

# O-GlcNAc transferase inhibitors: current tools and future challenges

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## Abstract

The O-linked N-acetylglucosamine (O-GlcNAc) post-translational modification (O-GlcNAcylation) is the dynamic and reversible attachment of N-acetylglucosamine to serine and threonine residues of nucleocytoplasmic target proteins. It is abundant in metazoa, involving hundreds of proteins linked to a plethora of biological functions with implications in human diseases. The process is catalysed by two enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) that add and remove sugar moieties respectively. OGT knockout is embryonic lethal in a range of animal models, hampering the study of the biological role of O-GlcNAc and the dissection of catalytic compared with non-catalytic roles of OGT. Therefore, selective and potent chemical tools are necessary to inhibit OGT activity in the context of biological systems. The present review focuses on the available OGT inhibitors and summarizes advantages, limitations and future challenges.

## Introduction

Protein O-linked N-acetylglucosamine (O-GlcNAc) post-translational modification is characterized by the attachment of N-acetylglucosamine moieties to serine and threonine residues of nucleocytoplasmic proteins in metazoa [1]. Global proteomic experiments have shown that hundreds of proteins are dynamically and reversibly O-GlcNAcylated in animals, with consequent modulation of gene expression [2], signal transduction [3], stress response [4] and protein stability [5]. Two enzymes, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), catalyse the attachment and the removal of N-acetylglucosamine respectively [6,7]. Both proteins are essential in animals with loss of their function causing severe abnormalities in early developmental stages, underlining a key role of O-GlcNAc in fundamental biological processes [8–10]. Altered O-GlcNAcylation profiles have been associated with several human pathologies, including diabetes [11], heart failure [12], Alzheimer's disease and other neurodegenerative disorders [13–15]. Nutrient availability appears to be one of the main regulators of OGT function, the high-energy donor substrate UDP-GlcNAc derives from glucose metabolism through the hexosamine biosynthetic pathway, a metabolic cascade parallel to glycolysis [16]. UDP-GlcNAc is also a donor substrate for the glycosyltransferases in the endoplasmic reticulum and Golgi involved in the synthesis of complex N-linked carbohydrates on

secreted proteins. There is evidence to suggest that OGT may have functions in addition to its glycosyltransferase catalytic activity, including the scaffolding and recruitment of proteins to specific sub-cellular compartments [17]. In addition, an unexpected intrinsic proteolytic activity has been reported recently [18]. Tissue-specific OGT knockout mice have been used to overcome the lack of viable total knockout animals [19]. Nevertheless, the concomitant loss of both catalytic and non-catalytic activities makes total or partial OGT depletion unsuitable for experiments aimed at dissecting these activities. Consequently, specific and potent OGT inhibitors are invaluable tools to explore the biological role of O-GlcNAcylation in eukaryotes without affecting the levels of the OGT protein itself. Several compounds have been designed and employed in the last few years, allowing important scientific advances in the O-GlcNAc field. Nevertheless, lack of specificity, off-target effects and/or limited cell permeability are still obstacles to overcome in the future. This review provides an overview of the current range of reported OGT inhibitors, exploring applications, limitations and future directions.

## Substrate analogues

The crystal structure of OGT and its catalytic mechanism have been previously determined: the donor substrate UDP-GlcNAc binds to the active site of the enzyme first, followed by the target polypeptide substrate [20,21]. UDP-GlcNAc itself also functions as the catalytic base, enabling the transfer of GlcNAc on to the target peptide serine/threonine [21]. Interestingly, the reaction product UDP inhibits OGT *in vitro* with an IC<sub>50</sub> of 1.8 μM, making it the most potent OGT inhibitor reported to date [21,22]. Nevertheless, it is unsuitable for cell biology studies because it is not cell-penetrant and is a substrate for a wide range of other enzymes.

**Key words:** inhibitor, O-linked N-acetylglucosamine (O-GlcNAc), O-linked N-acetylglucosamine (O-GlcNAc) transferase, post-translational modification, protein structure, signal transduction.

