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Molecular Interrogation to Crack the Case of O-GlcNAc

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Abstract

The O-linked β -*N*-acetylglucosamine (O-GlcNAc) modification, termed O-GlcNAcylation, is an essential and dynamic post-translational modification in cells. O-GlcNAc transferase (OGT) installs this modification on serine and threonine residues while O-GlcNAcase (OGA) hydrolyzes it. O-GlcNAc modifications are found on thousands of intracellular proteins involved in diverse biological processes. Dysregulation of O-GlcNAcylation and O-GlcNAc cycling enzymes has been detected in many diseases including cancer, diabetes, cardiovascular and neurodegenerative diseases. In this minireview, we will discuss recent advances in the development of molecular tools to investigate OGT and OGA functions and substrate recognition. We will also cover new chemical approaches to study O-GlcNAc dynamics and its potential roles in the immune system. We hope this minireview will encourage more research in these areas to advance understanding of O-GlcNAc in biology and diseases.

Graphical Abstract

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Recent advances in molecular approaches for O-GlcNAc study, with special emphasis on chemical tools to understand the functional roles and dynamic regulation of O-GlcNAcylation and its cycling enzymes (OGT and OGA).

Keywords

Glycosylation; Inhibitors; O-GlcNAc; Chemical Tools; Substrate Recognition

1. Introduction

The O-linked β -*N*-acetylglucosamine modification (O-GlcNAcylation) of proteins is an essential mechanism in dynamic regulation of cellular functions such as cell cycle and metabolic processes in response to nutrient and stress stimuli.^[1–7] This reversible modification is catalyzed by a single pair of enzymes.^[3] O-GlcNAc transferase (OGT) transfers β -*N*-acetylglucosamine (GlcNAc) from the sugar donor uridine diphosphate (UDP)-GlcNAc to serine and threonine residues on thousands of nuclear, cytoplasmic and mitochondrial proteins, while O-GlcNAcase (OGA) hydrolyzes this modification (Figure 1).^[8–13] Previous studies have shown that, in mammals, genetic knockout of OGT is embryonically lethal, and deletion of OGA is perinatally lethal.^[14,15] Given the essential roles of these enzymes and O-GlcNAc, it is not surprising that their dysregulation links to numerous diseases. For instance, increased O-GlcNAcylation has been found in various cancers, while genetic knockdown of OGT reduced cancer cell proliferation and metastasis *in vivo*.^[16–19] On the other hand, decreased O-GlcNAc level has been associated with neurodegenerative diseases.^[20] Interestingly, OGA inhibitors have shown to increase O-GlcNAcylation and ablate pathogenic markers of some of these disease states.^[21,22] Additionally, mutations in OGT are potential causes for X-linked Intellectual Disabilities,^[23–25] and a single nucleotide polymorphism in OGA could increase diabetes risk.^[26] Taken together, these studies have garnered attention toward OGT and OGA as potential drug targets in many challenging therapeutic areas.

Despite the significance of O-GlcNAc homeostasis in normal and disease states, our understanding of its dynamic regulation as well as its molecular functions remains obstructed. Methods for discerning the diverse roles of O-GlcNAc are in high demand. Herein, we provide a brief overview of recently developed OGT and OGA inhibitors as well as new techniques for understanding their mechanisms of substrate recognition. We will also cover novel chemical approaches to study the functions of O-GlcNAc and their emerging applications to the immune system. Due to space restrictions, we will selectively highlight recent chemical related methods for O-GlcNAc study in this minireview. For detailed information on the functional understanding of O-GlcNAc as well as molecular mechanisms and structural characterizations of O-GlcNAc processing enzymes, please refer to other excellent reviews.^[27–30]

2. Molecular Tools to Investigate OGT Functions and Substrate Recognition

OGT is the only glycosyltransferase that contains an extended N-terminal tetratricopeptide repeat (TPR) region, which has been proposed as an important feature for OGT-protein substrate binding (PDB: 1W3B).^[31] However, the molecular basis of how OGT recognizes and glycosylates numerous protein substrates is largely unknown. One focus of this section will highlight innovative techniques developed toward addressing this mystery. The other part of this section will discuss recently developed OGT inhibitors to investigate its functions. It is worth noting that many of these studies leveraged the structural and mechanistic information derived from a truncated OGT (OGT_{4.5}) construct containing the catalytic region and 4.5 of the 13.5 TPRs (PDB: 3PE3).^[32] In contrast to full-length OGT, OGT_{4.5} can be readily crystallized. A series of OGT_{4.5}-substrate and -product complex structures, along with kinetic studies, have established that OGT follows an ordered bi-bi mechanism, in which OGT binds to UDP-GlcNAc followed by peptide substrate for sugar transfer (Figure 2a).^[33–36] The structures of OGT_{4.5} also facilitated rational design of OGT inhibitors. In general, advances in understanding the structure and mechanism of OGT have greatly accelerated the development of new chemical tools.

2.1 Recently Developed OGT Inhibitors

Developing potent and selective OGT inhibitors is a challenging task, partially because of the large, hydrophilic OGT active site as well as the competition from high concentrations of intracellular UDP-GlcNAc that is a common sugar donor for many glycosyltransferases. Recent studies have applied a variety of strategies to address this challenge, leading to a better understanding of the function of this essential enzyme. One class of OGT inhibitors are bisubstrate conjugates, such as UDP-peptides, that attempt to mimic the binding features of both sugar donor- and acceptor-substrates. The first reported UDP-peptide conjugate, Goblin-1, was an acceptor peptide covalently coupled to UDP via a short alkyl linker (Figure 2b), displaying an IC₅₀ value of 18 μM *in vitro*.^[37] In 2018, Thiogoblin-1, an S-linked UDP-peptide conjugate (Figure 2b) in which the acceptor serine was replaced with cysteine, demonstrated more potent OGT inhibition than Goblin-1 (IC₅₀ = 2 μM).^[38] These compounds provided new chemical scaffolds to design OGT inhibitors, but unfortunately, they are typically unsuitable for cellular use due to their poor cell permeability.

