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Thio-Linked UDP–Peptide Conjugates as O-GlcNAc Transferase Inhibitors

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Supporting Information

ABSTRACT: O-GlcNAc transferase (OGT) is an essential glycosyltransferase that installs the O-GlcNAc post-translational modification on the nucleocytoplasmic proteome. We report the development of S-linked UDP–peptide conjugates as potent bisubstrate OGT inhibitors. These compounds were assembled in a modular fashion by photoinitiated thiol–ene conjugation of allyl-UDP and optimal acceptor peptides in which the single pair of serine was replaced with cysteine. The conjugate VTPVC(S-propyl-UDP)TA (IC₅₀ = 1.3 μM) inhibits the OGT activity in HeLa cell lysates. Linear fusions of this conjugate with cell penetrating peptides were explored as prototypes of cell-penetrant OGT inhibitors. A crystal structure of human OGT with the inhibitor revealed mimicry of the interactions seen in the pseudo-Michaelis complex. Furthermore, a fluorophore-tagged derivative of the inhibitor works as a high affinity probe in a fluorescence polarimetry hOGT assay.

The reversible attachment of β-N-acetylglucosamine (O-GlcNAc) to serine and threonine residues of nucleocytoplasmic proteins is a highly conserved and dynamic post-translational modification (PTM) found in higher eukaryotes. The transfer of O-GlcNAc from the donor substrate UDP-GlcNAc onto substrate proteins is catalyzed by the O-GlcNAc transferase (OGT), while the removal is catalyzed by the O-GlcNAc hydrolase (OGA). Both enzymes possess substantial substrate promiscuity, which enables this single pair of enzymes to control the O-GlcNAc proteome linked to the regulation of a range of cellular processes. Protein O-GlcNAcylation is essential for proper development of the animal embryo and mutations in the O-GlcNAc transferase have recently been associated with intellectual disability. Multiple metabolic pathologies, such as diabetes and cancer, as well as neurological diseases such as Alzheimer’s and Parkinson’s have been linked to dysregulation of protein O-GlcNAcylation.

Elucidation of the precise biological role(s) of protein O-GlcNAcylation is markedly hampered by the lack of potent and selective inhibitors of OGT. The unusual catalytic machinery of the enzyme, which only seems to be fully assembled on the binding of the donor substrate, has proven to be difficult to target. Previously, high-throughput compound screening studies have yielded cell penetrant small molecules like BZX and OSMI1. The former renders OGT inactive by cross-linking Lys and Cys in the active site. Given its reactivity, BZX may also target enzymes that contain similar Lys/Cys pairs. OSMI1 inhibits OGT through an as of yet unknown mechanism and was also shown to affect cell surface glycosylation. The most widely used OGT inhibitor, the per-acetylated derivative of 5-thio-N-acetylgalcosamine, hijacks the hexosamine biosynthetic pathway to yield the donor substrate analogue UDP-SS-GlcNAc that is inherently nonselective in the context of several other families of essential UDP-GlcNAc processing glycosyl transferases present in the cell.

Recently, we have reported the synthesis and kinetic evaluation of bisubstrate OGT inhibitors that have been conceived by tethering the anchoring UDP moiety of the donor substrate to the serine side chain of a series of heptapeptides derived from several human OGT (hOGT) acceptor proteins. The bisubstrate UDP–peptide conjugate VTPVS-(O-propyl-UDP)TA 1 acted as a competitive hOGT inhibitor in vitro (IC₅₀ = 18 μM).

To develop these UDP–peptide conjugates into useful chemical biology tools two problems need to be addressed. First, the potency needs to be increased to effectively compete with the donor substrate UDP-GlcNAc (Kₐ = 16 μM). Second, the intrinsic cell penetrance of these conjugates is limited by their size and the negative charge of the UDP moiety. To address the first problem, an expedient access to a diverse set of bisubstrate inhibitors was required to enable efficient structure–activity studies. To improve cell penetrance, we aimed to fuse the bisubstrate “warhead” to a selection of cell penetrating peptides (CPP) to achieve cellular uptake.