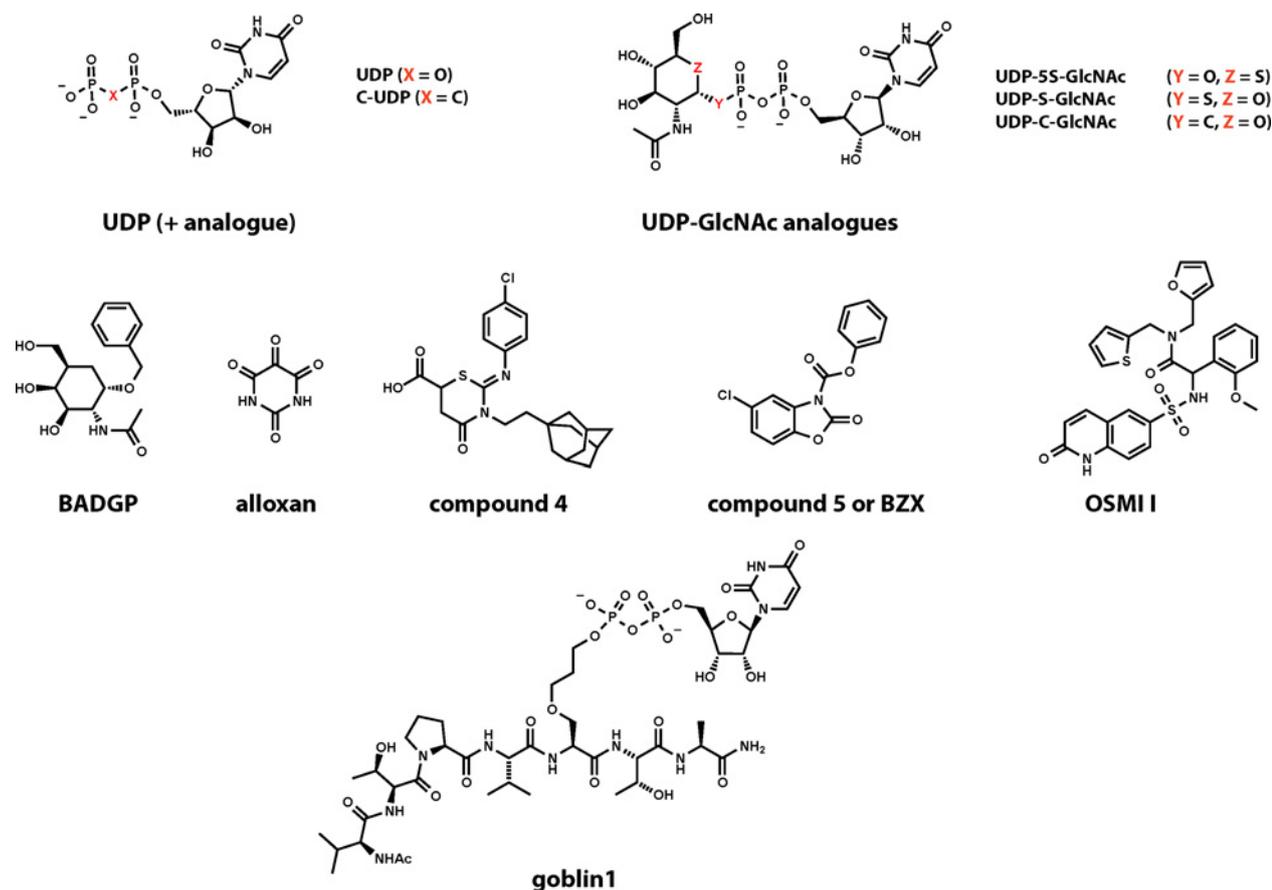
**Abbreviations:** BADGP, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside; BZX, benzoxazolinone; FOXM1, forkhead box protein M1; goblin, OGT bisubstrate-linked inhibitor; NFκB, nuclear factor kappa-light-chain enhancer of activated B cells; OGA, O-GlcNAcase; O-GlcNAc, O-linked N-acetylglucosamine; OGT, O-GlcNAc transferase; SGLT, sodium-coupled glucose transporter.