The OSMI compound series represents one of the most potent classes of OGT inhibitors reported to date. The core structure (quinolinone-6-sulfonamide) was initially identified from a high-throughput screen, displaying modest inhibition potency and cell permeability.^[39,40] Following extensive optimization, OSMI-4a (Figure 2b) was reported as a second generation in this class, showing remarkable binding affinity (~8 nM) to OGT *in vitro*.^[41] OSMI-4, an esterified precursor of OSMI-4a, displayed an EC₅₀ around 3 μM in cells. Notably, the crystal structures of OGT_{4.5} in complex with OSMI-4a and its analogues (PDBs: 6MA1, 6MA2, 6MA3, 6MA4, and 6MA5) revealed that the quinolinone-6-sulfonamide core is a faithful uridine mimic, providing invaluable information to guide the rational design of future generations of OGT inhibitors. From the structural studies, these

compounds occupy the space of both sugar and peptide substrates, suggesting their selectivity for OGT over other glycosyltransferases, though further validation is needed.

The elegantly designed sugar donor analogue UDP-5SGlcNAc represents another class of efficient OGT inhibitors (Figure 2b).^[42] By replacing the endocyclic oxygen atom of UDP-GlcNAc with sulfur, UDP-5SGlcNAc dramatically reduced the sugar transfer rate and effectively inhibited OGT with a K_i value of 8 μ M. To circumvent the cell permeability issue of UDP-5SGlcNAc, an acetylated metabolic precursor (Ac₄5SGlcNAc) was generated to inhibit OGT in cells.^[42] This design is clever, but due to the high level of structural similarity to UDP-GlcNAc, it has been reported that UDP-5SGlcNAc may also inhibit other glycosyltransferases utilizing the same sugar donor.^[40,42] To further improve the aqueous solubility of this inhibitor class, non-acetylated 5SGlcNAc with varying *N*-acyl substituents were tested.^[43] One of the most promising compounds, 5SGlcNHex (Figure 2b), was able to reduce O-GlcNAc levels in mice in a dose- and time-dependent manner. To our knowledge, 5SGlcNHex was the first OGT inhibitor that effectively reduced O-GlcNAc *in vivo*. Furthermore, it revealed an interesting correlation between decreased O-GlcNAc levels and impaired hormone production.

Most reported OGT inhibitors reversibly inactivate the enzyme, while covalent inhibitors have rarely been exploited to target OGT until recently. Covalent inhibitors offer potential advantages over reversible inhibitors, including increased efficacy and prolonged duration of action. They are also expected to better overcome the competition from high-concentration endogenous sugar donor.^[44] The first reported covalent inhibitor of OGT was BZX2 (Figure 2b).^[45] It contains a dicarbamate core that rapidly reacts with an essential active-site lysine followed by a nearby cysteine (PDB: 3TAX). This interesting double-displacement mechanism crosslinks the OGT active site, rendering it inactive. While BZX2 rapidly inhibits OGT *in vitro*, it is not suitable for cellular studies due to its extensive side reactivity.

Targeted covalent inhibitors are designed to react with poorly conserved residues on the target protein, creating distinct selectivity profiles compared to traditional covalent inhibitors.^[46] Recently, the first rationally designed targeted covalent inhibitor of OGT was reported.^[47] This development was inspired by the observation of a non-conserved, non-catalytic cysteine residue (C917) located in close proximity to the *N*-acetyl group of active-site bound UDP-GlcNAc. By introducing a suitable electrophilic functionality to the *N*-acetyl group of UDP-5SGlcNAc, the new compound (named UDP-ES1, Figure 2b) was found to specifically react with C917 and covalently inhibit OGT *in vitro* (PDB: 6E37). Interestingly, ES1, the metabolic precursor containing the same electrophilic functionality, provided sustained reduction of O-GlcNAcylation with OGT being directly validated as a major target in cells. Compared to one of the best reversible OGT inhibitors, Ac₄5SGlcNAc, ES1 was more effective and particularly suited for conditions with high intracellular concentrations of UDP-GlcNAc. Notably, ES1 was shown to inhibit OGT without significant impact on a functionally similar glycosyltransferase, epidermal growth factor domain-specific OGT (EOGT) which utilizes the same UDP-GlcNAc sugar donor.^[47–49] Specificity has been one of the major challenges in designing OGT inhibitors. This rationally designed targeted covalent inhibitor offers a new strategy to interrogate OGT functions and can potentially be exploited to investigate other glycosyltransferases.

During the past few years, several potent and selective OGT inhibitors have been successfully developed to investigate the functional roles of O-GlcNAc in cells. Future efforts are highly desirable to discover new OGT inhibitors for *in vivo* studies which may evaluate OGT as a potential therapeutic target.

2.2 New Strategies to Study OGT-Protein Substrate Recognition

Understanding how OGT interacts with various protein substrates is an essential but challenging task. OGT is able to glycosylate thousands of proteins without an apparent sequence motif and currently there is no structural information on OGT-protein substrate complexes. Towards addressing these challenges, new strategies have been developed.

2.2.1 Protein Microarrays—Protein microarrays have recently been applied to explore the substrate recognition of OGT.^[50] In this method, a commercially available microarray containing ~8,000 human proteins was incubated with UDP-GlcNAc and purified OGT (Figure 3). The O-GlcNAc-modified proteins were detected using an anti-O-GlcNAc antibody (CTD110.6). However, low levels of pre-existing O-GlcNAc modification and antibody epitope bias resulted in increased background and false-positive results. A second-generation detection method employed an azido analogue of UDP-GlcNAc (UDP-GlcNAz) for click chemistry-based fluorescent detection (Figure 3).^[51] This optimized method significantly decreased background signal and facilitated identification of an asparagine ladder in the TPR domain that is important for protein substrate binding. Interestingly, integration of the protein microarray with proteomic analysis further revealed a parallel aspartate ladder in the TPR region that contributes to OGT substrate selectivity.^[51,52] Protein microarray provides a highly efficient strategy to characterize OGT substrate recognition at similar protein concentrations *in vitro*. Consideration should be given to existing endogenous protein modifications like phosphorylation that may adversely influence the accuracy and reproducibility of the microarray results.