In our original approach, the phosphorylated “stretched serine” building block was incorporated into the backbone of a...
peptide with subsequent formation of the pyrophosphate bond in a reaction with an activated UMP derivative (Figure 1). To improve synthetic efficiency we sought an alternative modular strategy that would enable installation of tethered UDP as the single moiety on a variety of peptide backbones. Advantageously, the suggested convergent approach would also facilitate the assembly of the proposed CPP-bisubstrate inhibitors by solid phase peptide synthesis (SPPS) followed by attachment of the tethered UDP as the last synthetic step. As the chemical structure of the O-linked UDP−peptide conjugates showed limited opportunity for modular assembly, we instead opted to explore novel S-linked UDP−peptide conjugates that could be accessed by chemoselective cysteine modification with a thiol-reactive tethered UDP derivative (Figure 2). Evaluation of three mainstay techniques for carbon−sulfur bond formation such as Michael addition (Path A), phosphine promoted disulfide contraction26 (Path B), and "click" thiol−ene conjugation (TEC)27−30 (Path C) in the context of construction of such thiol reactive UDP derivatives revealed inherent limitations of the first two approaches (Figure 2). Indeed, introduction of a mandatory electron withdrawing group would disrupt the topological integrity of the linker in the proposed Michael type reactive derivative. In turn, disulfide contraction, widely used for preparation of S-linked glycoproteins, seemed to be less attractive for peptide substrates, as it would unavoidably result in the loss of the stereochemical integrity of the linking cysteine. In fact, only photoinitiated TEC reaction of cysteine containing peptides with simplistic allyl-UDP 4 held promise for an efficient assembly of the envisaged conjugates.

To synthesize the requisite allyl-UDP 4 the Bogachev−Kiessling31,32 protocol was used by coupling UMP N-methyl imidazolide 3 with the crude bis-triethylammonium salt of allyl phosphoric acid, prepared in turn from allyl bis-fluorene methyl phosphate 2 (Scheme 1, Scheme S8). The structure of 4 was unambiguously assigned by a set of spectroscopic methods (Figures S1−S3); the presence of a pyrophosphate bond was confirmed by a typical pair of doublets (σ −11, −12, JP,P = 21 Hz) in the 31P NMR spectrum. The photoinitiated TEC reaction between 4 and 20% molar excess of the substrate peptide VTPVCTA 5 was initially attempted at 10 mM in 0.1 M acetate buffer (pH 4) in the presence of 20% of different water-soluble photoinitiators VA-044, DMPA (50% MeOH additive), or LAP.33 The reactions were irradiated using an 8 W Camag TLC reader lamp at 366 nm and monitored by LC-MS. We found that LAP efficiently mediated the conjugation, resulting in clean formation of the expected product 6 (m/z 1175) after only 10 min (Scheme 2A).

On the other hand, <5% conversion was detected with VA-044, while no reaction took place with DMPA. The TEC reaction was equally efficient when no excess of the substrate peptide was present and with only 10% of the photoinitiator. No products were observed in the absence of LAP or without UV light. The reaction was scaled up to 0.05 mmol (20 mM in water), and the S-linked UDP−peptide conjugate 6 was isolated by preparative HPLC in 56% yield. The structure of the product was verified by 1H and 13C NMR spectra, proving the presence of the peptide backbone and the UDP moiety (Figures S4, S5). The 31P spectrum showed two broadened peaks.

**Scheme 1. Chemical Synthesis of Allyl-UDP**

| Reagents | Conditions | Yield |
|----------|------------|-------|
| (i) Et3N-DCM, 4:1, 16 h; (ii) a) Bogachev−Kiessling coupling, 3 h; b) MeOH, Et3N, H2O, 16 h, 44% |   |   |
singlets at $\sigma -10.94$ and $-11.48$, confirming the integrity of the pyrophosphate bond.

The inhibitory potency of 6 was assessed by the previously described fluorometric OGT activity assay.25 Unexpectedly, the S-linked UDP peptide conjugate 6 appeared to be an almost 10-fold more potent hOGT inhibitor ($IC_{50} = 2 \mu M$) than its O-linked progenitor 125 (Figure 3A). To provide a negative control and on the assumption that hOGT evolved to bind D-configured UDP, we synthesized the L-uridine congener 7 starting from allyl-L-UDP 4-L (Scheme 2A, Scheme S10), which showed no inhibition of hOGT (Figure 3A). Next, we demonstrated that 6 is also a hOGT inhibitor in a cell-free system, reducing protein O-GlcNAcylation in a dose-dependent manner by about 50% at 1 mM (Figure 3B, Figure S6).

Next, we attempted the assembly of the potentially cell penetrating bisubstrate hOGT inhibitors. To this end, starting from peptides featuring a VTPVCTA C-terminus and either Penetratin (RQIKIWFQNRRMKWKK - 8/9)34 or TAT (YGRKKRRQRRR - 10)35 peptides at the N-terminus, we synthesized S-linked UDP peptide conjugates 11−13 by TEC reaction with allyl-UDP 4 (Scheme 2C). Encouragingly, the conjugates 11 and 13 ($IC_{50}[13] = 5 \mu M$, Figure S7) were shown to be almost as potent hOGT inhibitors in vitro as the parent compound 6. However, neither 11 nor 13 had a noticeable effect on total O-GlcNAcylation in cell cultures at concentrations up to 1 mM (Figure S8). Moreover, microscopy of the HeLa cells treated with the 5-fluorescein thioureide (Flut) labeled conjugate 12 for 24 h revealed fluorescent puncta (Figure S8). Taken together, these data suggest that while 12 and, implicitly 11 and 13, could cross the cell membrane, they remain trapped in the early endosomes36,37 and therefore cannot target cytosolic hOGT.