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**Figure 1** | Chemical structures discussed in this review

UDP-5S-GlcNAc is administered as Ac<sub>4</sub>-5S-GlcNAc in *in cellulo* studies.



Synthesis of OGT substrate and product analogues led to the generation of the three compounds UDP-S-GlcNAc ( $IC_{50} = 93 \mu M$ ), UDP-C-GlcNAc ( $IC_{50} = 41 \mu M$ ) and C-UDP ( $IC_{50} = 9 \mu M$ ) [22] (Figure 1). C-UDP exhibited the highest potency in this group, but is not cell permeable and it may also affect other enzymes acting on UDP and its metabolites.

The uracil analogue alloxan (2,4,5,6-tetraoxypyrimidine; Figure 1) was the first OGT inhibitor reported and has been commonly used [23–25]. It is less potent than UDP ( $IC_{50} = 18 \mu M$ ) but is cell-permeable due to it being a substrate for glucose transporters [26]. Although alloxan has been used in several studies covering cell lines and animal models, the resulting insights are questionable due to a large number of off-target effects and general cellular toxicity due to its ability to generate reactive oxygen species (ROS) [27].

OGT inhibitors have also been obtained from the modification of the donor substrate UDP-GlcNAc. The cell-permeable compound Ac<sub>4</sub>-5S-GlcNAc can be administered to cells to hijack the hexosamine pathway towards the production of UDP-5S-GlcNAc (Figure 1), a donor substrate analogue that is utilized by OGT at a much lower rate compared with UDP-GlcNAc [28]. This compound

showed high potency with an  $EC_{50}$  of  $5 \mu M$  *in cellulo* and it has been used in a number of studies. As an example, Donovan et al. [29] have inhibited OGT with Ac<sub>4</sub>-5S-GlcNAc to investigate the role of O-GlcNAc in the modulation of the transcription factor Sp1, with interesting insights into the development of diabetic retinopathy [29]. Notably, reduced O-GlcNAcylation of Sp1 down-regulates the transcription of the pro-angiogenic factor VEGF-A (vascular endothelial growth factor A), involved in retinal vascularization [29]. Inhibition of OGT by Ac<sub>4</sub>-5S-GlcNAc provided insights into the role of OGT in the insulin response, showing a concerted role of O-GlcNAc and insulin-dependent signalling cascades on membrane lipid microdomains [30]. The compound has also been used on pancreatic cancer cells suggesting a correlation between hyper-O-GlcNAcylation and apoptosis via the modulation of the NF- $\kappa$ B anti-apoptotic transcriptional activity [31]. The main pitfall of Ac<sub>4</sub>-5S-GlcNAc is that it reduces the intracellular pool of UDP-GlcNAc by hijacking the hexosamine pathway. Therefore, it may affect other glycosyltransferases by either direct or indirect inhibition. As a consequence, N-glycosylation and extracellular glycan synthesis may be

impaired in cultured cell lines [32]. Finally, another similar compound is BADGP (benzyl-2-acetamido-2-deoxy- $\alpha$ -D-galactopyranoside; Figure 1). It has been shown to target glycosyltransferases in general and it also reduces the flux of the hexosamine biosynthetic pathway [33,34].