2.2.2 GlcNAc Electrophilic Probes—A unique class of chemical tools termed GlcNAc Electrophilic Probes (GEPs), containing an allyl chloride that extends from the *N*-acetyl group of UDP-GlcNAc, were recently reported (Figure 4).^[53] These probes can rapidly discriminate changes in both sugar- and protein-substrate binding of OGT mutants, compared to wild-type, by generating varied levels of modification on OGT C917 and the protein substrate (Figure 4). Both of these modifications can be easily detected using an azide-containing GEP (GEP1A) and an alkyne fluorophore in a click-chemistry-based in-gel fluorescence assay. This new technique is expected to accelerate the characterization of structural features and key residues of OGT that contribute to substrate binding and recognition. As an example, a patch of residues lining the inner surface of the TPR domain that contribute to substrate binding was identified using this assay.^[53] These results were further confirmed by orthogonal radiolabeled kinetic experiments. In general, the GEP1A fluorescence assay can be applied to rapidly screen OGT variants for differences in substrate binding, thus quickly assessing the functional role of OGT residues in protein substrate recognition and interaction.

Another interesting application of GEPs is to crosslink OGT with protein substrates *in situ*. Leveraging the ordered bi-bi mechanism (Figure 2a), a preincubation with OGT allows GEP1, an analogue of GEP1A without azido group (Figure 5), to react with OGT C917 before being transferred to the protein substrate, thus crosslinking OGT with its protein substrate during the sugar-transfer process.^[53] Combined with downstream analyses (*e.g.*, quantitative LC–MS/MS), this assay offers a unique strategy to enrich, identify, and characterize genuine substrates that transiently or weakly interact with OGT *in vitro*. As mentioned above, both protein microarray and GEPs are convenient and efficient methods to probe the substrate recognition modes of OGT. However, results should be considered carefully and paired with complementary validation experiments to determine their biological significance.

3. Structural Insights and OGA Inhibitors

Human OGA belongs to glycoside hydrolase family 84 (GH84). It is a multidomain protein with *N*-acetyl- β -D-glucosaminidase catalytic domain, stalk domain, and pseudo histone acetyltransferase (HAT) domain.^[10] The catalytic domain (but not other domains) of human OGA shares high sequence similarity with bacterial GH84 homologs. It was reported that the pseudo HAT domain of human OGA displayed histone acetyltransferase activity,^[54,55] but later structural studies on bacterial homologs of this domain suggested that it is a catalytically incompetent ‘pseudo’-HAT, as it lacks the key residues for binding acetyl coenzyme A.^[56,57] Thus, the function of the pseudo-HAT domain of human OGA remains elusive. Previous studies on enzyme kinetics and bacterial GH84 homolog structures supported that OGA applies a two-step substrate-assisted mechanism, forming a bicyclic oxazoline intermediate, to hydrolyze O-GlcNAc modifications (Figure 6a).^[58–61] This information has guided the rational design of several potent and selective OGA inhibitors and more recently, guided the engineering of GH84 enzymes into phosphorylases.^[62] As a significant advancement for the field, the structures of human OGA containing the catalytic and stalk domains were reported in 2017 (PDBs: 5TKE, 5UHK, and 5M7R).^[63–65] In contrast to previously reported bacterial homolog structures, human OGA forms an unusual arm-in-arm homodimer that creates two potential substrate-binding clefts (Figure 6b). Indeed, the structures of human OGA in complex with each of five distinct glycopeptide substrates revealed that the GlcNAc moieties of glycopeptides were nested identically in the catalytic pocket. More interestingly, the peptides were bound in a bidirectional but generally conserved conformation within the substrate-binding cleft, forming important contacts with OGA cleft surface residues (Figure 6b).^[63,66] This emerging new substrate binding mode of OGA will be invaluable to guide future design of novel inhibitors with improved substrate selectivity.

Development of selective, cell-permeable inhibitors of OGA is extremely important for understanding the functional roles of this enzyme and O-GlcNAc modification. To date, reported OGA inhibitors are predominantly transition-state mimics that reversibly bind in the active site. One of the first reported OGA inhibitors was PUGNAc (Figure 6c) which inhibits rat spleen derived OGA and human OGA with a K_i value of 52 nM and 46 nM, respectively.^[2,59] PUGNAc has been widely used as a cell permeable OGA inhibitor. However, it can also inhibit the most functionally similar enzyme to OGA in humans,

lysosomal β -hexosaminidase (belonging to glycoside hydrolase 20 family), with K_i of 36 nM.^[59]

The structure of a bacterial GH84 homolog in complex with PUGNAc inspired the rational design of GlcNAcstatin class of OGA inhibitors. GlcNAcstatin C (Figure 6c) exhibited a K_i value of 4.4 nM toward human OGA and modest selectivity over lysosomal β -hexosaminidase.^[67,68] Interestingly, GlcNAcstatin G (Figure 6c), bearing an elongated *N*-acetyl side chain, displayed a > 900,000-fold selectivity over lysosomal β -hexosaminidase while maintaining high potency for human OGA (K_i = 4.1 nM).^[69] This compound was shown to be cell permeable and induced cellular hyper-O-GlcNAcylation levels at low nanomolar concentrations.

