Communication

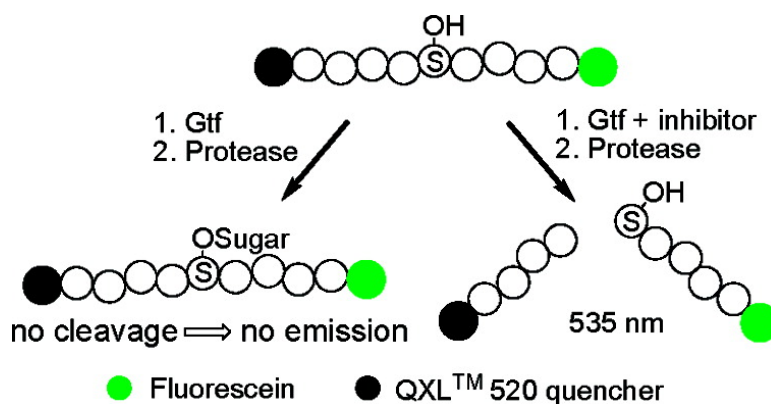# A Strategy to Discover Inhibitors of O-Linked Glycosylation

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A Strategy to Discover Inhibitors of O-Linked Glycosylation

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Glycosylation of proteins plays a role in a wide range of biological phenomena, from intercellular recognition to the regulation of gene expression. Changes in protein glycosylation occur in many diseases, but in most cases, a causal link between these changes and the observed pathologies has not been clearly established.[1] Specific, cell-permeable inhibitors of glycosyltransferases (Gtfs) would be useful for probing how changes in glycosylation levels or glycan composition affect cells and tissues.[2] High-throughput screening (HTS) has proven extremely useful for discovering cell-permeable small-molecule inhibitors for kinases and many other classes of enzymes, but it has rarely been used to discover Gtf inhibitors, mainly due to a lack of robust non-radiometric assay strategies to detect glycosylation.[3,4] Here we describe a general protease-protection assay to monitor the activity of Gtfs that initiate O-linked glycosylation (Table 1). We demonstrate that this approach can be readily adapted to monitor the activity of three such Gtfs, and we apply the strategy to screen ~124 000 small molecules against O-GlcNAc transferase (OGT), an enzyme that mediates a unique type of intracellular O-glycosylation involved in signal transduction.[5] Several low micro-molar inhibitors have been discovered and validated in a secondary assay. The assay strategy reported here should enable the rapid identification of inhibitors for many Gtfs that catalyze O-linked glycosylation.

Since one well-known function of protein glycosylation is to increase protein half-life by blocking access to proteolytic sites,[6] we speculated that the addition of a single sugar to a peptide might protect adjacent cleavage sites from proteolysis. Inspired by a widely used protein kinase assay,[7,8] we devised a protease-protection assay to monitor glycosylation. In this approach, a peptide substrate labeled with a FRET pair is subjected to glycosylation and then treated with a protease that discriminates between glycosylated and non-glycosylated peptides. The resulting FRET signal is measured to determine the amount of proteolysis, which is related to the degree of peptide glycosylation (Figure 1). The strategy is conceptually straightforward, but to implement it for a given Gtf, it is necessary to identify peptide substrate/protease pairs that satisfy three requirements: (1) the peptide must be a good substrate for the Gtf of interest; (2) the peptide must contain a unique protease site adjacent to the glycosylation site; and (3) there must be a large difference in the rates of proteolysis of the glycosylated and the unglycosylated versions of the peptide. We explored the feasibility of the strategy by applying it to three Gtfs that catalyze two distinct types of O-linked glycosylation. One enzyme, OGT, is a β-O-GlcNAc transferase that plays a central role in the hexosamine signaling pathway. Aberrant OGT activity has been linked to diabetic complications and other pathologies, and OGT inhibitors may be useful both as tools to understand hexosamine signaling and as therapeutic agents.[5] The other enzymes are α-O-GalNAc-transferase 1 and 2 (ppGalNAc T1 and T2), both of which initiate O-linked mucin-type glycosylation. Changes in the activity of

Table 1. Summary of Assay Development

sequence	protease	enzyme	$Z'_R{}^a$	$Z'_I{}^a$
STPV**S**SANMK	proteinase K	OGT	0.93	0.91
STPV**S**RANMK	proteinase K	OGT	0.95	0.90
EPGP**T**EAPK	proteinase K	ppGalNAc-T2	0.96	0.90
EDAV**T**PGPK	thermolysin	ppGalNAc-T1	0.96	ND

