage concentration of 20 mM. The UDP-Glo assay kit was purchased from Promega (WI, USA). CKII peptide (KKKYPGGSTPVSSANMM) was synthesized by ChinaPeptides (Shanghai, China). Metabolic labeling reagents Click-iT GlcNAz, Biotin-alkyne, Protein reaction buffer kit and O-GlcNAc enzymatic labeling system were from Invitrogen (CA, USA). All the compounds were dissolved in DMSO.

**Molecular modeling.** A total of 16 X-ray structures of human OGT (3PE3, 3PE4, 3TAX, 4AY6, 4CDR, 4GYW, 4GY, 4GZ3, 4N39, 4N3A, 4N3C, 4X19, 4XIF, 5BNW, 5C1D, 5HGV) bound to UDP, UDP-GlcNAc or an inhibitor from the Protein Data Bank were prepared as the receptor ensemble. The multiple receptor conformations for flexible docking is a practical shortcut to realize the receptor flexibility that could improve docking calculations49. These conformations exhibited considerable similarities, especially in the UDP binding pocket, but still had differences at the sidechains of many important residues (Fig. S5b). The 16 receptor structures were superimposed by UCSF Chimera to locate the same coordinate using a convenient docking box. A 22 × 22 × 22 Å box was set with the center next to the side chain of Pro559 to cover the UDP binding pocket of OGT.

Nearly 61,000 compounds from TCM Database@Taiwan were used as the ligand library. All molecules were predicted for ADME-Tox and other properties by the FAF-Drugs server. The whole library was filtered using the following properties: (1) 250 ≤ MW ≤ 600; (2) neutral and non-zwitterionic; (3) 2 ≤ ring systems ≤ 4; (4) hydrogen bond acceptors ≥ 1 and hydrogen bond donors ≥ 2. Concerning OGT conformational changes induced by UDP or other molecules, the free form structure of OGT was obtained, and no significant conformational change

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was found (Fig. S5c). Then, 4234 compounds were assessed by the virtual screening with the 16 conformations of OGT structures ensemble by Autodock Vina.

The average Vina scores of the lowest binding free energies for each ligand were re-ranked to obtain the compounds for further filter. The docking poses of Top 200 compounds with high average Vina scores were chosen for the ensemble cluster by UCSF Chimera. The biggest clusters of each compound were used for detailed research on the interaction between the ligand and receptors. The polar interaction with the key residues and regions, such as Q839, K842 and T921 in UDP binding pocket, were used as the necessary guideline. Meanwhile, the biological source and known bioactivity were used as reference.

All MD processes were produced by Gromacs 4.6.7 with an Amber99sb force field, and the parameters of ligands were calculated by Chimera and ACYPTE. The complex was solvated in a cubic TIP3P water box with 1 nm distance from the edge, which was neutralized by sodium ions. The temperature of the system was gradually heated to 300 K over 100 ps to perform the 5 ns NVT equilibration and 5 ns NPT equilibrations subsequently after two steps of energy minimization. Finally, the 10 ns MD simulations at 300 K and 1 atm were carried out with the LINCS algorithm to restrain the hydrogen positions at their equilibrium distances, which allowed the use of an integration time step of 2 fs. The binding free energy was calculated by the Molecular Mechanics-Poisson-Boltzmann Surface Area (MM-PBSA) method with g_mmpbsa, a plugin of Gromacs.

**Protein purification.** The plasmids encoding OGT were a gift from Professor David J. Vocadlo (Simon Fraser University, Canada). Mutants (N557A and K988A) of OGT were generated using the QuickChange site-directed mutagenesis kit (Stratagene). The gene encoding Nup62 was subcloned into a pET28a vector. The expression and purification of Nup62, the full length OGT and its mutants were performed following previously described protocols.

**Western/lectin blots.** All biotinylated lectins were purchased from Vector Laboratories (CA, USA). The O-GlcNAc specific antibody CTD110.6 was purchased from Covance (WI, USA). Anti-OGT, anti-OGA, anti-Nup62 and anti-GAPDH antibodies were purchased from Cell Signaling Technology (MA, USA). HRP-conjugated goat anti-mouse IgM, goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology (CA, USA). Streptavidin-HRP was purchased from Thermo (Shanghai, China). Samples were analyzed using standard procedures. Then, blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo).