In general, GlcNAcstatins represent a class of highly potent and selective OGA inhibitors. Further improvement of their aqueous solubility is expected to facilitate their broader applications.^[70]

NAG-thiazoline (Figure 6c), mimicking the oxazoline intermediate, demonstrates high potency towards both families 20 and 84 glycoside hydrolases.^[59,60] The modest selectivity of NAG-thiazoline toward OGA over lysosomal β -hexosaminidase has limited its application in more complex biological systems. Exploiting the unusually deep catalytic pocket of OGA (PDB: 2J4G), an NAG-thiazoline analogue (NButGT in Figure 6c) containing an extended propyl substituent demonstrated 1,500-fold selectivity for OGA over lysosomal β -hexosaminidase.^[59,60] Unfortunately, NButGT was later found to be unstable in solution over time. A major improvement on this class of compounds was made by introducing an amine to the alkyl chain of NButGT.^[71] The new compound, Thiamet-G (Figure 6c), was highly stable and offered favorable charge interactions with the essential catalytic residue Asp174 of OGA. It displayed high potency (K_i = 21 nM) and 37,000-fold selectivity over human lysosomal β -hexosaminidase. More recently, the structures of human OGA in complex with Thiamet-G (PDBs: 5UN9, 5UHL, and 5M7S) consistently demonstrated that this compound is an excellent transition-state mimic and engages in extensive interactions with OGA active-site residues.^[63–65] A notable feature of Thiamet-G is that it can penetrate through the blood-brain barrier and reduce Alzheimer's symptom in mouse models.^[72] While the exact role of Thiamet-G in potential treatment of neurodegenerative diseases remains elusive, it has been suggested that Thiamet-G-induced O-GlcNAc elevation may reduce the accumulation and aggregation of hyperphosphorylated tau protein and stimulate autophagy through an mTOR-independent pathway.^[73]

To further improve the central nervous system penetration properties of Thiamet-G, medicinal chemistry approaches have been applied to develop new OGA inhibitors. By reducing the topological polar surface area of Thiamet-G, MK-8719 (Figure 6c) exhibited excellent brain penetration while maintaining potent OGA inhibition and high selectivity.^[74] The X-ray crystal structure of human OGA complexed with MK-8719 (PDB: 6PM9) demonstrated a nearly identical binding conformation as bound Thiamet-G. Excitingly, MK-8719 was successfully advanced into first-in-human phase I clinical trials and granted orphan-drug designation for treatment of progressive supranuclear palsy by the US-FDA.^[75]

Alongside the compounds mentioned above, stereoisomeric iminocyclitols represent another interesting class of OGA inhibitors, which displayed low nanomolar inhibition of OGA *in vitro* and can even penetrate the blood–brain barrier in animal models.^[64,76] Notably, one of the most potent pyrrolidine iminocyclitol derivatives, VV347 (Figure 6c, $K_i = 8$ nM), is found to interact with multiple residues in the OGA substrate-binding cleft in addition to the commonly found active-site interactions (PDB: 5M7U).^[64] This represents a distinct binding mode compared to previously reported OGA inhibitors. Despite the attractive features, the selectivity of VV347 over lysosomal β -hexosaminidase remains unclear and the synthesis of these scaffolds is challenging.

While rationally designed OGA inhibitors have achieved great success, new assay development will continue to promote the discovery of novel OGA inhibitors with large chemical diversity and potentially different mechanisms of action. For example, recently developed Bis-Acetal-Based Substrates (BABS) efficiently reports OGA activity *in vitro* and in cell lysates^[77] and may be developed to facilitate screening of new inhibitors for OGA or other exo-acting glucosidases.^[78]

In summary, recent progress on OGA structural characterization and inhibitor development are expected to enhance the functional investigation of OGA and O-GlcNAc. Notably, some OGA inhibitors demonstrated promising therapeutic potential in neurodegenerative disease. However, challenges remain in elucidating the precise roles of OGA from homeostasis to pathogenesis, which requires the continued development of new chemical tools, combined with other approaches, to fully elucidate the regulatory mechanisms of OGA.

4. Chemical Approaches to Investigate O-GlcNAc Functions

The vast regulatory network of O-GlcNAc is still largely a mystery. One challenge arises from the lack of consensus sequence motif in O-GlcNAc modified proteins. Another challenge is due to the poor O-GlcNAc enrichment methods using traditional lectins and antibodies.^[11,79] In addition, there are limited methods to manipulate O-GlcNAc levels in a protein- or site-specific manner. This section will highlight the chemical approaches that have been developed to enrich, detect and interrogate O-GlcNAcylation. We will cover chemoenzymatic and metabolic labeling methods, progress of small-molecule receptors for β -O-GlcNAc and recent advances in generating naturally and engineered GlcNAcylated polypeptides.

4.1 Chemoenzymatic Labeling

O-GlcNAcylation typically occurs at low stoichiometry, making robust enrichment of O-GlcNAc essential for its sensitive detection. A widely used enrichment and detection method is chemoenzymatic labeling by β -1,4-galactosyltransferase (GalT), a human enzyme responsible for specific attachment of galactose (Gal) to terminal GlcNAc moieties.^[80] The specificity of this reaction was initially exploited for radiometric detection of protein O-GlcNAcylation status using ^3H -galactose.^[1] To extend the applications of this assay, the substrate binding pocket of GalT was enlarged by Y289L mutation. GalT-Y289L can accommodate galactose analogues with chemoselective functionalities, such as *N*-azido acetylgalactosamine (GalNAz) and 2-keto-Gal.^[80,81] This method has been successfully

employed for the selective enrichment and detection of endogenous O-GlcNAcylated proteins *in vitro*, with highest efficiency on denatured protein samples. Recently, this method has been adapted to detect O-GlcNAc levels in tumor biopsies, representing a new area for *ex vivo* applications.^[82] Despite the success, this technique is unsuitable for detection of dynamic O-GlcNAc *in vivo*, primarily because GalT-Y289L is inefficient at labeling GlcNAc groups on tertiary protein structures. New methods with improved biological compatibility and specificity will expand our knowledge of O-GlcNAc modification.