^{a} $Z'_R = Z'$ using coumarin/fluorescein. $Z'_I = Z'$ using QXL 520/fluorescein. The glycosylated residue is shown in bold. ND = not determined.
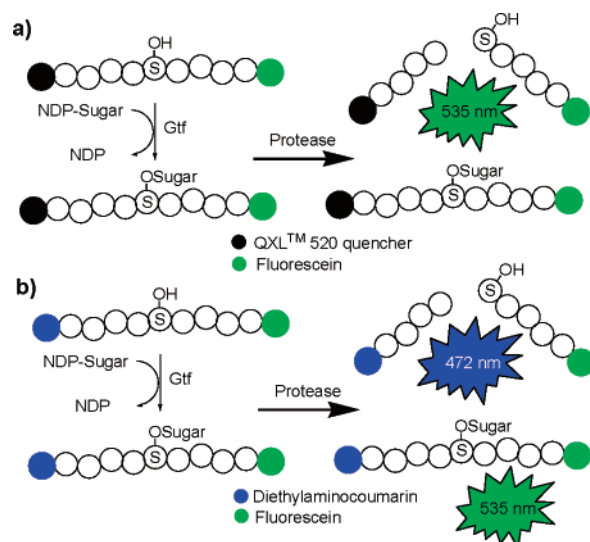

Figure 1. Schematic showing both protease-protection assay strategies. (a) In the fluorescence intensity approach, excitation light (485 nm) is absorbed when the peptide is intact. (b) In the ratiometric approach, light from coumarin emission (470 nm) is transferred to fluorescein and then emitted (535 nm) when the peptide is intact.

various ppGalNAc-Ts have been linked to cancer, and inhibitors of these enzymes may provide more information on their biological roles.[4,9,10] For each enzyme, we synthesized a small panel of peptide substrates containing fluorescein at one terminus and either a QXL 520 quencher (AnaSpec) or 7-diethylaminocoumarin dye at the other terminus to enable readout either by absolute fluorescence intensity or by the ratio of emission intensities, respectively. The peptide sequences were based on reported substrates[11,12] but contained changes in amino acids flanking the glycosylation site to introduce potential protease cleavage sites. The ability of the substituted peptides to serve as glycosyl acceptors was evaluated by LC/MS; those that were efficiently glycosylated were selected for further study. Fully glycosylated and unglycosylated variants of the selected peptides were combined with serial dilutions of several proteases, and the fluorescence emission was monitored. For each Gtf, we were able to identify a peptide substrate/protease pair for which there was *at least* a 100-fold difference between the protease concentrations required to cleave the unglycosylated and the
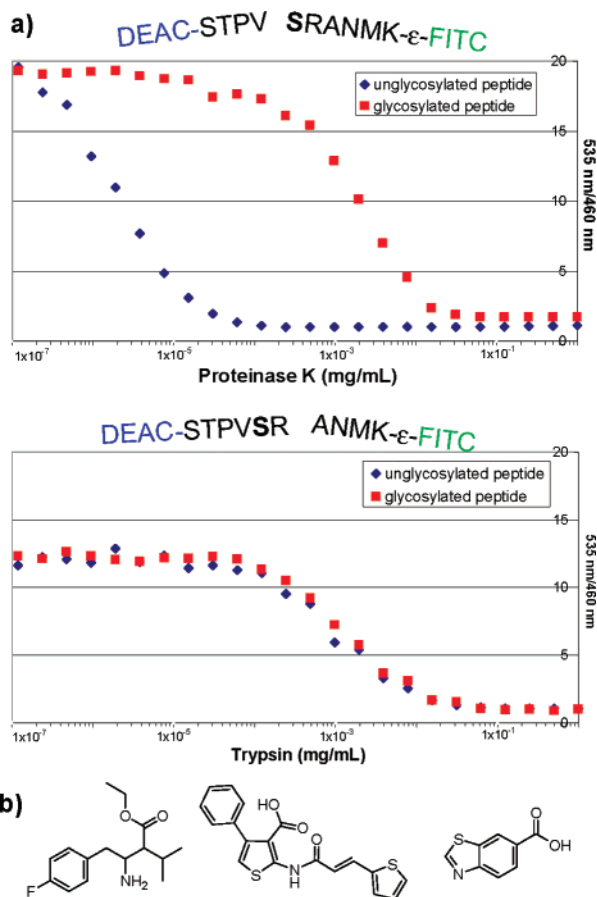
Figure 2. (a) The assay window with selective (top) and non-selective (bottom) proteolysis using an OGT acceptor peptide. The glycosylated residue is shown in bold. (b) A subset of validated OGT inhibitors discovered through HTS.

glycosylated peptides (Figure 2a). Known concentrations of glycosylated and unglycosylated peptides were then combined, and suitable proteases were added at concentrations that cleaved the unglycosylated peptides to completion without affecting the glycosylated peptides. The fluorescence intensity after proteolysis was linear throughout the entire glycosylation range, making the assay suitable for kinetic measurements (see Supporting Information).