**Immunocytochemistry.** COS7 cells were fixed in 4% paraformaldehyde at 37 °C for 15 min and washed 3 times with PBS. Cells were permeabilized using 0.1% Triton X-100 in PBS-T for 0.5 h and blocked with 5% goat serum in 1% bovine serum for 1 h at room temperature. Then, COS7 cells were incubated with primary antibodies against O-GlcNAc (1:50, CTD110.6) diluted in 5% goat serum in 1% bovine serum overnight at 4 °C. Secondary antibody (goat anti-mouse IgM, conjugated to FITC) were used to visualize the proteins. Cells were cover slipped with Vectashield Mounting Medium with DAPI (Thermo) and mounted onto slides. Image acquisition was performed on a Leica confocal microscope.

**OGT activity assay.** HPLC was used to preliminarily analyze the inhibition of OGT by the compounds. According to the previously described, the reaction condition was optimized. 200μM CKII, 300nM OGT, 1 mM UDP-GlcNAc, the compounds (100 μM) and buffer (150 mM NaCl, 1 mM EDTA, 2.5 mM tris(hydroxypropyl)phosphine, 25 mM Tris-HCl, pH 7.4) were mixed and incubated at room temperature for 1 h. After being precipitated by methanol, the reaction mixtures were loaded onto HPLC (the reverse-phase chromatographic column was Zorbax SB-C18 StableBond analytical column, 250 mm × 12.5 mm, 5 μm, Agilent) to quantify the yield of glycopeptide product. Mobile phase A consisted of 0.1% TFA in H2O and mobile phase B consisted of 0.1% TFA in MeCN. The components were eluted using a gradient (flow rate at 1 mL/min; at 0 min elution solvent mixture A/B = 70/30; wavelength = 214 nm). IC50 values were calculated using GraphPad 5 (n = 3).

A cell-free reaction system was used to determine the inhibition of O-GlcNAc level on a purified protein acceptor Nup62. Reaction mixtures containing 10μM Nup62, 1 mM UDP-GlcNAc, 500nM OGT, buffer (150 mM NaCl, 1 mM EDTA, 2.5 mM tris(hydroxypropyl)phosphine, 25 mM Tris-HCl, pH 7.4), and compounds were incubated at 37 °C for 1 h. Then, SDS-PAGE loading buffer was added and western blots were used to detect O-GlcNAc on Nup62.

OGT activity assay based on a UDP-Glo assay kit was performed as previously described. Following the manufacturer's protocol, assays were optimized and performed in white, flat bottom 384-well assay plate. CKII peptide was used as the acceptor. Reactions contained the following components: 250nM OGT, 125μM CKII and 40μM UDP-GlcNAc, and buffer (150 mM NaCl, 1 mM EDTA, 2.5 mM tris(hydroxypropyl)phosphine, 25 mM Tris-HCl, pH 7.4). Luminescence was measured in triplicate using a microplate luminometer. IC50 values were calculated using GraphPad 5.

**Cell viability, cytotoxicity assay.** Cell counting kit-8 (CCK-8) was used to evaluate the cell viability of cultured cells following the manufacturer's protocol. Inhibition rates were analyzed using GraphPad 5. PBMCs were treated with L01 or OSMI-1 and cytotoxicity was assessed using Annexin-V in a FACSCalibur flow cytometry system.

**Acute toxicity assay.** All procedures of the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the School of Life Science & Medicine, Dalian University of Technology and all experiments were conducted according to the relevant guidelines. Zebrafish embryos at 72-hours post-fertilization were selected for the acute toxicity assay. Zebrafish embryos were generated by natural...
pairwise mating and raising at 28.5 °C in embryo water. Zebrafish embryos were arrayed in 24-well plate (20 larvae per well) and incubated with 1 mL of embryo water per well containing various concentrations of L01 or OSMI-1 at 28.5 °C for 24 h. DMSO (0.1%, v/v) solution served as the control. The observation of zebrafish was made directly in the 24-well plate using an inverted dissecting microscope. The number of dead zebrafish in each concentration solution was recorded within 24 h, and the survival rate was calculated (%).

Statistical analysis. All data are presented as the mean ± s.e.m., n = 3. Data groups were compared by two-tailed Student’s t-test using the GraphPad Software. Differences between groups were considered statistically significant if P < 0.05. The statistical significance is denoted by asterisks (*P < 0.05, **P < 0.01; ***P < 0.001).

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Conceived and designed experiments: Yubo Liu and Jianing Zhang. Cell culture: Yubo Liu, Huang Huang and Qiong Wu. Performed western blot and immunoprecipitation: Yubo Liu, Yang Ren. Performed OGT activity assay: Yubo Liu and Yang Ren. Performed molecular modeling: Sijin Wu. Performed toxicity assay: Yu cao. Performed other experiments and analysis: Yubo Liu and Wenli Li. Wrote the paper: Yubo Liu and Jianing Zhang.

Additional Information
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