4.2 Metabolic Labeling

Metabolic labeling exploits the promiscuity of cellular machinery that can process substrate analogues, such as GlcNAc with chemical handles, to enable sensitive enrichment and detection of dynamic O-GlcNAc in living systems. One of the most popular methods is the use of unnatural monosaccharide precursors for metabolic labeling of O-GlcNAc, such as tetraacetylated *N*-azidoacetyl-glucosamine (Ac₄GlcNAz).^[83] This compound can enter cells and hijack the hexosamine biosynthetic pathway (HBP) to produce UDP-GlcNAz, which can be directly used by OGT to modify dynamic O-GlcNAcylation sites. The azido group on the GlcNAz moiety offers a handle to enrich and detect this modification by click-chemistry-based reactions. However, the efficiency of this metabolic labeling method is relatively low because Ac₄GlcNAz cannot transit the pyrophosphorylase step of the GlcNAc salvage pathway.^[83,84] This problem was circumvented by applying tetraacetylated *N*-azidoacetyl-galactosamine (Ac₄GalNAz), which can be efficiently converted to UDP-GalNAz and then epimerized into UDP-GlcNAz for O-GlcNAc labeling.^[84]

Recently, the Ac₄GalNAz metabolic incorporation method has been coupled to genome-wide mapping to investigate how O-GlcNAc regulates gene expression in fly and mammals.^[85] Dynamically O-GlcNAcylated proteins were labeled by metabolic feeding of Ac₄GalNAz at varied time points. Bioorthogonal coupling of the azido sugar replaced the traditional antibody enrichment in chromatin immunoprecipitation-sequencing (ChIP-seq), which allowed for time-resolved monitoring of chromatin-associated O-GlcNAcylated protein dynamics. Intriguingly, these results suggest that chromatin-associated O-GlcNAcylated proteins are frequently involved in gene activation. In another study, similar Ac₄GalNAz labeling and bioorthogonal conjugation has been applied to demonstrate how O-GlcNAcylation dynamically regulates core translational factors.^[86]

Another interesting application of metabolic feeding is for live-cell fluorescent labeling of O-GlcNAcylated proteins. In order to achieve this, a UDP-GlcNAc fluorescent analogue was developed for direct single-step metabolic labeling.^[87] The glucosamine nitrobenzoxadiazole (GlcN-NBD) was fed into cells, converted to UDP-GlcN-NBD by the HBP and demonstrated widespread labeling in the cytoplasm and nucleus of fixed cells. Though this probe is unsuitable for imaging living samples due to high fluorescence background, with some improvements, this method has the potential to monitor spatiotemporal activity of OGT in its native environment.

These metabolic labeling strategies are well suited for detection of dynamic O-GlcNAcylation. However, they cannot detect endogenous O-GlcNAc on sites with low turnover rates. Additionally, the per-O-acetylated azido and alkynyl sugars (such as

Ac₄GalNAz) may spontaneously and non-enzymatically modify cysteine side chains to generate artificial S-glycosylation (Figure 7a).^[88] Endogenous S-glycosylation has been reported and OGT displays some activity to transfer GlcNAc to cysteine residues in mammalian cells.^[88–90] However, the non-enzymatic side reactions of per-O-acetylated sugar analogues can potentially introduce false positives in mapping O-GlcNAcylation and S-GlcNAcylation sites.^[88,91]

A recent study found that O-acetyl groups on the unnatural monosaccharides are the main cause for artificial S-GlcNAcylation.^[88] Toward addressing this issue, un-acetylated sugars were initially used to avoid the artificial S-glycosylation in glycoproteomic profiling. However, due to the poor cell permeability of un-acetylated sugar, this method required feeding more than 10-fold higher sugar concentrations to achieve modest levels of O-GlcNAc labeling. More recently, 1,3-di-O-propionyl-*N*-azidoacetyl-galactos-amine (1,3-Pr₂GalNAz) was reported as a next generation metabolic probe for glycan labeling (Figure 7b).^[91] This probe can enter cells and be converted to UDP-GlcNAz for highly efficient and specific O-GlcNAc labeling without introducing artificial S-glycosylation. 1,3-Pr₂GalNAz has been successfully applied to investigate the role of O-GlcNAcylation in regulating the self-renewal and pluripotency of mouse embryonic stem cells. It was discovered that O-GlcNAcylation increases the stability of a critical transcription factor, ESRRB, and promotes its binding with OCT4 and NANOG pluripotency regulators. As mentioned above, metabolic labeling accounts for newly added O-GlcNAc modifications after treatment. Coupling this strategy with methods that can specifically detect endogenous O-GlcNAc will aid in a more comprehensive understanding of O-GlcNAc biology.

4.3 Small-molecule Receptors for GlcNAc

Traditional methods (*e.g.*, lectins and anti-O-GlcNAc antibodies) lack the desired specificity and binding affinity for O-GlcNAc detection.^[92–94] Thus, novel approaches with improved endogenous O-GlcNAc detection are highly desirable. Recently, a catalytically impaired mutant OGA homolog from *Clostridium perfringens* (CpOGA^{D298N}) was successfully applied to enrich and detect endogenous O-GlcNAcylated substrates.^[95–97] The CpOGA^{D298N} presents increased binding affinities over traditional methods, though it shows biased affinity for Ser-O-GlcNAc compared to Thr-O-GlcNAc peptides based on MS profiling of the embryonic *Drosophila* O-GlcNAc proteome.^[97] Small-molecule synthetic receptors have emerged as a promising new research direction to address some of these needs. These receptors recognize carbohydrates similarly to lectins but have the potential to be developed into more versatile tools that can selectively bind to a particular monosaccharide of interest.

Synthetic receptors with modest selectivity and affinity have been designed for glucopyranosides^[92,94,98–108] and mannosides^[109–113] These receptors present similar architecture that include a set of parallel aromatic surfaces (ground and roof) to provide CH- π interactions with carbohydrate CH groups (Figure 8a).^[93,94] The aromatic groups are connected by a series of polar spacers (pillars) to prevent twisting or collapse. More importantly, the polar spacers offer hydrogen bonding interactions with polar equatorial substituents (*e.g.*, -OH groups) of the carbohydrate to form a “temple-like” structure.

Interestingly, a receptor for free GlcNAc molecules (racemic mixture) was reported with a binding constant of $K_a = 1.28 \times 10^3 \text{ M}^{-1}$ (Figure 8b).^[80] More excitingly, the best receptor for β -OMe-GlcNAc (Figure 8c) offered K_a of $1.82 \times 10^4 \text{ M}^{-1}$ in aqueous solution and demonstrated 11.7-fold binding selectivity over α -OMe-GlcNAc and no binding to glucose.^[94] We anticipate this great progress will encourage future development of more specific and water-soluble receptors to interrogate O-GlcNAc modifications *in vitro* and in cells.