### HTS-derived inhibitors

An alternative strategy for the discovery of OGT inhibitors has been the application of high-throughput screening against a large library of drug-like compounds [35]. This screen was designed to detect compounds that compete with sugar nt binding and identified two compounds: 3-(2-adamantanylethyl)-2-[(4-chlorophenyl)azamethylene]-4-oxo-1,3-thiazaperhydroine-6-carboxylic acid, also known as 'compound 4' or ST045849 and 4-methoxyphenyl 6-acetyl-2-oxobenzo[d]oxazole-3(2H)-carboxylate, referred to as 'compound 5' or BZX [35] (Figure 1). The inhibition efficiency of the two molecules was tested *in vitro* against full-length OGT constructs showing  $IC_{50}$  of 53  $\mu$ M for compound 4 and 10  $\mu$ M for compound 5. A study showed that OGT inhibition by compound 4 decreased pancreatic  $\beta$ -cell development and reduced insulin production [36]. Another recent study revealed the importance of O-GlcNAcylation in gluconeogenesis and maintenance of embryonic stem cell self-renewal [37]. Compound 4 has also provided interesting data about the effect of O-GlcNAc on protein stability. Increased global glycosylation in renal proximal tubule cells prevented the degradation of the Na<sup>+</sup>/glucose transporters SGLT1/2 in hypoxic conditions [38]. This study suggests a protective role of O-GlcNAc towards ischaemia-induced renal damage. BZX has been used to treat breast cancer cells, showing anti-growth and anti-invasion effects through the modulation of the transcription factor FoxM1 [39]. The latter, in particular, regulates the expression of the proteins p27<sup>Kip1</sup> and matrix metalloproteinase-2 (MMP-2), involved in cell cycle regulation and cell invasion respectively [39]. OGT inhibition by BZX also decreased the expression of genes associated with DNA replication and the cell cycle and reduced the stability of the oncogene c-Myc in human prostate cell lines [40]. Furthermore, the compound has been used to reveal a role of O-GlcNAcylation in the vascular contractile response via the modulation of the RhoA/Rho-kinase pathway [41]. However, it has been previously shown that BZX is a covalent/suicide inhibitor and it cross-links the active site of OGT (Lys<sup>842</sup> and Cys<sup>917</sup>) through a double-displacement mechanism, with potential toxic and off-target effects [42].

A novel, small cell-permeable molecule inhibitor, OSMI-1 (Figure 1), has been recently designed basing on the high-throughput screen mentioned above. The  $IC_{50}$  for OGT inhibition is 2.7  $\mu$ M and the compound reduced global O-GlcNAc levels in different mammalian cell lines [32]. This small molecule inhibitor has recently been used to study the role of O-GlcNAc in the replication of Herpes Simplex Virus (HSV), suggesting that reduced OGT activity decreases viral replication rates [43]. Although OSMI-1 minimally affected

surface glycan synthesis, it affected cell viability, suggesting the presence of off-target effects [32].