We next adapted the assay to a high-throughput format in order to screen for inhibitors of OGT. We used the fluorescence intensity approach (Figure 1a) for high-throughput screening because it allows for longer wavelength excitation, which greatly reduces the number of false positives related to library compound autofluorescence (see Supporting Information). After identifying concentrations of OGT and substrates required to achieve ∼80% glycosylation in 2 h in a 384 well plate and optimizing the assay protocol, we screened 124 226 compounds in duplicate over 2 days at the Institute for Chemistry and Cell Biology at Harvard Medical School. The duplicate data sets were remarkably self-consistent, and most assay plates yielded Z' factors between 0.7 and 0.8.[13] Given the high Z' factor, reproducibility, and assay linearity, the screening data could be treated quantitatively, and the RFU for each well was converted into a percent glycosylation. Eighty-four compounds that inhibited OGT activity by 30% or more were identified, corresponding to a hit rate of 0.065%. Of these 84 hits, 38 (45%) were confirmed by an orthogonal radiometric assay,[3a] and nine of the confirmed hits had IC$_{50}$ values between 0.9 and 20 μM, making

these the most potent OGT inhibitors described to date (Figure 2b).[3a] Moreover, there was a good correlation between primary and secondary hit potencies (see Supporting Information). The low overall hit rate and high percentage of validated hits suggests that typical sources of false positives did not contribute significantly to the hit set.[13] As already noted, library compound autofluorescence was minimized through the use of a high excitation wavelength. Promiscuous or non-selective inhibitors, which result in false positives in many other assays,[14] were entirely absent, presumably because such compounds would prevent proteolytic cleavage as well as Gtf activity, resulting in a negative well. The assay remains robust even with low product conversion ($Z' > 0.5$ at 10% glycosylation), which suggests that low enzyme concentrations or non-optimal acceptor substrates can be used for screening.

There is a pressing need for small-molecule tools to modulate the activity of specific Gtfs in cells and in vivo,[2] but there has been no clear path to such compounds. The HTS strategy described here can be adapted to screen most, if not all, Gtfs that initiate O-linked glycosylation, including enzymes involved in proteoglycan and mucin-type glycan synthesis, O-GlcNAcylation, and Fringe/Notch signaling, as well as bacterial virulence.[1,15,16] Screening these enzymes against large, diverse small-molecule libraries should lead to the identification of selective Gtf inhibitors, providing compounds to test the potential of non-substrate-based small molecules for dissecting glycosylation pathways in cells.

References

(1) (a) Ohtsubo, K.; Marth, J. D. *Cell* **2006**, *126*, 855−67. (b) Spiro, R. G. *Glycobiology* **2002**, *12*, 43R−56R.
(2) Prescher, J. A.; Bertozzi, C. R. *Cell* **2006**, *126*, 851−4.
(3) A high-throughput substrate-displacement strategy has been applied to screen two UDP-GlcNAc transferases. See: (a) Gross, B. J.; Kraybill, B. C.; Walker, S. *J. Am. Chem. Soc.* **2005**, *127*, 14588−9. (b) Helm, J. S.; Hu, Y.; Chen, L.; Gross, B.; Walker, S. *J. Am. Chem. Soc.* **2003**, *125*, 11168−9. (c) Hu, Y.; Helm, J. S.; Chen, L.; Ginsberg, C.; Gross, B.; Kraybill, B.; Tiyanont, K.; Fang, X.; Wu, T.; Walker, S. *Chem. Biol.* **2004**, *11*, 703−11.
(4) A moderate-throughput ELISA assay that detects glycosylation has been used to screen for inhibitors of mucin-type O-linked glycosylation. See: Hang, H. C.; Yu, C.; Ten Hagen, K. G.; Tian, E.; Winans, K. A.; Tabak, L. A.; Bertozzi, C. R. *Chem. Biol.* **2004**, *11*, 337−45.
(5) Hart, G. W.; Housley, M. P.; Slawson, C. *Nature* **2007**, *446*, 1017−22.
(6) Varki, A. *Glycobiology* **1993**, *3*, 97−130.
(7) Rodems, S. M.; Hamman, B. D.; Lin, C.; Zhao, J.; Shah, S.; Heidary, D.; Makings, L.; Stack, J. H.; Pollok, B. A. *Assay Drug Dev. Technol.* **2002**, *1*, 9−19.
(8) This is the Z'-Lyte assay from Invitrogen. See: http://www.invitrogen.com/content.cfm?pageid=9866.
(9) Ten Hagen, K. G.; Fritz, T. A.; Tabak, L. A. *Glycobiology* **2003**, *13*, 1R−16R.
(10) Brockhausen, I. *EMBO Rep.* **2006**, *7*, 599−604.
(11) Gerken, T. A.; Raman, J.; Fritz, T. A.; Jamison, O. *J. Biol. Chem.* **2006**, *281*, 32403−16.
(12) Kreppel, L. K.; Hart, G. W. *J. Biol. Chem.* **1999**, *274*, 32015−22.
(13) (a) Inglese, J.; Johnson, R. L.; Simeonov, A.; Xia, M.; Zheng, W.; Austin, C. P.; Auld, D. S. *Nat. Chem. Biol.* **2007**, *3*, 466−79. (b) Zhang, J. H.; Chung, T. D.; Oldenburg, K. R. *J. Biomol. Screen.* **1999**, *4*, 67−73.
(14) McGovern, S. L.; Helfand, B. T.; Feng, B.; Shoichet, B. K. *J. Med. Chem.* **2003**, *46*, 4265−72.
(15) Peter-Katalinic, J. *Methods Enzymol.* **2005**, *405*, 139−71.
(16) Jank, T.; Giesemann, T.; Aktories, K. *Glycobiology* **2007**, *17*, 15R−22R.

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