The design of small-molecule receptors for carbohydrates is very challenging. For one, the large excess of aqueous solution strongly competes with polar interactions between the receptor and carbohydrate. Another challenge arises in achieving sufficient binding specificity as carbohydrates are chiral and carry only slight variations in their functional groups. While there may still be a long way to go, small-molecule receptors and other types of synthetic receptors^[114] with high binding affinity, specificity, and solubility will open up new adventures for O-GlcNAc study.

4.4 O- and S-GlcNAcylated Polypeptides

An important role of O-GlcNAc is stabilization of peptide and protein substrates. Engineered O-GlcNAc peptides originated from the need to understand the mechanisms of stabilization. This strategy was later employed to make synthetic O- and S-GlcNAcylated polypeptides to gain a deeper understanding of the site-specific role of O-GlcNAc and to enhance the properties of glycosylated molecules.

4.4.1 Synthetic Peptides with Site-Specific O-GlcNAcylation—The low stoichiometry and high heterogeneity of O-GlcNAcylation across different sites of a protein renders a significant challenge in studying the precise role of this modification. Solid-phase peptide synthesis combined with expressed protein ligation has been used to generate homogenous, site-specifically O-GlcNAcylated peptides and short proteins for *in vitro* functional studies.^[115,116] It has been demonstrated that synthetic O-GlcNAcylated α -synuclein resists calpain-mediated cleavage and subsequent proteolysis, suggesting higher overall stability.^[115,117] Interestingly, synthetic α -synuclein with site-specific O-GlcNAcylation revealed that individual modification sites have differential impacts on protein aggregation, a hallmark of Parkinson's disease.^[117–119] For example, O-GlcNAcylation on several sites of α -synuclein demonstrated significant inhibition of its aggregation and toxicity without disrupting its normal biological functions in neuronal cell culture.^[115,116,118,120,121] These findings raise interesting questions about the protective role of O-GlcNAc in neurodegeneration and open the synucleinopathy therapeutic arena to compounds that affect O-GlcNAcylation. The application of these synthetic strategies is straightforward but, due to the difficulty of refolding synthetic proteins into their native 3D conformations, is generally limited to small proteins without significant tertiary structure like α -synuclein.

4.4.2 Engineered GlcNAc Modifications—Effective stabilization of peptide and protein by O-GlcNAc is exemplified by microtubule-associated protein tau, which has multiple O-GlcNAc modification sites. It was found that increasing the O-GlcNAc level of tau can enhance the solubility of this protein and reduce its pathogenic aggregates.^[72] As

another mechanism for stabilization, O-GlcNAcylation counteracts ubiquitination of many natural substrates, including the inflammation controlling FOXP3 transcription factor, further enhancing protein stability.^[122] Therefore, it is hypothesized that more general installation of artificial O-GlcNAc modifications on peptides and proteins may enhance their stability. This concept was successfully tested with two clinically relevant peptides that are not naturally O-GlcNAcylated, glucagon-like peptide-1 and parathyroid hormone. Both peptides demonstrated enhanced stability in serum and improved *in vivo* activity with engineered O-GlcNAc modifications.^[123] Interestingly, incorporating an unnatural amino acid further increased their stability, suggesting that strategic integration of these complementary methods may provide synergistic improvement of peptide properties and clinical efficacy.

While the synthetic peptide strategy has achieved great success in studying site-specific O-GlcNAc functions *in vitro*, it is generally not suitable for *in vivo* applications as O-GlcNAc residues can be readily hydrolyzed by OGA. S-GlcNAc is structurally similar to O-GlcNAc, but has improved stability and resistance to OGA catalyzed hydrolysis.^[89,90,124] Hence, S-GlcNAc may serve as a stable O-GlcNAc mimic.

Recently, site-specific incorporation of S-GlcNAc in live cells has been achieved by leveraging CRISPR–Cas9 technology.^[124] Natural O-GlcNAcylation sites (serine or threonine residues) are mutated to cysteine which can be glycosylated by endogenous OGT. This strategy was applied in mouse embryonic stem cells to generate S-GlcNAcylated OGA. In contrast to above mentioned O-GlcNAc enhanced protein stability, this study found that site directed S-GlcNAcylation reduced OGA half-life while maintaining its hydrolase activity. As S-GlcNAc modification could be more stable than O-GlcNAc, a more detailed study of site-specific GlcNAcylation, at prolonged time scale, was possible. This technique also brings minimal perturbation to the native system, avoiding the need for artificial enzymatic machinery or treatment with chemical probes. Nevertheless, the experimental data derived from S-GlcNAc mimics need to be interpreted carefully as S-GlcNAc interacts with OGA homolog (and possibly other interacting molecules) with substantially lower binding affinity than O-GlcNAc,^[124] and in some cases the cysteine mutation of O-GlcNAc sites may induce undesired effects on the modified proteins. Despite these considerations, this study is expected to promote future development of new techniques to accelerate understanding the role of site-specific O-GlcNAcylation in living systems.

5. Chemical Glycoimmunology

One of the most complex and finely tuned regulatory networks is the human immune system. An arsenal of adaptive and innate immune responses is responsible for sensing, identifying and removing potentially harmful and pathogenic substances. Though only recently appreciated, it is no surprise that O-GlcNAcylation plays a key role in dynamically regulating these processes. Previous reports have explored the emerging links between O-GlcNAc and the immune response.^[125–128] Here we will discuss the latest findings and future directions of chemical tools that will enhance our understanding of O-GlcNAc in the immune system. For recent reports that cover in depth how the immune system is affected by O-GlcNAc modifications, please refer to other reviews.^[129,130]