### Bisubstrate inhibitors

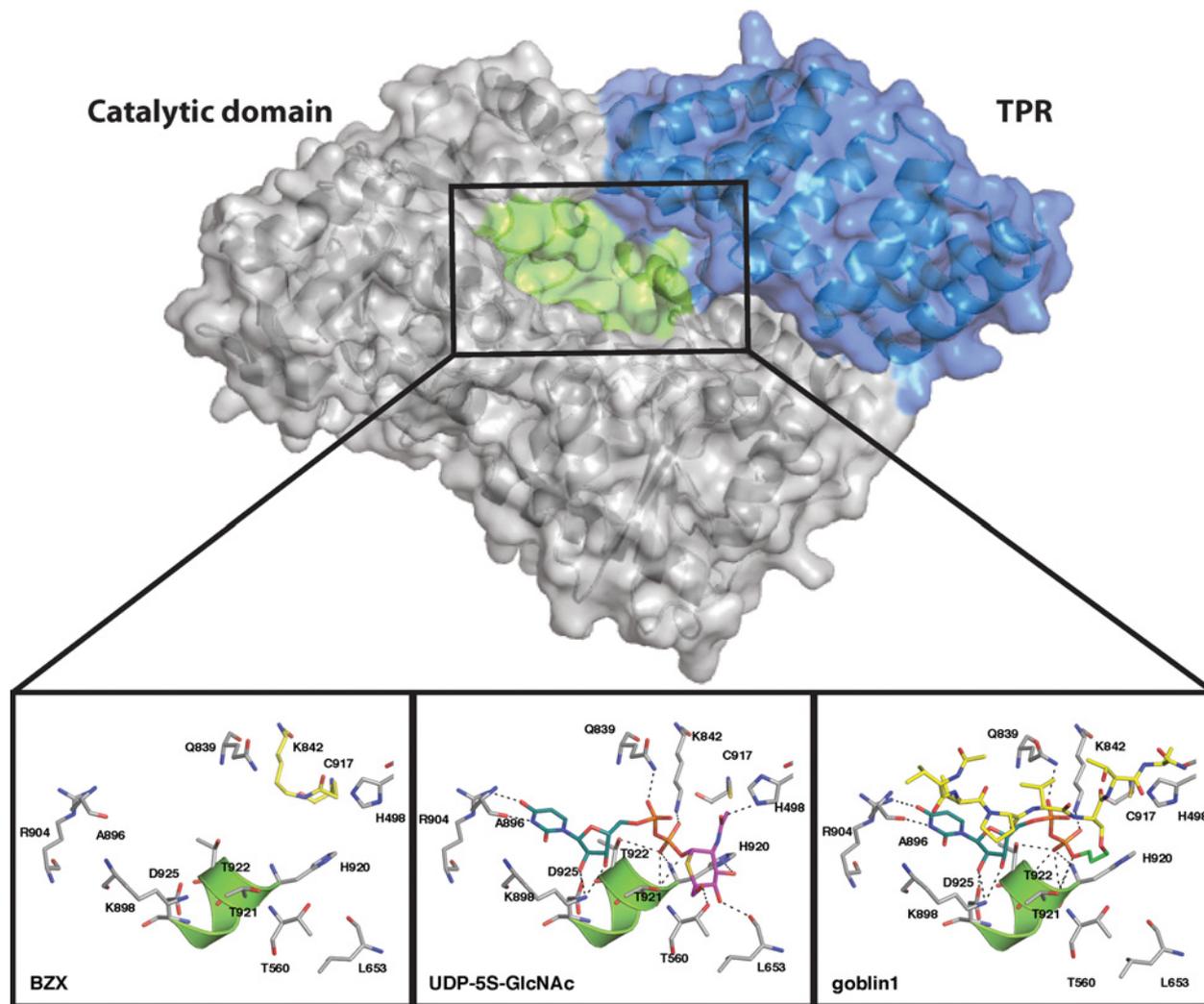
Recent advances in our understanding of the OGT catalytic mechanism have facilitated the rational design of competitive inhibitors. Novel compounds deriving from both OGT donor and acceptor substrate have been constructed, aiming to achieve selective inhibition. The inhibitors goblin1 (OGT bisubstrate-linked inhibitor 1; Figure 1) and goblin2 have been created by connecting an acceptor peptide to a UDP through a short linker that replaces the GlcNAc moiety. These compounds exhibited low micromolar affinity for OGT and inhibited O-GlcNAcylation of peptides and protein substrates *in vitro* ( $IC_{50}$  = 18  $\mu$ M for goblin1) [44]. A limited exploration of the selectivity of these molecules by testing on the bacterial GlcNAc transferase *SmNodC* revealed no inhibition. Although the specificity of goblin towards OGT remains to be further investigated, the major current limitation with this class of compounds is the lack of cell permeability. Therefore the compound has not been yet employed for cell biology applications but it has been used *in vitro* for the inhibition and the study of an OGT orthologue from the early metazoan *Trichoplax adhaerens* [45]. Future experiments need to address this lack of cell permeability, perhaps through the attachment of a cell-penetrant peptide and possibly other intracellular localization signals to target the compound to specific cell compartments.

### Common features of OGT inhibitor binding modes

Structural data are available for four of the recently published human OGT inhibitors: UDP, UDP-5S-GlcNAc, compound 5 and goblin1, allowing a closer examination of their binding mode (Figure 2). Not surprisingly, UDP, UDP-5S-GlcNAc and goblin1 possess an identical binding mode forming an extensive array of polar interactions and hydrogen bonds to the same key residues in the active site of the OGT (i) to the uracil ring, (ii) the 2- and 3-hydroxyls of the ribose and (iii) the  $\alpha$ - and  $\beta$ -phosphates. In particular, the  $\beta$ -phosphate forms the largest number of interactions with the active site, to the backbone and side chains of His<sup>920</sup>, Thr<sup>921</sup>, Thr<sup>922</sup> and Lys<sup>842</sup> as well as the electrostatic dipole of the  $\alpha$ -helix harbouring the first three of these residues (Figure 2). Interestingly, the GlcNAc moiety forms few interactions with the enzyme and has previously been shown to barely contribute to binding [21]. This was further confirmed by the bisubstrate inhibitor goblin1, where the GlcNAc sugar ring has been replaced by a three-carbon linker, retaining low micromolar binding towards OGT [44]. Strikingly, UDP is still the most potent OGT inhibitor reported to date. Structurally guided mutagenesis experiments have shown that Lys<sup>842</sup> is of paramount importance for the binding of UDP to OGT as well as catalytic activity [21], suggesting that the positively charged lysine residue tethers the reaction product in the active site. Furthermore, Lys<sup>842</sup> may be responsible for the unusual conformation of the nt sugar in the active