Discovery of the potent hOGT binder 6 also offers an opportunity for development of a sensitive hOGT fluorescence polarimetry assay (FP). We explored the fluorescently tagged derivative 17 of the inhibitor as a high affinity FP probe. To this end, the peptide 14 was efficiently transformed into corresponding S-linked UDP−peptide conjugate by TEC reaction with allyl-UDP 4. Next, the N-terminal 6-amino-hexanoyl (ahx) residue was tagged with 5(6)-fluorescein NHS ester to give the requisite 5(6)-fluorescein carboxamide (Floc) labeled derivative 17 (Scheme 2B).

Figure 3. (A) Dose−response curves of inhibition of hOGT activity in the presence of increasing concentrations of 6 and 7. Errors shown represent the s.e.m. of three replicates. (B) Western blot analysis of cell lysate in the presence and absence of 6, 7, and UDP-5S-GlcNAc. HeLa cell lysates were O-GlcNAc stripped by the potent bacterial O-GlcNAcase CpOGA, whose activity was then blocked by the potent OGA inhibitor GlcNAcstatin G. These stripped lysates were then re-O-GlcNAcylated by recombinant OGT and blotted for protein O-GlcNAcylation using the RL2 antibody. HSP60 was used as a loading control.

\[ \text{Scheme 2. Photoinitiated Thiol−Ene Conjugation of Cysteine Containing Peptides with Allyl-UDP} \]

\[ ^{a} \text{(i) 8 W 366 nm, 10 min, 50−55%; (ii) 5(6)-fluorescein NHS ester, DMF, 0.15 M NaHCO}_3, 3 \text{ h, 85%}. \]
sequence was performed with 14 and allyl-L-UDP 4-L to give the negative control compound 18.

Direct binding affinity of compound 17 to hOGT was measured, yielding a $K_i$ of 1.6 $\mu$M, whereas no quantifiable binding could be detected for the corresponding negative control 18 (Figure 4A). The observed value is in good agreement with the $IC_{50}$ of 2 $\mu$M established in our activity assay.

To verify whether the probe 17 could be used in a workable FP hOGT assay we demonstrated that the available competitive inhibitors UDP-5S-GlcNAc24 and 1 and 11 displace 17 from the complex with the enzyme in a dose-dependent manner (Figure 4B). The derived values of $K_i = 7 \pm 2$ $\mu$M (1) and $K_i = 5 \pm 2$ $\mu$M (UDP-5S-GlcNAc) were in agreement with the previously reported affinities, validating this assay.

Next, we used this assay platform to examine the effect of the peptide sequence on the inhibitory potency of the S-linked UDP--peptide conjugates. We compiled a set of peptides derived from the known hOGT substrate proteins RB2, TAB1, CK2, and KER, as well as a previously published hOGT progenitor 1 (Table 1). The topology of the heptapeptides in the 7-SSC subseries (19–23) was chosen to replicate that of the peptide 5. The nonapeptides 9-SSC (24–26) were designed with a centrally located Cys. The pair of the tridecapeptides 27 and 28 was included to explore distant regions of the binding groove. The TEC reaction of allyl-UDP 4 with all substrates worked routinely well to give an array of the novel S-linked UDP--peptide conjugates 29–38 (Table 1, Table S1) that were evaluated by the modified FP assay (Table 1, Figure 5).

| sequence | origin/Cys position | Cys peptide | product | $K_i$ (nM) |
|----------|---------------------|-------------|---------|------------|
| 1        | VTPVCTA             | RB2_7-SSC   | 5       | 1.3 ± 0.8  |
| 2        | VTPVCTA             | pseudo RB2_7-SSC | 19 | 12.8 ± 4.5 |
| 3        | SVPYCSA             | TAB1_7-SSC  | 20      | 2.3 ± 1.6  |
| 4        | VTPVCSA             | CK2_7-SSC   | 21      | 1.2 ± 1.3  |
| 5        | VTPVCSA             | SEQ_7-SSC   | 22      | 1.3 ± 0.5  |
| 6        | PEFTCRS             | KER_7-SSC   | 23      | 12.5 ± 7   |
| 7        | VTPVCTATH           | RB2_9-SSC   | 24      | 12.7 ± 2.3 |
| 8        | SVPYCSAQ5           | TAB1_9-SSC  | 25      | 15 ± 2.3   |
| 9        | PEFTCRSA            | KER_9-SSC   | 26      | 56 ± 32    |
| 10       | PVCTAHTHSLRSLH      | RB2_13-SSC  | 27      | 80 ± 46    |
| 11       | KENSAVPVCTA         | RB2_13-S11C | 28      | 1.8 ± 1.3  |