5.1 O-GlcNAc in the Immune System

The link between O-GlcNAc and human immunity has been traditionally studied using conventional genetic and biochemical approaches. OGT has been shown to play an important role in the adaptive immune response by glycosylating regulators in T and B cell activation.^[122,131–133] For instance, targeted deletion of OGT in mature B cells resulted in impaired B cell receptor activation and signaling.^[132] In line with this, during T cell activation, a variety of important receptors (such as Notch) and transcription factors (like c-Myc) are all O-GlcNAcylated.^[131] Interestingly, O-GlcNAc has shown to affect both pro- and anti-inflammatory responses, primarily through modulating transcription factor activities. For example, in pro-inflammatory responses, O-GlcNAcylation of the p65 subunit of the NF- κ B complex has been found to regulate NF- κ B-mediated expression of inflammatory cytokines.^[134] Paradoxically, O-GlcNAcylation of RIPK3, a member of the TNF receptor-1 signaling complex, has been implicated in anti-inflammatory response through NF- κ B antagonism.^[135] These results suggest a delicate balance of O-GlcNAc homeostasis in inflammation, wherein both increased and decreased levels of O-GlcNAc trigger a hyperinflammatory response through distinct mechanisms. It follows that dysregulation of any of these well-balanced systems could lead to pathogenesis. A recently described example showed that O-GlcNAcylation of STAT3, a critical transcription factor in inflammatory signaling, normally acts as a negative regulator of IL-10 production.^[136] However, aberrant modification of this transcription factor was linked to a variety of inflammatory diseases.

These studies underline the vast complexity of O-GlcNAcylation in the immune response. They also highlight the wealth of opportunities that could become available for scientific inquiry and therapeutic development. Hence, new tools are required that can effectively dissect this complexity to uncover a full picture of the role O-GlcNAc plays in the immune system.

5.2 Chemical Strategies for O-GlcNAc Glycoimmunology

Chemical tools that complement traditional approaches have recently been applied to study O-GlcNAc in immune cells. For example, OGA inhibitors (*e.g.*, NButGT) have been used to investigate the role of normal and increased O-GlcNAcylation on transcription factors like p65 in the NF- κ B complex.^[137] This study found that O-GlcNAc regulates NF- κ B signaling in coordination with phosphorylation and acetylation in healthy and disease cells. Another application of OGA inhibitors is to increase the O-GlcNAc stoichiometry for more sensitive detection through proteomic analysis. Thiamet-G has been used to enhance the proteomic profiling of activated B cells which implicated the O-GlcNAcylation of lymphocyte-specific protein-1 (Lsp1) as a key regulator of B cell activation and apoptosis.^[133] As mentioned before, these inhibitors affect global O-GlcNAc profiles, therefore, more targeted probes will be needed to interrogate the complex networks of O-GlcNAc in these living systems.

Chemoenzymatic labeling, representing another type of chemical tool, has also been employed in the immune system. For example, *N*-(4-pentynoyl)-alkyne galactosamine (GalNA1) followed by proteomic analysis was used to compare protein glycosylation in CD8⁺ T cells before and after activation.^[138] This study discovered that plastrin-2 and 40S

ribosomal protein S23, both of which are involved in a variety of transcription and translation processes, were dynamically regulated by O-GlcNAc. However, due to the inherent challenges of O-GlcNAc detection (low stoichiometric abundance and low ionization efficiency), more sensitive approaches are needed to uncover more complete O-GlcNAc profiles in the immune system.

Another technique with higher sensitivity of glycan detection, named Isotope Targeted Glycoproteomics (IsoTaG), was developed for the identification and characterization of metabolically labeled glycopeptides^[139] and was recently applied to study activated T cells.^[140] This method metabolically labels glycosylated proteins with an isotopically coded chemical tag (Figure 9). The unique isotopic abundance signature allows for selective tandem MS fragmentation of tagged glycopeptides, independent of their abundance in the complex sample. The IsoTaG technique is particularly effective for low abundance proteins lacking consensus sequence motifs, as is the case of O-GlcNAcylated proteins. This method identified approximately 2,000 O-linked glycopeptides in activated T cells, most of which were located in the cytoplasm or nucleus with a small portion in the secretory pathways.^[140] As part of this study, quantitative LC-MS/MS analysis revealed substantial changes in O-GlcNAc between control and activated samples, suggesting a functional significance of O-GlcNAcylation in T cell activation.

While the precise role of O-GlcNAc is largely unclear in immune cells, avenues for developing O-GlcNAc-based immunotherapies are beginning to form. Traditionally, immunotherapy is conceived as interventions comprised of hormones, antibodies, enzymes and immune cells. Recently, modulating cell surface O-linked glycans has become a new direction in this area. O-linked glycans are known to play crucial roles in regulating protein stability, signal transduction, and cell-cell interactions.^[133,141] O-linked glycosylation of transmembrane proteins is essential for the development of broadly applicable diagnostic cancer biomarkers.^[142] New approaches for cancer immunotherapy include precise editing of the cell glycocalyx.^[143] With this strategy, antibody–enzyme conjugates selectively remove sialic acid moieties, directing immune cells to specifically kill the desialylated cancerous cells. Similar technologies can conceivably be developed to modulate O-GlcNAcylation on intracellular proteins for therapeutic use. The briefly described chemical strategies combined with longstanding genetic and biochemical approaches create a foundation for future understanding of the precise role of O-GlcNAc in the immune system and for the development of potential O-GlcNAc-directed immunotherapies.

6. Summary and Outlook

In the past few years, significant progress has been made in the O-GlcNAc research field. New OGT inhibitors with higher potency, specificity and cell permeability have been successfully developed. Future research is expected to develop even better OGT active-site inhibitors for *in vivo* studies and to develop substrate-specific inhibitors that can avoid perturbing the global O-GlcNAc profile. These studies will benefit from a better understanding of how OGT interacts with its protein substrates. The discovery of parallel asparagine and aspartate ladders in the OGT TPR domain represents an important step toward understanding how OGT selectively recognizes substrate proteins. Disruption of

these substrate recognition modes has been linked to a variety of diseases. For example, OGT variants containing missense and splice-site mutations in the TPR domain have been recently connected to X-linked disabilities.^[24,25,144–147] OGT remains active with these mutations, however, changes in the TPR can destabilize the super-helical structure and affect substrate binding.^[25,145,148] It is expected that future research will continue to uncover a more complete picture delineating how OGT recognizes such amazingly diverse protein substrates. These efforts will provide new insights into the molecular mechanism of OGT-substrate recognition and will facilitate development of new OGT inhibitors for biomedical use.