**Figure 2 | Structure of the human OGT in complex with inhibitors discussed in this review (PDBID 4GYI, 4CDR and 3TAX)**

OGT active site residues are shown as grey sticks. The  $\alpha$ -helix exhibiting the electrical dipole that interacts with the  $\beta$ -phosphate of UDP-5S-GlcNAc and goblin1 is shown as green cartoon. Hydrogen bonds formed between the inhibitors and the OGT are shown as black dashed lines. BZX forms a covalent link between the Lys<sup>842</sup> and Cys<sup>917</sup> residues and is shown as yellow sticks. UDP-5S-GlcNAc, being a substrate analogue, binds to OGT in the same way as the donor substrate. The UDP moiety is shown as teal sticks and the GlcNAc pyranoside as magenta sticks. The bisubstrate inhibitor goblin1 is shown as a stick model with the peptide part coloured in yellow, the C-3 linker in green and the UDP moiety in teal.



site, compared with other glycosyltransferases of the same family [21]. The importance of Lys<sup>842</sup> is further supported by the binding mode of compound 5 [35]. This inhibitor forms a covalent link between Lys<sup>842</sup> and Cys<sup>917</sup> of OGT via a double-displacement mechanism [42], rendering the enzyme inactive.

Future inhibitors should be designed towards exploiting the key interactions of Lys<sup>842</sup> with the donor substrate, as targeting this residue has shown the largest effect on reduction in activity [21]. Additional pockets that may be exploited are the GlcNAc-binding pockets as well as the peptide-binding groove, which has been shown to preferably bind specific peptide sequences [46].

## Concluding remarks

O-GlcNAc modification is abundant, essential and involved in fundamental biological processes. Its functional role in animals is essential but at the same time poorly understood. Genetic approaches have revealed that OGT disruption is lethal early in development. Knockdown approaches may be unsuitable due to the enzyme possessing both catalytic and scaffolding roles that remain to be dissected. For this purpose, different categories of OGT inhibitors have been designed in the last 15 years. The donor substrate analogues Ac<sub>4</sub>-5S-GlcNAc and BADGP, might reduce the flux through the hexosamine pathway and reduce the amount of intracellular UDP-GlcNAc with potential side effects on glycan synthesis.

Other compounds that have been constructed to target OGT directly can be classified into two categories: small molecule and bisubstrate inhibitors. The first group mimics and competes with the donor substrate and includes UDP, alloxan, compound 4, BZX and OSMI-1. However, most of these are likely to possess off-target toxicity. To obviate the presence of non-specific effects, the second category, including the bisubstrate inhibitors goblin 1 and 2, has been recently developed combining elements of both substrates. However, although these large molecules bind OGT with micromolar affinity, they are not cell-permeable and therefore as yet unsuitable for *in vivo* studies. All the compounds discussed in this review have been employed for several applications in multiple biological systems, including *in vitro* assays, mammalian cell lines, *ex vivo* tissues and primary cultures. Their use has allowed important scientific advances linking O-GlcNAc metabolism to cancer, diabetes, neuronal function and development. However, the potential off-target effects complicate the interpretation of any data generated using these inhibitors. Future work is necessary to improve the current tools or design novel molecules to create a cell-permeable, potent and selective OGT inhibitor suitable for cultured cell lines and animal models. A compound with these features will be necessary for further accurate exploration of the biological role of O-GlcNAc. In addition, considering the potential involvement of O-GlcNAc in many physiological processes, such a compound may represent a potential therapeutic tool against several pathologies, such as diabetes and cancer.

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