The collated data (Table 1) suggest that conjugates derived from the heptapeptides 7-SSC cover the minimal structure of the bisubstrate inhibitor until there is proline in the −2 position, as the binding potency remains in the range of 1 ± 0.5 $\mu$M (Table 1, Table S1) that were evaluated by the modified FP assay (Table 1, Figure 5).

The nonapeptides 9-SSC (24–26) were designed with a centrally located Cys. The pair of the tridecapeptides 27 and 28 was included to explore distant regions of the binding groove. The TEC reaction of allyl-UDP 4 with all substrates worked routinely well to give an array of the novel S-linked UDP--peptide conjugates 29–38 (Table 1, Table S1) that were evaluated by the modified FP assay (Table 1, Figure 5).
complex of hOGT with UDP-SS-GlcNAc and an acceptor peptide (Figure 6). The largest atomic shift between the UDP moieties of conjugates 1 and 6 and the corresponding substrates/substrate analogues is 0.7–0.8 Å. Shifts were also observed between the positions of the linking oxygen and sulfur (0.8 Å) and the positions of the linker C3 atoms (1.1 Å). Overall, in the conjugate 1 the linker adopts a synclinal conformation (dihedral angle O−C1−C2−C3 of 72°), while in 6 the linker adopts an antiperiplanar conformation (dihedral angle S−C1−C2−C3 171.4°). This difference may contribute to the increased potency of 6, as the antiperiplanar conformation of the thio-propyl linker seen in 6 is energetically more favorable than the synclinal conformation of the oxy-propyl linker in 1.

In conclusion, we developed a modular synthetic approach to S-linked UDP−peptide conjugates using a photoinitiated TEC reaction between allyl-UDP and cysteine containing peptides. A series of conjugates sharing the XXPXC(S-propyl-UDP)XX scaffold showed binding potency toward hOGT in the 1 ± 0.5 μM range in vitro, which places them among the most potent hOGT inhibitors reported to date. The increased potency of the conjugate VTPVC(S-propyl-UDP)TA in comparison with its O-linked progenitor correlates with the more relaxed conformation of the thiopropyl linker. We have shown that the chosen S-linked conjugate inhibits recombinant hOGT activity in HeLa cell lysates and successfully assembled two prototypes of the cell-penetrating bisubstrate hOGT inhibitors that feature Penetratin and TAT peptides C-terminally extended with the VPTVC(S-propyl-UDP)TA motif. Finally, we developed a practical variant of the fluorescence polarimetry hOGT assay using a fluorescently labeled derivative of the bisubstrate conjugate as a high affinity probe suitable for high-throughput screening. Further optimization of the structure of the novel hOGT bisubstrate inhibitors as well as evaluating of different types of cell penetrating vehicles could lead to further improvements of this scaffold.

ASSOCIATED CONTENT

S Supporting Information
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Synthetic procedures and spectral data for all new compounds, methods, figures, and tables (PDF)

Accession Codes
X-ray diffraction data and refined structures have been deposited with the Protein Data Bank under accession codes 5NPS (1) and 5NPR (6).

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Author Contributions
K.R., V.S.B., and D.M.F.v.A. conceived the study. V.S.B. performed chemical synthesis; K.R. performed structural biology, steady-state kinetics, and fluorescence polarimetry experiments; A.G. and R.T. performed Western blot experiments; R.T. performed fluorescence microscopy experiments; K.R., V.S.B., and D.M.F.v.A. interpreted the data; K.R., V.S.B., and D.M.F.v.A. wrote the manuscript with input from all authors.

Notes
The authors declare no competing financial interest.

Additional supporting research validation reports of the deposited structures for this article may be accessed at no charge at http://wwpdb.org/validation/2016/XrayValidationReportHelp.
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REFERENCES

(1) Torres, C. R., and Hart, G. W. (1984) Topography and polypeptide distribution of terminal N-acetylgalosamine residues on the surfaces of intact lymphocytes. J. Biol. Chem. 259, 3308–3317.