Recent landmark advances in human OGA structures and inhibitor development will promote the functional elucidation of OGA in physiological and pathological processes. Similar to OGT, it remains unclear how OGA recognizes various glycoprotein substrates. Better understanding of the roles of OGA substrate-binding cleft and other domains is expected to inform design of substrate-specific inhibitors. These probes are expected to facilitate further discoveries in OGA functional significance and the molecular impacts of its dysregulation in diseases.

To modulate O-GlcNAc levels, most of the strategies reported to date have relied on directly targeting its cycling enzymes. However, O-GlcNAc regulation can go beyond aiming at OGT or OGA. For instance, CRISPR-Cas9 genome editing and small-molecule inhibitors have been coupled to knockout glutamine fructose-6-phosphate amidotransferase (an essential enzyme in the HBP) and block the production of UDP-GlcNAc.^[149] This approach resulted in low cellular O-GlcNAc levels and suppressed cancer cell proliferation. This study demonstrates that genetic and chemical approaches can work in harmony to regulate O-GlcNAc modification of living systems. However, the extent that inhibiting the HBP impacts overall cell homeostasis is yet to be determined. In parallel, chemoenzymatic and metabolic labeling approaches exploiting exogenous sugar analogues to enrich and detect O-GlcNAcylated proteins have proven powerful in studying complex biology from transcription and translation to the immune system. Forthcoming technologies to track O-GlcNAc *in vivo* will be highly desirable, especially those that can monitor and manipulate O-GlcNAc in a protein-specific, site-specific, and localization-specific manner, for elucidating the precise roles of O-GlcNAc in biology and its potential in therapeutic development.

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Figure 1.

The diverse range of cellular functions regulated by dynamic O-GlcNAcylation and a few examples of techniques for detecting and modulating this modification. O-GlcNAc transferase (OGT) utilizes uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), the end product of the hexosamine biosynthetic pathway (HBP), to modify various proteins while O-GlcNAcase (OGA) removes these modifications. OGT and OGA inhibitors have been developed to investigate their functional roles. Other chemical tools, such as metabolic labeling, have also been applied to study O-GlcNAc dynamics.

Figure 2.

(a) The ordered bi-bi mechanism of OGT-catalyzed O-GlcNAcylation. UDP: Uridine diphosphate. G in red hexagon: GlcNAc. **(b)** Chemical structures of OGT inhibitors. Yellow background highlights the two reported covalent inhibitors of OGT, while the other inhibitors in this panel function reversibly.

Figure 3.

Protein microarray to study OGT substrate preferences with two different detection methods. Top: microarrays were incubated with OGT and UDP-GlcNAc, then O-GlcNAc-modified proteins were detected using a monoclonal anti-O-GlcNAc primary antibody (CTD110.6) and a fluorescent secondary antibody. Bottom: Arrays treated with OGT and UDP-GlcNAz were reacted with ADIBO-biotin and stained with fluorescent streptavidin. The chemical structures of UDP-GlcNAc and UDP-GlcNAz are shown on a gray background.

Figure 4.

GEP1A fluorescence assay to screen OGT variants for differences in substrate binding. Wild-type (WT) and mutant OGT generate distinct modification patterns following the reaction with GEP1A and a protein substrate. The patterns can be detected using click-chemistry-based in-gel fluorescence assay to discern whether mutations in OGT affects sugar binding or protein substrate binding. *Cys*: C917 residue of OGT. The chemical structure of GEP1A is shown on a gray background.

Figure 5.

GEPI1 enables crosslinking of OGT with protein substrates *in situ*, which facilitates the detection and characterization of OGT-substrate interactions following downstream analyses. The chemical structure of GEPI1 is shown on a gray background.

Figure 6.

(a) The two-step substrate-assisted catalytic mechanism of OGA. Yellow highlights the oxazoline intermediate. R: Serine or threonine of a peptide or protein. (b) Crystal structure of human OGA in complex with glycopeptide substrates demonstrating a conserved conformation in the substrate-binding cleft of OGA. Left panel: The catalytic domain and stalk domain of one OGA_{cryst} monomer are shown as ribbon in orange and cyan, respectively; the sister monomer is shown as ribbon in gray. Five glycopeptides (shown as spheres in the indicated colors) bound in the OGA substrate-binding cleft are overlapped: p53 (PDB: 5UN8), ELK1 (PDB: 5VVT), TAB1 (PDB: 5VVU), α-crystallin (PDB: 5VVV), and Lamin B1 (PDB: 5VVX). Right panel: Enlarged view of glycopeptides bound in the substrate-binding cleft of OGA (boxed area). The substrate-binding cleft formed by the catalytic domain of one OGA monomer and the stalk domain of the sister monomer is shown as surface in gray and cyan, respectively. The GlcNAc moieties of the glycopeptide substrates are shown as sticks in black with semitransparency and the peptides are shown as ribbon in the indicated colors. (c) The chemical structures of representative OGA inhibitors.

Figure 7. Metabolic labeling approaches to study dynamic O-GlcNAcylation. **(a)** Acetylated metabolic precursor Ac_4GalNAz leads to artificial and non-enzymatic S-glycosylation. **(b)** The 1,3-Pr₂GalNAz can be converted to UDP-GlcNAz in cells and provide high efficiency and specificity for O-GlcNAc labeling.

Figure 8.

Small-molecule receptors for carbohydrates. **(a)** General design framework for small-molecule “temple” receptors. Non-polar or aromatic interactions are shown in blue. Polar interactions are shown in orange. **(b)** Free GlcNAc (racemic mixture) receptor. **(c)** β -OMe-GlcNAc receptor.

Figure 9.

The Isotope Targeted Glycoproteomics (IsoTaG) strategy for O-GlcNAc profiling. Ac_4GalNAz precursor (shown in magenta) is fed into cells and converted into UDP-GlcNAz (shown in red) for OGT catalyzed glycosylation. Following click-chemistry conjugation (shown in orange) of GlcNAz and IsoTaG alkynyl biotin probe (shown in green), GlcNAz modified proteins can be enriched via biotin and glycopeptides with an IsoTaG signature will be selected for tandem MS fragmentation.