(2) Hart, G. W., Housley, M. P., and Slawson, C. (2007) Cycling of O-linked beta-N-acetylgalosaminide on nucleocytoplasmic proteins. Nature 446, 1017–1022.

(3) Dong, D. L., and Hart, G. W. (1994) Purification and characterization of an O-GlcNAc selective N-acetyl-D-galosaminidase from rat spleen cytosol. J. Biol. Chem. 269, 19321–19330.

(4) Hahne, H., Sobottki, N., Nyberg, T., Helm, D., Borodkin, V. S., van Aalten, D. M., Agnew, B., and Kuster, B. (2013) Proteome wide purification and identification of O-GlcNAc-modified proteins using click chemistry and mass spectrometry. J. Proteome Res. 12, 927–936.

(5) Chalkley, R. J., Thalhammer, A., Schoepfer, R., and Burlingame, A. L. (2009) Identification of protein O-GlcNAcylation sites using electron transfer dissociation mass spectrometry on native peptides. Proc. Natl. Acad. Sci. U. S. A. 106, 8894–8899.

(6) Webster, D. M., Teo, C. F., Sun, Y., Wloka, D., Gay, S., Klonowski, K. D., Wells, L., and Dougan, S. T. (2009) O-GlcNAc modifications regulate cell survival and epiboly during zebrafish development. BMC Dev. Biol. 9, 28.

(7) Watson, L. J., Long, B. W., DeMartino, A. M., Brittian, K. R., Readnower, R. D., Brainard, R. E., Cummins, T. D., Annamalai, L., Hill, B. G., and Jones, S. P. (2014) Cardiomyocyte Ogt is essential for postnatal viability. Am. J. Physiol Heart Circ Physiol 306, H142–153.

(8) Gambetta, M. C., Oktaba, K., and Muller, J. (2009) Essential Role of the Glycosyltransferase Sxt/Ogt in Polycystin Repression. Science 325, 93–96.

(9) Bouzzi, H., Lesca, G., Trujillo, C., Alwasiyah, M. K., and Munnich, A. (2015) Nonsyndromic X-linked intellectual deficiency in three brothers with a novel MED12 missense mutation. J. Biol. Chem. 292, 8948–8963.

(10) Willems, A. P., Gundogdu, M., Kempers, M. J. E., Giltay, J. C., Pfundt, R., Ellerink, M., Loza, B. F., Fujikschot, J., Ferenbach, A. T., van Gassen, K. L. I., et al. (2017) Mutations in N-acetylgalosaminide (O-GlcNAc) transferase in patients with X-linked intellectual disability. J. Biol. Chem. 292, 12621–12631.

(11) Willems, A. P., Gundogdu, M., Kempers, M. J. E., Giltay, J. C., Pfundt, R., Ellerink, M., Loza, B. F., Fujikschot, J., Ferenbach, A. T., van Gassen, K. L. I., et al. (2017) Mutations in N-acetylgalosaminide (O-GlcNAc) transferase in patients with X-linked intellectual disability. J. Biol. Chem. 292, 12621–12631.

(12) McLarty, J. L., Marsh, S. A., and Chatham, J. C. (2013) Post-translational modification product by O-linked N-acetylgalactosamine: Its role in mediating the adverse effects of diabetes on the heart. Life Sci. 92, 621–627.

(13) Banerjee, P. S., Ma, J. F., and Hart, G. W. (2015) Diabetes-associated dysregulation of O-GlcNAcylation in rat cardiac mitochondria. Prog. Natl. Acad. Sci. U. S. A. 112, 6050–6055.

(14) Itkonen, H. M., Minner, S., Guldvik, I. J., Sandmann, M. J., Tsourlakis, M. C., Berge, V., Svindland, A., Schlimm, T., and Mills, I. G. (2013) O-GlcNAc Transferase Integrates Metabolic Pathways to Regulate the Stability of c-Myc in Human Prostate Cancer Cells. Cancer Res. 73, 5277–5287.

(15) Li, Z. H., and Yi, W. (2014) Regulation of cancer metabolism by O-GlcNAcylation. Glycoconjugate J. 31, 185–191.

(16) Singh, J. P., Zhang, K. S., Wu, J., and Yang, X. Y. (2015) O-GlcNAc signaling in cancer metabolism and epigenetics. Cancer Lett. 356, 244–250.
Pathak, S., Alonso, J., Schimpl, M., Rafie, K., Blair, D. E., Borodkin, V. S., Schuttelkopf, A. W., Albarbarawi, O., and van Aalten, D. M. F. (2015) The active site of O-GlcNAc transferase imposes constraints on substrate sequence. *Nat. Struct. Mol. Biol.* 22, 744–U133.